Characterising Novel Pathways in Testis Determination Using Mouse Genetics

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Key Words
Gonadal development · Knockout mice · Mouse genetics · Sex reversal · Testis determination

Abstract
Mammalian sex determination is the process by which the bipotential embryonic genital ridge commits to either the testicular or ovarian fate. The existence of a gonad primordium with 2 possible developmental outcomes, depending on the presence or absence of the Y chromosome, remains a fascinating paradigm for examining the manner in which genes cooperate to establish cell fate and regulate differentiation. Development of the mouse gonad offers a unique model for the investigation of sex determination, characterised by powerful transgenic, genomic and other experimental resources for the study of the molecular control of organogenesis. This review focuses on recent progress in our understanding of mouse testis determination, with an emphasis on multi-locus studies of mutant alleles aimed at constructing pathways of interacting gene products. Studies in 2 broad areas have been especially revealing: (i) identification of pathways required for the appropriate expression of Sry, and (ii) characterisation of the antagonistic interactions between the core testis- (SRY-SOX9-FGF9) and ovary- (RSPO1-WNT4-CTNNB1-FOXL2) determining gene regulatory networks. We discuss these advances with an eye on emerging themes in mammalian sex determination.

The study of cell fate determination is of paramount importance in developmental biology, not least given the current imperative to rationally manipulate the fate of stem cells in vitro for therapeutic applications in medicine. The mammalian gonad is unique in being bipotential: cell lineages of the early gonad respond to extrinsic and intrinsic signals to commit to one of 2 organogenetic fates: testis or ovary. This bipotentiality, its genomic, epigenomic and proteomic basis, is likely to shed much light on our understanding of the molecular underpinnings of lineage commitment and differentiation. The mouse is a powerful tool in experimental developmental biology because of the ease with which its genome can be manipulated and examined. Much progress has been made in recent years in identifying key molecules that function in the commitment to testis and ovary development in mammals [e.g. reviewed in Jakob...
and Lovell-Badge, 2011; Quinn and Koopman, 2012; Warr and Greenfield, 2012; Ono and Harley, 2013]. One theme that has emerged by studying the consequences of gene ablation in the mouse is the antagonism that exists between these 2 developmental pathways, from embryo to adult. In this review, we will concentrate on recent progress in our understanding of embryonic testis determination afforded by the study of mouse mutants and detailed phenotypic analyses. In particular, it focuses on multi-locus studies and the attempts to construct developmental pathways from such data, both upstream and downstream of activity of the Y-chromosomal testis-determining gene, Sry, in the gonadal soma.

**Regulating Sry Expression**

The Y-linked gene SRY is possibly one of the most enigmatic in the mammalian genome. Its transcript is so rare that it was not detectable in conventional cDNA libraries when its structure in the genital ridge was first investigated; and its protein product, an HMG-box transcription factor, underpins sex determination in most mammals but is not highly conserved and lacked any established target genes in vivo for almost 20 years [Whitfield et al., 1993; Hacker et al., 1995; Jeske et al., 1995; Hiramatsu et al., 2008; Sekido and Lovell-Badge, 2008; Kashimada and Koopman, 2010]. Most intriguingly, its expression is so transient in the mouse gonad that it is detectable for just a handful of hours in a given gonadal somatic cell [Sekido et al., 2004; Wilhelm et al., 2005; Hiramatsu et al., 2008]. Although a number of transcription factors have been implicated in the control of Sry expression [reviewed in Sekido and Lovell-Badge, 2009; Kashimada and Koopman, 2010; Warr and Greenfield, 2012], the molecular basis of this remarkably dynamic expression profile, at the cellular and tissue level, is still a matter of great interest and considerable ignorance. Recent studies are, however, beginning to shed more light on this topic (see overview in fig. 1), and some key Sry regulators are discussed below.

**CBX2: A Link between Epigenetics and Sex Determination**

CBX2 (also known as M33) is a polycomb group (PcG) protein found in the polycomb-repressive complex 1 (PRC1). PRC1 acts to control gene expression during cell-fate specification and differentiation through chromatin modifications, although it is unclear whether the underlying mechanism involves repression of transcriptional machinery or regulation of chromatin structure [Simon and Kingston, 2009]. CBX proteins are thought to mediate the recognition of methylated histones by PRC1 via their conserved chromodomains, though other molecular interactions and roles are reported [Blus et al., 2011].

