Spring 2018 – Systems Biology of Reproduction
Lecture Outline – Sex Determination
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesdays & Thursdays
January 23, 2018
Week 3

Sex Determination

- History
- Jost model of sexual differentiation
  - Chromosomal sex
  - Gonadal sex
  - Phenotypic sex
- Gonadal development systems
  - Cell biology
  - Required genes
- How does chromosomal sex dictate gonadal sex?
  - Molecular cloning of testis-determining factor(s) (e.g. SRY)
  - Interactions of SRY and SOX genes
  - X chromosome sex determining factor DSS/DAX
  - Interactions SRY, SOX, DAX, SF1, and DMRT
- How does gonadal sex dictate phenotypic sex?
  - Müllerian Inhibitory Substance (MIS)
  - Androgen induced male differentiation
- Abnormal sexual differentiation
  - New potential sex determination genes
- Mechanisms of sex determination in other species

Required Reading

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that are more credible than currently available projections. ■

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In retrospect

Twenty-five years of the sex-determining gene

The discovery that the gene *SRY* on the mammalian Y chromosome drives testis development marked a turning point in the decades-long quest to understand the genetic underpinnings and evolution of sex determination.

JENNIFER A. MARSHALL GRAVES

It has long been known that a testis-determining factor (TDF) on the Y chromosome kick-starts testis development in humans and other mammals. The testes make hormones, and these hormones make the embryo male. Twenty-five years ago, Sinclair *et al.*[1] reported in *Nature* that TDF was the gene *SRY*. This discovery opened up the surprisingly intricate genetic pathway that determines whether a baby is born a boy or a girl. It also led to an understanding of how genes on the Y chromosome evolved, and of the impact of this key evolutionary event.

Until the 1980s, there was no viable candidate sex-determining gene. Just where was TDF located? What kind of product did it encode? What did it do? During the 1980s, the position of TDF was narrowed down to a small region on the short arm of the Y chromosome, when it was found that some males had XX chromosomes that harboured a small piece of the Y, whereas some females had XY chromosomes that lacked bits of the Y — these added and deleted regions of Y were assumed to contain the TDF sequence. The race was then on to find TDF.

In 1987, the geneticist David Page and his associates[2] identified the first coding gene on the human Y, called *ZFY*. The gene looked like a winning candidate: it was in the right place; it was expressed in the testis; and it was evolutionarily conserved in other placental mammals, such as monkeys, mice, dogs and horses. But in 1988, PhD students in my laboratory[3], Andrew Sinclair and Jamie Foster, mapped *ZFY* to a non-sex chromosome (an autosome) in marsupials, which are a separate branch of mammals. A few months later, it was found[4] that, although *ZFY* is expressed in mouse sperm precursors, it is absent from the other cells of the testis, where a true TDF must be expressed to exert a sex-determining effect.

Sinclair joined a renewed hunt for human TDF in the laboratory of geneticist Peter Goodfellow, using DNA from XY males that had even smaller pieces of the Y than had previously been studied. This was slow and frustrating work, because the Y chromosome is full of repetitive sequences and so specific regions are hard to pinpoint. It was 1990 before they found[1] a small coding gene close to the end of the Y chromosome (Fig. 1). Noncommittally they called the gene *SRY*, for sex region on the Y. The final proof that *SRY* was the TDF came from the discovery of *SRY* mutations in XY females[5] and from the demonstration that adding *Sry* to XX mice was sufficient to induce male development[6]. *SRY* was located on the Y in other placental mammals and, thankfully, even in marsupials[7].

Researchers in the field imagined that identifying TDF would rapidly lead to an understanding of how it worked, and would point to other genes in the sex-determining pathway. But 25 years on, it has become clear that the pathway kick-started by *SRY* is complex, full of checks and balances.

Initially, *SRY* proved a puzzle because it was unlike any known gene. It turned out to be a member of a previously unidentified family, now called the SOX genes. Painstaking biochemical studies of the SRY protein revealed that it bound to a certain DNA sequence and bent it at an angle, presumably to bring other sequences — or the proteins bound to them — into proximity, promoting or inhibiting transcription[8]. The discovery of a different

50 Years Ago

The Royal Society Anniversary Address by Lord Florey, O.M., P.R.S. Perhaps the deployment of Government resources is the modern equivalent of events in the early days of the Society when Fellows contributed—or sometimes did not contribute—a shilling a week towards demonstrating experiments at meetings. There never was enough money … At the moment it is considered to be desirable to give free medicine to all. The application of free calamine lotion to the irritated skins of the populace may be more important than administering to the needs of irritated scientists; but this sort of judgement is in the realm of politics … it has long been the policy of the Society to have symposia and lectures … the popularity of such gatherings has brought difficulties … on one occasion, we had to migrate to the lecture theatre of the Shell Building on the South Bank … one consequence of this peripatetic existence has been that we have had to procure a coffin-like box for the transport of the mace, and I am sure that our original Fellows, and even Charles II himself, might have been somewhat astonished at the adventures of their royal emblem.
From *Nature* 18 December 1965

100 Years Ago

The Romanes Lecture … was a scathing indictment of the ineptitude of the lawyer-politicians who possess a dominating influence on national affairs … To the neglect of science, and the excessive predominance in Parliament and the Government of men with the spirit of the advocate to whom all evidence which will not support their case is unwelcome, Prof. Poulton ascribes the chief mistakes in the conduct of the war.
From *Nature* 22 November 1915

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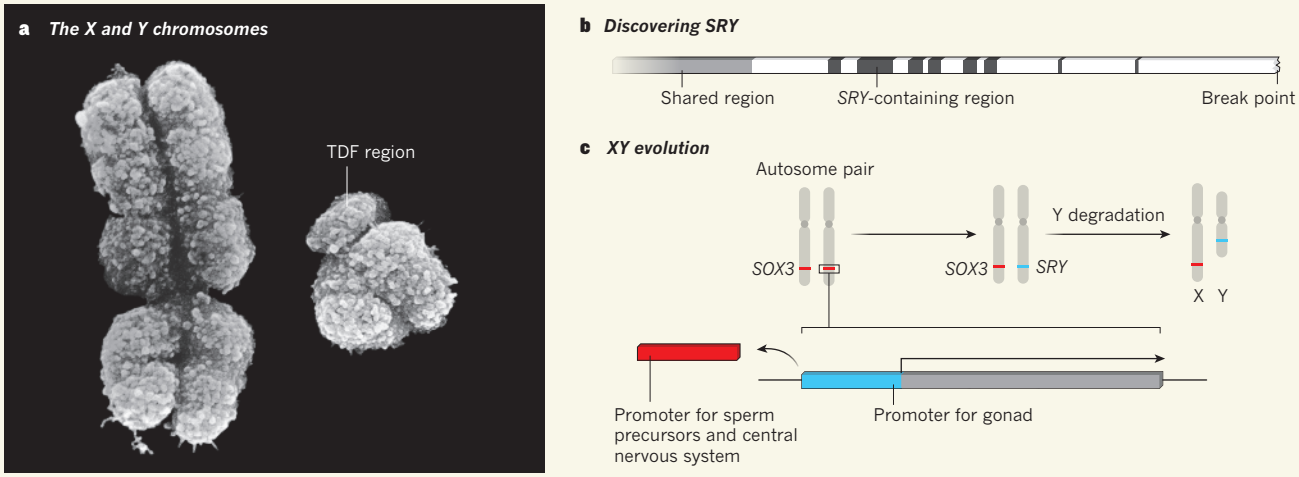

Figure 1 | An evolving understanding of sex. **a**, In humans, sex is based on the presence or absence of the Y chromosome, seen here with its larger partner, X. The testis-determining factor (TDF) that drives male development was known to lie on the short arm of Y, but its identity was a mystery. **b**, In 1990, Sinclair *et al.*[1] found two males with only a small piece of Y, which had been broken and fused to the X. They scoured the 35,000 base pairs between the break points and the region at the tip of the Y that is shared with the X, finding several regions (black) that were specific to the Y. One of these regions contained the TDF gene, *SRY*. **c**, This discovery led to an understanding of how X and Y evolved. The gene *SOX3* was located on a pair of non-sex chromosomes (autosomes) in the ancestors of mammals. A promoter sequence drove expression of *SOX3* in sperm precursors and the central nervous system. The promoter on one copy of *SOX3* was replaced with a sequence that drives expression in the undifferentiated gonad (a tissue that can develop into either an ovary or a testis). This expression pattern allowed the new gene, *SRY*, to direct testis development. Over time, genes not needed for male development were degraded on this chromosome, giving rise to the Y. (Part **b** adapted from ref. 1.)

SOX gene that was disrupted in XY female babies with a severe bone deformity[9,10] revealed that this gene, *SOX9*, is the binding target of SRY protein. *SOX9* is now known to be a master regulator of sex determination throughout the vertebrates.

Studying the mutations that cause sex reversal in humans, mice, goats or dogs (the same pathway is active in all mammals) has proved a successful strategy for identifying many genes in the sex-determination pathway. Gradually, a network of genes that are regulated by, or regulate, *SRY* or *SOX9* has been constructed, and their function tested by mutating the genes in mice[11]. Some genes promote testis formation, some maintain it, and yet others oppose them. This pathway and its control is still being explored. Our improved understanding has helped us both to answer fundamental scientific questions and to diagnose and treat many babies who are born with disorders of sex determination[12].

The other major line of research enabled by the identification of *SRY* was the evolution of sex genes and chromosomes. The hunt for *SRY* in marsupials revealed that mammals have an *SRY*-related gene on the X chromosome, *SOX3*, which was proposed to be the ancestor of *SRY*[13]. This idea is supported by human and mouse data[14] that showed that misexpression of *SOX3* in the undifferentiated gonad (a tissue can develop into either an ovary or a testis, depending on the signals it receives) drives male development in XX embryos. *SRY* probably evolved from *SOX3* when its 5′ region was replaced by a promoter sequence that drove expression in the gonad (Fig. 1).

Although it might seem counterintuitive that the testis-determining factor evolved from the X chromosome, it has since emerged[15] that 20 of the 27 genes on the male-specific part of the human Y evolved from genes on the X. Thus, the Y is basically a degraded X chromosome. This supports the hypothesis that sex chromosomes originate when one member of an autosome pair acquires a sex-determining gene. Nearby genes then also acquire a sex-specific function, crossing over between the chromosome pair is suppressed to keep the male-specific gene package together, and the genetically isolated region on the sex-specific chromosome degrades rapidly[15,16].

"It has become clear that the pathway kick-started by* SRY *is complex, full of checks and balances."

The mammalian XY sex pair was probably defined by the evolution of *SRY*. Vertebrate phylogeny puts the age of *SRY* and the XY pair at between 166 million and 190 million years old. Furthermore, rapid speciation in other lineages that have undergone sex-chromosome turnover raises the possibility that acquisition of *SRY* might have driven the divergence of the egg-laying monotreme mammals from the rest of the mammalian lineage — monotremes have a bizarre, complex sex-determination system that is related to bird sex chromosomes[17].

The future of the Y chromosome is now hotly debated. Evidence suggests that the mammalian Y will disappear in just a few million years if gene loss continues at the same rate as in the past[18]. It has already disappeared in two groups of rodents, and *SRY* has been replaced by another gene from the sex-determining network[19]. The primate Y seems more stable[20], but will eventually erode away. Humans may be in for another round of sex-chromosome turnover — and maybe speciation — if and when *SRY* finally bows out. ■

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Review

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Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpators

Amaury Herpin[1,2] & Manfred Schartl[1,3,*]

Abstract

Sexual dimorphism is one of the most pervasive and diverse features of animal morphology, physiology, and behavior. Despite the generality of the phenomenon itself, the mechanisms controlling how sex is determined differ considerably among various organismic groups, have evolved repeatedly and independently, and the underlying molecular pathways can change quickly during evolution. Even within closely related groups of organisms for which the development of gonads on the morphological, histological, and cell biological level is undistinguishable, the molecular control and the regulation of the factors involved in sex determination and gonad differentiation can be substantially different. The biological meaning of the high molecular plasticity of an otherwise common developmental program is unknown. While comparative studies suggest that the downstream effectors of sex-determining pathways tend to be more stable than the triggering mechanisms at the top, it is still unclear how conserved the downstream networks are and how all components work together. After many years of stasis, when the molecular basis of sex determination was amenable only in the few classical model organisms (fly, worm, mouse), recently, sex-determining genes from several animal species have been identified and new studies have elucidated some novel regulatory interactions and biological functions of the downstream network, particularly in vertebrates. These data have considerably changed our classical perception of a simple linear developmental cascade that makes the decision for the embryo to develop as male or female, and how it evolves.

Keywords Dmrt1; ovary; SRY; testis; transcription factor
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See the Glossary for abbreviations used in this article.

Introduction

Developmental cascades are generally headed by evolutionary conserved master regulators that determine the developmental fate of a cell lineage toward distinct tissues or organs during embryogenesis. In contrast, determination of the development of the reproductive organs does not follow this rule. Studies over the last decades have revealed that the gene-regulatory cascades triggering sexual differentiation from worms and flies to mammals are composed of substantially different factors. In particular, a remarkable diversity of master sex-determining genes that govern the genetic hierarchies has become apparent. On the other hand, the downstream components seemed to be evolutionarily more conserved and appear to converge on the regulation of a few central common effectors. A well-known example illustrating this paradigm is the master sex-determining gene of mammals, the *SRY* gene. A corresponding homolog has not been detected outside of therian mammals (Marsupials and Placentalia). Conversely, those genes that act downstream of *SRY* as transcription factors (*SOX9*, *DMRT1*) or signaling pathways (TGF-β/Amh, Wnt4/β-catenin, Hedgehog), and genes involved in *SRY* regulation (*SF1*, *WT1*) have homologs with a known or presumed role in gonadogenesis or gonadal differentiation in many vertebrate species, and some even in non-vertebrate deuterostomes and protostomes. These findings suggested that a central paradigm of sex determination is that "masters change, slaves remain".

This appealing global rule was quickly commonly accepted, in particular as the diversity at the top was confirmed experimentally [1–3]. Remarkably, some master sex-determining genes were recurrently identified and became the "usual suspects" for future studies in the search for master regulators (Table 1). All of these are genes, or duplicates and paralogs of genes, which were previously known to act in the regulatory network of gonad development. Much progress has also been made in understanding some of the regulatory interactions of the networks or cascades governed by the long known master sex-determining genes as well as, although to a lower extent, for the newly detected ones. We review here the current knowledge about the different molecules that have been demonstrated

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Glossary

Amh
Anti-Müllerian hormone
Autosome
On contrary to a sex chromosome, autosomal chromosomes are chromosomes that are not involved in primary sex determination
Csd
Complementary sex determiner
CTD
C-terminal domain
DKK1
Dickkopf-related protein 1
Dmd3
Doublesex and Mab-3 domain family member 3
DMRT1 or 3
Doublesex and Mab-3 related transcription factor 1 or 3
Dosage sensitive gene
Gene where the amount of gene product that determines the phenotype is dependent on the number of copies. Two copies are usually sufficient to establish the phenotype, while one is not (haploinsuffiency). For example, in birds two copies of the *Dmrt1* gene trigger male gonadal development, while one copy is not sufficient to make a male and then leads to female development
Dsx
Doublesex
Environmental sex determination (ESD)
When the sex of an individual is driven by different external factors including temperature, pH, social interactions (dominance, stress. . .)
Esr1
Estrogen receptor 1 is the human estrogen receptor alpha
Fem
Feminizer
FGF9
Fibroblast growth factor 9
Foxl2
Forkhead box transcription factor L2
Fru
Fruitless
Fst
Follistatin
Gene regulatory network
Set of interactions between different regulators (DNA, RNA, proteins) leading to their interdependent modulation of expression and regulation
Genotypic sex determination (GSD)
When the sex of an individual is triggered by its genotype only (can be mono or polygenic)
Gonadal maintenance
Establishment of a genetic programm in order to maintain the fate and differentiation state of the different cellular types composing the gonad, keeping either the male or female identity
Gsdf
Gonadal soma derived factor
Her-1
Hermaphroditization of XO-1
Hetero-/homo- gamety
When individuals produce gametes with either different sex chromosomes (hetero-) or similar sex chromosomes (homo-). It is refered to male heterogamety when males produce X and Y chromosome-containing gametes or female homogamety for females producing only X chromosome-containing gametes (XX-XY sex determination system, like in most mammals). For instance in birds, snakes and butterflies males are (ZZ) homogametic and females (ZW) heterogametic (ZZ-ZW sex determination system)
Heteromorphic sex chromosomes
When sexual chromosomes are morphogically distinguishable (different degrees of heteromorphism exist, depending on the age of the sex chromosomes)
Hhip
Hedgehog-interacting protein
HMG
High mobility group
irf9
Interferon regulatory factor 9
Mab-3
Male abnormal 3
masc
Masculinizer
Master sex-determining gene
A gene (not necessarily coding for a protein) responsible for the initial trigger leading to sex determination
Neofunctionalization
The process by which a gene changes its function or adds a new one by mutations that change the structure of its gene product and/or its expression pattern
Nix
Male-determining factor in the mosquito *Aedes aegypti*
NTD
N-terminal domain
piRNA
PIWI-interacting RNA
Primordial germ cells
In the embryo the precursors of the stem cells that will give rise to the germ cell lineage. During sex determination and gonad differentiation they become committed to either produce male or female germ cells as spermatogonia or oogonia, which after meiosis will become the gametes. Primordial germ cells continiously express a certain set of genes in order to maintain their unique undifferentiated/pluripotent state
Ptch
Patched
Rspo1
R-spondin 1
Sdc
Sex determination and dosage compensation defective
SdY
Sexual dimorphic on the Y chromosome
Sex chromosome
Chromosome involved in the primary sex determination. They usually harbour a master sex determining gene/trigger
Sex determination
Primary mechanism leading to the expression of the phenotypic sex. Sex determination is mostly triggered either by the genome (genotypic sex determination) or by the environment (environmental sex determination)
Sexual differentiation
Developmental consequence of the sex determination process. Regroups the events dealing with internal and external genitalia and secondary sex characters
SF1
steroidogenic factor–1
Somatic gonad
The non-germ line component of the gonad. The somatic gonad consists of mainly two characteristic cell types in female: the granulosa and theca cells of the ovary and three specific cell types in the testis: Sertoli, Leydig and peritubular myoid cells
SOX9
Sry-related HMG box 9

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SRY
Sex determining region Y
STRA8
Stimulated by retinoic acid gene 8
Sxl
Sex lethal
TAD
Transactivation domain
TESCO
Testis-specific enhancer core
TGF-β
Transforming growth factor beta
Therian mammals
Non-egg-laying = marsupials and placental mammals
TRA
Transformer
Wnt
Wingless-related MMTV integration site
WT1
Wilm's tumor gene 1
Xol
XO lethal

Table 1. Master sex-determining genes in vertebrates.

Master SD gene	Organism	SD system	SD gene ancestor	SD gene generated from ancestor by	Ancestor gene function
SRY	Therian mammals	XY	Sox3	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
Dmrt1	Birds	WZ	Dmrt1	Allelic diversification	Transcription factor, key role in male sex determination and differentiation
DM-Y	*Xenopus laevis*	WZ	Dmrt1	Gene duplication	Transcription factor, key role in male sex determination and differentiation
Dmrt1bY	Medaka (*Oryzias latipes, O. curvinotus*)	XY	Dmrt1	Gene duplication	Transcription factor, key role in male sex determination and differentiation
SdY	Rainbow trout (*Oncorhynchus mykiss*)	XY	Irf9	Gene duplication	Interferon response factor, no gonadal function known
GsdfY	Luzon ricefish (*Oryzias luzonensis*)	XY	Gsdf	Allelic diversification	TGF-β factor, important role in fish gonad development
Sox3Y	Indian ricefish (*Oryzias dancena*)	XY	Sox3	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
amhY	Perjerrey (*Odontesthes hatcheri*)	XY	Amh	Gene duplication	Anti-Muellerian hormone, growth factor
amhr2Y	Fugu (*Takifugu rubripes*)	XY	Amh receptor 2	Allelic diversification	Type II receptor for Amh, important function in gonad development, medaka mutant shows sex reversal
Dmrt1	Chinese tongue sole (*Cynoglossus semilaevis*)	WZ	Dmrt1	Allelic diversification	Transcription factor, key role in male sex determination and differentiation
GsdfY	Sablefish *(Anoplopoma fimbria*)	XY	Gsdf	Allelic diversification	TGF-β factor, important role in fish gonad development

to determine sex in a variety of animals and what has been learned about the maintenance of the sexual identity of ovary and testis.

