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# Epigenetic regulation of male fate commitment from an initially bipotential system

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## Abstract

A fundamental goal in biology is to understand how distinct cell types containing the same genetic information arise from a single stem cell throughout development. Sex determination is a key developmental process that requires a unidirectional commitment of an initially bipotential gonad towards either the male or female fate. This makes sex determination a unique model to study cell fate commitment and differentiation *in vivo*. We have focused this review on the accumulating evidence that epigenetic mechanisms contribute to the bipotential state of the fetal gonad and to the regulation of chromatin accessibility during and immediately downstream of the primary sex-determining switch that establishes the male fate.

### Keywords

Epigenetics; Sex determination; Cell fate commitment; Plasticity; Development; Gonad

## 1. Introduction

Vertebrates share a common template to achieve development of two distinct sexes. Initially, male and female embryos are indistinguishable. During development, the embryonic gonad forms with the unique ability to differentiate into two alternative organs: testes (males) or ovaries (females) (Fig. 1A). Gonadal differentiation diverges based on a genetic or environmental switch that activates one pathway and represses the other. This system presents an interesting model to explore the various levels of regulation involved in fate commitment of single cells and the coordination of the entire cell community that makes up the bipotential gonad.

One interesting characteristic of vertebrate sex determination is the plastic ability to switch between a male and female fate. For example, in most reptilian species, the incubation temperature of the egg controls the sex of the offspring, and switching eggs between temperatures during a critical window of development results in a switch to the opposite sex. Many fish can undergo sex reversal as adults, and although mammals do not undergo full sex

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reversal, the removal of certain key transcription factors can cause a switch in the identity of female cells to male cells (Ottolenghi et al., 2007; Uhlenhaut et al., 2009) or male cells to female cells (Matson et al., 2011). Importantly, gonadal cells do not switch randomly to another fate, but specifically to their developmental alternative fate.

Although multiple mechanisms are interwoven in the process of fate commitment and canalization as one sex or the other, epigenetic regulation is emerging as an important component. Epigenetics, the study of changes to gene function without changes in the DNA sequence, is capable of imposing a stable differentiation state throughout cell division. Between 1940 and 1956, Conrad Waddington proposed the concept of an epigenetic landscape to describe the process of cell fate commitment during development. He envisioned that a cell progresses towards a differentiated state through a series of fate decisions that are stabilized by changes to its epigenome. These changes maintain cell-type-specific gene expression patterns that channel the cell along certain pathways while eliminating other possible pathways the cell might take (Waddington, 1940, 1942, 1956). In this review, we will consider several lines of evidence in the field of vertebrate sex determination that suggest that epigenetic regulation is a key element in the process of commitment to and canalization of the male or female fate.

## 2. Are XX and XY chromatin landscapes created equal?

For many years the term "primary sex determination" has been used in vertebrates to refer to the fate decision in the bipotential gonad that leads to the commitment to testis or ovary fate. This is because (1) the first microscopic differences between male and female development occur in the gonad, and (2) once the gonad is committed to testis or ovary fate, the hormonal secretions of the testis or ovary are the dominant influence on male and female secondary sex characteristics.

However, for all heterogametic species, genetic sex (the presence of XX/XY or ZZ/ZW chromosomes) is established at fertilization long before the gonad forms, differentiates, and produces hormones. The presence of heteromorphic sex chromosomes leads to disparities in gene dosage between males and females. In flies and worms, the mechanisms that control dosage compensation are directly linked to sex determination (Cline and Meyer, 1996). In mammals, an XX/XY heterogametic species, the process of dosage compensation is not obviously linked to primary sex determination. To balance the difference in dosage of genes on the X between males and females, one X chromosome in females is inactivated in the late blastocyst stage through heterochromatin formation and spreading (Zhao et al., 2008; Pinter et al., 2012). At one time it was believed that X-inactivation silenced all genes on the inactive X. However, in recent years it has become clear that around 15% of X-linked genes in humans (Carrel and Willard, 2005) and 3.3% of X-linked genes in mice (Yang et al., 2010a) escape inactivation and are expressed at higher levels in females than in males. Conversely, males express Y-linked spermatogenesis genes that are not shared with females. Therefore, XX and XY cells harbor differences attributable to their sex chromosome complement. It is clear that if a testis is induced in an XX embryo, the embryo will develop as a phenotypic male despite the fact that the genetic sex of each cell is female (Koopman et al., 1991; Vidal et al., 2001; Qin et al., 2004; Polanco et al., 2010). This means that gonadal

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sex is dominant in mammalian systems. Nonetheless, the sex chromosome complement of vertebrate cells could influence their differentiation before or after steroid-producing gonads develop. In fact there is clear evidence in birds that this is the case (McQueen and Clinton, 2009).

Interestingly, a number of X escapees and Y-linked genes play crucial roles in chromatin regulation (Table 1). Chromatin is regulated at many levels by epigenetic mechanisms, such as DNA methylation and histone modifications that can alter the 3D organization of chromatin within the nucleus. Changes to chromatin structure ultimately modulate transcription factor accessibility to DNA binding sites, thereby regulating gene expression. Examples of Y-linked and X escapee genes in this category will be reviewed below.

### 2.1. Y-linked chromatin modifiers

The Y chromosome is the smallest chromosome and Y-encoded genes mostly function in male sex determination, testis development and fertility. Amongst the Y-encoded genes are a few chromatin modifiers: the histone demethylase *Uty* (ubiquitously-transcribed TPR gene on the Y chromosome), the H3K4-specific histone demethylase *Jarid1d* (Jumonji, AT-Rich Interactive Domain 1d), and in humans, the histone acetyltransferase *CDY* (Chromodomain protein Y-linked) (Dhanoa et al., 2016). Of these, *Uty* and *Jarid1d* have homologues on the X chromosome, and are reviewed in the following section.

*CDY* is exclusively expressed in the testes. The CDY family of proteins contains two conserved domains: a chromodomain, which interacts with the repressive H3K9me histone modification (Kim et al., 2006a), and a catalytic histone acetyltransferase domain, which has high affinity for H4 (Lahn et al., 2002). However, this catalytic domain has also been found to recruit histone deacetylases (HDACs) (Caron et al., 2003). As histone acetylation correlates with transcriptional activity, *CDY* may function as both a transcriptional activator and a repressor. It is speculated that *CDY*-mediated hyperacetylation of H4 may play an important role in facilitating the transition from histones to protamines, nuclear proteins that replace histones in sperm (Lahn et al., 2002). However, whether *CDY* can regulate transcription at a genome-wide level through its interaction with H3K9me and HDACs remains to be elucidated.