Embryos lacking Cbx2 have previously been reported to exhibit XY gonadal sex reversal, thereby providing a link between PcG-mediated control of gene expression and testis determination [Katoh-Fukui et al., 1998]. A recent publication describes experiments aimed at analysing the phenotypic abnormalities in Cbx2 knockout gonads in further detail [Katoh-Fukui et al., 2012]. Firstly, the authors show that Sry expression is almost undetectable in XY Cbx2-deficient gonads at 11.5 dpc. Secondly, they report that reduced proliferation of mesenchymal cells likely causes gonadal hypoplasia in the Cbx2 knockout embryos. Finally, they describe the use of transgenic rescue experiments in which forced gonadal expres-

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**Fig. 1.** The mouse testis- and ovary-determining pathways and their mutual antagonism. Genes required for normal Sry expression are shown in the top left, including a GADD45γ/MAP3K4/p38/GATA4 pathway (grey arrow). Within the large blue arrow are shown the core testis-determining genes Sry, Sox9, Fgf9 and Fgfr2 and their positive (arrow) or negative (hammered line) interactions. These negative (inhibitory) interactions are almost exclusively with elements of the ovary-determining pathway of Rspo1, Wnt4/β-catenin and Foxl2 (large pink arrow), which themselves inhibit the testis-determining genes, primarily Sox9. The existence of possible additional targets, or uncertainty concerning an interaction, is shown by a question mark next to an arrow or hammer line. Arrows and hammer lines do not necessarily indicate a direct interaction between the named genes/proteins.
sion of Sry and Sox9 from heterologous promoters is sufficient to rescue sex reversal in Cbx2-deficient XY gonads, though hypoplasia persists. These data indicate that CBX2 likely functions upstream of the regulation of Sry expression and also suggest that any links between cell proliferation, growth and the likelihood of testis determination are complex. Interestingly, forced expression of proliferation, growth and the likelihood of testis determination are complex. Interestingly, forced expression of Sry did not induce testis development in XX Cbx2 knockout gonads, despite the near normal expression levels of SRY in these gonads at 11.5 dpc detected by immunohistochemistry. The mechanistic basis of this phenomenon remains unclear.

The authors were unable to establish a direct link between CBX2 and Sry expression by using chromatin immunoprecipitation (ChIP) to detect occupancy of putative Sry regulatory regions by CBX2 protein; but neither direct nor indirect effects have been excluded. It also remains unclear whether CBX2 is acting to activate or silence gene expression at target loci during testis determination. Nevertheless, the role of CBX2 in regulating Sry expression in mouse, and possibly in humans [Blason-Lauber et al., 2009], provides an entry-point into the study of the epigenetic control of gene expression required for testis determination. Such studies will also benefit from insights gained by examining the mechanistic role of PRC1 in coordinating the timing of entry into meiosis of primordial germ cells in XX embryonic gonads [Yokobayashi et al., 2013].

MAPK Signals Are Decisive in Mouse and Human Testis Determination

The category of known sex-determining proteins has historically been dominated by transcription factors, such as SRY and SOX9, and, to a lesser extent, secreted signalling molecules, such as FGF9 and WNT4. Piecing together the molecular pathways that regulate testis and ovary development requires identifying the full panoply of functional classes of proteins, and possibly non-coding RNAs, that participate. Forward genetic screens in the mouse, allied to chemical mutagenesis, are an increasingly important tool for the identification of novel loci regulating specific aspects of phenotype, especially given the relative ease with which causative mutations can be identified with current genomics resources [Bull et al., 2013]. The first demonstration of a role for mitogen-activated protein kinase (MAPK) signal transduction in mammalian sex determination originated with an N-ethyl-N-nitrosourea (ENU)-based, forward genetic screen for mouse loci regulating gonad morphogenesis [Bogani et al., 2009]. Embryos homozygous for the boygirl (byg) mutation, a null allele of the gene encoding the kinase MAP3K4, exhibit XY gonadal sex reversal on the C57BL/6J (B6) background. This sex reversal is associated with dramatic down-regulation of Sry expression at 11.5 dpc, resulting in activation of the ovary-determining pathway and ovary formation. However, the cellular defect in the byg/byg XY gonad was unclear given the ubiquitous expression of Map3k4 and the potential variety of cellular and molecular pathways that might impact on Sry expression [Bradford et al., 2009; Sekido and Lovell-Badge, 2009].