Master sex-determining genes: case studies from Sox and DM domain factors to emerging "unusual" suspects

From Sry down to Sox3 across vertebrates

SRY belongs to a family of transcription factors, which are characterized by an evolutionary conserved high-mobility group (HMG box) DNA-binding domain flanked by weakly conserved N- and C-terminal sequences. In mice, both, gain- and loss-of-function studies have shown that *SRY* is not only sufficient but also necessary for triggering testis development [4,5]. With the exception of only two species (the mole vole *Ellobius* [6] and the spiny rat [7]) which have probably lost the gene), *SRY* is the universal master male sex regulator of all therian mammals [8]. Cytogenetic and comparative molecular studies of mammalian sex chromosomes provided evidence that SRY most probably arose after two major events: (i) a dominant mutation of the *SOX3* allele (giving rise to the proto-Y) as well as (ii) fusion of the gene with regulatory sequences from another gene already located on the X chromosome [9] (Fig 1). Necessarily occurring before the divergence of the therian lineage, these events could be estimated to have happened ~146–166 million years ago [10,11]. Sharing an overall identity of 67% at the amino acid level and up to 90% identity when specifically considering the HMG DNA-binding domain, the X-chromosomal *SOX3*-encoded protein is most similar to SRY [12]. Consistent with this hypothesis, the expression of *SOX3* has been documented in the developing gonads of mice, chicken [13], fish [14], and frog [15]. Only the absence of *SOX3* expression

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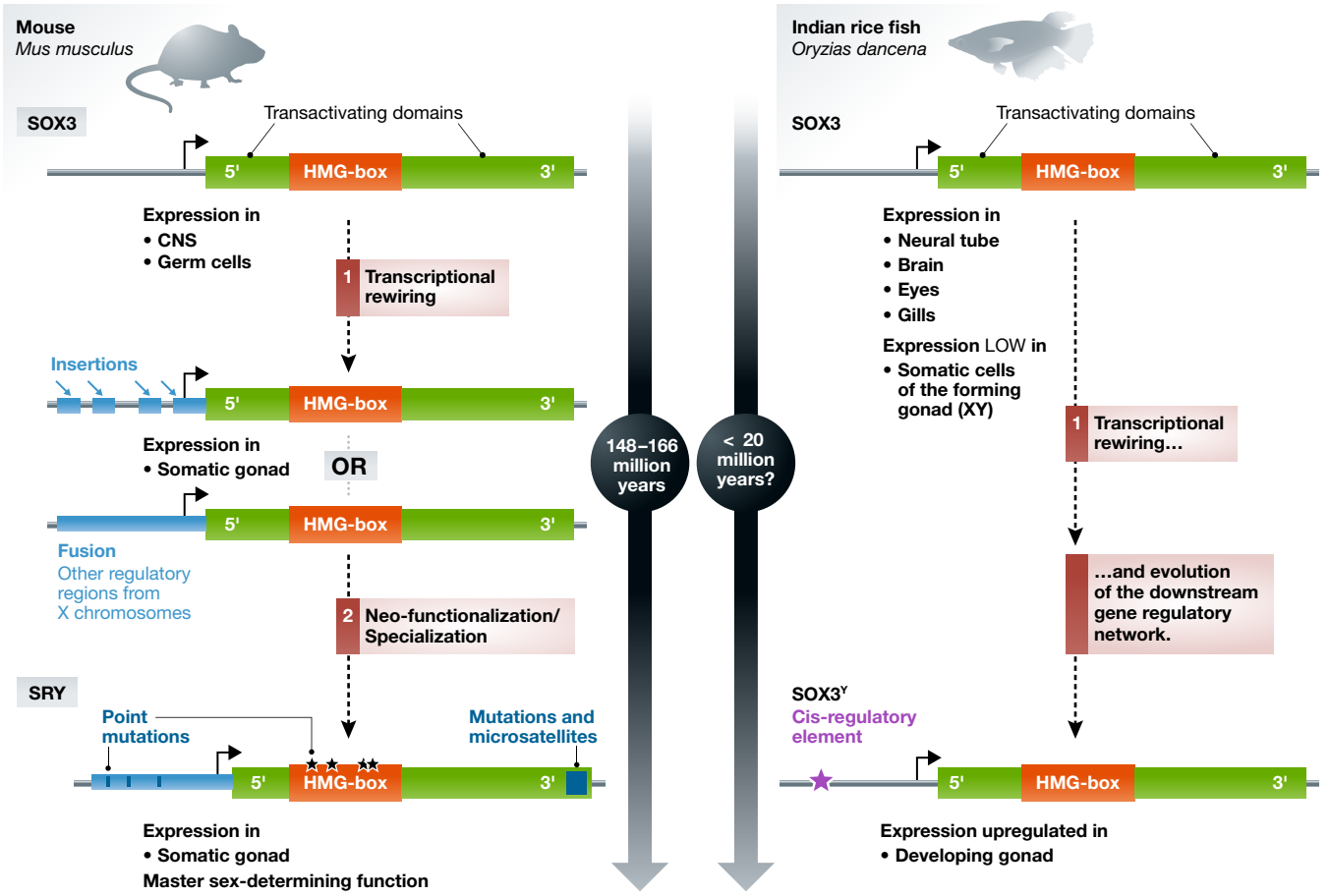

Figure 1. Independent evolution of *SOX3* genes toward a master sex-determining function in mice and Indian rice fish.
While SRY appears to be restricted to the therian mammals, evidence accumulates that SOX3 has independently been recruited as a "precursor" of master sex-determining genes also outside mammals. Hence, although not *a priori* destined to have a direct function during sex determination, common mechanisms of evolution seem to be repeatedly employed. Given that SOX3 is not generally expressed during gonadal induction or during gonadal development, the first step toward a sex-determining function is a transcriptional rewiring in order to acquire a timed pattern of expression compatible with sex determination. Such transcriptional rewiring, although not unique to SOX3 (see Dmrt1bY in medaka fish for example [56]), generally involves either fusion of the gene to new promoters or insertions of transposable elements into their pre-existing promoter, bringing in *cis*-regulatory elements compatible with the timing of gonadal induction. Interestingly and surprisingly, it seems that at least in mice and rice fish, this step alone was sufficient to endow SOX3 with a sex-determining function. Usually, the transcriptional rewiring steps seem to be accompanied by neo-functionalization or functional specialization processes. These include specialization of the protein activity itself in therian mammals (adapted from reference [20]) or more surprisingly adaptation of the downstream gene-regulatory network (target genes) in the Indian rice fish.

in the developing marsupial gonad is not consistent with a conserved role in mammalian sex determination [16,17]. Although SOX3 has no obvious primary function in sex determination, as the *Sox3* knockout mice have no gonadal phenotype [18], the clear evolutionary relationship between SOX3 and SRY raised the question whether gain-of-function point mutations may account for SOX3-induced XX male sex reversal in mice or humans. This has been shown only recently using a transgenic mouse model in which ectopic expression of SOX3 in the developing XX gonads resulted in complete XX female to male sex reversal [19]. Interestingly, the XX gonads of the transgenic hemizygous mice (Tg/+) did not only display an up-regulation of *Sox9* but also started to differentiate Sertoli cells, forming testis cords together with the appearance of a male-specific vasculature. Interestingly, using co-transfection assays it was shown that, similar to SRY, SOX3 only modestly transactivated the SOX9 testis-specific enhancer "TESCO" element [20] and synergistically interacted with steroidogenic factor-1 (SF1). Interestingly, the development of SOX3-triggered testes in XX animals was not possible in the absence of *Sox9*. In the same direction, patients displaying XX female to male sex reversal due to rearrangements of the genomic regions encompassing the regulatory sequences of *SOX3* have been reported [19]. Together, these data suggest that gain of function of SOX3 during gonadal development can in principle substitute for SRY to trigger testis development. These findings provide functional evidence supporting the long-standing hypothesis that SOX3 is the evolutionary precursor of SRY (Fig 1). It is also reasonable to postulate that rearrangements of the *SOX3* gene might be an underappreciated cause of XX female to male sex reversal in human patients [19].

While SRY appears to be specific to the therian mammals, there is accumulating evidence that *SOX3* has spawned independently other sex chromosomes outside mammals. Though being expressed in the ovary of frogs [21] without any sex-determining function determined so far, *sox3* might be involved in the switch responsible

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for sex determination in the Japanese wrinkled frog (*Rana rugosa*). Members of this species are either ZW or XY depending on which side of the island they are located [22]. Curiously, the Z and X chromosomes are not only homologous but share many genes with the X chromosome of humans including the *sox3* gene. Further molecular characterization and genetic mapping could disclose the presence of a Y-specific allele for *sox3* [23,24]. So far, this is an intriguing finding, but further studies are needed to ascertain a function for *sox3* in the sex developmental decision process of the embryonic gonad. If *sox3* has such a function, then the next question would be how the different genetic systems (ZW or XY) impact on *sox3* function.

Stronger evidence comes from the Indian ricefish (*Oryzias dancena*) (Fig 1), in which the XY sex chromosome pair also shares homology with the human X, including the presence of the *sox3* gene [14]. Using positional cloning to identify the sex-determining locus, it was found that the male-specific region on the Y chromosome harbors a *cis*-regulatory DNA segment that up-regulates expression of the Y-chromosomal copy of *sox3* during gonadal development (Fig 1). Sex reversal of XX fish transgenic for the regulatory segment linked to *sox3* to become males, and fish with targeted deletion of the Y-chromosomal *sox3* gene developing as females confirmed its major role during sex determination. Furthermore, it was demonstrated that Sox3 initiated testicular differentiation by up-regulating expression of *gsdf*, a gene highly conserved in fish male sex differentiation pathways [14]. Interestingly, a BAC clone carrying the *sox3* gene of *O. dancena* was not able to induce male gonadal development in the closely related species O. *latipes*, which has a different male sex determination gene. This supports the hypothesis that the acquisition of Sox3 function as a master sex-determining gene has occurred with a concomitant change in the downstream gonadal gene-regulatory network (Fig 1). Taken together, the results provided strong evidence for the recruitment—even in distantly related species—of Sox3 into the pathway leading to male gonadal development.

SRY reveals plasticity of sex-determining mechanisms among mammals Despite substantial variations in expression profiles, structure, and amino acid sequences within mammals, the function of SRY to activate a conserved target gene—SOX9—during testis development appears to be conserved [20]. SRY directly binds to the TESCO sequence of the *SOX9* gene [20]. Once activated, the SOX9 protein initiates the differentiation of somatic precursors into Sertoli cells that will then coordinate the gonadal development toward testes [25]. In the absence of *SOX9* activation, the fetal gonad will develop toward ovaries. While the function of *SRY* as a regulator of *SOX9* appears to be conserved, the molecular details underlying transcriptional regulation of *SOX9* by *SRY* [26] are not fully known and their conservation among mammals has not been deeply investigated. Such information would be important to evaluate whether under a conserved master determiner, the subordinate network is strictly conserved as well or shows variation in its regulatory interactions.

In contrast to most known transcriptional activators, most SRY proteins that have been studied in different mammalian species do not exhibit a well-defined transactivation domain (TAD). For instance, the N- and C-terminal domains (NTD and CTD) flanking the evolutionary conserved DNA-binding domain of human SRY are poorly preserved and do not seem to display any intrinsic transactivation activity [27]. Hence, it is assumed that the transcriptional activation of the human *SOX9* gene by SRY is possible only after the recruitment of a transactivating protein partner through its NTD and/or CTD sequences [28]. However, mouse SRY does not only lack the NTD but also displays an unusual CTD made of a bridge domain together with a poly-glutamine (polyQ) tract encoded by a CAG-repeat microsatellite [27]. It has recently been shown that this poly-glutamine domain does not only prevent mouse SRY from proteasomal degradation, but additionally functions as a bona fide TAD. Due to the fact that it allows the direct transcriptional induction of Sox9, this poly-Q domain plays a central role for the male-determining function of SRY *in vivo* [27]. Such data suggest that during evolution, mouse SRY has gained a functional unit, which is absent in other mammals [27]. Given such important transactivating properties for that poly-Q CTD in mice, it is puzzling that SRY proteins from either human or goat lacking a TAD are able to induce testicular development in transgenic XX mice embryos [29,30]. It appears reasonable to consider that both human and goat SRY proteins are able to bind to the highly conserved mouse TESCO target sequence using their respective DNA-binding HMG boxes. For the activation of *SOX9* transcription, it is assumed that transactivation is then mediated after the recruitment of a third TAD-containing protein partner. It can be further hypothesized that acquisition of a poly-glutamine stretch after insertion of a CAG microsatellite in a rodent ancestor made the recruitment of a transactivating partner unnecessary. Consequently, it is assumed that mouse SRY's ability to employ such a transactivating partner was lost during evolution. This assumption is supported by the observation that the acquisition of the poly-glutamine stretch is concomitant with an increase of variation in different parts of the SRY protein. These include the loss of the NTD as well as accumulation of deleterious amino acid substitutions in the HMG box [31]. Though no longer required, the third partner protein—probably a pleiotropic effector—may still be expressed at the sex determination stage. It would then potentially enable human and goat SRYs to trigger male gonadal development when expressed in transgenic mice. This reveals an unanticipated level of plasticity of the molecular mechanisms in the implementation of the primary sex-determining signal even among mammals. Identification of such putative partners of SRY may help in understanding human primary sex reversal pathologies, which are not explained by alterations in the known players of male sexual development [32].

Roles of DM domain factors in sex determination, differentiation, and gonadal maintenance

DMRT1, wherever you look Among the evolutionary conserved downstream effector genes of genetic sex-determining cascades, the DMRT gene family holds an outstanding position. This family is involved in sexual development of organisms as phylogenetically diverse as mammals, birds, fish, frogs, flies, worms, and corals [33–38] (Figs 2 and 3). Characterized by a highly conserved DNA-binding core motif—known as the DM (Doublesex and Mab-3) domain—, DMRT proteins act as transcription factors. Initially described to be involved in sex determination in worms and flies, they have been shown to regulate diverse aspects of somatic sexual dimorphism in these organisms. The ability to functionally

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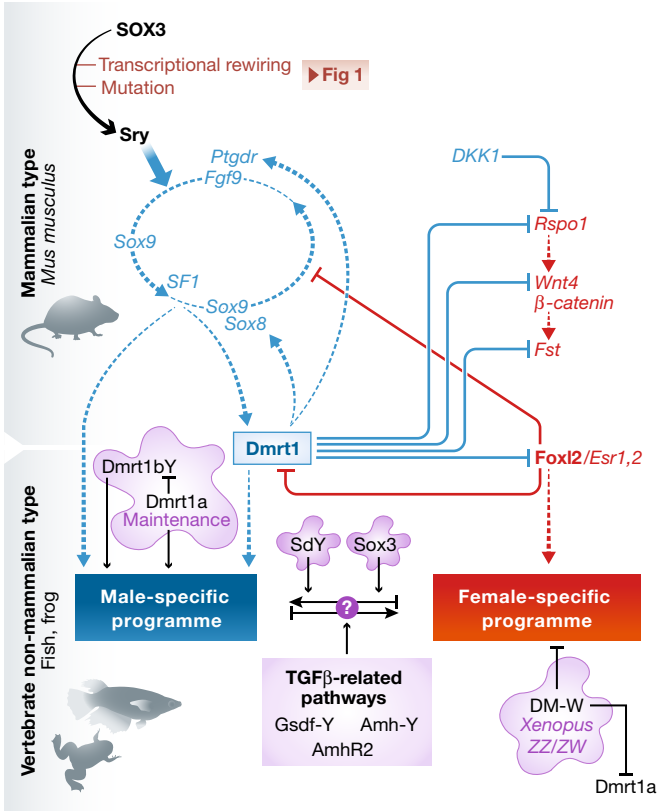

Figure 2. Gene-regulatory network of gonadal sex induction and maintenance in vertebrates.
Schematic representation of main interactions within the regulatory network. In gonadal fate determination of mammals, Sry initiates activation of the male pathway (blue) through up-regulation of Sox9. Dmrt1 is not only important for keeping the male pathway on but also in suppressing the two female networks (red). These two female networks involve Foxl2 as well as the Wnt/β-catenin signaling pathways. Maintenance of gonadal identity in the differentiated gonads is a result of the cross-inhibition activities of Dmrt1 and Foxl2. A critical equilibrium between these conflicting pathways underlies the bipotentiality of the gonadal somatic cells. Tipping the balance into one direction or the other will regulate the gonadal fate as a consequence of the activation of the male or female pathways. Solid lines define negative regulations. Dashed lines designate positive regulations. Beside the *Sry* ancestor *Sox3* and *Dmrt1*, other genes (pink) can become the master sex-determining genes by similarly impacting on the seesaw between the male and female programme.

substitute for each other across species led to the picture that sex determination cascades might—at least partially—rely on preserved molecules and pathways [37] (Figs 2 and 3; Table 1). Consistent with this, many of the DMRT homologs so far characterized among metazoans have been shown to be predominantly expressed during the development of the primordial gonad [35]. Interestingly, DM domain genes have also recently been described to be primarily involved in gonadal differentiation of the male flatworm (*Schmidtea mediterranea*) [39]. Similarly, in the water flea *Daphnia magna*, a crustacean with environmental sex determination, DMRT homologs have been found to trigger the switch in male versus female development of many dimorphic structures [40]. Thus, this widespread gene family appears to be directly involved in sexual development in all major animal groups. Nevertheless, DM domain factors were long considered as one of the underdogs of sexual determination because of their recurrent subordinate role in the cascade. A deeper interest in the field of sex determination for this group of genes only came with the discovery of a *dmrt1* homolog located on the Y chromosome of the medakafish (*Oryzias latipes*). Resulting from a gene duplication of the autosomal *dmrt1a* gene, it was designated *dmrt1bY* [41] or *dmy* [42]. It is the only functional gene in the Y-specific region of the sex chromosome, and it was shown to be not only necessary but also sufficient for triggering male development (see also Fig 2).

In humans, haploinsufficiency of the genomic region that includes *DMRT1* and its paralogs *DMRT2* and *DMRT3* leads to XY male to female sex reversal [43]. This suggested that the *DMRT1* gene is an important dosage-sensitive regulator of male development in vertebrates. In chicken and other avian species and in a fish, the smooth tongue sole (*Cynoglossus semilaevis*) [44]), *DMRT1* is located on the Z chromosome, but absent from W, and shows the expected expression pattern for a dosage-dependent male sex-determining gene of birds [45] and flatfish. In chicken, it was demonstrated through RNA interference experiments that *DMRT1* is indeed required for male gonad development [45]. While in these organisms *DMRT1* acts as a dosage-dependent male determiner, in *Xenopus laevis*, a duplicated copy of *dmrt1* on the W, which lacks the dimerization domain, appears to fulfill its function as a dominant-negative version. It is proposed to interfere with the transcriptional activation of the target genes of Dmrt1 and thus acts as a suppressor of male development [46].

Remarkably, all these *DMRT1* genes have acquired their new roles as master sex determination genes through different mechanisms: via gene duplication and translocation in medaka, duplication, translocation and truncation in *Xenopus*, or loss of function of the W allele in birds or tongue sole (Table 1).

In mice, it is apparent that *Dmrt1* is not required for male primary sex determination since newborn Dmrt1 mutants are males with testes [36]. However, *Dmrt1* is required for male gonadal differentiation of somatic cells and germ cells [47–49]. This is a parallel situation to mammalian *Foxl2* [50], which plays a conserved role in ovarian development but in mouse (opposed to some other mammals, including human and goat [51]) is not required for initiation of female development (see [52] for review). Targeted deletion of mouse *Dmrt1* and also of the autosomal *dmrt1a* of medaka, which is not involved in primary male sex determination, have revealed a major role in male gonad maintenance: when *Dmrt1* is lost, even in adults, this triggers sexual cell-fate reprogramming, in which male Sertoli cells trans-differentiate into their female counterparts, the granulosa cells [49]. This is accompanied by testicular reorganization toward a more ovarian morphology [49]. Ectopic DMRT1 expression in the ovary silenced the female sex-maintenance gene *Foxl2* and reprogrammed juvenile and adult granulosa cells into Sertoli-like cells, triggering formation of structures, which resemble male seminiferous tubules [53]. In the same direction, deletion of the *dmrt1* gene in medaka resulted in transition of the developing testis to ovary [54]. Hence, DMRT1's range of action is not limited to function in initiating the male gonadal phenotype during early development but also accounts for the livelong active repression of the two "anti-testis" pathways of FOXL2 and WNT4/β-catenin [49], and can do so even in the absence of the testis-determining genes *SOX8* and *SOX9* (Fig 2). Additionally, mRNA profiling revealed that DMRT1 activates many testicular genes and

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down-regulates ovarian genes [53]. Interestingly, transient expression of DMRT1 has also been reported in the fetal gonad of both sexes. The involvement in the regulation of germ cell development in testes and ovaries indicates that DMRT1 has different functions in males and females [55].

DMRT1 is required in female germ cells for entry into meiotic prophase, and in male germ cells for the control of mitotic arrest until birth [55]. Control of the decision to enter meiosis versus mitotic arrest is mediated by the ability of DMRT1 to selectively modulate retinoic acid signaling through context-dependent regulation of STRA8. DMRT1, for example, directly represses STRA8 transcription during testicular differentiation [55]. Thus, a picture emerges where DMRT1 controls a regulatory network that on the one hand can drive sexual fate and on the other hand can maintain the program of sexually differentiated cells, depending on the cellular context.

DMRT1, a jack-of-all-trade From studies in mouse and medaka [49,53,54,56,57], it is emerging that *DMRT1* holds a key position as the master switch or gatekeeper controlling the cell fate of the somatic cells of the gonads in female and male [33,34,53,58,59]. If this is so, then one could ask, why such a complex regulatory network upstream of *DMRT1* would be necessary to flip the switch, because numerous examples indicate that DMRT1 can do it on its own as for instance in birds, *Xenopus* and medaka [41,42,45,46]. *DMRT1* orthologs in these species appear to have undergone mutational events causing either loss or gain of function. Such altered DMRT1 activity may have favored evolutionary transitions leading to new genetic sex determination systems (see [59] for review). The ability of DMRT1 to toggle Sertoli/granulosa cell fate supports the hypothesis that loss- or gain-of-function mutations in *DMRT1* can elevate it into a master sex-determining role. Such mutations would help to promote changes between genetic sex determination mechanisms that are commonly observed among vertebrates.

DMRT1 is one of the sex determination network genes that appears more often also as master regulator (Table 1). It can be hypothesized that its strategic position at the interface of sex determination and the process of sex-specific gonadal differentiation, integrating a developmental fate decision with activation of organ differentiation programmes (Fig 2), made *DMRT1* suitable to be selected either as new controller at the top or at least for being one of the few key genes to be regulated.