### 2.2. X escapee chromatin modifiers

*Utx* (ubiquitously-transcribed TPR gene on the X chromosome) is an X-linked gene that escapes X-inactivation in both mice and humans (Greenfield et al., 1998). This gene encodes a histone demethylase, which specifically removes the repressive histone modification H3K27me3 (Hong et al., 2007). *Utx* is critical for embryonic development, as *Utx*-null females die at midgestation (Shpargel et al., 2012; Welstead et al., 2012; Wang et al., 2013). *Utx* has a homolog on the Y chromosome, known as *Uty*. Although early *in vitro* studies showed that *Uty* was catalytically inactive (Lan et al., 2007), *Utx*-null males are viable and fertile (Shpargel et al., 2012; Welstead et al., 2012; Wang et al., 2013). Furthermore, *Uty*-null males phenocopy *Utx*-null males, suggesting that *Utx* and *Uty* are functionally redundant (Wang et al., 2013). Consistent with this interpretation, a more recent study found that human *Uty* is an active demethylase, but has reduced activity due to point mutations that

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affect substrate binding (Walport et al., 2014). Alternatively, UTX and UTY may function through H3K27me3-independent mechanisms as activators, by interacting with RBBP5, part of an H3K4me3-methyltransferase complex, and the SWI/SNF chromatin remodeler BRG1 (Shpargel et al., 2012). However, even though *Utx* and *Uty* may have similar functions, their expression levels and patterns differ in XX and XY embryos (Xu et al., 2008b), suggesting that they may regulate chromatin in a sex-specific manner.

*Jarid1c* (Jumonji, AT-Rich Interactive Domain 1c) is an X escapee that encodes an H3K4-specific histone demethylase that plays an important role in brain development and function. *Jarid1c* is more highly expressed in XX than XY mice, independent of whether an ovary or a testis is present (Xu et al., 2008a). Importantly, expression of its Y-linked homolog *Jarid1d* is unable to compensate for loss of *Jarid1c* despite its similar function, suggesting that sex-specific expression of this histone demethylase may contribute to differences in brain development and function such as neurite length and aggressive behavior (Xu et al., 2008a; Lee et al., 2007).

Another X escapee, *MeCP2* (methyl CpG-binding protein 2), plays a crucial role in brain development. Although *MECP2* is not itself a chromatin modifier, it has a high binding affinity to methylated CpGs and ability to interact with epigenetic machinery (Hendrich and Bird, 1998). Once bound, *MECP2* can regulate chromatin structure by recruiting HDACS, heterochromatin protein 1 (HP1), H3K9 histone methyltransferases, the DNA demethylase MBP1, and the chromatin remodeler ATRX amongst others, and in one case was able to alter higher-order chromatin structure by inducing chromatin looping (Nan et al., 1998; Agarwal et al., 2007; Fuks et al., 2003; Becker et al., 2013; Nan et al., 2007; Kernohan et al., 2014).

Although there is currently no phenotypic evidence that these sex-linked chromatin modifiers directly regulate primary sex determination, sexually dimorphic expression of chromatin modifiers could directly affect the activity of many genes leading to sex-specific transcriptional programs. Whether XX and XY cells initiate development with different chromatin landscapes, and whether these differences have significant phenotypic consequences in fetal or adult life is an area of active research using models that can disentangle genetic and hormonal effects (Burgoyne et al., 1995; Arnold, 2009).

## 3. The chromatin landscape at the bipotential stage

Despite differences in sex-chromosome complements, XX and XY fetal gonads are initially bipotential, and the somatic cells that compose the gonad have the full ability to differentiate into either Sertoli cells (males) or pregranulosa cells (females) (Fig. 1A) (Albrecht and Eicher, 2001). Consistent with their bipotential nature, both XX and XY progenitor cells co-express genes later associated with Sertoli- and granulosa-cell development (Nef et al., 2005; Munger et al., 2013). In other pluripotent systems, such as embryonic stem cells (ESCs), chromatin has an open configuration, which is associated with active transcription due to accessibility of transcription factor binding sites and transcription start sites. This open configuration confers pluripotent cells with a unique plasticity that enables differentiation into a wide range of lineages (Atlasi and Stunnenberg, 2017). In ESCs,

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promoters of developmental genes are often marked by both the active H3K4me3 and the repressive H3K27me3 histone modifications (Box 1) (Bernstein et al., 2006a; Azuara et al., 2006). Despite their antagonistic functions, this “bivalency” plays a key role in pluripotency by maintaining genes in a poised state for rapid activation or repression upon differentiation. As pluripotent cells differentiate, chromatin becomes more restricted by epigenetic deposition of DNA methylation and histone modifications that establish heritable cell-type-specific gene expression patterns (Atlasi and Stunnenberg, 2017).

Based on this model, one hypothesis is that the chromatin landscape in XX and XY bipotential progenitor cells is similar, enabling equal access to promoters of sex-determining genes and regulatory elements of both sexes, and that after sex-determination, the chromatin landscape becomes more restricted to canalize either the male or female fate and repress the alternative pathway. Investigation of this hypothesis revealed that, similar to ESCs, key sex-determining genes that are co-expressed in XX and XY mouse progenitor cells are bivalent, possibly contributing to the bipotential nature of these cells (Fig. 1B) (Garcia-Moreno et al., unpublished). The upregulation of the male or female pathway is accompanied by mutually antagonistic mechanisms that repress the alternate cell fate (Kim et al., 2006b; Ottolenghi et al., 2007; Chassot et al., 2008; Uhlenhaut et al., 2009; Matson et al., 2011). At the chromatin level, this transition is reflected in a reorganization of histone marks around sex determining genes. Genes associated with the female pathway lose their repressive mark when the ovarian pathway is activated, and genes associated with the male pathway lose their repressive mark when the testis pathway is activated. However, ovary-determining genes that become transcriptionally repressed when the gonad commits to a male fate remain bivalent, similar to the fate of testis-determining genes in the ovary (Fig.1B) (Garcia-Moreno et al., unpublished). Maintaining the alternate cell fate in a state poised for activation possibly contributes to the unique capacity of supporting cells to transdifferentiation from Sertoli cells to pregranulosa cells and vice-versa, even during adulthood (Uhlenhaut et al., 2009; Matson et al., 2011). As bivalency is a conserved epigenetic mechanism (Lesch et al., 2016), it will be interesting to determine whether bivalent sex-determining genes confer plasticity to non-mammalian systems that can switch between the male and female fate in response to environmental stimuli.

Another study performed DNaseI hypersensitive site sequencing (DNaseI-seq) and chromatin immunoprecipitation followed by sequencing (ChIP-seq) for H3K27ac, a histone modification indicative of active enhancers, to generate a profile of the chromatin landscape in purified mouse Sertoli cells at embryonic day 13.5 (E13.5), soon after commitment to the male pathway (Maatouk et al., 2017). DNaseI-seq identified 28,133 nucleosome-depleted regions (NDRs) (corresponding to regulatory elements) that were unique to Sertoli cells when compared to six other tissues. As expected, these NDRs were commonly located near Sertoli-specific genes, implying that they represent regulatory elements that drive Sertoli cell differentiation. Interestingly, pregranulosa-specific genes had a similar enrichment of Sertoli-unique NDRs in their neighborhood in Sertoli cells, suggesting that the chromatin architecture between Sertoli and pregranulosa cells is similar and may reflect their shared progenitor state. However, H3K27ac-positive NDRs were significantly enriched only around Sertoli-specific genes. Together these findings indicate that as sex-determining pathways diverge, genes in Sertoli cells that are associated with the male pathway lose their repressive

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H3K27me3 mark and acquire H3K27ac. Simultaneously, genes associated with the female pathway remain bivalent in Sertoli cells, and retain NDRs in their enhancer/promoter regions. It is likely that these H3K27ac-negative NDRs (inactive enhancers) associated with pregranulosa-specific genes are bound by repressors that block upregulation of the female-determining pathway.