One approach to determining how MAP3K4 signalling operates in testis determination is to search for determinants of MAP3K4 activity: molecules that interact with, and activate, this kinase and offer spatiotemporal specificity to MAPK signalling. Just such an approach resulted in the identification of Gadd45g, an autosomal testis-determining gene that encodes a MAP3K4-interacting protein [Gierl et al., 2012; Warr et al., 2012], GADD45γ and its paralogs GADD45α and GADD45β have been implicated in a number of physiological roles, including the cellular stress response and active DNA demethylation [Hollander and Fornace, 2002; Niehrs and Schäfer, 2012; Schäfer et al., 2013]. Moreover, GADD45γ has been shown to associate with and activate MAP3K4 in other contexts and thereby activate MAPK signalling [Chi et al., 2004; Miyake et al., 2007]. Careful expression analysis of Gadd45g revealed a transient but dynamic expression profile in somatic cells of both the XX and XY gonad between 10.5 and 13.5 dpc, reminiscent of the spatiotemporal expression profile of Sry itself. Previous analyses of Gadd45g-deficient mice on a mixed genetic background had not revealed any anomalies of development [Hofmeyer et al., 2001]; however, on both the C57BL/6J [Warr et al., 2012] and C57BL/6N [Gierl et al., 2012] genetic backgrounds, loss of Gadd45g causes embryonic XY gonadal sex reversal, confirming that both of these B6 substrains are sensitised to disruptions to testis determination. The sex reversal in both cases is characterised by a delay to Sry expression and a consequent reduction in transcript and protein levels at 11.5 dpc, suggesting that GADD45γ acts upstream of Sry in the testis-determining pathway.

The association between GADD45 and active DNA demethylation prompted analyses of the reported tissue-specific differentially methylated region (T-DMR) of the Sry proximal promoter [Nishino et al., 2004] in wild-type and mutant gonadal somatic cells: the existence of the T-DMR was confirmed, but no appreciable differences in methylation were found between mutant and controls [Gierl et al., 2012; Warr et al., 2012]. These negative data
do not exclude a role for GADD45γ in demethylation of other regulatory regions of Sry, or of other loci. However, an alternative mechanism was sought for the impact of loss of GADD45γ, and its reported role in the activation of MAP3K4 naturally led to an analysis of MAPK signalling. A deficit in the levels of activated (phosphorylated) p38 MAPK was detected in Gadd45g- and Map3k4-deficient gonads at 16-tail somites (around 11.25 dpc), suggesting a role for this kinase in testis determination. This was established, firstly, by the use of chemical antagonists and agonists of p38 MAPK signalling in ex vivo models of testis development [Gierl et al., 2012]; secondly, conditional gene targeting showed that loss of both the p38a and p38b MAPK isoforms resulted in XY gonadal sex reversal in vivo, again associated with reduction in Sry levels [Warr et al., 2012]. Moreover, phosphorylation of the known Sry transcriptional regulator and MAPK target, GATA4, was also reduced in mutant gonads, suggesting a role for a GADD45γ/MAP3K4/p38 pathway in activating GATA4 and thereby initiating timely expression of Sry.

It has been estimated that MAPKs may have around 200–300 substrates each [Cuadrado and Nebreda, 2010], so the identification of additional bona fide targets of p38 MAPK signalling in the developing gonad is likely to be important for our understanding of signal integration during testis determination. p38 MAPKs have emerged as important modulators of gene expression through their recruitment to chromatin and regulation of chromatin modifiers and remodellers [Lluis et al., 2006; Cuadrado et al., 2010; Ferreiro et al., 2010]. Notably, the PcG protein RNF2, involved in chromatin remodelling, is thought to be a substrate of p38 MAPK [Rao et al., 2009]. Given the role of CBX2 and GADD45 proteins in epigenetic modulation, and the fact that MAP3K4 can activate the histone acetyltransferase, CBP, in trophoblast stem cells [Abell et al., 2011], it is probable that epigenomic profiling of somatic cells during sex determination, in both mutant and wild-type gonads, will be fruitful area of future study. Moreover, the role of non-coding RNAs in gonad development, widely implicated in epigenetic regulation, remains to be determined [Chen et al., 2012]. The dynamic expression profiles of genes such as Gadd45g and Sry require some mechanistic explanation at the level of chromatin; however, this will require the genome-wide epigenomic analysis of very limiting amounts of tissue from multiple embryonic stages, e.g. somatic cells isolated from the XY and XX mouse gonad between 10.5 and 14.5 dpc. The efficiency of standard technologies such as ChIP will need to improve for this to be possible, although progress here is apparent [Ng et al., 2013]. Alternatively, the promise of in vitro reprogramming may yet allow the routine production of large numbers of cells with properties very close to those of embryonic pre-Sertoli/Sertoli cells [Buganim et al., 2012]. Substitution of a cell line model or analysis of other non-limiting tissues, while permitting straightforward use of the available technology, is unlikely to yield data that are as relevant to our understanding of sex determination in vivo.