Emerging suspects from gonadal TGF-β signaling

The anti-Müllerian hormone (Amh) is a growth factor from the TGF-β family and plays a major role in mammals for the degradation of the Müllerian duct-forming part of the female reproductive tract in male embryos. It is not required for mouse testis development. However, in non-mammalian vertebrates, it appears to play a central role in testis formation. For instance, in chicken embryonic gonads, AMH is expressed much higher in males and is predicted to be responsible for organizing the early testis in birds [60]. In the medaka *hotei* mutant, Amh signaling is disrupted by a mutation in the type II receptor for Amh. As a consequence, a male to female sex reversal with an over-proliferation of germline stem cells occurs [61].

Although being clearly a subordinate member of the sex regulatory network in mammals and at least in those species that make use of *DMRT1* as master regulator of male development, the Amh/Amh-receptor system has, like *DMRT1*, sometimes made it to the top (Table 1). In the pejerrey, a freshwater fish species from Patagonia, a duplicated version of the *amh* gene became the male sex-determining gene on the Y chromosome [62], reminiscent of the situation for *dmrt1* in medaka fish. In the pufferfish, *Fugu rubripes*, the receptor for Amh exists in two versions that differ by one amino acid (H384D) in the kinase domain [63]. The 384^{His} allele is a Fugu-specific (conserved in several other pufferfishes) mutation that confers lower activity to the receptor and is encoded on the X chromosome [63]. Thus, a quantitative difference in Amh signal transduction in females, which are homozygous for the mutant, versus males, which have kept one allele of the wild-type receptor on their Y, is responsible for male development [63]. Like in the medaka *hotei* mutant [61], low signaling from the receptor is connected to feminization of the gonad.

Gonadal soma-derived factor (Gsdf) is another growth factor from the TGF-β family that is closely related to Amh. It is only found in fish, and its biochemical function is not well studied. It is assumed to have a role in male gonad development due to its exclusive expression in the early differentiating testis of all fish looked at so far [64–68]. Despite its proposed role in the downstream regulatory network, *gsdf* has made it up to the top in *Oryzias luzonensis* [69] a sister species to medaka, and most likely also in the sablefish [70].

Taken together, it appears that certain genes, which are members of the regulatory network, namely *sox3*, *dmrt1*, and TGF-β signaling components, can become the master sex-determining gene independently again and again, while other important components of the sex-determining pathways have not appeared as masters so far (Fig 2 and Table 1). Whether we just have to wait for the analyses of primary genes for sexual development in more species, in order to put genes like *foxl2*, *sox9*, *sox8*, *wnt4*, etc., on the list of usual suspects, or whether there is a biological reason that makes some genes more prone to become the top regulator, is currently unsolved. We could imagine that some genes remain "too difficult to recruit" as master regulators, for instance if they have also non-reproductive but vital functions in other organs. In such case, interferences between a duplicated new master gene and its homolog may not be tolerated, except for the case that the neo-gene would have an appropriate gonad-specific regulation as soon as the founder event occurs. Many of those genes that did not appear as master sex determiners so far indeed have important functions in other tissues and organs.

Recurrent actors in invertebrate sex determination

The invertebrate ancestors of DMRT1 DM domain-containing genes have been shown to be primarily involved in gonad differentiation in a flatworm [39] and to direct male versus female development of dimorphic structures in water flea [40]. Interestingly, this functional convergence is common among insects (see [3,71–73] for reviews). In *Drosophila*, the initial trigger of sex is dependent on the ratio of the number of X chromosomes versus the haploid autosome complement (X:A). In the female situation, an X:A ratio of one will enable the transcription of the *Sex lethal* gene (*Sxl*), a splicing regulator. The SXL protein will then promote the female-specific splicing of *Transformer* (Tra), a direct downstream target, and lead to the production of functional TRA proteins. Similarly, a complex made of TRA and TRA-2 proteins will then favor the female-specific

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splicing of the *Doublesex* (*Dsx*, the *Dmrt1* homolog) gene transcripts. This results in the production of the female-type DSX protein DSX^F, which initiates up-regulation of the downstream gene-regulatory network for female development. In males, an X:A ratio of 0.5 will prevent the production of the SXL protein and, by default, results in the production of the male-specific splice form of the *Tra* gene. This splice variant translates into a non-functional protein due to a premature stop codon. In the absence of TRA, by default the male-specific splice form from the *Dsx* gene will be produced. The male-type DSX protein DSX^M will then orchestrate the downstream gene-regulatory network for male development [71,74] (Fig 3). Orthologs of *Drosophila dsx* have been identified and studied in a large number of insects [75–77]. Mediation of alternative sex-specific splicing of *dsx* by TRA and TRA2 is also widely conserved in insects although variations of the sex determination systems occur [3], suggesting that different molecular mechanisms involving splicing activators or repressors are employed to preferentially generate sex-specific variants of *dsx* mRNA [78].

Despite considerable efforts, similar sex-specific alternative splicing events in the molecular regulation of sex determination of vertebrates have not been shown. Conceptually similar is the fact that DSX translates the sexual determination process of a cascade of alternative splicing events into the transcriptional control of a large number of sex-specific effector genes. Similarly, DMRT1 in vertebrates appears to hold such a "translational" function at the interface where a fate-determining signal is put into effect at the level of sex-specific somatic cell differentiation (Figs 2 and 3).

In invertebrates, the homologs of vertebrate *Dmrt1* (e.g. *Dsx* in *Drosophila* and *Mab3* in *C. elegans*) are typical downstream factors of sex determination and so far, it is not reported that a DM domain

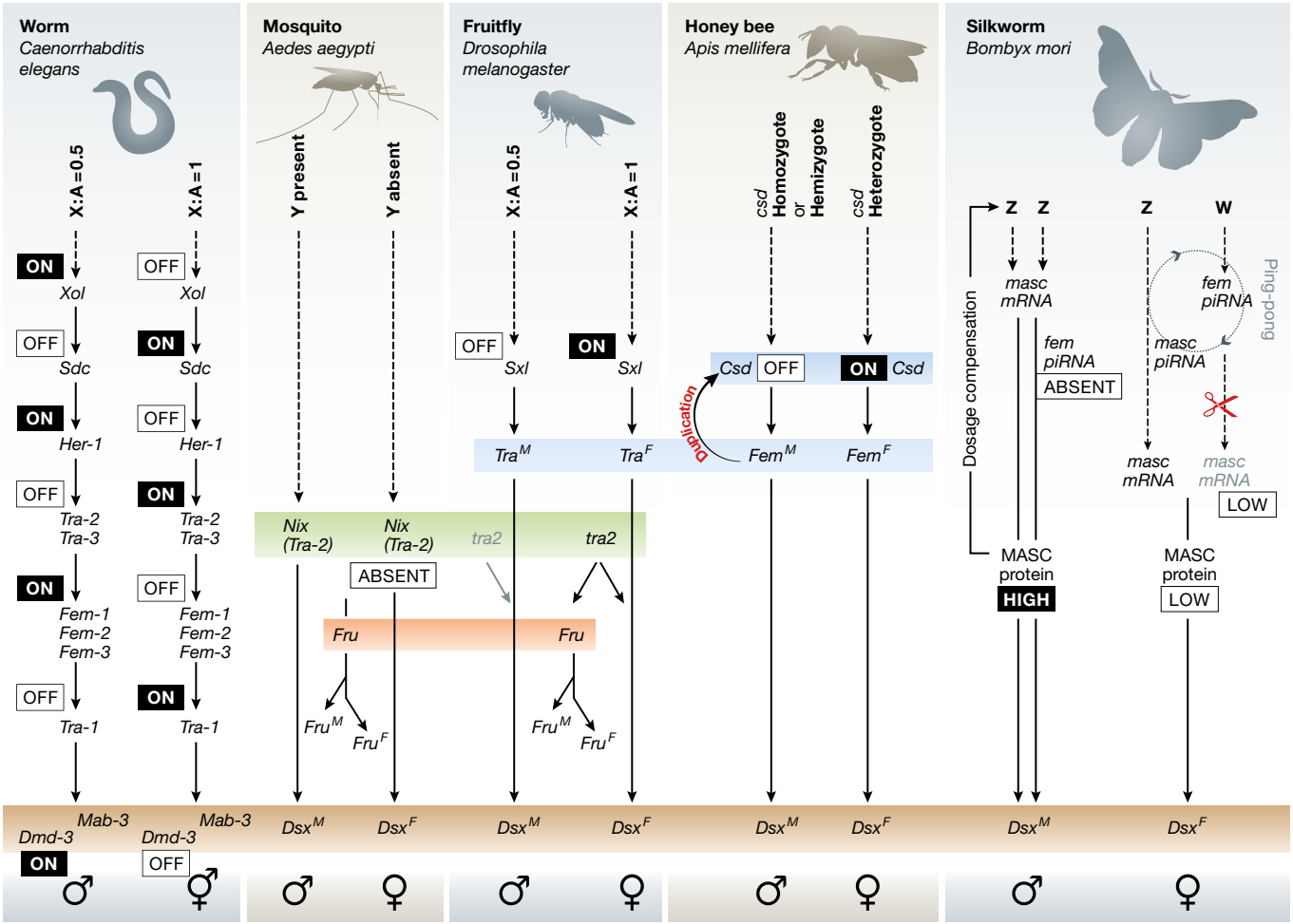

Figure 3. Sex-determining cascades in *C. elegans* and some insects.
Molecular and genetic pathways leading to the formation of the gonad in the worm *C. elegans*, the mosquito *Aedes aegypti*, the fly *D. melanogaster*, the honey bee *A. mellifera*, and the silkworm *B. mori*. Conservation of the *Dsx*, *Mab-3* and *Dmrt1*, *Tra*-like, (*Tra-2*), or *Fru* homologs is designated with either pale brown, pale blue, pale green, or pale orange boxes, respectively. *Tra-(1, 2* or *3)* of *C. elegans* are not phylogenetically related to *Tra* of *Drosophila*. *Fem-(1, 2* or *3)* of *C. elegans* are not phylogenetically related to *fem* of *Bombyx mori*. In *C. elegans* and *D. melanogaster*, a ratio between X chromosomes and autosomes determines the sex. This leads to the on/off state of *Xol* or *Sxl*, respectively. Heterozygosity turns on *Csd* in the honeybee *Apis melifera*, leading to female development, and hemizygosity or homozygosity leaves *Csd* unexpressed and produces a drone. In the mosquito (*Aedes aegypti*), sex determination is triggered by a dominant male determiner (*Nix*). *Nix* is a distant homolog of the splicing factor *Tra-2* of *Drosophila* and likely regulates the sex-specific splicing of *Fru* and *Dsx*. Sex in the silkworm *Bombyx mori* is controlled via a ZW sex chromosome system. Produced only from the sex-determining locus on the W, the piRNAs suppress the male sex-determining factor MASC.

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gene has made it up to the top in any invertebrate species [3]. But like in vertebrates, genes that are known as downstream members in one species can also usurpate a position as an initial genetic trigger in another species [3]. In insects, paralogs of the gene *tra* that is a well-studied component of the sex determination cascade in *Drosophila*, evolved as the master sex-determining switch gene in the housefly (*Musca domestica*), a wasp (*Nasonia vitripennis*), and the honeybee (*Apis mellifera*) [72,79,80]. In this regard, studies about complementary sex determination in the honeybee give exciting insights into how molecular diversity of regulatory pathways can evolve [81,82], as discussed in more detail below.

Complementary sex determination in honeybees uses a conserved module from chromosomal sex determination Genetic sex determination in the honeybee does not depend on the presence of hetero- or homomorphic sex chromosomes with different genetic compositions but rather follows a haplodiploid mode. Males develop from haploid unfertilized eggs, while diploid fertilized eggs develop into females. Hence, male or female sexual development occurs as the result of a signal originating from either a single or two different alleles from one gene, called *complementary sex determiner* (*Csd*) (Fig 3). Consequently, maleness or femaleness is determined by either homo-, hemi-, or heterozygosity of the *Csd* locus. The *Csd* gene products belong to an arginine-/serine-rich protein family. Interestingly, the C-terminal end of Csd also displays high similarity with the TRA protein, an essential downstream genetic factor of the sex-determining pathway in *Drosophila* ([81] and Fig 3).

Intriguingly and in contrast to the situation in *Drosophila* with *Tra* and other downstream genes (see Fig 3), neither transcriptional nor splicing variations of the *Csd* gene could be detected as sex-specific triggers. It is currently presumed that the regulation of the downstream regulatory network is mediated by the tendency of the CSD proteins to form heterodimers. Interestingly, the sex determination locus of the honeybee harbors a second gene also required for sex determination: *feminizer* (*Fem*) [82]. Further, phylogenetic studies revealed that *Fem*—as *Csd*—is also a close homolog of the *Tra* gene from *Drosophila*. It has been shown that *Csd* arose after duplication of the *Fem* gene 10–70 million years ago while the honeybee lineage was specifying. Knockdown experiments using RNA interference (RNAi) of either *Csd* or *Fem* resulted in female to male phenotypic sex reversions, implying that both factors are required for sex determination in the honeybee downstream of sex-specific splicing of the *Fem* gene by the CSD protein ([81,83] and [3] for review).

The situation in the honeybee resembles the roles of *dmrt1* in medaka and *Xenopus* and of *amh* in the pejerrey: A highly conserved downstream component of the network underwent a gene duplication, and then, one of the duplicates evolved a new function at the top of the cascade (Figs 2 and 3).

Another usurpator in mosquito? In the yellow fever mosquito, *Aedes aegypti*, like *Drosophila* a member of the order Diptera, sex is dependent on the presence or absence of a Y chromosome. Recent work has uncovered the molecular nature of the male-determining gene [84]. Intriguingly, this gene, called *Nix*, shows some sequence similarity to the *Tra-2* gene. This gene in *Drosophila melanogaster* is a downstream member of the sex determination cascade. Further downstream in the fruitfly cascade are the *Fru* and *Dsx* genes, and also in *Aedes aegypti*, both genes are regulated by the *Tra-2* homolog *Nix* (Fig 3). It is tempting to propose that in the mosquito, we have another example of a subordinate sex determination gene that has made it to the top.

The "unusual" suspects

All the above discussed cases of turnovers and novel master sex determiners include genes that have been previously known as components of downstreams sex determination networks, for example, from mouse, human, *Drosphila*, and *C. elegans*. Unexpectedly, there are two recent reports on sex-determining genes which were neither known nor suspected to be involved in the molecular regulation of this process.

An immune-related gene evolved into the master sex-determining gene in rainbow trout In the rainbow trout *Oncorhynchus mykiss*, a gene expressed only in the testis, predominantly during testicular differentiation, was recently characterized [85]. Localized at the sex-determining locus, this gene was named *sdY* for sexual dimorphic on the Y chromosome. Astonishingly and unlike other master sex-determining genes characterized so far, *sdY* has no homology with any known gene in sex determination pathways but with an immunity-related gene, the interferon regulatory factor *irf9* [85]. *SdY* arose by duplication and truncation of the autosomal *irf9* gene (Table 1). It lost the DNA-binding domain but preserved its protein–protein interaction domain. So far, the molecular mechanism through which SdY triggers male gonad development is unknown.

A single female-specific piRNA is the primary determiner of sex in the silk worm Sex in the silkworm *Bombyx mori* and all butterflies is determined by a ZW sex chromosome system. The W chromosome lacks any protein-coding genes but consists predominantly of transposons and non-coding RNAs. The only transcripts produced from the sex-determining region on the W are PIWI-interacting RNAs (piRNAs). After deep sequencing and isolation of dimorphically expressed RNAs, the *Fem* piRNA (*Fem* standing for "feminizing factor") was shown to be specifically expressed in females at all stages of development [86]. Furthermore, *Fem* piRNA targets and cleaves the *Masculinizer* (*Masc*) RNA molecule transcribed from a gene located on the Z chromosome. Interestingly, MASC, a CCCH-type zinc finger protein, favors male-specific splicing of *Bm-dsx*, leading to male development [86]. Hence, in ZW embryos, *Masc* RNA level is down-regulated by *fem* piRNAs, inhibiting male development. By default, female-specific splicing of *Bm-dsx* then occurs, triggering female development [86] (Fig 3). Interestingly, genetic inhibition of *Masc* resulted in the premature death of ZZ embryos before they hatched. In light of this observation, it was shown that the MASC protein is necessary for dosage compensation in order to lower Z gene transcription in ZZ embryos to the same level as in ZW embryos [86]. Whether or not this sex determination pathway is conserved across all lepidopterans remains to be explored, but coupling two important mechanisms namely sex determination and dosage compensation within the same genetic pathway and additionally distributing their genes onto the sex chromosomes should strongly promote evolutionary conservation.

SdY from rainbow trout and *Fem* piRNA are paradigms showing that unrelated genes are able to acquire *de novo* sex-determining functions. It can, however, not be excluded that they are representing

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factors of the sex determination regulatory network that have been overlooked so far.

Plasticity of the downstream sex determination regulatory network

What happens when "masters change"? The slogan "slaves remain" could imply that not much happens downstream of the changing master sex determiner. However, the findings on the diversity of SRY structure and its way to act as a transcriptional activator (see above) indicate that even under the same master gene, the regulatory interactions of the network undergo changes and that biology is not that simple.

In *Drosophila*, it has been shown that at the very downstream end of the sex determination, cascade pathways diverge by cooption of new effector genes [73] explaining the divergence of secondary sex characters between species. In vertebrates, some transcription factors like DMRT1, FOXL2, SOX9, and components of pathways such as Rspo1/Wnt/Fst or Hedgehog of the gonadal gene-regulatory network are well conserved on the DNA sequence level; however, their specific functions, regulations, and interplays can be substantially different. In medaka, down-regulation of the Hedgehog pathway by Dmrt1bY was shown [87]: Transcription of the Hedgehog receptor Ptch-2 in medaka testis is down-regulated by Dmrt1bY/Dmrt1a, while the antagonist Hhip is up-regulated [87]. The Hedgehog pathway is usually up-regulated by DMRT1 in mammals. It appears that despite its necessity for mammalian testis induction and development and later on in regulating Leydig and myoid cell function [88–90], the Hedgehog pathway might not only be dispensable during medaka male gonadogenesis and maintenance, but needs to be suppressed by *DMRT1* genes.

For R-spondin 1 (Rspo1), preferential ovarian expression is generally described. However, such strict female dimorphism was not observed in zebrafish [91], where the gene is also expressed in adult testes. Here, Rspo1 has a crucial role in testis cell proliferation [92] and it has further been shown to be involved in skin and mammary gland differentiation in mammals [93]. Follistatin (*Fst*) expression in the mouse co-localizes with *Foxl2* in the ovary [94], but in rat, it is expressed very broadly in germ and somatic cells of the testis [95]. Sparse expression of *fst* was also noted in the interstitial cells of the medaka testis, together with an up-regulation of *fst* expression *in vitro* after transfection of *dmrt1a* [87].

SOX9 has been shown to be expressed in the developing testes of all vertebrate embryos examined so far (see [60] for review). However, whereas SOX9 is upstream of AMH in mammals, the reverse applies in birds, and in medaka, Sox9 even appears to be not involved in primary sex determination at all [96,97]. In mammals, the current understanding is that SRY acts together with SF1 to activate *SOX9*, while in return, *SRY* is turned off by SOX9. *SOX9* further maintains its expression in an autoregulatory loop. SF1 is still required, but SRY becomes dispensable later during development [20]. In non-mammalian vertebrates, Sox9 activation must then rely on other factors than Sry. Intuitively, one could think that DM domain genes might have taken over. However, in chicken embryos, *DMRT1* expression is occurring at least 2 days before that of *SOX9* [60], implying that other genes mediating the DMRT1 signal to *SOX9* are involved. In medaka *sox9b*, the homolog of tetrapod *sox9* genes is rather involved in germ cell function than gonad determination although being expressed in the somatic part of the primordial gonads [96]. In addition, while in mammals, SOX9 activates the expression of *FGF9* [98], the gene does not exhibit any sexually dimorphic expression in chicken [60] and has even been lost in fish [99]. It is obvious that the gonadal function of *SOX9* underwent several changes during vertebrate evolution.

Genetic networks are indeed more complex than a straight top-down scenario. We have to add now that the differences in gene expression do not only reflect differences in cell biology and morphogenesis of the gonads but definitively are also the consequences of changes in the initial trigger for activating the network. That master sex-determining genes are prone to regulatory putsches in order to acquire an upstream position might only be possible because of the flexibility of the downstream gene-regulatory network. Hence, while Graham proposed a few years back that "Masters change, slaves remain" [1], it is now time to change this paradigm: "When masters change, some slaves remain, others are dismissed or acquire new tasks, and new ones can be hired".

Conclusions and perspectives

The variability and plasticity of the mechanisms that govern the development of the gonads is unmet by any other organ systems or tissues. While for instance the *Pax6* gene that is a master regulator of mammalian eye development is highly conserved (ectopic expression of human *PAX6* is able to induce eye development in *Drosophila* [100]), the downstream components of this cascade are not conserved (the induced eye is a typical composite insect eye). Surprisingly, it appears to be the other way round for sex determination genes. The evolution of genetic interactions in the sex-determining pathways and cascades is characterized by a relative conservation at the bottom and an apparent diversity at the top. This was explained in a classical hypothesis by A. Wilkins with an evolutionary scenario in which these hierarchies during evolution build up from a common downstream component (Sox or DM domain factors for instance), which acquires new upstream regulators. Those new additions would naturally vary in different evolutionary lineages [101]. Recent studies on the molecular identification of such upstream regulators and the downstream regulatory network, some of which provided the backbone for this review, brought new insights into how sexual development is regulated in different organisms, and how new sex determiners have evolved.