## 4. Epigenetic regulation of the primary switch

The balance between male and female fate in the early gonad is disrupted by master regulators of sex-determination that act as a “switch” to activate the male pathway and repress the female pathway. There is increasing evidence that these primary switches are epigenetically regulated. Below, we will review how the primary mammalian switch *Sry* is epigenetically regulated during sex determination, and provide evidence that the epigenetic regulation of master regulators of male sex-determination is conserved in non-mammalian systems.

### 4.1. The mammalian switch, Sry

In mammals, it is the presence of the Y-encoded transcription factor *Sex–determining Region Y (Sry)* that directs the bipotential fetal gonad towards the male fate (Sinclair et al., 1990; Gubbay et al., 1990; Koopman et al., 1991). *Sry* expression breaks the initial male-female balance in bipotential progenitors and directs differentiation of Sertoli cells through a downstream signaling cascade (Albrecht and Eicher, 2001). In XY mice, *Sry* is transiently expressed between E10.5 and E12.5 (Hacker et al., 1995). Although there are still unanswered questions about the events leading to the activation of *Sry*, a number of epigenetic modifiers have been identified.

**4.1.1. Regulation of Sry mediated by DNA methylation—**DNA methylation, which in mammals occurs primarily at cytosines located next to guanines (CpG), plays a crucial role during development by epigenetically controlling gene silencing and establishing cell-type-specific gene expression patterns during differentiation (Razin and Cedar, 1984). The methylome of the Y chromosome was first explored by Nishino et al. (2004). This group generated an *in vivo* methylation profile of XY mouse embryos throughout development using bisulfite sequencing, a standard technique that identifies methylated CpGs. There are 16 CpGs in the 4.5 kb region upstream of *Sry* that cluster into two regions. Region I contains four CpGs and overlaps the TSS of a circular transcript of *Sry*, which is believed to be untranslated (Dolci et al., 1997), whereas Region II contains seven CpGs and overlaps the TSS of the linear transcript of *Sry*, which is translated into a functional protein from E10.5–E12.5 in XY fetal gonads (Jeske et al., 1995). Bisulfite sequencing revealed that both regions were hypomethylated at the blastocyst stage and became hypermethylated in E8.5 embryos in which *Sry* is not yet expressed. At the time of *Sry* activation (E11.5), both regions lost methylation in the gonad, but not in other tissues such as the liver. Finally, at E15.5, Region II became re-methylated, whereas Region I remained hypomethylated. It is unlikely that hypomethylation at Region I is of functional significance, as this region did not induce activity in an *in vitro* promoter assay (Nishino et al., 2004). However, the possible three-dimensional structure of the inverted repeats around this locus may interfere with *in vitro*

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promoter assays (Gubbay et al., 1990; Capel et al., 1993). Nonetheless, this work established a clear anti-correlation between promoter CpG methylation and *Sry* expression in the gonad throughout sex determination (Fig. 2A).

To investigate the mechanistic link between methylation and *Sry* expression, Nishino et al. established an *in vitro* system by culturing primary cells isolated from E11.5 XY gonads (Nishino et al., 2004). Introduction of previously *in vitro*-methylated constructs containing either the circular or linear promoter regions strongly suppressed the activity of ectopically introduced *Sry*. These results clearly showed that *Sry* gene expression can be epigenetically regulated through DNA methylation-mediated silencing.

In mammals, DNA methylation occurs primarily at the CpG site. Non-CpG methylation, which occurs at CNG sites in which N can be any nucleotide, is more common in plants (Gruenbaum et al., 1981), bacteria (Yoder et al., 1997; Casadesus and Low, 2006) and yeast (Pinarbasi et al., 1996), but was observed at CC(A/T)GG motifs in various mammalian systems (Malone et al., 2001; Franchina and Kay, 2000; Agirre et al., 2003; Imamura et al., 2005). Interestingly, the promoter for both the circular and linear transcripts of *Sry* (regions I and II) contains a CCTGG motif (Nishino et al., 2011). In the gonad, this motif has the opposite methylation dynamic than its neighboring CpG sites, and coincides with *Sry's* temporal expression pattern. While CpG sites transiently lose methylation at E11.5, CCTGG sites are transiently methylated (Fig. 2A). An *in vitro* promoter assay further showed that CCTGG methylation can induce *Sry* activity, suggesting that non-CpG methylation is associated with active transcription and may play an important role in *Sry* activation. However, although the CCTGG site at Region II was conserved in six mouse strains, two of these strains (C57BL/6 and 129S1) did not exhibit CCTGG methylation at E11.5, suggesting that non-CpG methylation may be background-dependent and raises the possibility of strain-specific differences in epigenetic regulation of *Sry* (Nishino et al., 2011).

Although it is well established that DNA methylation strongly correlates with *Sry's* activity, the mechanisms by which its promoter CpG sites are dynamically methylated during such a precise window of development have not been described. This is partly due to the fact that *in vivo* disruption of DNA methyltransferases (DNMTs) leads to early embryonic lethality (Li et al., 1992; Okano et al., 1999). Warr and colleagues hypothesized that GADD45g, a protein that can induce gene expression by recruiting DNA repair factors to demethylate target genes (Barreto et al., 2007; Niehrs and Schafer, 2012), was involved in *Sry's* activation based on the observation that loss of *Gadd45g* caused complete male-to-female sex reversal in mice due to failure to activate *Sry* (Warr et al., 2012). However, loss of *Gadd45g* did not alter the normal methylation status of *Sry* during sex determination, suggesting that it does not activate *Sry* through promoter demethylation, but must function through an alternative mechanism (Gierl et al., 2012). Despite this, the same study found that demethylation at *Sry's* promoter begins as early as 5–6 tail somites, and is therefore one of the earliest male-specific molecular events preceding *Sry* expression.

Interesting data concerning the epigenetic regulation of sex determination came from cloned animals that were generated by somatic cell nuclear transfer (SCNT). Cloned animals exhibit anomalies of sex differentiation despite having a normal karyotype (Inoue et al.,

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2009). For example, canine SCNT results in male-to-female sex reversal in almost 23% of cases (Jeong et al., 2016). A genetic analysis of *SRY*+ XY DSD dogs found no genetic differences that could account for sex reversal. However, the *SRY* locus in XY DSD dogs was hypermethylated compared to control XY dogs (Jeong et al., 2016). This hypermethylation corresponded with reduced expression of *SRY* (in addition to other downstream male-determining genes), and an upregulation of the ovarian developmental pathway. Although this case is specific to an artificial system, it strongly supports the idea that epigenetic regulation can control the sex-determination network.

Not much is known about the role that DNA methylation plays in regulating the human *SRY* gene. In one study, an epigenetic profile of the Y chromosome was generated from blood cells of two individuals from distinct populations (Singh et al., 2011). A CTCF transcription factor binding site was found in the promoter region of *SRY*, which overlapped an unmethylated CpG site. CTCF binds chromatin insulators and acts as a transcriptional repressor. Interestingly, this sitewas enriched for repressive histone modifications, pointing to the possibility that the human *SRY* gene may be silenced through recruitment of repressors and not through DNA methylation. However, caution should be taken when drawing conclusions based on analysis of a non-gonadal cell type that does not usually express *SRY*.