The identification of MAP3K1 mutations in familial and sporadic instances of 46,XY disorders of sex development (DSD), including cases of 46,XY complete gonadal dysgenesis (sex reversal), suggests some role for MAPK signalling in human testis determination [Pearlman et al., 2010]. No definitive MAP3K1 null alleles causing 46,XY DSD have been reported, i.e. stop-codons causing premature truncation of the MAP3K1 polypeptide. Moreover, Map3k1 is not required for mouse testis determination [Warr et al., 2011]. Together, these observations suggest that sex-reversing MAP3K1 mutations may be gain-of-function alleles, somehow disrupting MAPK signalling at a crucial phase of human testis determination. This could be tested by introduction of such mutations into the mouse genome. Of course, which MAPKs are actually required for human testis determination, and at which stages, remains unclear and requires the identification of loss-of-function alleles in cases of 46,XY DSD. Nevertheless, it is our prediction that the genetic pathway leading to SRY activation may provide a promising source of candidate genes that, if mutated appropriately, lead to 46,XY DSD in the human population.

Insulin and IGF1 Receptors and Sry Expression

A previous study reported XY gonadal sex reversal in triple knockout embryos lacking insulin receptor (INSR), IGF type I receptor (IGF1R) and insulin receptor-related receptor (IRR) [Nef et al., 2003]. This sex reversal was attributed to a reduction in Sry expression. A recent follow-up study has now demonstrated that loss of only INSR and IGF1R is sufficient to cause XY gonadal sex reversal, in addition to growth retardation [Pitetti et al., 2013]. This sex reversal is characterised by a dramatic loss of Sry expression at 11.5 dpc, although transcripts are detectable by 12.5 dpc, indicating an actual delay in expression. One interesting aspect of this phenotype is the delay in ovarian differentiation observed in both XY and XX mutant gonads: key ovarian markers, such as FOXL2 and SCP3, are not detectable until 16.5 dpc. What is the basis of this delay in differentiation? Expression profiling of SF1-posi-
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| DOI: 10.1159/000358402 |

Determination of pro-ovary genes such as Wnt4. These data indicate a role for SOX9 in inhibiting the ovarian pathway of development, although the molecular basis of this remains unclear. The canonical WNT/β-catenin signalling pathway promotes ovary differentiation [Vainio et al., 1999; Maatouk et al., 2008] and SOX9 may directly inhibit β-catenin activity, as it does during chondrogenesis [Akiyama et al., 2004; Topol et al., 2009]. Alternatively, it may act to inhibit expression of the ovary determinant, Foxl2 [Wilhelm et al., 2009; Georg et al., 2012]. Interestingly, after testis determination, SOX9 appears to be dispensable for testis cord differentiation due to the presence of the related protein, SOX8, revealing how functional redundancy can complicate interpretation of normal gene function [Barrionuevo et al., 2009; Georg et al., 2012].