The "bottom-up hypothesis" formulated by Wilkins has to be revisited now taken into account the discoveries of the new master regulators. It seems that the master regulator/switch is not necessarily elected from the existing cascade usurping the top position but could be equally recruited from outside to accomplish a new sex-determining function after neo-functionalization. We also have to modify the hypothesis as we now know that in vertebrates, unlike in invertebrates, sex determination is not brought about by a simple linear cascade, but by a complex network of multiple regulatory interactions. Such a network might offer multiple opportunities where a newly added factor can trigger the outcome of the network signal toward male or female. There is also evidence accumulating

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that regulatory cascades can become shorter, rather than being topped up, when a new sex determiner appears, for example, in honeybees [72,102].

Gonad development appears to cope well with such changes of primary triggers as the many examples of different master sex regulators show, which finally all guarantee the developmental switch to either a testis or ovary. An intriguing situation has been recently reported for zebrafish, where the laboratory strains used worldwide have all lost their original sex-determining chromosome, but still produce normal males or females [103]. New upstream sex determiners appear to evolve quickly in those domesticated strains—similar to a situation in the other small aquarium fish model, the medaka [104]—which might take care in the future of the current sex bias observed at present for many laboratory strains. These are instances of "evolution in action," which offer prospects to observe in the laboratory how new sex determiners evolve and to obtain insights into the underlying molecular mechanisms. Certainly, we also need more information from different species about their master sex-determining gene and how it acts on the downstream regulatory network to obtain a reasonable understanding of the variety of sex-determining mechanisms.

Somehow unexpected are the accumulating findings that also the downstream network is not as strictly conserved as the "masters change, slaves remain" paradigm was imposing. Whether these differences in the expression pattern and function are related to specific adaptations of varying reproductive biology is a challenging question for the future. On the other hand, such changes may be due to the impact of the new upstream regulator. Intriguingly, even in a setting of the same master sex-determining genes, intricate differences downstream can be found, as seen for SRY in different mammals. It has also been argued that genetic networks, including sex determination, in general can change randomly without necessarily impacting on the final phenotype and thus evolve neutrally (see Sidebar A). Again, we need more details on the molecular biology of the sex-determining networks from different organisms; for instance, on a comparative basis from birds, *Xenopus* and those fish that all use *dmrt1* as their common master sex-determining gene.

Unexpectedly, it turned out that sex determination is not only needed as the molecular switch for the undifferentiated gonad primordium to develop either as testis or ovary, but that the sexual identity of the gonadal soma needs to be maintained as long as the organ has to provide its function(s). In vertebrates, two genes that appear to have a more downstream function in the determination network of the embryo are the top players here: *DMRT1* and *FOXL2*. The dichotomous developmental potency of the gonadal soma is apparently kept throughout the entire life. The reason for this is unknown. In particular among fishes, hermaphroditic species are common. Those fish can switch during their reproductive life from one sex to the other. Whether these organisms have found a way to make a controlled use of the livelong plasticity of the gonad or whether the plasticity seen even in the mammalian gonads is a relic of an evolutionary past are just two questions that emerge from those new findings.

The recent progresses reviewed here have considerably increased our understanding of the diverse molecular mechanisms underlying the amazing variation and plasticity of sexual development, and we might so far just only see the tip of the iceberg.

Sidebar A: Evolutionary concepts for the diversity of sex determination mechanisms

Sex determination is a very basal and ubiquitous developmental process, and the fact that it is so variable even between closely related organisms poses many fascinating questions. Molecular biologists are most interested to understand how these different mechanisms work, what factors are involved, upstream and downstream, and how they are regulated to bring about the amazing plasticity of the respective genetic cascades and networks. These are the so-called proximate causes of the observed variability. Organismic biologists focus more on the "ultimate" causes that lead to the changes from one to the other sex determination mechanism within and between certain lineages. A number of scenarios and hypotheses have been put forward to explain which evolutionary forces could favor such transitions and turnovers [105].

One explanation is that a mutation, which creates a new sex determination mechanism, gives a fitness advantage to its carriers. Then, by natural selection, this mutation will sweep through the population and take over, while the previous mechanism is lost [106]. Such new mutations could for instance alter the sex ratio, and if the ecological conditions favor such a bias, this mutation will be beneficial. As another example, a new sex determination mechanism might for instance be more efficient under certain ecological conditions, for example, works faster or is less or more susceptible to environmental influences.

If sex is determined through sex chromosomes, a common feature is the reduction of recombination around the sex-determining gene, which spreads out from there over almost the entire chromosome and finally fully arrests. As a consequence, deleterious loss-of-function mutations will accumulate in genes on the chromosomes carrying the sex locus [107]. Hence, such a chromosome will become less fit in evolutionary terms because of its mutational load, and once these disadvantages accumulate to a critical level, an emerging "younger" and less degenerated sex chromosome can take over [108].

Another hypothesis is based on linkage of sex-determining genes to other genes that favor one sex or are antagonistic to the other sex [109]. Many examples exist for such genes, which for instance are involved in gonad development or sexual dimorphism. If such a gene is closely linked to a gene that can influence the developmental decision toward male or female, the sex-determining gene will be co-selected as a hitchhiker and enjoy the fitness advantage that the linked sex beneficial or sexually antagonistic gene has under conditions of natural or sexual selection.

Rather than postulating a fitness advantage for the emerging novel sex determination mechanism, it is also considered that neutral or non-adaptive processes of genetic drift, mutation, and recombination can be instrumental. Such hypotheses are based on an analysis by M. Lynch how in general genetic networks can evolve [110]. He pointed out that only the final gene product of a genetic network or cascade produces a phenotype, which is exposed to selection. Thus, many changes in the upstream system can occur without necessarily altering the finally expressed phenotype. These changes can become fixed in a population by random genetic drift. As a result, the regulatory network has changed, but the phenotype will be constant. Such considerations were then applied to the genetic cascades and networks that govern sex determination [102]. Indeed, the final outcomes of the sex determination process are morphologically and functionally surprisingly similar in related groups of organisms, which have very different master sex regulators [111].

For all of these theoretical explanations, which appear to be to a certain extent opposing or even contradictory, examples to support them can be found. A single one obviously cannot explain all the different cases of sex determination systems and the multitude of turnovers and transitions. Rather than being alternatives, they may be complementary to explain the biodiversity of mechanisms that make the undifferentiated gonad anlage of an embryo to develop toward testis or ovary. To further our understanding of the trajectories that lead to the evolution of diverse mechanisms, we need not only detailed molecular knowledge about the proximate causes of such diversity but also more information about the ecology and population genetics under which they occur.

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Sidebar B: In need of answers

(i) What are the protein partners of SRY in human and goat that directly activate Sox9 expression?
(ii) Are the differences in the expression pattern and function of the genes in the downstream cascades or networks related to specific adaptations of varying reproductive biology? Or are they the result of neutral evolution and genetic drift?
(iii) Have the naturally occurring hermaphroditic species of fish found a way to make a controlled use of the livelong plasticity of the gonad? Or is the plasticity seen in the mammalian gonads a relic of an evolutionary past?
(iv) What are the evolutionary forces driving the outstanding high variability of molecular and genetic mechanisms of sex determination? Is this all due to stochastic variation? Or is there a global (so far unknown?) reason? Or do all evolutionary mechanisms postulated so far cooperate, with differing importance depending on the species or phylogenetic lineage?
(v) Are Sox3 and Irf9 in vertebrates and *Fem* piRNA components of the downstream sex determination cascades or networks that have been overlooked so far?
(vi) Why do some members of the regulatory networks of sexual development frequently become master sex-determining genes while others never appear at the top position?

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Conflict of interest

The authors declare that they have no conflict of interest.

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Spring 2018 – Systems Biology of Reproduction
Lecture Outline – Sex Determination
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesdays & Thursdays
January 23, 2018
Week 3

Sex Determination

- History
- Jost model of sexual differentiation
  - Chromosomal sex
  - Gonadal sex
  - Phenotypic sex
- Gonadal development systems
  - Cell biology
  - Required genes
- How does chromosomal sex dictate gonadal sex?
  - Molecular cloning of testis-determining factor(s) (e.g. SRY)
  - Interactions of SRY and SOX genes
  - X chromosome sex determining factor DSS/DAX
  - Interactions SRY, SOX, DAX, SF1, and DMRT
- How does gonadal sex dictate phenotypic sex?
  - Müllerian Inhibitory Substance (MIS)
  - Androgen induced male differentiation
- Abnormal sexual differentiation
  - New potential sex determination genes
- Mechanisms of sex determination in other species

Required Reading

Graves (2015) Nature 528:343-344.

Herpin and Schartl (2015) EMBO Reports 16:1260-1274.

Spring 2018 – Systems Biology of Reproduction
Discussion Outline (Sex Determination)
Michael K. Skinner – Biol 475/575
January 25, 2018
Week 3

Sex Determination

Primary Papers:

1. Yamauchi et al (2013) Science 343:69-72
2. Zhao et al (2015) Development 142:1083-1088
3. Bhandari et al (2012) PLoS ONE 7:e43380

Discussion

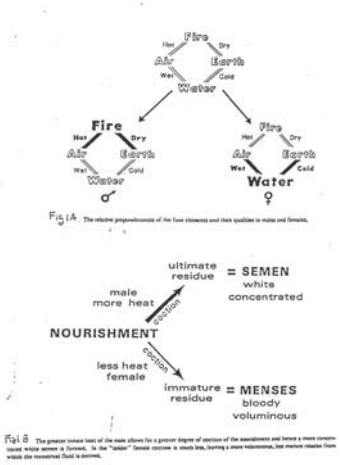
Student 4: Reference #1 above
- What are the genes on the Y required?
- What was the experimental design and methods?
- What conclusions are made on the future fate of the Y?

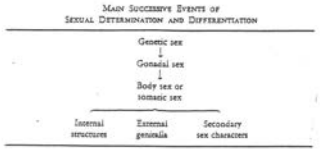
Student 5: Reference #2 above
- What is the Dmrt1 gene?
- How does Dmrt1 cause sex reversal to male?
- What is the relationship of SRY and Dmrt1?

Student 6: Reference #3 above
- What are the downstream targets of SRY?
- What was the method used to identify the targets?

Is SOX9 the only target of SRY that is important?

History and Jost





Jost Model -

Alfred Jost, University of Paris

1940's & 1950's

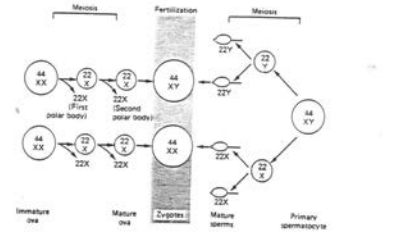
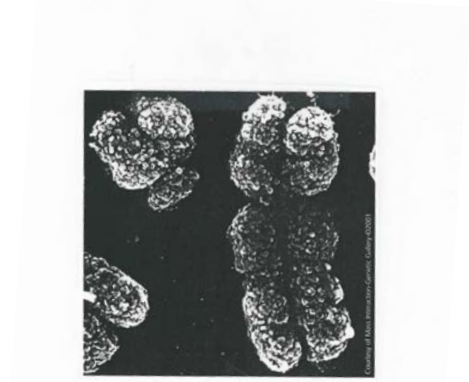
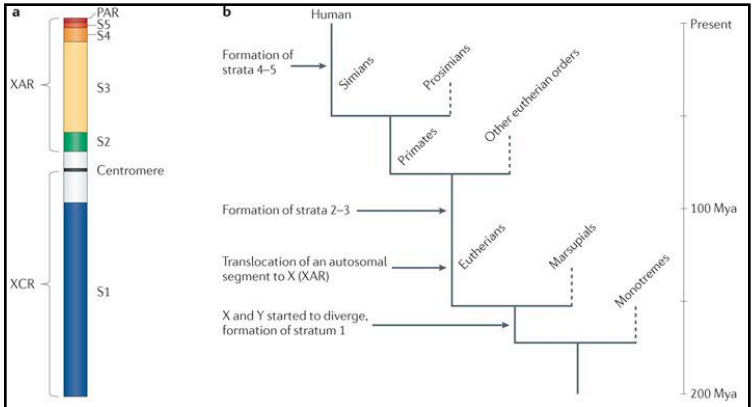


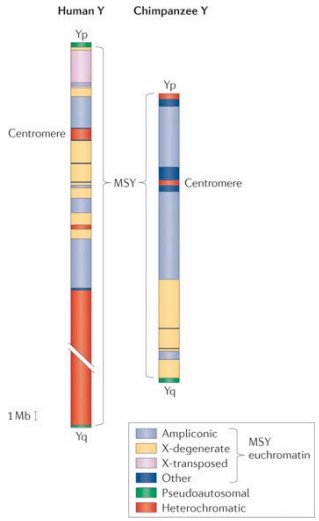
Figure 23–1. Basis of genetic sex determination. In the case of the 2-stage meiotic division in the female, only one body survives as the mature ovum. In the case of the male, the meiotic division results in the formation of 4 sperms, 2 containing the X and 2 the Y chromosome. Fertilization thus produces a 44XY (male) zygote or a 44XX (female) zygote.



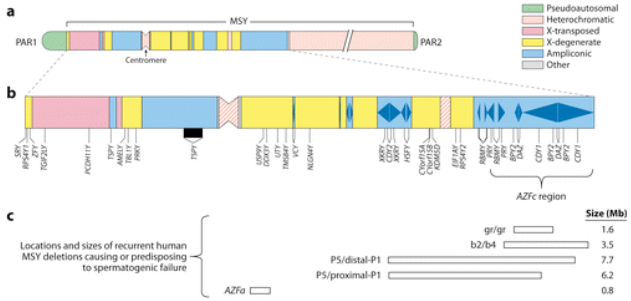
Neutrality? The default starting point for human embryos may be a neutral one, not male or female as previously thought.



a | A schematic illustration of the human X chromosome. The evolutionary history of this chromosome includes a region defined as the X-conserved region (XCR) that is present on the X chromosome of marsupials but is autosomal in monotremes. This indicates that in therians (placental mammals plus marsupials) sex chromosomes started to evolve sometime between therians' split from monotremes (about 170 million years ago) and the split between eutherians (placental mammals) and marsupials (about 150 million years ago). The X-added region (XAR) is of autosomal origin and is considered to have been added to the human X chromosome after the split between eutherians and marsupials but before the radiation of eutherians (150 million–100 million years ago). The pseudoautosomal region (PAR) recombines with a corresponding region on the Y chromosome during male meiosis. S1–S5 denote the five evolutionary strata that have been identified. Each stratum tentatively represents the result of stepwise cessation of recombination between the proto-sex chromosomes. b | A tree showing the approximate timing of events associated with the evolution of the human X chromosome. Mya, million years ago.

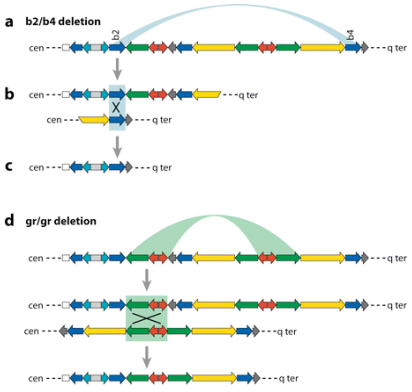


The human and chimpanzee Y chromosomes differ considerably in organization and structure despite the recent divergence of the two lineages (about 6 million years ago). Ampliconic regions contain massive palindromic arrays of multi-copy testis-specific genes. The X-degenerate regions denote segments homologous to the X chromosome that were present on the proto-sex chromosomes before they started to diverge into X and Y. The X-transposed region on the human Y chromosome is a segment transposed from the X chromosome to the Y chromosome in the human lineage after the split from chimpanzee. Most parts of the human and chimpanzee Y chromosomes do not recombine during male meiosis and are referred to as the male-specific region on Y (MSY). The figure is modified, with permission, from Ref.

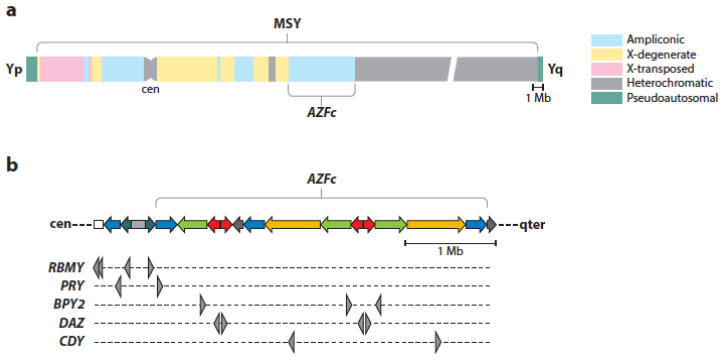


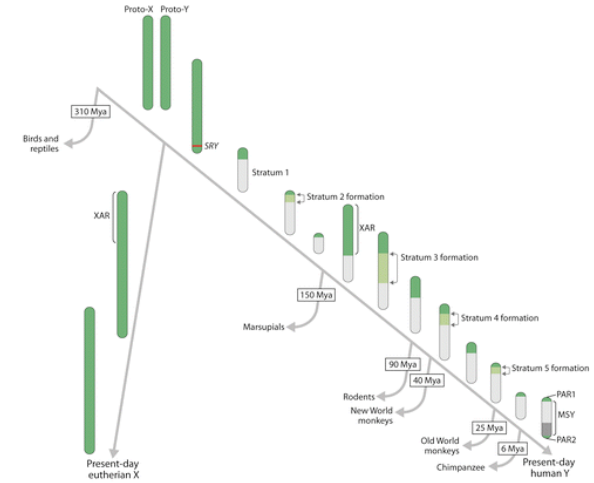
Hughes JF, Rozen S. 2012.
Annu. Rev. Genomics Hum. Genet. 13:83–108

Sequence classes, genes, and palindromes on the human Y chromosome. (a) Schematic representation of the entire human Y chromosome, with the male-specific region (MSY) indicated. (b) A more detailed representation that focuses on the euchromatic MSY and excludes the major heterochromatic block on Yq. Palindrome arms are shown as pairs of dark blue triangles. Arms belonging to the same palindrome are indicated by adjacent, opposite-facing triangles. Positions of protein-coding genes are indicated directly below the chromosome by vertical lines. The TSPY gene family, indicated by a rectangle below the chromosome, is arranged in a tandem array with 23–64 copies of a 20.4-kb repeat unit. Each repeat unit contains a single copy of the TSPY gene. (c) Locations and sizes of recurrent deletions within the human MSY that cause (or, in the case of the gr/gr deletion, predispose to) spermatogenic failure. Abbreviations: PAR1, short-arm pseudoautosomal region; PAR2, long-arm pseudoautosomal region.



Mechanisms by which b2/b4 and gr/gr deletions arise. (a) The organization of amplicons (large, nearly identical segmental duplications) that make up the AZFc region. Amplicons of the same color have DNA sequences that are 99.82%–99.97% identical to one another. b2/b4 deletions are caused by ectopic recombination and crossing over between the b2 and b4 repeats in the AZFc region. (b) Schematic representation of the b2/b4 crossover, which could occur either within a single chromatid or between sister chromatids. (c) The product of the b2/b4 crossover, an extremely abbreviated variant of the AZFc region from which 3.5 Mb have been removed. (d) Ectopic crossing over between green or red amplicons, causing gr/gr deletions. Abbreviations: cen, direction of centromere; q ter, direction of long-arm terminus of the chromosome.





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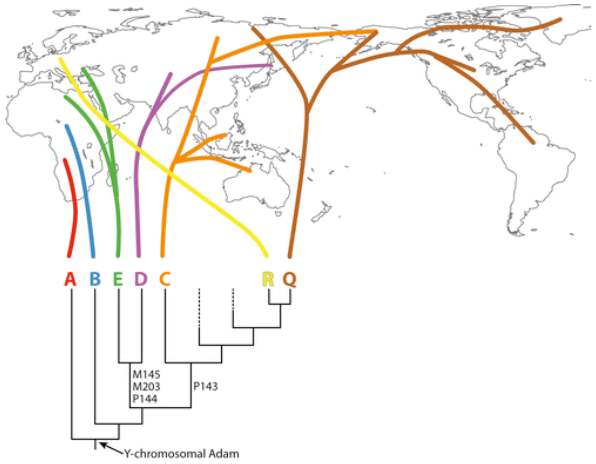

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Gonadal Sex Determination

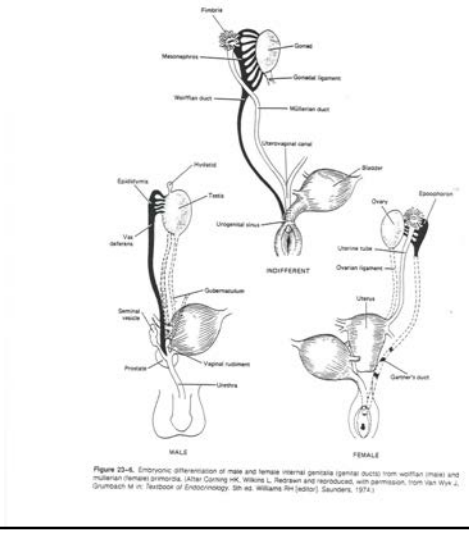


Figure 23–6. Embryonic differentiation of male and female internal genitalia (genital ducts) from wolffian (male) and müllerian (female) primordia. (After Corning HK, Wilkins L. Redrawn and reproduced, with permission, from Van Wyk JJ, Grumbach M in: Textbook of Endocrinology. 5th ed. Williams RH [editor]. Saunders, 1974.)