**4.1.2. Regulation of Sry mediated by histone modifications—**Evidence that histone modifiers are critical regulators of mammalian sex determination came from the observation that loss of the chromobox protein homologue 2 (*Cbx2*) leads to complete male-to-female sex reversal and development of hypoplastic gonads in mice (Katoh-Fukui et al., 1998) and humans (Biason-Lauber et al., 2009). CBX2 is part of the Polycomb-group (PcG) of proteins that typically function as transcriptional repressors and play a critical role in regulating gene silencing during development (Simon and Kingston, 2009; Margueron and Reinberg, 2011). The PcG proteins assemble into two complexes: the Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). Canonically, PRC2 first catalyzes H3K27me3 at the promoter of its target genes. PRC1 then binds H3K27me3 through its CBX2 subunit, and maintains gene repression through chromatin compaction (Bernstein et al., 2006b; Lau et al., 2017). Sex-reversal in XY *Cbx2* mutants is characterized by a failure to upregulate *Sry* and its downstream target *SRY*-box 9 (*Sox9*) (Katoh-Fukui et al., 2012). As sex-reversal was rescued by forced expression of *Sry*, CBX2 was proposed to act as an early activator of *Sry* expression (Katoh-Fukui et al., 2012). Although some subunits of the PcG proteins have been reported to drive active transcription of certain target genes (Jacob et al., 2011; Mousavi et al., 2012; Herz et al., 2012), CBX2 has not been shown to act directly as an activator. Therefore, a role for CBX2 as an activator was unexpected. Recent evidence suggests that PRC2 may be involved in “transcriptional focusing” by spatially confining H3K27me3 + silent domains (Lu et al., 2017). It is possible that disruption of the PcG complex, such as loss of CBX2, may lead to aberrant spread of these domains resulting in silencing of underlying genes, possibly including *Sry*. Another possibility is that CBX2 induces *Sry* expression indirectly by repressing a repressor. Because the male and female pathways are mutually antagonistic, stabilization of the male fate requires active repression

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of female-determining genes, raising the possibility that CBX2 has a wide-spread role in the repression of the female-pathway, which may otherwise block male fate commitment.

Further evidence for the epigenetic regulation of *Sry* mediated through histone modifiers came from studies of the histone demethylase *Jmjd1a*. *Jmjd1a*-deficient mice exhibit male-to-female sex reversal (Kuroki et al., 2013). *Jmjd1a* encodes a histone demethylase that acts specifically on the repressive H3K9me2 histone modification. ChIP for H3K9me2 and the active histone modification mark H3K4me2 was performed in purified progenitor cells from gonads of XY mice at E11.5 when *Sry* expression is at its peak. As opposed to control XY mice, the *Sry* locus of *Jmjd1a*-deficient mice retained high enrichment of the repressive H3K9me2 mark, and had low enrichment of the active H3K4me2 mark. Consistently, *Sry* expression levels were reduced in mutant mice. This suggests that *Jmjd1a*-dependent demethylation of H3K9me2 is required for subsequent H3K4me2 accumulation at *Sry's* promoter, leading to an open chromatin conformation and *Sry* activation (Fig. 2A). Consistent with this observation, a separate study found that the *Sry* promoter has low levels of H3K9me3 and is enriched for the active H3K4me3 modification in E12.5 mouse testes. In contrast, adult testes have high H3K9me3 and low H3K4me3 enrichment, suggesting that the *Sry* locus regains H3K9me3 after it is silenced, and that this mark maintains long-term repression of *Sry* throughout adulthood in mice (Sinha et al., 2017). Recently, sex-reversal of *Jmjd1a*-deficient micewas rescued by disrupting the GLP/G9a H3K9 methyltransferase complex, pointing towards this complex as the one that catalyzes H3K9me2 at the *Sry* locus (Fig. 2A) (Kuroki et al., 2017). This study highlights how various epigenetic factors interact to fine-tune the expression of *Sry* by balancing the levels of H3K9me2 at its promoter.

In addition to accumulation of H3K4me2 at *Sry's* promoter, activation of *Sry* requires deposition of H3K27ac by the CREB-binding protein (CBP) and its paralogue p300 (Fig. 2A) (Carre et al., 2017). A recent study found that deletion of either gene in gonadal somatic cells results in partial male-to-female sex reversal, whereas loss of three of the four *p300/Cbp* functional alleles leads to complete male-to-female sex reversal. ChIP showed reduced levels of H3K27ac at the promoter of *Sry* in *p300/Cbp* mutants at E11.5, corresponding to reduced *Sry* and *Sox9* expression levels. This study indicates that this histone/lysine acetyltransferase complex is a key component in the activation of *Sry* during testis development.

Interestingly, epigenetic profiling of human blood cells showed that the human *SRY* gene promoter was enriched for the active H3K9ac mark and the repressive H3K27me3 mark, but H3K9me3 was only enriched over the gene body (Singh et al., 2011). The conflicting H3K9ac and H3K9me3 modifications may be a reflection of mixed cell populations, as *SRY* is expressed in the B-cell lineage but not in other blood lineages. Studies of human gonadal lineages are required to understand which chromatin modifiers are involved in *SRY* activation during sex determination in humans. However, there is evidence that disruption of epigenetic regulation can lead to disorders of sexual development (DSDs). Consistent with the mouse studies mentioned above, one XY phenotypically normal girl with hypoplastic ovaries was found to have inherited two loss-of-function mutations in the *CBX2* gene (Biason-Lauber et al., 2009). In a separate case, an *SRY*+ XY woman with streak gonads had a hyperacetylated *SRY* locus relative to control XY males (Mitsuhashi et al., 2010). This

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hyperacetylation coincided with prolonged *SRY* activity, which the authors suggested might destabilize the downstream male-determining network, although further experiments are required to fully explain this case.

### 4.2. Evidence for epigenetic regulation of the key switch gene, Dmrt1, in other vertebrates

Several new lines of evidence suggest that epigenetic regulation of key switch genes may be a conserved element of sex determination pathways in different species. *Dmrt1* (doublesex and mab3-related transcription factor 1) is a highly conserved transcription factor that plays a critical role in vertebrate sex determination (Matson and Zarkower, 2012) and has been shown to act as the master sex-determining gene in many non-mammalian systems (Masuyama et al., 2012; Smith et al., 2009; Yoshimoto et al., 2008). In mice, *Dmrt1* is not required for testis-development; however, it is essential for maintaining the Sertoli cell phenotype in adult testes, as loss of *Dmrt1* causes transdifferentiation of Sertoli cells into pregranulosa cells (Matson et al., 2011). Although there are currently no studies investigating the epigenetic regulation of *Dmrt1* in mammals, its epigenetic regulation has been investigated in the half-smooth tongue sole, chickens, and the red-eared slider turtle.