A recent study shows that the mutually antagonistic testis- and ovary-determining pathways can be jointly disrupted by gene inactivation in such a way that testis development is possible even in the absence of the master testis-determining genes, Sry and Sox9 [Lavery et al., 2012]. RSPO1 promotes ovary development through its activation of WNT/β-catenin signalling [Tomizuka et al., 2008]. Loss of RSPO1 causes delayed, partial female-to-male gonadal sex reversal in XX mice, associated with up-regulation of Sox9 expression around birth [Chassot et al., 2008]. One prediction from these data is that, given the putative role of SOX9 in inducing testis development in RSPO1-deficient XX mice, removal of both RSPO1, a key ovary determinant, and SOX9, a reliable testis inducer, would result in failure of both ovary and testis differentiation. However, XX fetuses lacking both RSPO1 and SOX9, in addition to SRY, exhibit delayed development of ovotestes and hypoplastic testes [Lavery et al., 2012]. What might account for this? Expression profiling revealed activation of expression of both Sox10 (at P0) and Sox8 (at P12) in double knockout gonads. Both genes belong with Sox9 in the SoxE gene family and are associated with testis development; indeed, ectopic expression of Sox10 in XX embryos can induce testis differentiation [Polanco et al., 2010]. This Sox8/Sox10 gene activation in Rspt1/Sox9 double knockout gonads implies inhibition of expression of these genes by either SOX9 or RSPO1 in normal circumstances; most likely RSPO1, given that it promotes ovary differentiation. Finally, removal of both Sox9 and Sox8 results in failure of normal testis cord differentiation after the sex determination stage and progressive transdifferentiation of mutant Sertoli cells to an ovarian-like fate, associated with strong down-regulation of Sox10 expression [Barrionuevo et al., 2009; Georg et al., 2012]. Thus, SOX8 and SOX9 are required for Sox10 ex-

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expression in the fetal testis, but the absence of Rspo1 presumably obviates this requirement for SOX9. The molecular basis of these genetic relationships needs further investigation.

Such circumstances tell us much about how sex-determining gene regulatory networks respond robustly to catastrophic genetic insult and will no doubt shed light on the aetiology and pathology of some cases of 46,XY DSD in humans. Of course, the phenotypic consequences of gene ablation experiments reflect the response of the whole mouse genome to the insults: the role of mutation-dependent, strain-specific modifiers of phenotype has been reported before in the context of Sox gene function [Hosking et al., 2009]. It appears that the sex-determining mechanism, like others, has evolved so as to permit gonadogenesis to proceed even in the absence of proteins that are known to function under normal circumstances. This is evidence of the strong canalisation of the sex-determining mechanism: either ovary or testis development is a required outcome. Formation of some intermediate tissue is, in an evolutionary sense, to be avoided. Delayed commitment to a specific cell fate, as reported in one other mutant discussed above, is associated to varying degrees with the deletion of the Foxl2 and Dmrt1 genes in adult mouse gonads that ultimately results in somatic cell reprogramming [Uhlenhaut et al., 2009; Matson et al., 2011]. In the case of Sertoli cell-specific deletion of Dmrt1, a switch occurs from predominantly SOX9-positive Sertoli cells at P7 to predominantly FOXL2-positive cells by P28: the testis-to-ovary reprogramming is extended over a significant period of time [Matson et al., 2011]. This may reflect the complexity of in vivo cellular reprogramming that occurs in these abnormal genetic contexts when certain gene products that are normally expressed in a cell lineage at a particular stage do not appear, or suddenly disappear. These delays are perhaps reminiscent of the time taken to reprogram somatic cells into pluripotential stem cells in vitro [Muraro et al., 2013]. Future studies will be important to address why sex reversal is still an outcome of such adult gene deletions, as opposed to the adoption of some other cell fate, and how similar the genomic and transcriptomic contexts are of adult and embryonic gonadal cell lineages. The relationship between gonad reprogramming and late-onset disease will also be important to examine.

**FGF Signalling: Positive or Negative Role?**

FGF signalling was first shown to play an important role in testis determination when XY gonadal sex reversal of Fgf9-deficient mouse embryos was reported [Colvin et al., 2001]. Loss of Fgf9 in XY gonads results in loss of testicular markers and inappropriate expression of ovarian-determining genes such as Wnt4 [Kim et al., 2006]. The gonadal receptor for FGF9, FGFR2, has an established role in testis determination based on data from conditional gene ablation experiments. Whilst such analyses are beset by incomplete deletion of floxed alleles, often based on the variable performance and specificity of Cre deleter lines, 2 separate studies reported defects in testis determination upon gonadal deletion of Fgfr2 using distinct Cre lines [Kim et al., 2007; Bagheri-Fam et al., 2008].

A reduction in the abundance of Sox9 transcripts in Fgf9 homozygous mutant gonads suggested a role for FGF9 in regulating Sox9 expression [Colvin et al., 2001]. Further studies revealed that initial up-regulation of Sox9 at 11.5 dpc occurred normally in Fgf9 homozygous mutant gonads; however, SOX9 was not detectable at 12.5 dpc, indicating a role for FGF9 in maintenance of Sox9 expression [Kim et al., 2006]. In contrast, Sry expression at 11.5 dpc was unaffected in Fgf9-deficient gonads. The relationship between Sox9 and Fgf9 was shown to be one of mutual dependence, since in gonads lacking Sox9 generated by conditional gene targeting, Fgf9 expression was greatly reduced or absent [Kim et al., 2006].