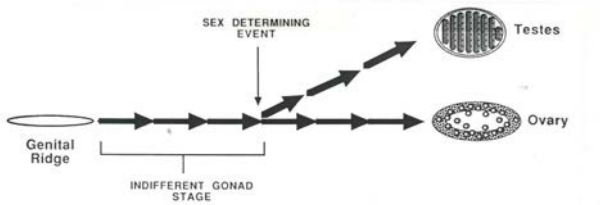
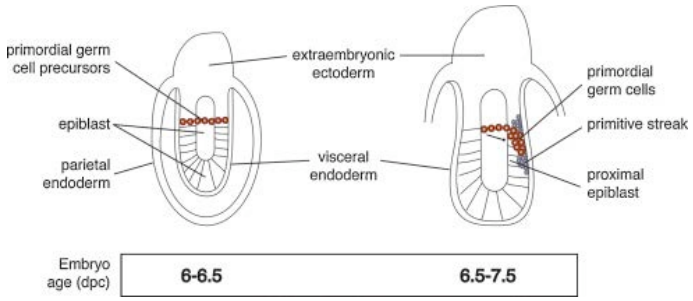
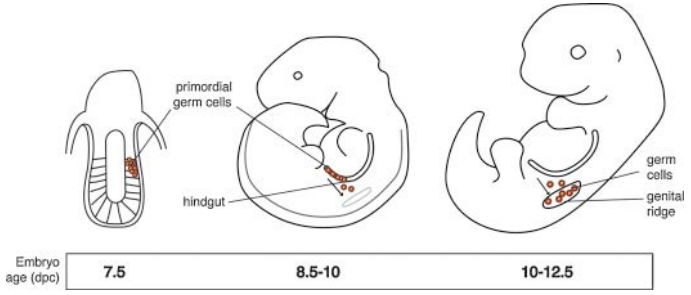
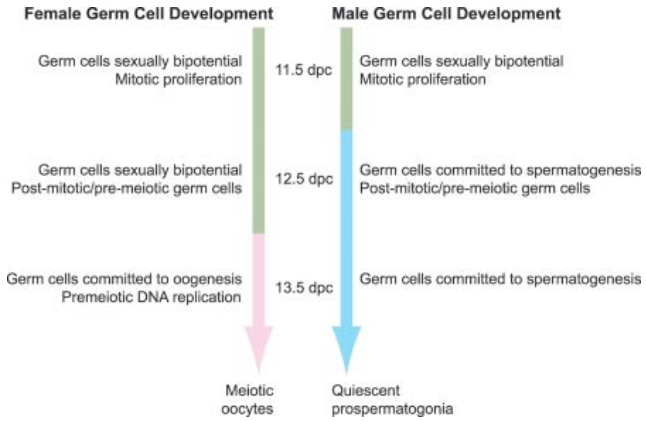
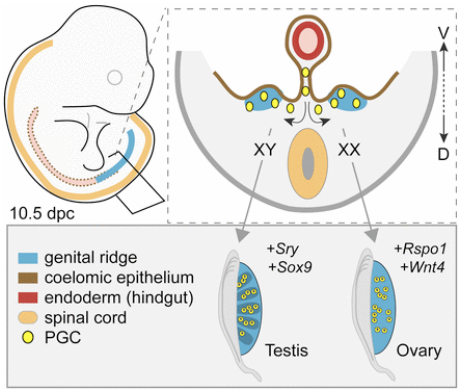


Fig. 1. Diagrammatic representation of gonadal development in mammals. The development of the genital ridge into a testis or ovary proceeds via a series of differentiation steps (heavy arrows). During the indifferent gonad stage, differentiation steps are considered common to both males and females. After the sex-determining event, differentiation diverges and a series of male specific steps leads to testicular development.









The establishment of the bipotential genital ridges and gonadal sex determination. In mammals, the genital ridges (blue) typically appear as longitudinal outgrowths along the surfaces of the mesonephroi within the coelomic cavity. In mice, they emerge at ~10 dpc through recruitment of cells from the overlying coelomic epithelium (brown). Primordial germ cells (yellow) colonize the genital ridges (arrows) after leaving the hindgut (red) via the dorsal mesentery. At this stage in development, the genital ridges are bipotential and can differentiate into testes or ovaries, depending on genetic cues. From ~10.5 dpc, the Y-linked sex-determining gene *Sry* is expressed in XY genital ridges and initiates *Sox9* expression and testis differentiation. In the absence of *Sry*, as in XX genital ridges, ovary differentiation is initiated by the action of genes such as *Rspo1* and *Wnt4*. (D) Dorsal; (V) ventral.

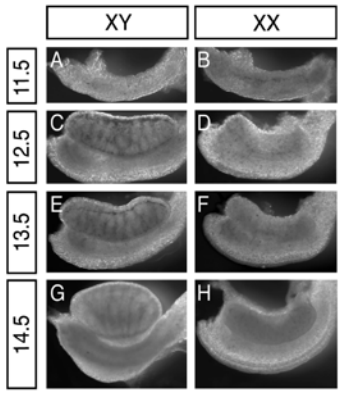


FIGURE 1. Morphological differentiation of mouse embryonic gonads. Whole-mount images of dissected mouse fetal gonads. **A,C,E,G,** XY gonads; **B,D,F,H,** XX gonads. **A,B,** 11.5 dpc; **C,D,** 12.5 dpc; **E,F,** 13.5 dpc; **G,H,** 14.5 dpc.

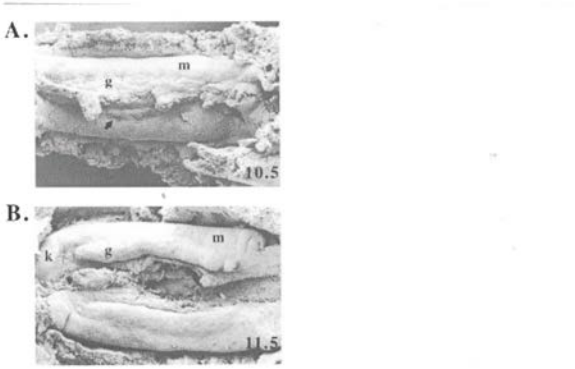


Fig. 2. Scanning electron micrographs of the urogenital ridge. (A) At 10.5 d.p.c. the urogenital ridge fills most of the body cavity between the limb buds. m, mesonephros; g, genital ridge region; the arrow indicates a pore on the coelomic surface of the genital ridge region. (B) By 11.5 d.p.c. the gonad (g) in the mesonephric region of the ridge and kidney (k) in the metanephric region of the ridge are distinct from the mesonephros. (m) No sexual dimorphisms are apparent. Modified from Fig. 8, Capel and Lovell-Badge (1993). Reproduced by permission from the Publishers (JAI Press).

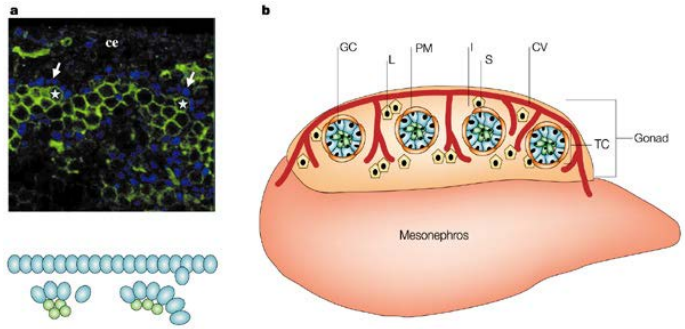
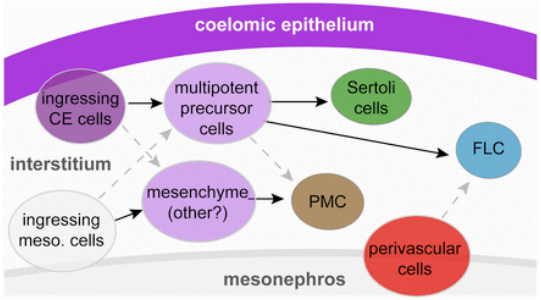


Figure 2 | **Compartmentalization of the testis.** **a** | At the earliest stages of testis organogenesis (11.75–12.0 days post coitum; dpc), Sertoli cells (stained with SF1 antibody; blue) polarize and begin to aggregate around clusters of primordial germ cells (stained with PECAM antibody; asterisk) to initiate development of testis cords. ce, coelomic epithelium. **b** | Between 11.5–12.5 dpc, the cells of the testis are organized into two functional compartments: testis cords (TC) and the interstitial space (I) outside the cords. Within testis cords, Sertoli cells (S; blue) surround germ cells (GC; green). A basal lamina is deposited between Sertoli cells and peritubular myoid cells (PM). The interstitial compartment contains Leydig cells (L; yellow) and the coelomic vessel (CV; red), with branches that extend between cords.



Known and proposed origins of the testicular cell lineages. The cells of the nascent genital ridges originate primarily from the overlying coelomic epithelium but also from the subjacent mesonephros. A subset of ingressing coelomic epithelial cells differentiates into Sertoli cells following *Sry* expression. Some of these supporting cells are also believed to differentiate into FLCs. It is unclear whether cells originating from the mesonephros contribute toward somatic cells other than blood endothelium, but they very likely contribute to the mesenchyme. The origin of PMCs remains unknown, but it is likely that they differentiate from a subset of mesenchymal cells or yet unidentified precursor cells of the testis interstitium. A second origin for the FLCs has also been proposed to include perivascular cells located at the gonad–mesonephric junction.

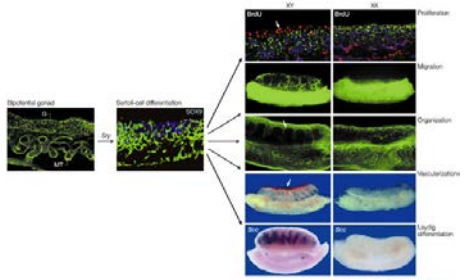
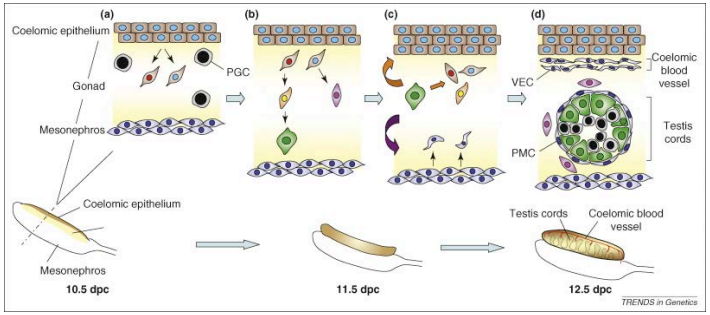
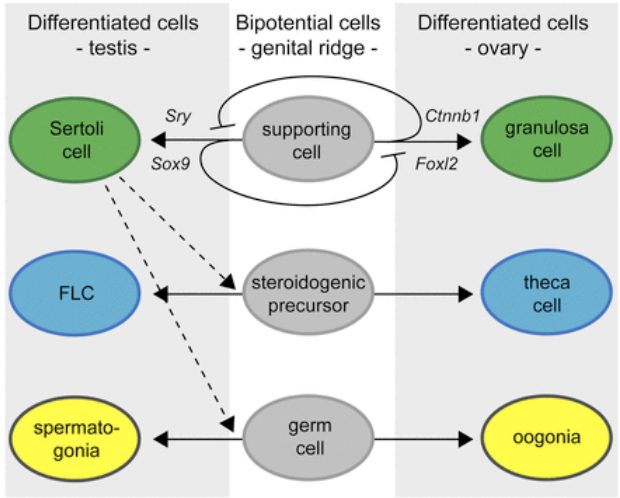
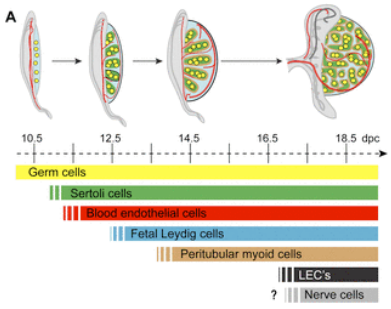
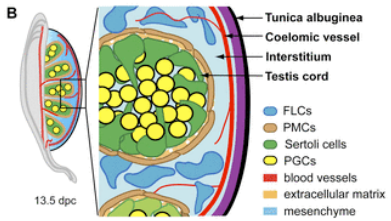


Figure 4 | **Cellular events downstream of *Sry* rapidly organize testis structure.** At the bipotential stage (10.5–11.5 days post coitum; dpc), no obvious morphological features distinguish XX and XY gonads. Antibodies against laminin (green) outline all cells in the gonad (G) and also label the basal lamina of mesonephric tubules (MT) in XX and XY samples. In XY gonads, *Sry* upregulates nuclear SOX9 (blue) in pre-Sertoli cells, and initiates Sertoli-cell differentiation by 11.5 dpc (germ cells and vasculature are labelled with platelet endothelial cell adhesion molecule (PECAM); green). Between 11.5–12.5 dpc, male-specific pathways activate marked morphological and cellular changes in the XY gonad (left column) that do not occur in the XX gonad (right column). These include an upregulation of proliferation in coelomic epithelial cells (measured by BrdU incorporation; red, arrow); migration of cells from the mesonephros (detected in recombinant cultures between a wild-type gonad and a mesonephros in which all cells express GFP; green); structural organization of testis cords (detected by laminin deposition; green); male-specific vascularization (red; blood cells are visible in the light microscope; arrow); and Leydig-cell differentiation (detected by RNA *in situ* hybridization for the steroid enzyme, *Scc*). BrdU image pair repoduced with permission from Ref. 29 © (2000) The Company of Biologists Ltd. XY migration image and vascular image pairs reproduced with permission from Ref. 77 © (2002) Elsevier Science.









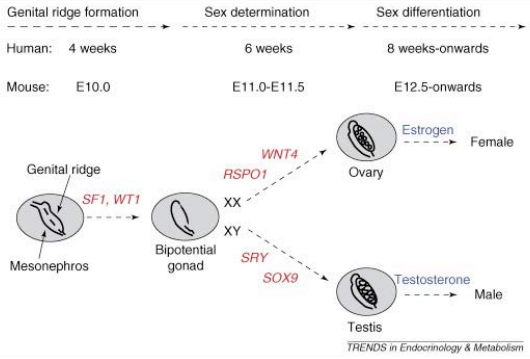
GENES REQUIRED TO OBTAIN BIPOTENTIAL GONAD

- Found with knockout mice or mutant human tissues not having gonad form from genital ridge.

WT1 - Wilms' Tumor, WAGR Syndrome, Frasier Syndrome, Denys-Drash Syndrome
- sex reversal/ different pathologies
- four zinc finger domains
- 16 different products from gene, 11 p13

SF1- Steroidogenic Factor 1, orphan nuclear steroid receptor
- knockout cause lack gonad
- mutation cause sex reversal
- influence MIS and DAX-1 expression

LIM1 - LIM Homeobox gene Lhx9
- knockout cause lack gonad
- LIM knockout cause lack SF1 (? Upstream)



BIOLOGY OF REPRODUCTION 62, 132–142 (2000)

Role of Neurotropins in Rat Embryonic Testis Morphogenesis (Cord Formation)[1]

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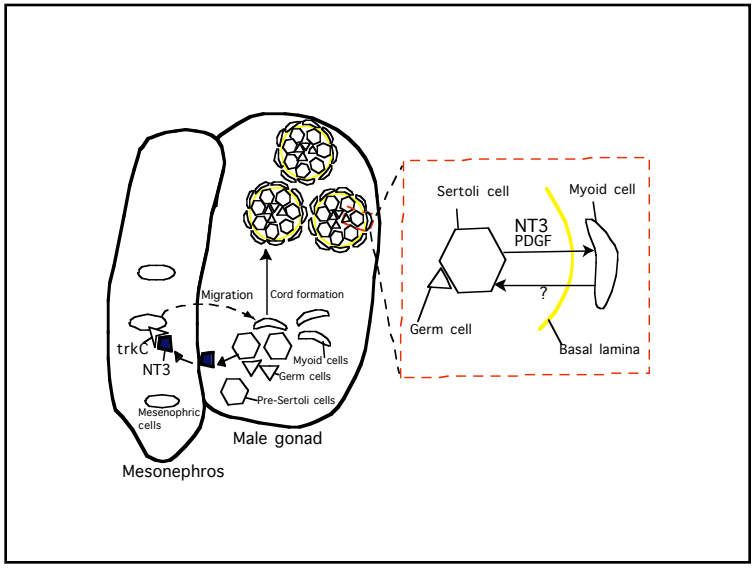
ABSTRACT

The process of seminiferous cord formation is the first morphological event that differentiates a testis from an ovary and indicates male sex determination. Cord formation occurs by embryonic Day 14 (Day 0 = plug date; E14) in the rat. A series of experiments were conducted to determine if neurotropins and their receptors are important for the process of rat embryonic cord formation. The expression of low affinity neurotropin receptor (p75/LNGFR) was determined by immunohistochemistry on sections of both testis and ovary from E13 through birth (Day 0, P0) with an antibody to p75/LNGFR. The staining for p75/LNGFR was present in the mesonephros of E13 gonads and in a sex-specific manner appeared around developing cords at E14 in the embryonic testis. At birth, staining for p75/LNGFR was localized to a single layer of cells (i.e., peritubular cells) that

amined to determine if there were any morphological differences in the testis. NT3 knockouts appeared to have normal cord morphology in E15 and E17 testis. TrkC knockout mice also had normal cord morphology in E14 and P0 testis. Both NT3 and trkC knockout-mice testis had less interstitial area than wild-type controls. In addition, the trkC knockout mice have an increased number of cells expressing p75LNGFR within the cords when compared to controls or NT3 knockout mice. Combined observations suggest compensation between the different neurotropin ligands, receptors, and/or possibly different growth factors for this critical biological process. In summary, results suggest a novel nonneuronal role for neurotropins in the process of cord formation during embryonic rat testis development. The hypothesis developed is that neurotropins are involved in the progression of male sex differentiation and are critical for the induction of embryonic testis cord formation.



Male-to-Female Sex Reversal in Mice Lacking Fibroblast Growth Factor 9

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Durham, North Carolina 27710

Summary

Fgfs direct embryogenesis of several organs, including the lung, limb, and anterior pituitary. Here we report male-to-female sex reversal in mice lacking *Fibroblast growth factor 9* (*Fgf9*), demonstrating a novel role for FGF signaling in testicular embryogenesis. *Fgf9*$^{-/-}$ mice also exhibit lung hypoplasia and die at birth. Reproductive system phenotypes range from testicular hypoplasia to complete sex reversal, with most *Fgf9*$^{-/-}$ XY reproductive systems appearing grossly female at birth. *Fgf9* appears to act downstream of *Sry* to stimulate mesenchymal proliferation, mesonephric cell migration, and Sertoli cell differentiation in the embryonic testis. While *Sry* is found only in some mammals, *Fgfs* are highly conserved. Thus, *Fgfs* may function in sex determination and reproductive system development in many species.

Introduction

The family of at least 22 Fibroblast growth factors (FGFs) regulates multiple developmental processes (Ornitz and Itoh, 2001). *Fgf9* is widely expressed in mouse embryos (Colvin et al., 1999). To determine the essential in vivo functions of *Fgf9*, we generated mice homozygous for a targeted deletion of *Fgf9* (*Fgf9*$^{-/-}$). *Fgf9*$^{-/-}$ mice die at birth, apparently due to lung hypoplasia (Colvin et al., 2001). We observed that females were dramatically overrepresented among *Fgf9*$^{-/-}$ embryos, and subsequent analysis revealed novel functions for *Fgf9* in sex determination and testicular embryogenesis.

Male and female mouse gonads at embryonic day 11.0 (E11.0) are morphologically identical "indifferent gonads" medial to each mesonephros. By E13.5, the testis is twice the size of the ovary and exhibits morphologically complex testicular cords (Figures 1A and 1B). Three male-specific events are known to direct early testicular genesis: cell proliferation, cell migration, and testicular cord formation. An increase in proliferation at the coelomic lining of the gonad (the coelomic epithelium) occurs between E11.2 and E12.1. This proliferation gives rise to Sertoli cells (a supporting cell lineage) early on and to interstitial cells throughout this period (Schmahl et al., 2000). Cells contributing to the interstitium, including vascular endothelial cells and peritubular myoid cells, migrate into the testis from the mesonephros and are required for testicular cord formation (Buehr et al., 1993; Martineau et al., 1997). Testicular cord development begins at about E12.2 with clustering of Sertoli and germ cells, followed by rearrangement so that Sertoli cells surround the germ cells. Testicular cords isolate male germ cells from interstitial cells, and prevent male germ cells from entering meiosis (Magre and Jost, 1991). Ovarian germ cells, which are not enclosed by supporting cells, progress by E13.5 to the first meiotic division (McLaren and Southee, 1997).

The testis regulates further male reproductive development. Until E13.5, both sexes have Mullerian and Wolffian ducts in each mesonephros. Sertoli cells produce Mullerian inhibiting substance (MIS) (Münsterberg and Lovell-Badge, 1991). MIS causes regression of the Mullerian ducts, which, in the absence of MIS, form the oviducts, uterus, and upper vagina. Interstitial Leydig cells produce testosterone, which induces formation of Wolffian duct derivatives, including the epididymis, vas deferens, and seminal vesicles. In females, the absence of testicular MIS and testosterone results in development of Mullerian structures and regression of the Wolffian ducts. Targeted deletion of *Mis* or its receptor results in development of Mullerian structures in XY mice (Behringer et al., 1994; Mishina et al., 1996).