**4.2.1. Epigenetic regulation of dmrt1 in fish—***dmrt1* is highly conserved in fish and plays an important role in male sex determination (Marchand et al., 2000; Guo et al., 2005). The half-smooth tongue sole *Cynoglossus semilaevis* is a flatfish that is a fascinating model to study the epigenetic regulation of sex determination, as it has both genetic- and temperature-dependent sex-determining mechanisms. The genetic sex of this species is determined by its Z and W chromosome composition: males carry two Z chromosomes, whereas females carry a Z and a W chromosome. In this species, dosage of the Z chromosome is the sex-determining factor. Despite the inheritance of sex chromosomes, ~14% of ZW females develop as males under normal conditions (22 °C), and the incidence of sex reversal can increase to ~73% when fish are reared at high temperatures (28 °C) (Chen et al., 2014). These sex-reversed fish are “pseudomales” that can reproduce with normal females. Importantly, the offspring of pseudomales and normal females maintain a high sex-reversal rate even under normal conditions (22 °C), suggesting that the ability to sex reverse can be inherited.

Shao et al. generated a DNA methylation profile of female, male and pseudomale fish to investigate the role of DNA methylation in the transition from genetic to environmental sex determination (Shao et al., 2014). This study identified *dmrt1*, which is Z-encoded in the tongue sole (Chen et al., 2014), as a critical gene that responds to temperature change through DNA methylation. Consistent with its expression pattern, *dmrt1* maintains low methylation levels throughout life in male gonads, but is increasingly methylated in female gonads starting at the time of sex determination and throughout adulthood. In ZW pseudomales, the *dmrt1* gene has low methylation levels similar to ZZ males, and is therefore active and able to drive testis-development. This male-specific methylation is then inherited in the ZW offspring of ZW pseudomales and ZW females, accounting for a higher rate of sex-reversal compared to ZW offspring between normal ZZ males and ZW females. This finding suggests that temperature can override genetic sex-determination by directly

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altering the epigenetic marks at *dmrt1*, and that in this species where methylation is not stripped from the genome in the early embryo, this effect is transgenerational.

**4.2.2. Epigenetic regulation of DMRT1 by MHM in chickens—**As in the half-smooth tongue sole, chickens have a ZZ (male) and ZW (female) sex chromosome composition. *DMRT1* is also a Z-linked gene in chickens that is more highly expressed in males than in females. Overexpression of *DMRT1* in chick ZW gonads induced the male pathway (Lambeth et al., 2014), whereas knockdown of *DMRT1* led to feminization of male gonads (Smith et al., 2009).

A region neighboring *DMRT1* is differentially methylated between males and females (Teranishi et al., 2001). In males, this region is hypermethylated (known as the male hypermethylated region or MHM) and is transcriptionally inactive, whereas in females, this region is hypomethylated and transcribed into a long non-coding RNA. In addition to being hypomethylated, the female MHM locus is enriched for the active H4K16ac mark (Bisoni et al., 2005) and has an open chromatin configuration (Itoh et al., 2011). MHM is hypothesized to inhibit *DMRT1* expression in females, as the MHM transcript accumulates at the site of transcription adjacent to *DMRT1* (Teranishi et al., 2001) (Fig. 2B). Consistent with this hypothesis, injection of exogenous MHM into rooster testicles led to a strong downregulation of *DMRT1* and a paling of the comb color, indicative of a change in sex hormones. Importantly, transcriptional levels of other key sex-determining genes such as *AMH* and *SOX9* were unaffected, suggesting that MHM specifically regulates *DMRT1* (Yang et al., 2010b).

**4.2.3. Epigenetic regulation of Dmrt1 by KDM6B in turtles—**The red-eared slider turtle *Trachemys scripta* lacks heteromorphic sex chromosomes. Instead, the temperature of incubation acts as a sex determinant. During embryogenesis, turtles develop as males if eggs are exposed to lower temperatures (26 °C), and as females if they are exposed to higher temperatures (32 °C). Prior to sex determination, *Dmrt1* expression in the embryonic gonads is sexually dimorphic, with higher levels at MPT (male-producing temperature) than at FPT (female-producing temperature) (Kettlewell et al., 2000; Czerwinski et al., 2016). Recently, *Dmrt1* was shown to be both necessary and sufficient to initiate male sex determination in *T. scripta* (Ge et al., 2017). Moreover, *Dmrt1* responds rapidly to temperature shifts, which establishes it as a master regulator of temperature-dependent sex determination. Interestingly, DNA methylation of the *Dmrt1* promoter region is inversely correlated with its expression and fluctuates in response to temperature changes. For example, the methylation level of the *Dmrt1* promoter greatly increased when eggs were shifted from MPT to FPT. In contrast, shifting eggs from FPT to MPT resulted in decreased promoter DNA methylation and gene activation (Ge et al., 2017) (Fig. 2C). This suggests that, similar to the tongue sole flatfish, temperature can regulate both sex-determining gene expression and promoter methylation. However, although the previous studies show an inverse correlation between CpG promoter methylation and *Dmrt1* expression, whether DNA methylation is a cause or a consequence of expression changes in temperature-dependent systems has not been established.

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Transcriptome sequencing of *T. scripta* embryonic gonads at MPT and FPT revealed that in addition to *Dmrt1, Kdm6b* (or *Jmjd3*) is also upregulated at MPT and its expression levels fluctuate in response to temperature changes (Czerwinski et al., 2016). *Kdm6b* is an H3K27me3-specific demethylase that can activate target genes through the removal of this repressive histone modification. Loss of *Kdm6b* in MPT gonads results in a complete shift from the male to the female sexual trajectory, suggesting that it also plays a key role in male sex-determination (Ge et al., in press). Knockdown of *Kdm6b* resulted in a significant downregulation of *Dmrt1* expression in MPT gonads, pointing towards *Dmrt1* as a downstream target of *Kdm6b*. Consistent with this hypothesis, ChIP-qPCR revealed that the *Dmrt1* promoter had low levels of H3K27me3 in MPT gonads in which *Kdm6b* and *Dmrt1* expression levels are high. In contrast, the *Dmrt1* promoter was enriched for H3K27me3 in FPT gonads in which *Kdm6b* and *Dmrt1* expression levels were low. Importantly, H3K27me3 levels at *Dmrt1* increased upon loss of *Kdm6b*, suggesting that *Kdm6b* activates *Dmrt1* by removing H3K27me3 at MPT leading to activation of this switch gene that induces male sexual development (Fig. 2C). This study provides the first direct mechanistic link between epigenetic and temperature regulation of sex determination.

## 5. Sry and other Sox genes as epigenetic regulators

In the developing testis, *Sry's* primary function is to upregulate *Sox9* expression (Sekido and Lovell-Badge, 2008). *Sox9* is part of the *Sry*-type HMG box (Sox) family, a group of proteins with over 60% similarity to the SRY high-mobility group (HMG) box region (Denny et al., 1992a). In mammals, several Sox genes, including *Sox9*, can replace *Sry* (Zhao and Koopman, 2012). For example, directed expression of *Sox9* in XX mice induces testis development, demonstrating that *Sox9* is sufficient for male sex determination (Vidal et al., 2001). Consistent with this, duplication of *SOX9* in XX humans can lead to female-to-male sex reversal (Cox et al., 2011). On the other hand, *Sox9*-null XY gonads develop as ovaries (Barrionuevo et al., 2006), and loss-of-function mutations of the human *SOX9* gene can lead to female development (Kwok et al., 1995; Stoeva et al., 2011). These studies highlight the role of *Sox9* as a critical regulator of male sex determination and Sertoli cell development.