Whilst initially interpreted as a genetic interaction characterised by positive feedback, recent data suggest a more complex interaction between Fgf9 and Sox9, involving Wnt4 [Jameson et al., 2012a]. Given that Wnt4 expression is activated in Fgf9- and Fgfr2-deficient XY gonads, there was a possibility that deletion of Wnt4 would rescue sex reversal in these mutants. Generation of mice and embryos lacking both Fgf9 and Wnt4 (as well as Fgfr2 and Wnt4) revealed that testis development could proceed in the absence of these FGF and WNT signalling components. Thus, while FGF9 signalling is required to inhibit the ovarian genetic pathway (in particular the WNT pathway) in XY gonads, these double knockouts suggest that it does not act directly or positively to maintain SOX9 expression and control other aspects of testicular morphogenesis. As in the case of testis development in the absence of SRY and SOX9, these data do not contradict the known role for FGF9/FGFR2 in testis determination; they show that the requirement for this role is dependent on the activity of other genes, in this case, Wnt4. Given the examples discussed earlier, in which related genes are up-regulated in some gene knockouts, thereby potentially offering a mechanistic explanation for sex determination in the absence of key genes, it would be interesting to know whether, for example, alternative FGF ligands are activated in Fgf9/Wnt4 double knock-
outs. In this context, it is noteworthy that Fgf10 is expressed in the XY and XX mouse gonad at 11.5 dpc [Hiramatsu et al., 2010]. These data also reveal how difficult it is to infer the structure of pathways from genetic analyses before the phenotypic consequences of all combinations of mutant loci have been analysed. Figure 1 depicts a possible pathway relating SRY/Sox9/Fgf9 functions and ovary-determining genes. Finally, it is interesting to note that an essentially negative role for Fgf9 in suppressing canonical Wnt signalling would suggest that its paracrine role in rapidly spreading the testis-determining signal from its initial source in the centre of the gonad to the poles [Hiramatsu et al., 2010] is one designed to extinguish an ovarian fate which the gonadal soma may otherwise be primed to adopt [Jameson et al., 2012b].

**Conclusion**

It is likely that future progress in sex determination research will involve the delineation of ever more elaborate pathways in testis and ovary development, with more antagonistic interactions evident between them. This will necessitate further investigation of the remarkable and unexpected plasticity of cell identity in the adult testis, which requires Dmrt1, and no doubt other pro-testis genes, for its maintenance [Matson et al., 2011]. Study of epigenomic mechanisms that underlie dynamic expression patterns and detailed characterization of mutual antagonism at the molecular level will also be central. Given the focus on the circuitry of gene regulation that is apparent in current research, it is also probable that the connections between the whirl of transcription factors and the expression of other functional classes of protein will be belatedly established. In this way, details of the molecular basis of cellular differentiation during later gonad morphogenesis will emerge as part of a coherent picture of mammalian gonad development and function, with mouse genetics, genomics and phenomics as integral parts of the story of its elaboration.

**Acknowledgements**

The authors apologise to colleagues for any published data not discussed in this review. Such omissions are due to space constraints. We also thank Steve Thomas for assistance in the production of figure 1 and other members of the Greenfield laboratory at Harwell for useful comments on the manuscript.

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Sex Dev 2014;8:199–207

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Note Added in Proof

Two recent publications reveal even more complexity in the genetic pathways required for Sry expression in the mouse. Firstly, the importance of controlled epigenetic modifications in testis determination is further underlined by the report of XY gonadal sex reversal in mice lacking the histone demethylase, JMJD1A (KDM3A) [Kuroki et al., 2013]. This report describes the role of JMJD1A in positively regulating Sry expression by demethylation of the inhibitory histone mark, H3K9me2, at chromatin of the Sry locus. This may be an important step in the deheterochromatinization of this Y-linked locus prior to the recruitment of transcription factors. Secondly, the role of the transcription factors SIX1 and SIX4 in sex determination is demonstrated by XY gonadal sex reversal in embryos lacking both proteins [Fujimoto et al., 2013]. SIX1 and SIX4 are reported to regulate Fog2 (Zfpm2) expression, which is itself required for expression of Sry.

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