Testicular expression of *Sry*, a transcription factor gene on the Y chromosome, is essential for increased proliferation in, and mesonephric cell migration into, the mouse testis (Capel et al., 1999; Schmahl et al., 2000). *Sry* is expressed in mouse testis between E10.5 and E12.5 (reviewed by Koopman, 1999) and is necessary and sufficient to induce male development. Deletion of *Sry* generates XY ovaries and mice with a female phenotype (Lovell-Badge and Robertson, 1990; Gubbay et al., 1990), and addition of an *Sry* transgene generates XX males (Koopman et al., 1991). A potential downstream target of *Sry* is *Sox9*, an autosomal transcription factor expressed in Sertoli cells (reviewed by Swain and Lovell-Badge, 1999). Mutations in *SRY* and *SOX9* have been identified in human XY females with gonadal dysgenesis (Sinclair, 1996).

Other than *Sry* and *Sox9*, mediators of early testicular genesis have been elusive. Mouse models of impaired testicular embryogenesis are limited. Mice lacking *M33* (a mouse homolog of *Polycomb*) exhibit XY sex reversal, but early growth of both XX and XY gonads is severely impaired (Katoh-Fukui et al., 1998). Mice lacking *Sf1*, *Wt1*, *Lim1*, *Emx1*, or *Lhx9* (Birk et al., 2000) exhibit agenesis or early regression of gonads of both sexes (reviewed by Capel et al., 1999; Koopman, 1999). Thus, any role for these molecules later in mouse testis development remains obscure, although mutation of human *SF1* is associated with incomplete XY gonad develop-

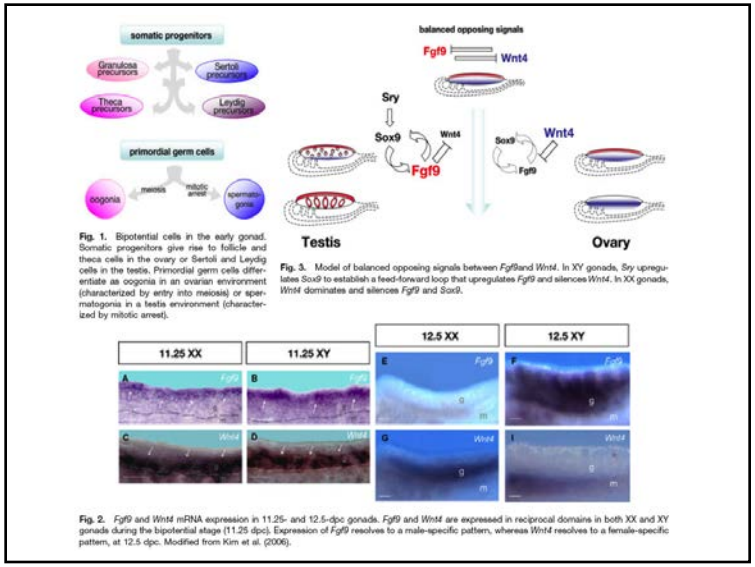


Fig. 1. Bipotential cells in the early gonad. Somatic progenitors give rise to follicle and theca cells in the ovary or Sertoli and Leydig cells in the testis. Primordial germ cells differentiate as oogonia in an ovarian environment (characterized by entry into meiosis) or spermatogonia in a testis environment (characterized by mitotic arrest).

Fig. 3. Model of balanced opposing signals between *Fgf9* and *Wnt4*. In XY gonads, *Sry* upregulates *Sox9* to establish a feed-forward loop that upregulates *Fgf9* and silences *Wnt4*. In XX gonads, *Wnt4* dominates and silences *Fgf9* and *Sox9*.

Fig. 2. *Fgf9* and *Wnt4* mRNA expression in 11.25- and 12.5-dpc gonads. *Fgf9* and *Wnt4* are expressed in reciprocal domains in both XX and XY gonads during the bipotential stage (11.25 dpc). Expression of *Fgf9* resolves to a male-specific pattern, whereas *Wnt4* resolves to a female-specific pattern, at 12.5 dpc. Modified from Kim et al. (2006).

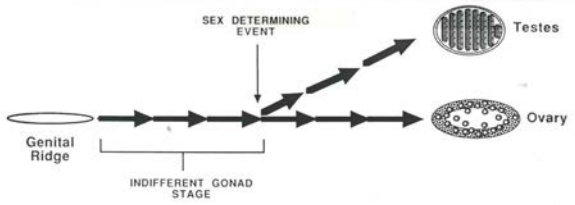


Fig. 1. Diagrammatic representation of gonadal development in mammals. The development of the genital ridge into a testis or ovary proceeds via a series of differentiation steps (heavy arrows). During the indifferent gonad stage, differentiation steps are considered common to both males and females. After the sex-determining event, differentiation diverges and a series of male specific steps leads to testicular development.

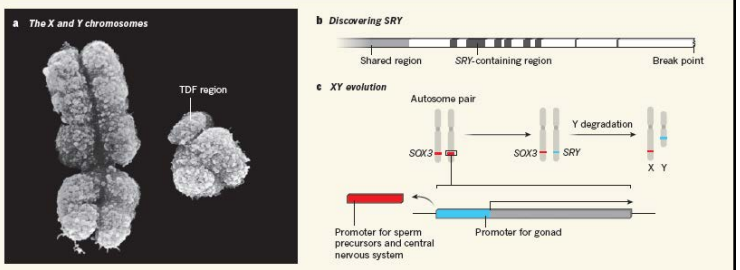
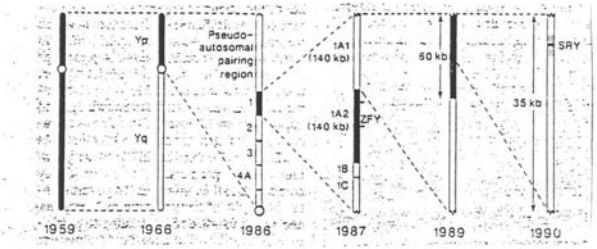
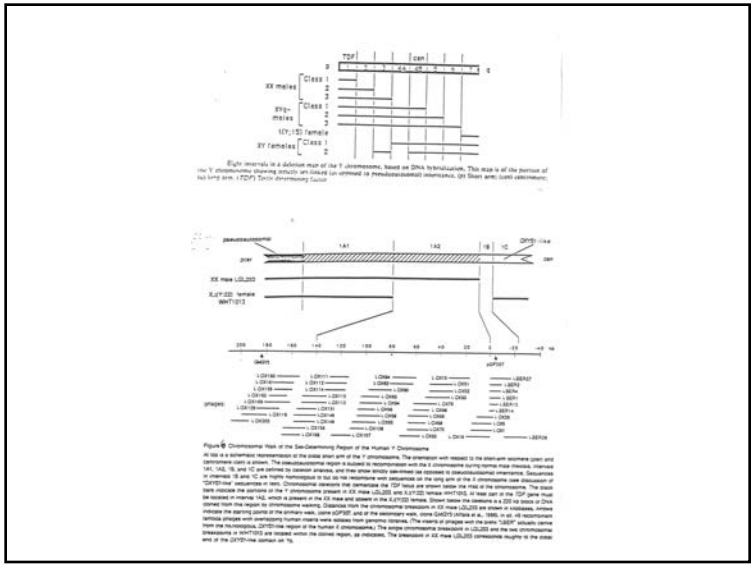
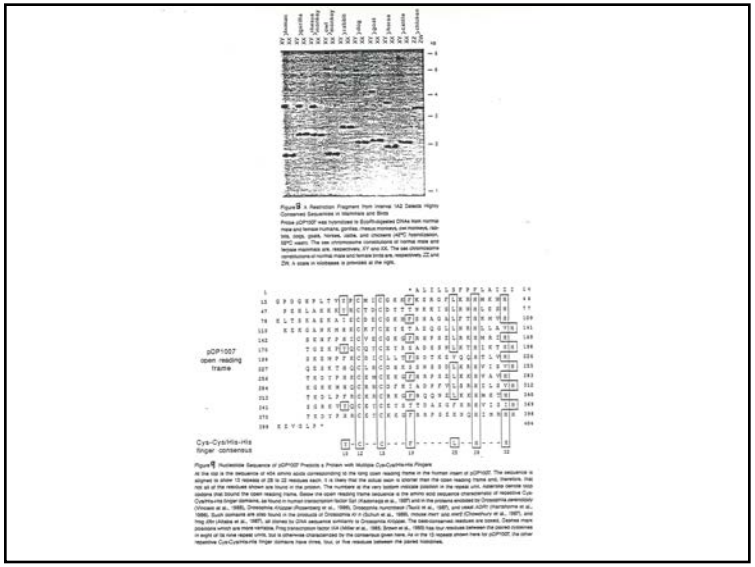


Figure 1 | An evolving understanding of sex. a, In humans, sex is based on the presence or absence of the Y chromosome, seen here with its larger partner, X. The testis-determining factor (TDF) that drives male development was known to lie on the short arm of Y, but its identity was a mystery. b, In 1990, Sinclair *et al.*[1] found two males with only a small piece of Y, which had been broken and fused to the X. They scoured the 35,000 base pairs between the break points and the region at the tip of the Y that is shared with the X, finding several regions (black) that were specific to the Y. One of these regions contained the TDF gene, *SRY*. c, This discovery led to an understanding of how X and Y evolved. The gene *SOX3* was located on a pair of non-sex chromosomes (autosomes) in the ancestors of mammals. A promoter sequence drove expression of *SOX3* in sperm precursors and the central nervous system. The promoter on one copy of *SOX3* was replaced with a sequence that drives expression in the undifferentiated gonad (a tissue that can develop into either an ovary or a testis). This expression pattern allowed the new gene, *SRY*, to direct testis development. Over time, genes not needed for male development were degraded on this chromosome, giving rise to the Y. (Part b adapted from ref. 1.)







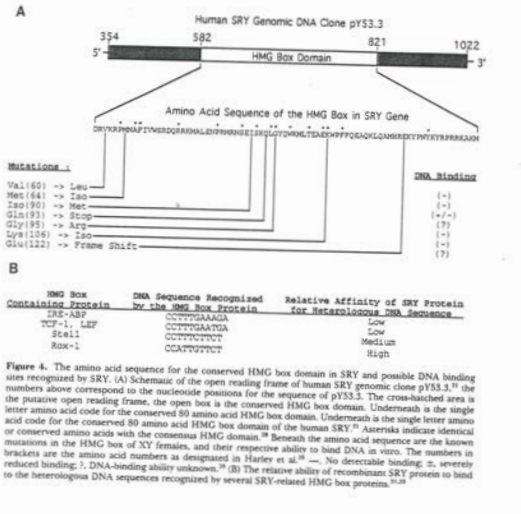
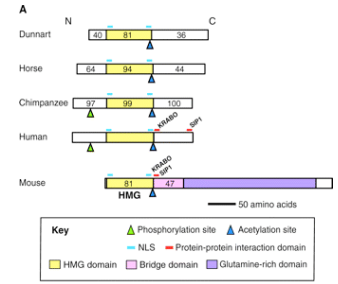
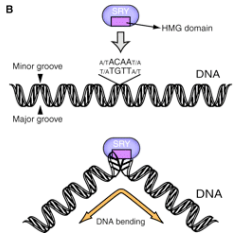


Figure 6. The amino acid sequence for the conserved HMG box domain in SRY and possible DNA binding sites recognized by SRY. (A) Schematic of the open reading frame of human SRY genomic clone pY53.3,[35] the numbers above correspond to the nucleoside positions for the sequence of pY53.3. The cross-hatched area is the putative open reading frame, the open box is the conserved HMG box domain. Underneath is the single letter amino acid code for the conserved 80 amino acid HMG box domain. Underneath is the single letter amino acid code for the conserved 80 amino acid HMG box domain of the human SRY.[35] Asterisks indicate identical or conserved amino acids with the consensus HMG domain.[36] Beneath the amino acid sequence are the known mutations in the HMG box of XY females, and their respective ability to bind DNA in vitro. The numbers in brackets are the amino acid numbers as designated in Harley et al.[35] —, No detectable binding; ±, severely reduced binding; ?, DNA-binding ability unknown.[36] (B) The relative ability of recombinant SRY protein to bind to the heterologous DNA sequences recognized by several SRY-related HMG box proteins.[31,33]





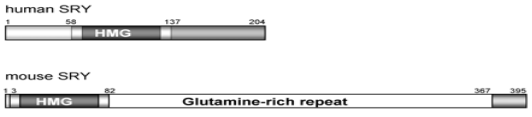


FIG. 1. Comparison of the human and mouse SRY protein domains. Shown are the protein domains of the human and mouse SRY proteins, showing the conserved HMG domain (HMG) and the glutamine-rich domain present in mice but absent in humans.

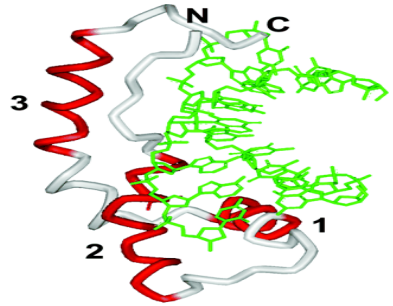
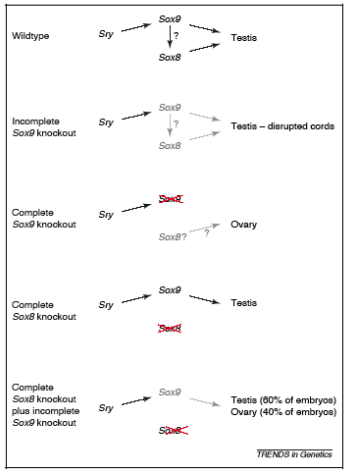
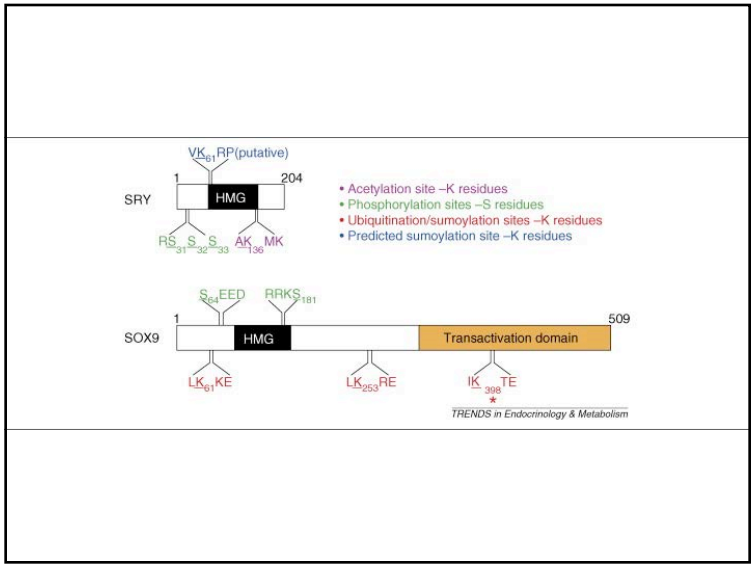
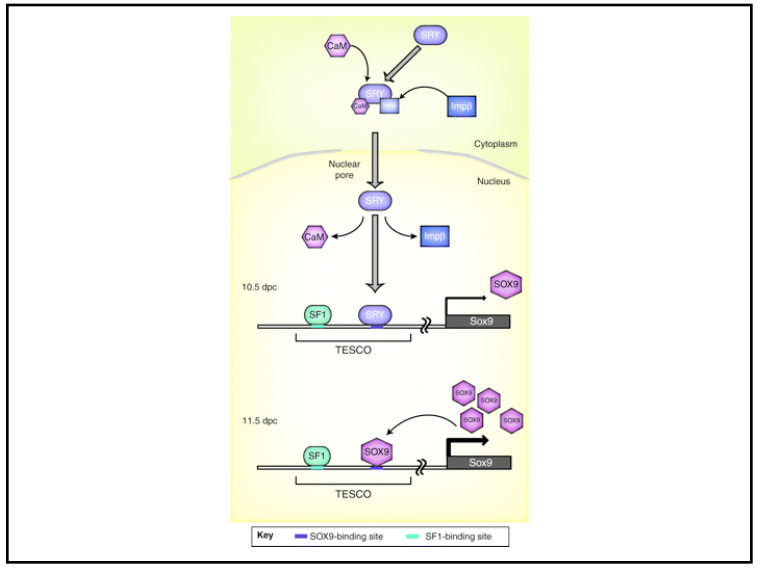


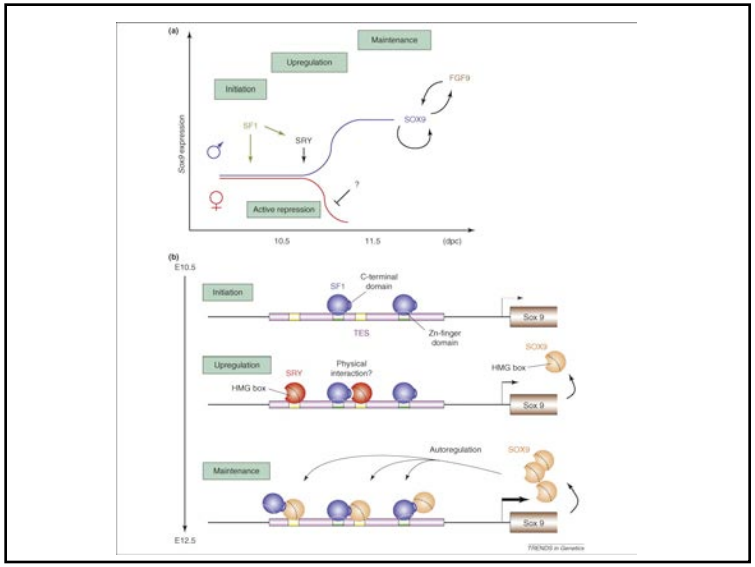
FIG. 2. A model of the structure of the SRY HMG box bound to DNA. The nuclear magnetic resonance structure of the SRY HMG domain, showing the three x-helices and the L-shaped conformation and binding to the minor groove of DNA causing it to bend and severely unwind (35).

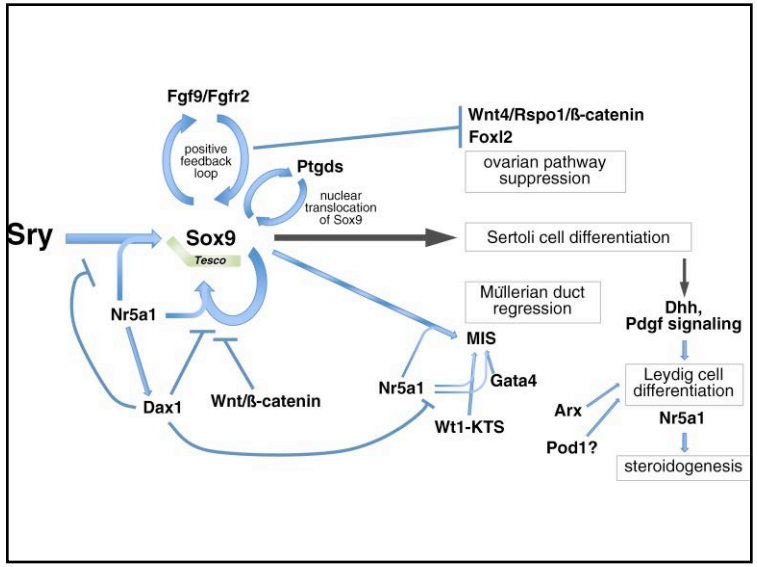
Sex determination: a tale of two *Sox* genes

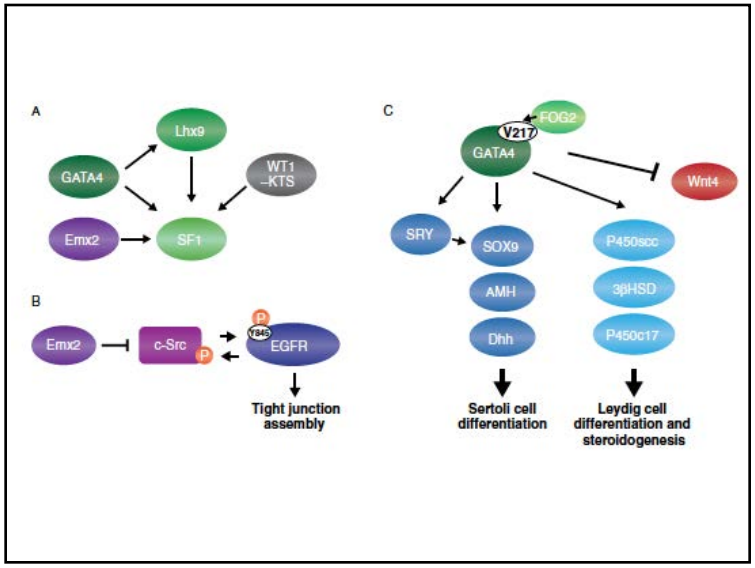



VK61RP(putative)
SRY
1
204
HMG
RS31S32S33
AK136MK
• Acetylation site –K residues
• Phosphorylation sites –S residues
• Ubiquitination/sumoylation sites –K residues
• Predicted sumoylation site –K residues
S64EED
RRKS181
SOX9
1
509
HMG
Transactivation domain
LK61KE
LK253RE
IK398TE
TRENDS in Endocrinology & Metabolism


SRY
CaM
Impβ1
Cytoplasm
Nuclear pore
Nucleus
10.5 dpc
SOX9
SF1
Sox9
TESCO
11.5 dpc
Key
SOX9-binding site
SF1-binding site


(a)
Maintenance
Upregulation
Initiation
FGF9
SOX9
SF1
SRY
Active repression
Sox9 expression
10.5
11.5
(dpc)
(b)
E10.5
Initiation
C-terminal domain
SF1
TES
Zn-finger domain
Sox 9
Upregulation
SRY
Physical interaction?
HMG box
SOX9
Maintenance
Autoregulation
E12.5


Fgf9/Fgfr2
positive feedback loop
Ptgds
nuclear translocation of Sox9
Wnt4/Rspo1/ß-catenin
Foxl2
ovarian pathway suppression
Sry
Sox9
Tesco
Sertoli cell differentiation
Müllerian duct regression
Dhh, Pdgf signaling
Nr5a1
MIS
Leydig cell differentiation
Dax1
Wnt/ß-catenin
Nr5a1
Gata4
Wt1-KTS
Arx
Pod1?
Nr5a1
steroidogenesis



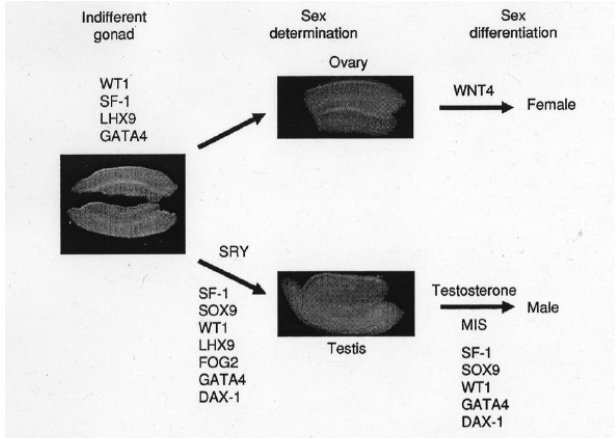


FIGURE 1. Molecular and hormonal determinants of mammalian sex determination and differentiation. The testis (male) and ovary (female) arise from a common precursor, the biopotential or indifferent gonad. The testis-determining factor (SRV) is essential for male gonad development. The other key factors involved in mammalian sex development are also indicated.