Both SRY and SOX9 are transcription factors (TFs) that recognize and bind a specific DNA sequence (Denny et al., 1992b). Although TFs typically act as direct activators when binding to DNA motifs of target genes, below we will review several studies that support a model in which SRY and SOX9 can themselves function as epigenetic regulators.

Despite *Sry* acting as the primary male-determining switch in mice and humans, sequence conservation between the *mSry* and *hSRY* genes is low and restricted to 79 amino acids that encode the HMG box, suggesting that this region is of high functional significance (Sinclair et al., 1990; Ferrari et al., 1992). The HMG box of SRY has an L-shaped structure formed by an extended segment and three α-helices (Read et al., 1993). Contrary to most TFs that bind the major groove of DNA, the SRY HMG box binds the minor groove of DNA inducing a 60°2013;70° bend in the double helix (Fig. 3A). In addition to DNA bending activity, HMG1 proteins can also bind to DNA cruciform structures, suggesting that SRY could also mediate attachment sites in chromatin loops (Fig. 3B) (Ferrari et al., 1992). This

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ability to directly modulate the 3D architecture of DNA leads to the speculation that SRY and other HMG-box containing TFs, such as the Sox genes, are core determinants of cell-type-specific chromatin landscapes. In support of this, it has been suggested that SRY might mediate effects at a distance by mechanically displacing factors associated with chromatin near the point of flexure. Alternatively, DNA bending might bring remote sites in close contact with each other to facilitate the interaction of transcription factors, or to promote the establishment of chromatin loops (Fig. 3A) (Bianchi and Beltrame, 1998). Importantly, the majority of sex-reversing point mutations in the *SRY* gene fall within the HMG box-encoding region (Berta et al., 1990). These mutations cause alterations to the SRY protein that reduce or ablate DNA binding affinity, suggesting that DNA binding and bending is of critical functional importance (Jager et al., 1992; Harley et al., 1992; Pontiggia et al., 1994; Murphy et al., 2001; Assumpcao et al., 2002).

In addition to directly bending DNA and altering the 3D conformation of chromatin, SRY and SOX9 can act as epigenetic regulators by interacting with chromatin-modifying complexes. In E11.5 fetal gonads, SRY interacts with Krüppel-associated box only (KRAB-O) protein and its obligatory co-repressor Krab-associating protein 1 (KAP1) (Oh et al., 2005; Peng et al., 2009). KAP1 then recruits heterochromatin protein 1 (Hp1), HDACs and the SETDB1 methyltransferase, which function as gene silencers by creating a repressive chromatin environment. Therefore, *Sry* may have a dual function in early sex determination by activating male-determining genes through direct binding to regulatory elements (such as binding to the testis-specific enhancer TESCO upstream of *Sox9* (Sekido and Lovell-Badge, 2008)), and repressing the female pathway through recruitment of KRAB-O/KAP1 chromatin-mediated repression machinery. Interestingly, KRAB-O knockdown in *Sry*-expressing cultured cells resulted in reduced levels of *Sox9*, suggesting that KRAB-O may also mediate *Sry* function. However, KRAB-O knockdown mice exhibited normal testis development, possibly due to redundancy from over 100 KRAB genes that are expressed in the mouse fetal gonad (Polanco et al., 2009).

In addition to p300/CBP-mediated activation of *Sry* through H3K27ac deposition, SOX9 can itself epigenetically regulate target genes by interacting with this histone acetyltransferase complex (Tsuda et al., 2003). In human chondrocytes, p300/CBP significantly enhanced *Sox9*-mediated transcription of target genes through p300-mediated histone acetylation (Tsuda et al., 2003; Furumatsu et al., 2005). Although these studies were not performed in the developing gonad, it is possible that *Sox9* functions during sex determination by acetylating SOX9 binding sites and recruiting additional activators to Sertoli-specific genes and regulatory regions. In this way, SRY and SOX9 may play critical roles during sex determination by epigenetically regulating downstream targets that further promote the divergence of the male pathway from the bipotential state.

## 6. Conclusions

In vertebrates, the formation of a bipotential gonad is the first and critical step for the development of sexually dimorphic organisms. The unique ability of the gonad to engage one of two possible developmental pathways during sex determination provides a valuable model to study cell fate decisions and transitions.

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Accumulating evidence suggests that various levels of epigenetic regulation underlie the resolution of cell fate in the early gonad by fine-tuning the timing and expression levels of sex-determining genes. The plasticity of the bipotential gonad is reflected in (and perhaps fostered by) a poised chromatin landscape that resolves through the activation of genes associated with one fate, and the maintenance of bivalency at genes associated with the opposite fate. At present, commitment to one fate or the other does not appear to be associated with an epigenetic lock-out of the alternative pathway. Instead, evidence suggests that maintenance of cell fate depends on TFs such as SOX9, FOXL2 and DMRT1. Dependence on TFs rather than more stable repressive mechanisms may not be unusual between lineages that originate from a common precursor, especially when there is an evolutionary advantage to maintaining plasticity. In the case of the supporting cell lineage in the gonad, one possibility is that this is an evolutionary holdover from species that undergo sex reversal, but this idea has not been investigated.

The activation of genes associated with the male fate is initiated by transcription factors such as SRY and DMRT1. Although TFs initiate transcriptional changes, their ability to bind DNA is modulated by the chromatin landscape. In addition, *Sry* and *Dmrt1* are themselves epigenetically regulated throughout sex determination, and in the case of *Sry*, can function as a downstream epigenetic regulator itself. The interlacing of TFs and the chromatin landscape during cell fate commitment and maintenance is an important area of future study, which will benefit from the unique characteristics of the bipotential gonad.

For years it has been speculated that species lacking genetic cues are able to convert external signals into a phenotypic output through changes in their epigenome. Recent evidence supports this idea. The advent of whole-genome sequencing coupled with small scale techniques that assay the chromatin landscape have been instrumental in advancing our understanding of the epigenetic mechanisms that regulate primary sex-determining signals in species such as fish and reptiles. We expect this field to expand significantly in the next few years.

Although we have limited this review to studies of the initial steps in the sex-determination cascade, further studies are sure to uncover additional levels of epigenetic regulation that further promote the canalization and maintenance of the male and female pathways. In addition to advancing our understanding of the basic biological principles underlying molecular and cellular fate commitment and development, increasing our understanding of the epigenetic landscape during sex determination has implications for our ability to diagnose human disorders of sex development with unknown etiologies. Currently ~80% of patients do not receive a genetic diagnosis. In many of these undiagnosed cases, the cause may escape conventional SNP screens because it is epigenetic in nature. Epigenetic studies can be expected to define critical regulatory regions beyond the coding genome.

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**Box 1**

Histone modifications mentioned in this review that mark promoters of active or silenced genes.