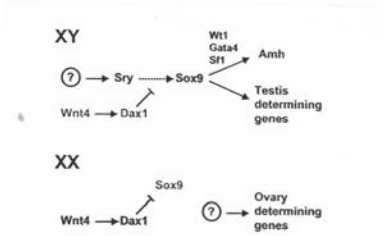
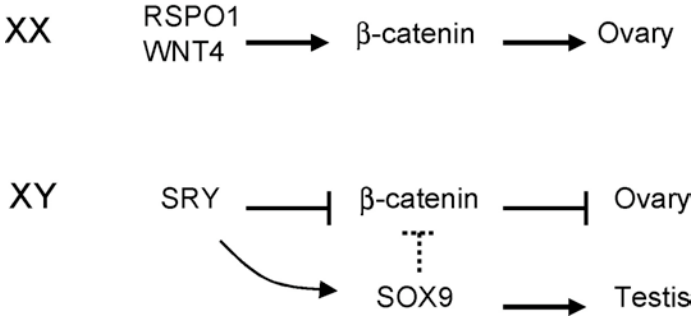
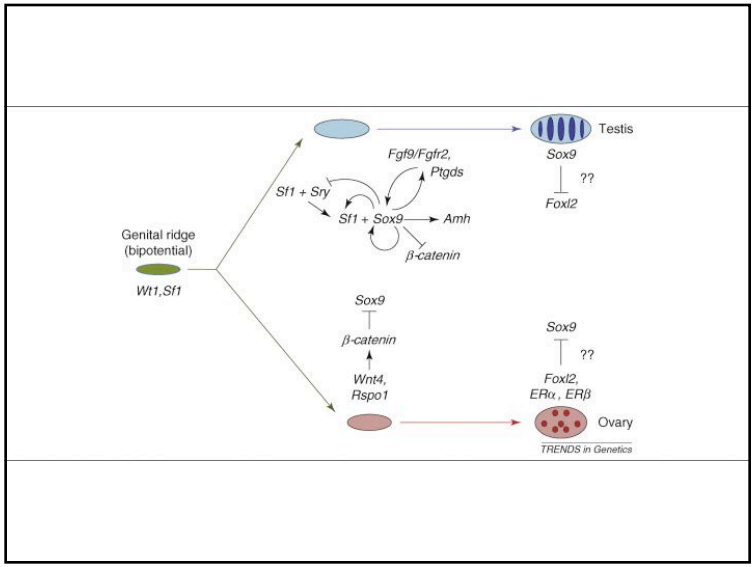
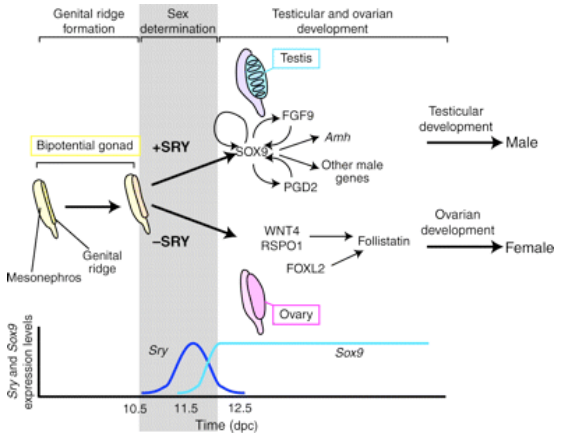


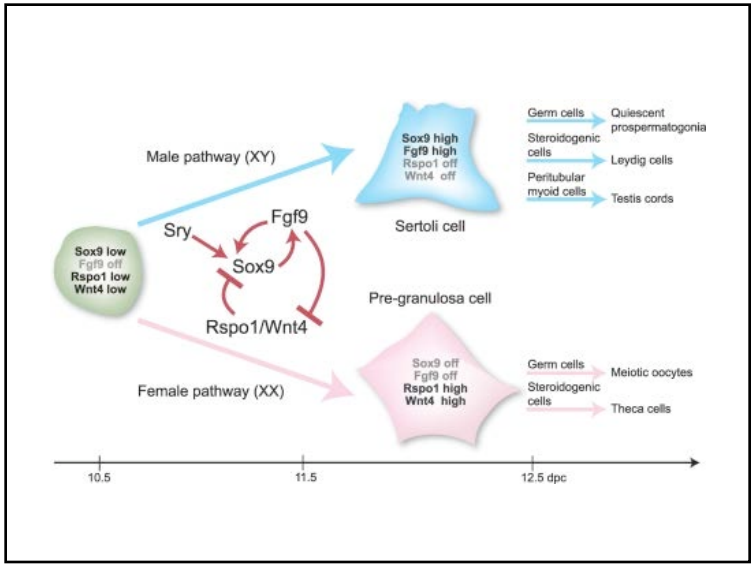
Figure 1. Molecular Pathways Controlling Sex Determining in XY and XX Mammals

Positive regulation is indicated by an arrow, whereas inhibition is represented by a blunt-ended line. The nature of the regulatory relationship between *Sry* and *Sox9*, represented by a dotted arrow, is not yet clear. Some of the unknown elements are indicated by question marks.



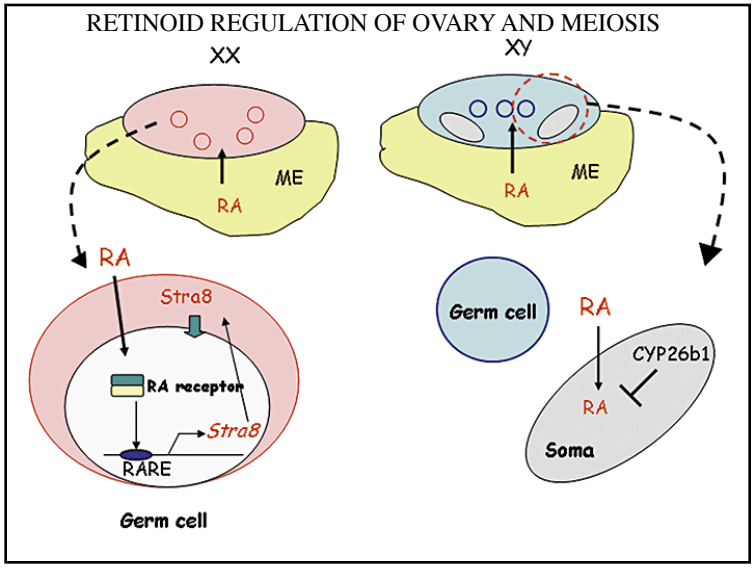


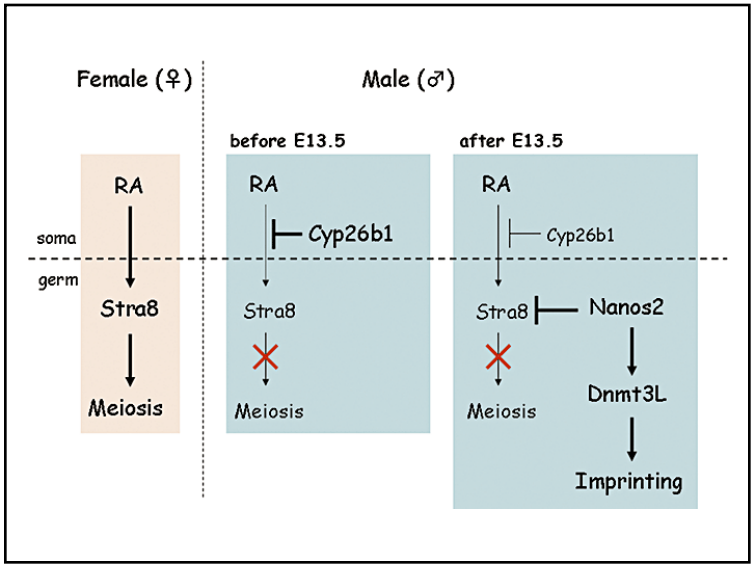


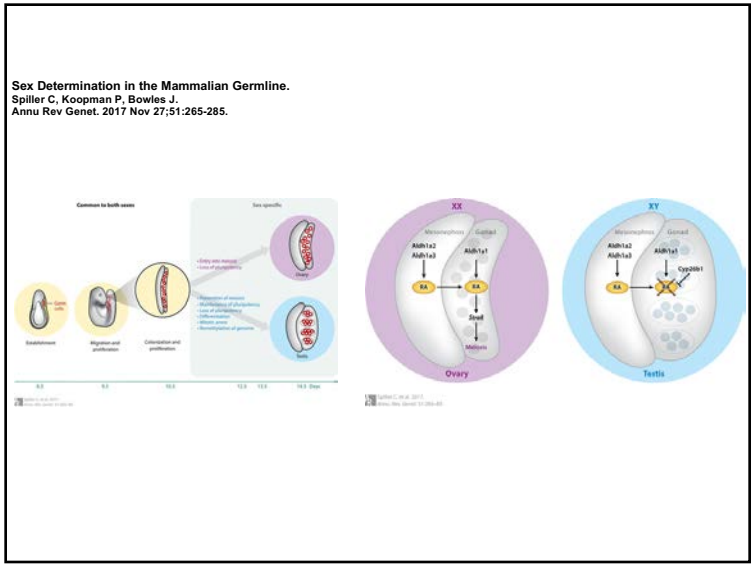


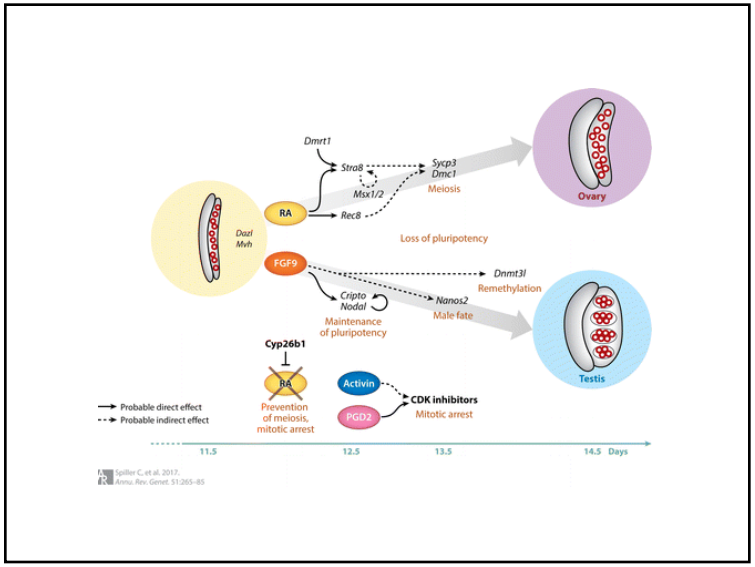
SOX4 regulates gonad morphogenesis and promotes male germ cell differentiation in mice.
Zhao L, Arsenault M, Ng ET, Longmuss E, Chau TC, Hartwig S, Koopman P.
Dev Biol. 2017 Mar 1;423(1):46-56.

Abstract
The group C SOX transcription factors SOX4, -11 and -12 play important and mutually overlapping roles in development of a number of organs. Here, we examined the role of SoxC genes during gonadal development in mice. All three genes were expressed in developing gonads of both sexes, predominantly in somatic cells, with Sox4 being most strongly expressed. Sox4 deficiency resulted in elongation of both ovaries and testes, and an increased number of testis cords. While female germ cells entered meiosis normally, male germ cells showed reduced levels of differentiation markers Nanos2 and Dnmt3l and increased levels of pluripotency genes Cripto and Nanog, suggesting that SOX4 may normally act to restrict the pluripotency period of male germ cells and ensure their proper differentiation. Finally, our data reveal that SOX4 (and, to a lesser extent, SOX11 and -12) repressed transcription of the sex-determining gene Sox9 via an upstream testis-specific enhancer core (TESCO) element in fetal gonads, raising the possibility that SOXC proteins may function as transcriptional repressors in a context-dependent manner.


RETINOID REGULATION OF OVARY AND MEIOSIS
XX
XY
ME
RA
ME
RA
RA
Stra8
RA receptor
Stra8
RARE
Germ cell
Germ cell
RA
CYP26b1
RA
Soma

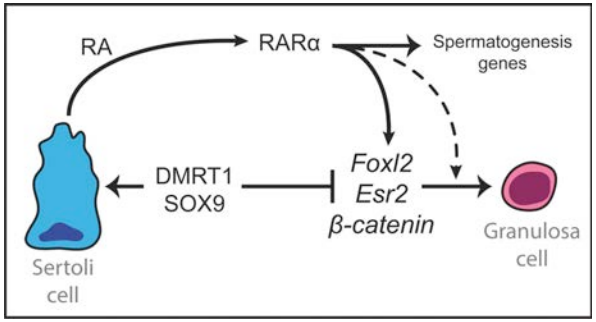

Female (♀)
Male (♂)
before E13.5
after E13.5
soma
germ
RA
Stra8
Meiosis
RA
Cyp26b1
Stra8
Meiosis
RA
Cyp26b1
Stra8
Nanos2
Meiosis
Dnmt3L
Imprinting


Sex Determination in the Mammalian Germline.
Spiller C, Koopman P, Bowles J.
Annu Rev Genet. 2017 Nov 27;51:265-285.
XX
XY
Ovary
Testis


Dmrt1
Stra8
Sycp3
Dmc1
Msx1/2
Meiosis
Ovary
RA
Rec8
Dazl
Mvh
Loss of pluripotency
FGF9
Dnmt3l
Remethylation
Cripto
Nodal
Nanos2
Male fate
Maintenance
of pluripotency
Testis
Cyp26b1
RA
Activin
CDK inhibitors
PGD2
Mitotic arrest
Prevention
of meiosis,
mitotic arrest
Probable direct effect
Probable indirect effect
11.5
12.5
13.5
14.5 Days

DMRT1 protects male gonadal cells from retinoid-dependent sexual transdifferentiation.

Minkina A, et al. Dev Cell. 2014 Jun 9;29(5):511-20.



Model for role of DMRT1 in sex maintenance

DMRT1 directly represses transcription in Sertoli cells of potential feminizing genes including *Foxl2, Esr2*, and the *Wnt/β-catenin* pathway genes *Wnt4* and *Rspo1* (Matson et al., 2011). This paper shows that DMRT1 thereby allows Sertoli cells to produce RA that is necessary for spermatogenesis without causing RARα to activate these feminizing genes, which also activate one another. The model also indicates that it is possible, based on data from other systems, that RARα synergizes with products of some of the feminizing genes to drive transdifferentiation. In addition to the genes shown, DMRT1 also represses *Cyp19a1/aromatase*, which makes estradiol that stimulates ER activity (Matson et al., 2011).

TABLE 1 Genes in mammalian sex determination and early gonadal differentiation known at the year indicated

1990	*1995*	*2001*
SRY	*SRY*	*SRY*
	WT1	*WT1*
	SF1	*SF1*
	DAX1	*DAX1*
	SOX9	*SOX9*
	LHX1(LIM1)	*LHX1(LIM1)*
		EMX2
		DMRT1
		M33
		GATA4
		LHX9
		VNN1
		FGF9
		WNT4

Genes are listed chronologically, in the order of their first implication in sex determination during the time intervals 1991–1995 and 1996–2001. For references, see Koopman (2001) and text.

Table 1 Genes involved in the male and female sex determination pathway

Gene	Protein function	Gain- and loss-of-function phenotypes: Human syndrome	Gain- and loss-of-function phenotypes: Mouse models	References
Genes involved in initial development the bipotential gonad				
Emx2	Transcription factor	–	Aberrant tight junction assembly, failure in genital ridge formation (LOF)	Kusaka *et al.* (2010)
Gata4	Transcription factor	Ambiguous external genitalia, congenital heart disease (LOF)	Failure in thickening of the coelomic epithelium, defective initial formation of genital ridge (LOF)	Lourenço *et al.* (2011), Manuylov *et al.* (2011) and Hu *et al.* (2013)
Wt1	Transcription factor	Denys-Drash, Frasier syndrome (LOF)	Disruption of seminiferous tubule and somatic cell apoptosis, XY sex reversal (LOF)	Kreidberg *et al.* (1993), Hammes *et al.* (2001) and Gao *et al.* (2006)
Lhx9	Transcription factor	–	Failure in genital ridge formation (LOF)	Birk *et al.* (2000)
Sf1	Nuclear receptor	Embryonic testicular regression syndrome, gonadal dysgenesis	Delayed organization of male testis cord, failure in genital ridge formation (LOF)	Park *et al.* (2005) and Lin *et al.* (2007)
Genes involved in the regulation of SRY expression during primary sex determination				
Gata4/Fog2	Transcription/cofactor	–	Apparent XY gonadal sex reversal (LOF)	Tevosian *et al.* (2002)
Gadd45γ	Stress-response protein	–	XY sex reversal (LOF)	Gierl *et al.* (2012) and Warr *et al.* (2012)
Map3k4	Kinase	–	XY sex reversal (LOF)	Bogani *et al.* (2009)
Cbx2	Transcription factor	XY ovarian DSD, XY sex reversal (LOF)	XY sex reversal (LOF)	Biason-Lauber *et al.* (2009) and Katoh-Fukui *et al.* (2012)
Il, Irr, Igf1r	Membrane receptor	–	XY sex reversal (LOF)	Nef *et al.* (2003)
Genes involved in male testis determination pathway				
SRY	Transcription factor	Turner syndrome, Klinefelter syndrome, XY sex reversal (LOF)	XY sex reversal (LOF); XX sex reversal (GOF)	Ford *et al.* (1959), Jacobs & Strong (1959), Koopman *et al.* (1989) and Gubbay *et al.* (1990)
Sox9	Transcription factor	Campomelic dysplasia XY sex reversal (LOF)	Abnormal Sertoli cell differentiation, XY sex reversal (LOF); XX sex reversal (GOF)	Foster *et al.* (1994), Huang *et al.* (1999), Vidal *et al.* (2001), Chaboissier *et al.* (2004) and Barrionuevo *et al.* (2006)
Amh	Hormone	XY sex reversal (LOF)	XY sex reversal (LOF)	
Fgf9	Growth factor	XY sex reversal (LOF)	XY sex reversal (LOF)	Kim *et al.* (2006) and Jameson *et al.* (2012)
Dmrt1	Transcription factor	XY gonadal dysgenesis, XY sex reversal (LOF)	Postnatal feminization in XY mice, defective seminiferous tubule (LOF)	Matson *et al.* (2012)

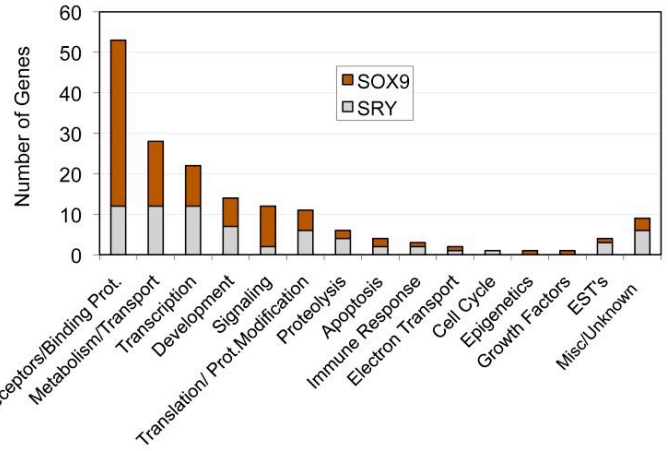
Table 1 *Continued*

Gene	Protein function	Gain- and loss-of-function phenotypes: Human syndrome	Gain- and loss-of-function phenotypes: Mouse models	References
Genes involved in female ovary determination pathway				
Wnt4	Signaling molecule	Ambiguous genitalia (GOF)	Failure in the formation of coelomic vessel and germ cell, degeneration of the female reproductive tract, partial XX sex reversal (LOF)	Jordan *et al.* (2003) and Yao *et al.* (2004)
β-catenin	Transcription factor	–	Partial XX sex reversal (LOF)	Chassot *et al.* (2008)
Rspo1	Growth factor	Palmoplantar hyperkeratosis, skin carcinoma, complete XX sex reversal (LOF)	Development of ovotestes, partial XX sex reversal (LOF)	Parma *et al.* (2006) and Chassot *et al.* (2008)
Foxl2	Transcription factor	BPES (LOF)	Premature ovarian failure, ablation of the primordial follicle pool, partial XX sex reversal (LOF)	Uhlenhaut & Treier (2006) and Ottolenghi *et al.* (2007)
Dax1	Nuclear receptor	XX sex reversal (LOF)	XX sex reversal (LOF)	Swain *et al.* (1998) and Meeks *et al.* (2003)

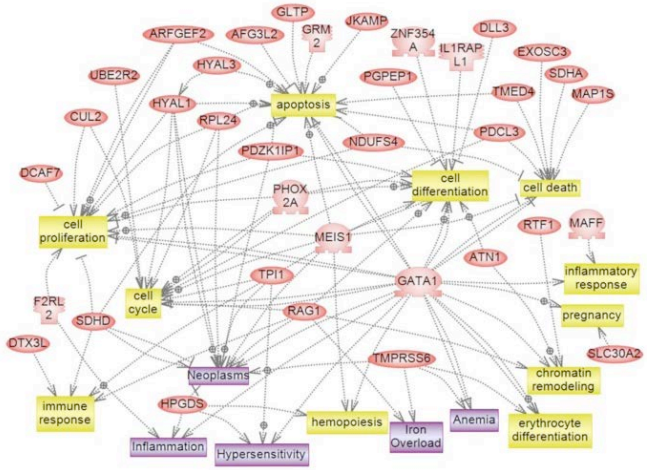
–, no mutations have been identified to date in human sexual reversal patients; GOF, gain of function; LOF, loss of function; BPES, blepharophimosis/ptosis/epicanthus inversus syndrome.