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| Active genes | Silenced genes |
| --- | --- |
| H3K4me2/3<br>H3K27ac<br>H3K9ac<br>H4K16ac | H3K27me3<br>H3K9me2/3 |

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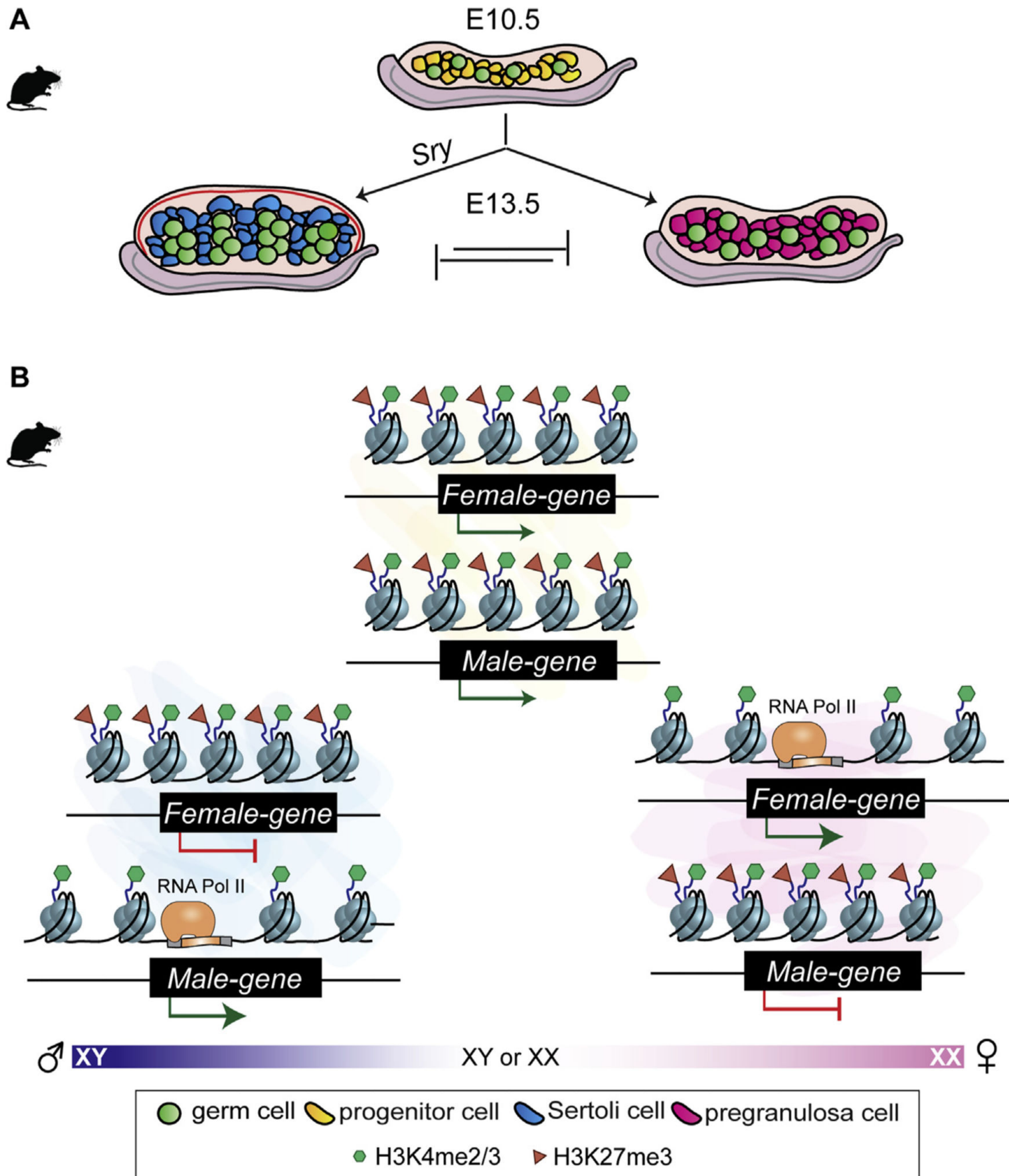


**Fig. 1.**

A) Overview of mammalian sex determination. In mice, gonads form at E10.5 and are indistinguishable between XY and XX individuals. The somatic progenitor cells that compose the fetal gonad (top) are initially bipotential. Following expression of the Y-encoded *Sry* gene at E11.5, progenitor cells differentiate into Sertoli cells and gonads develop as testes (left). In absence of *Sry*, progenitor cells differentiate into pregranulosa cells and gonads develop as ovaries (right). Commitment to the male or female fate requires repression of the alternate pathway. B) The chromatin landscape at the bipotential stage. Male- and female-determining genes are bivalent and co-expressed at low and similar levels

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in progenitor cells (top, yellow). Upregulation of male-determining genes leading to differentiation of Sertoli cells (left, blue) is accompanied by loss of the repressive H3K27me3 mark, whereas repressed female-determining genes remain bivalent. Upregulation of female-determining genes leading to differentiation of pregranulosa cells (right, pink) is accompanied by loss of the repressive H3K27me3 mark, whereas repressed male-determining genes remain bivalent.

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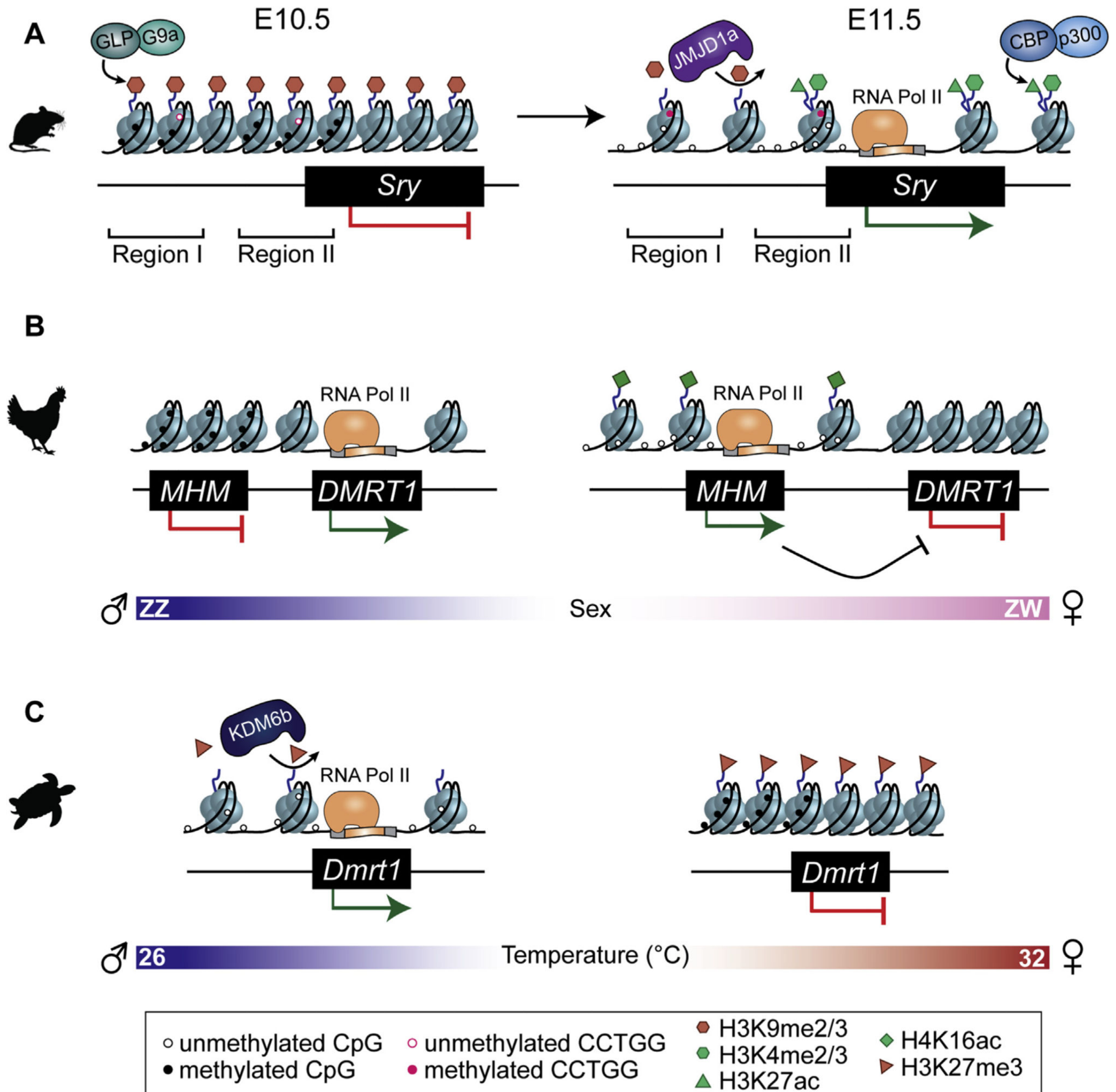


**Fig. 2.**

A) Epigenetic regulation of *Sry* in mice. Prior to sex determination (left) *Sry* is repressed and enriched for the repressive GLP/G9a-mediated H3K9me2 histone modification. Region I overlaps the TSS of an untranscribed circular *Sry* transcript, and Region II overlaps the TSS of a functional linear *Sry* transcript. At E10.5, regions I and II contain methylated CpGs, and unmethylated CCTGG sites. At E11.5, *Sry* activation requires *Jmjd1a*-mediated removal of H3K9me2, CBP/p300-mediated deposition of H3K27ac, and accumulation of the active H3K4me2 at its promoter. CpGs at region I and II become demethylated, and CCTGG sites become methylated. B) Epigenetic regulation of *DMRT1* in chickens. In males (ZZ, left), a region neighboring the male-determining gene *DMRT1* is hypermethylated (MHM)

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and repressed. In females (ZW, right), MHM is hypomethylated and enriched for the active H4K16ac histone modification. The open chromatin conformation of MHM in females enables transcription of a lncRNA that inhibits expression of *DMRT1*. C) Epigenetic regulation of *Dmrt1* in red-eared slider turtles. During embryogenesis, turtles develop as males if eggs are incubated at lower temperatures (blue, left) and as females at higher temperatures (red, right). At male-producing temperatures, *Kdm6b* is upregulated. KDM6b-mediated removal of the repressive H3K27me3 and demethylation of CpGs at the promoter of *Dmrt1* is required for its activation and subsequent testis development. At female-producing temperatures, enrichment of H3K27me3 and CpG methylation at the promoter of *Dmrt1* inhibits its expression and leads to ovary development.

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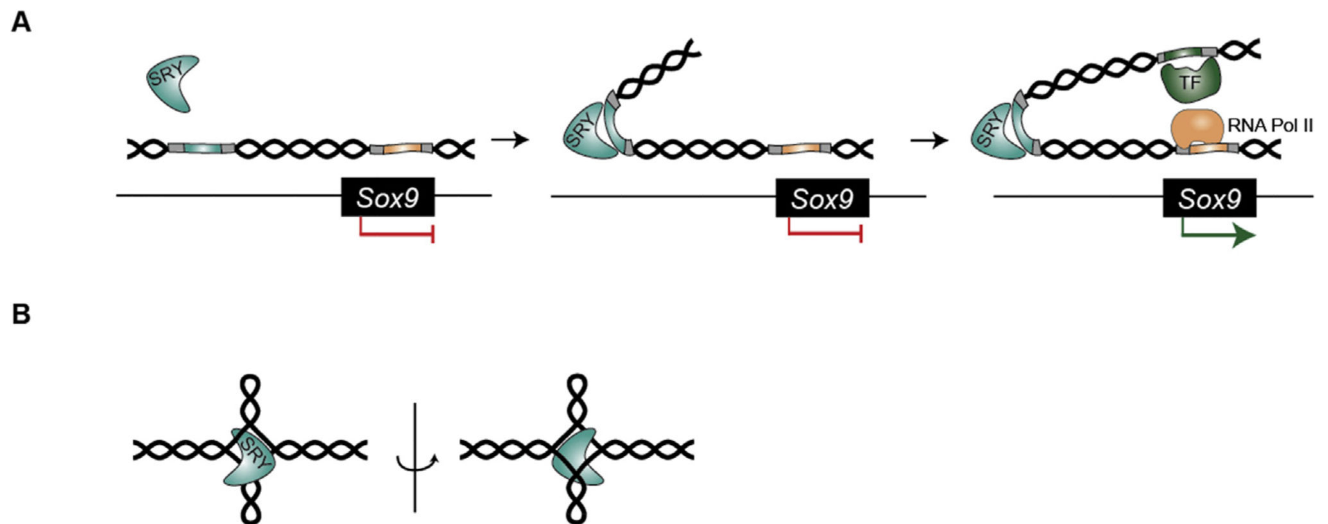


**Fig. 3.**

Overview of SRY mechanism in mammals. A) *Sry* encodes a transcription factor that recognizes a specific DNA-binding motif (blue box). SRY contains an L-shaped HMG box that binds the minor groove of DNA and induces a 60–70° bend in the double helix (middle panel). It is speculated that DNA bending by SRY may mediate effects at a distance by promoting chromatin looping, which brings transcription factors in close proximity to TSS. Depicted is a putative example of SRY-mediated activation of *Sox9* by binding to an upstream enhancer (right panel). B) The HMG box of SRY can bind to DNA cruciform structures regardless of DNA sequence.

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**Table 1**

Y-linked and X escapee chromatin modifiers.

| Gene | Molecular Function | References |
|---|---|---|
| **Y-linked chromatin modifiers** | | |
| *Uty* | H3K27me3-specific histone demethylase, interacts with RBBP5 and BRG1 (Shpargel et al., 2012) | Lee et al., 2007; Wang et al., 2013; Walport et al., 2014 |
| *Jarid1d* | H3K4-specific demethylase | Lee et al., 2007; Xu et al., 2008a |
| *CDY* (human) | H4-specific hyperacetylation, interacts with methylated H3K9 through its chromodomain, and recruits HDACs | Lahn et al., 2002; Kim et al., 2006a; Caron et al., 2003 |
| **X escapee chromatin modifiers** | | |
| *Utx* | H3K27me3-specific histone demethylase, interacts with RBBP5 and BRG1 | Hong et al., 2007; Xu et al., 2008b; Shpargel et al., 2012; Welstead et al., 2012; Wang et al., 2013 |
| *Jarid1c* | H3K4-specific demethylase | Iwase et al., 2007; Xu et al., 2008a |
| *MeCP2* | Binds methylated CpGs, recruits HDACs, HP1, H3K9 methyltransferases, the DNA-demethylase MBD2, and the chromatin remodeler ATRX, and promotes CTCF binding and formation of higher-order chromatin loops | Hendrich and Bird, 1998; Nan et al., 1998; Agarwal et al., 2007; Fuks et al., 2003; Becker et al., 2013; Nan et al., 2007; Kernohan et al., 2014 |