Bhandari RK, Haque MM, Skinner MK. (2012) Global genome analysis of the downstream binding targets of testis determining factor SRY and SOX9. PLoS One. 7(9):e43380.

A major event in mammalian male sex determination is the induction of the testis determining factor Sry and its downstream gene Sox9. The current study provides one of the first genome wide analyses of the downstream gene binding targets for SRY and SOX9 to help elucidate the molecular control of Sertoli cell differentiation and testis development. A modified ChIP-Chip analysis using a comparative hybridization was used to **identify 71 direct downstream binding targets for SRY and 109 binding targets for SOX9**. Interestingly, only 5 gene targets overlapped between SRY and SOX9. In addition to the direct response element binding gene targets, a large number of atypical binding gene targets were identified for both SRY and SOX9. Bioinformatic analysis of the downstream binding targets identified gene networks and cellular pathways potentially involved in the induction of Sertoli cell differentiation and testis development. The specific DNA sequence binding site motifs for both SRY and SOX9 were identified. Observations provide insights into the molecular control of male gonadal sex determination.

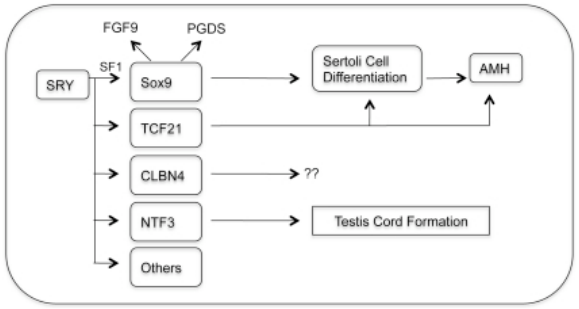


SRY direct binding target genes and their association to functional cellular processes



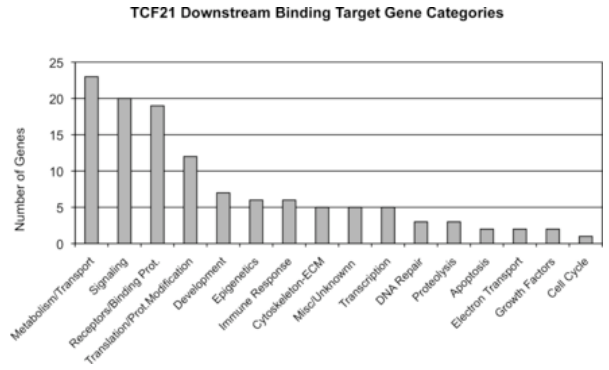
Bhandari RK, Sadler-Riggleman I, Clement TM, Skinner MK. (2011) Basic helix-loop-helix transcription factor TCF21 is a downstream target of the male sex determining gene SRY. PLoS One. 6(5):e19935.

The cascade of molecular events involved in mammalian sex determination has been shown to involve the SRY gene, but specific downstream events have eluded researchers for decades. The current study identifies one of the first direct downstream targets of the male sex determining factor SRY as the basic-helix-loop-helix (bHLH) transcription factor TCF21. SRY was found to bind to the Tcf21 promoter and activate gene expression. Mutagenesis of SRY/SOX9 response elements in the Tcf21 promoter eliminated the actions of SRY. SRY was found to directly associate with the Tcf21 promoter SRY/SOX9 response elements in vivo during fetal rat testis development. TCF21 was found to promote an in vitro sex reversal of embryonic ovarian cells to induce precursor Sertoli cell differentiation. TCF21 and SRY had similar effects on the in vitro sex reversal gonadal cell transcriptomes. Therefore, SRY acts directly on the Tcf21 promoter to in part initiate a cascade of events associated with Sertoli cell differentiation and embryonic testis development.

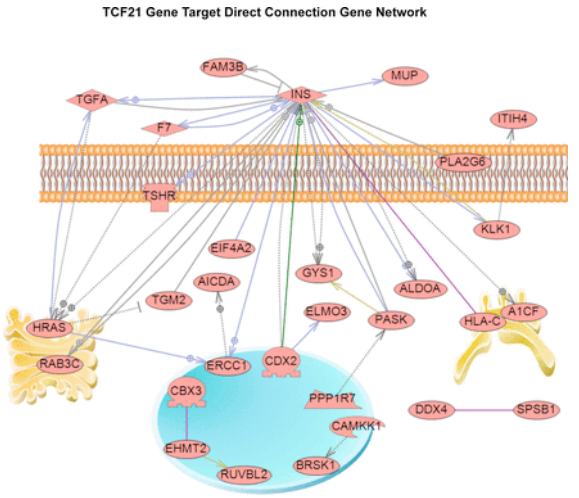


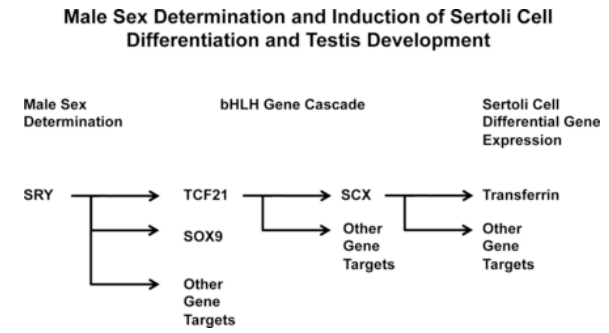
Summary of SRY downstream genes.
Proposed downstream actions of SRY on *Sox9* and *Tcf21* genes, along with *Clbn4, Ntf3*, and others yet to be identified. TCF21 induction of Sertoli cell differentiation and expression of marker genes such as *Amh* indicated. Combined actions of SRY and SF1 on *Sox9* expression and actions on *Fgf9* and *Pgds* expression indicated.

Bhandari RK, Schinke EN, Haque MM, Sadler-Riggleman I, Skinner MK. (2012) SRY induced TCF21 genome-wide targets and cascade of bHLH factors during Sertoli cell differentiation and male sex determination in rats. Biol Reprod. 87(6):131.



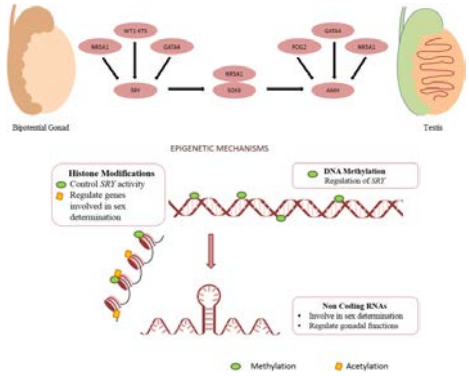
TCF21 binding target gene functional categories. Total numbers of target genes associated with a specific category are presented on the y-axis and gene functional categories on the x-axis.



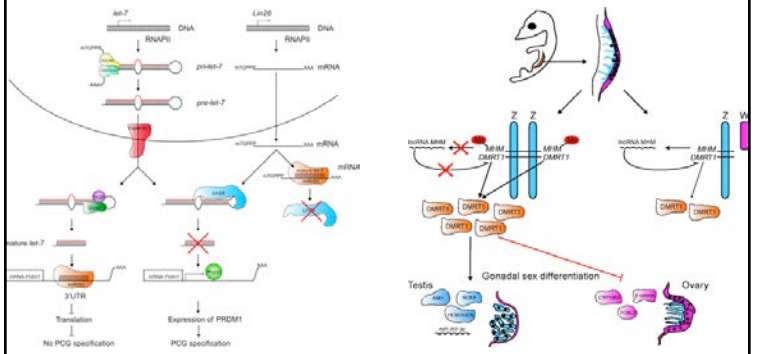


Schematic diagram of the hypothesized cascade of bHLH transcription factors involved in Sertoli cell differentiation and gonadal sex determination.

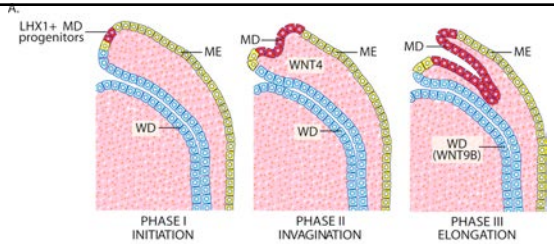
Genetic and epigenetic effects in sex determination.
Gunes SO, Metin Mahmutoglu A, Agarwal A.
Birth Defects Res C Embryo Today. 2016 Dec;108(4):321-336.

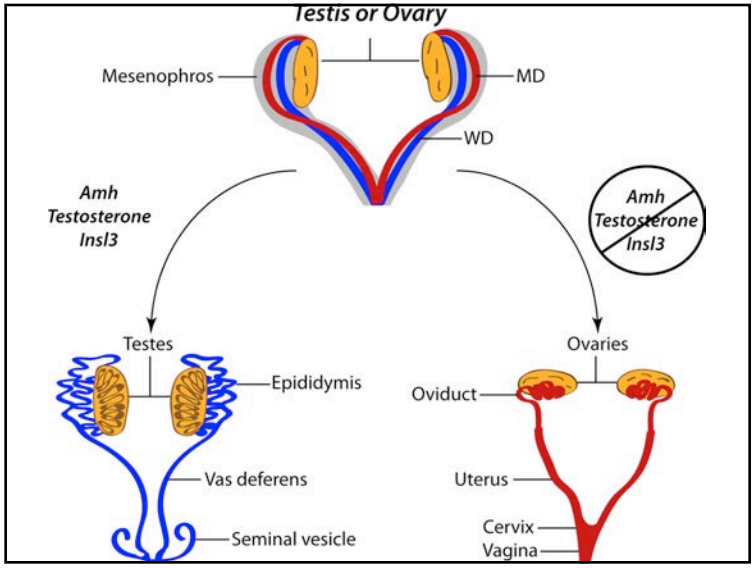


The role of non-coding RNAs in male sex determination and differentiation.
Rastetter RH, Smith CA, Wilhelm D.
Reproduction. 2015 Sep;150(3):R93-107.



Sex Differentiation





Role Testosterone -

1)Wolffian Duct development

2) Male Reproduction Genitalia

3) External Genitalia

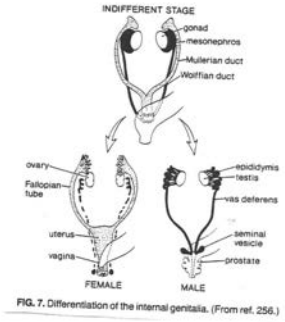
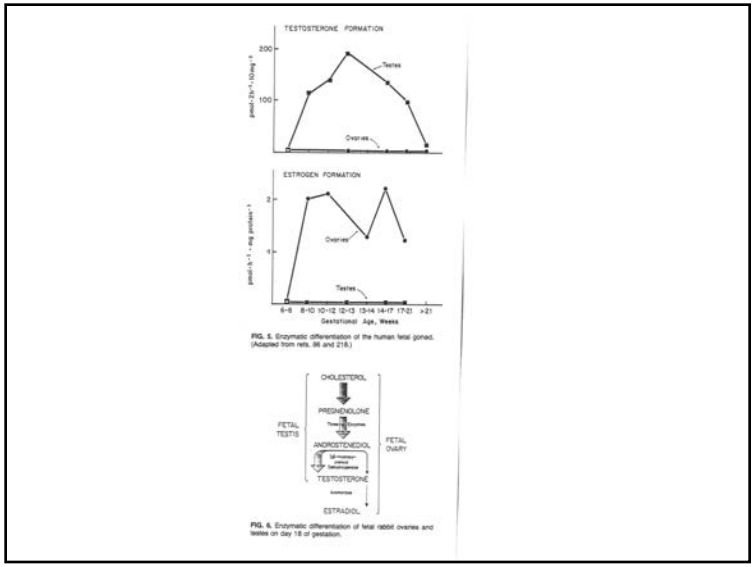


FIG. 7. Differentiation of the internal genitalia. (From ref. 256.)



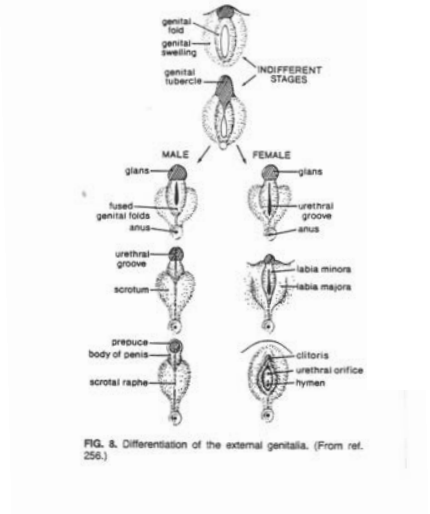


FIG. 8. Differentiation of the external genitalia. (From ref. 256.)

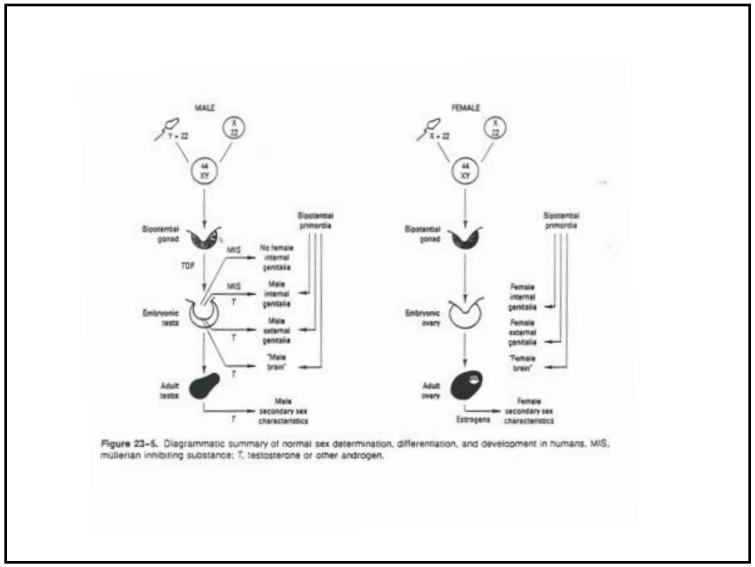
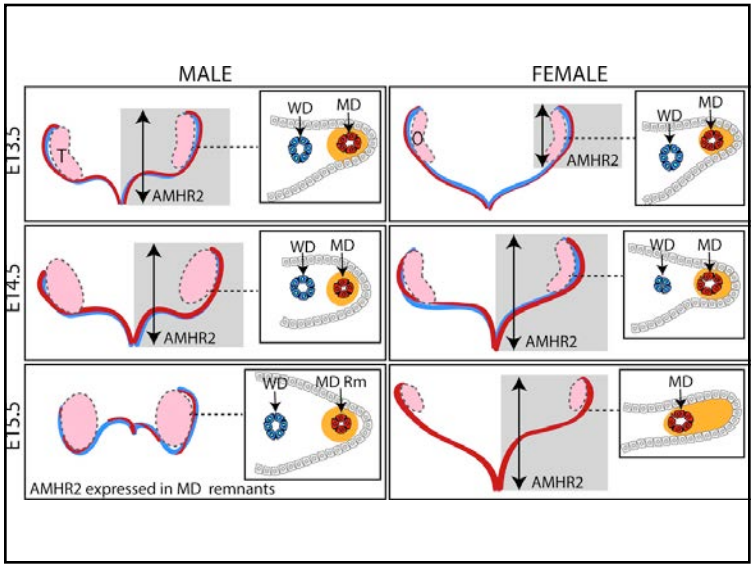
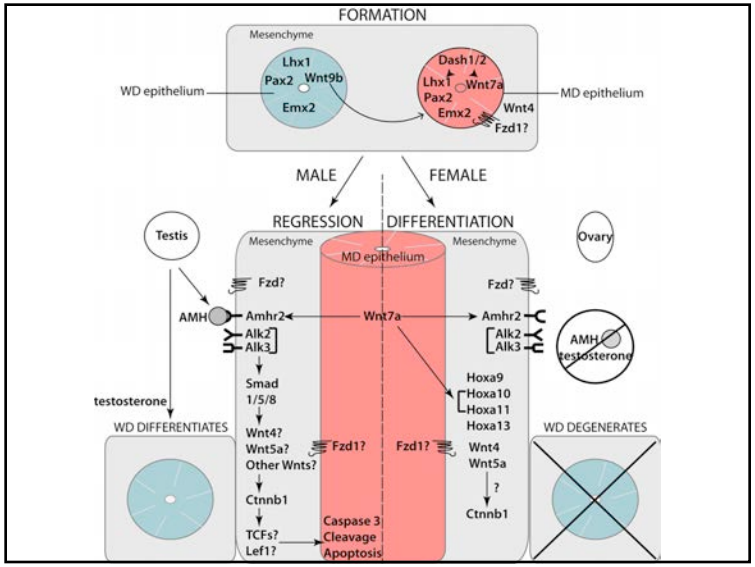
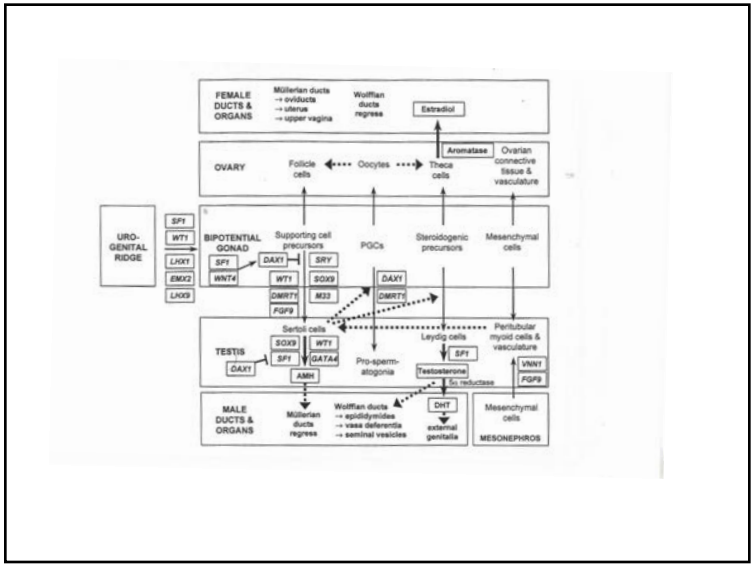


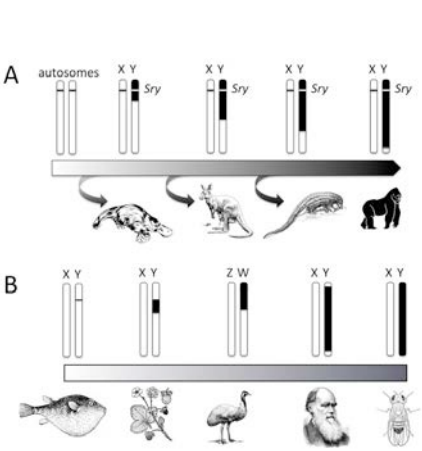
Figure 23–5. Diagrammatic summary of normal sex determination, differentiation, and development in humans. MIS, müllerian inhibiting substance; T, testosterone or other androgen.



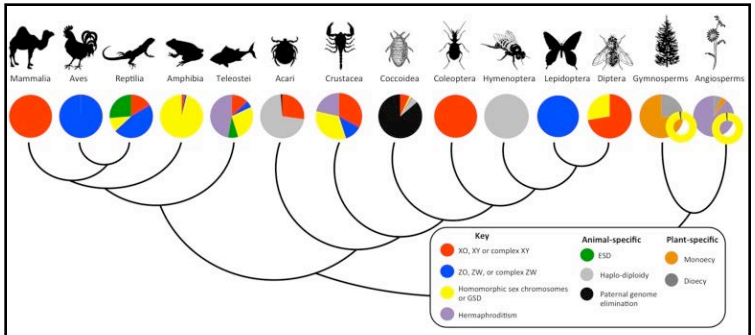




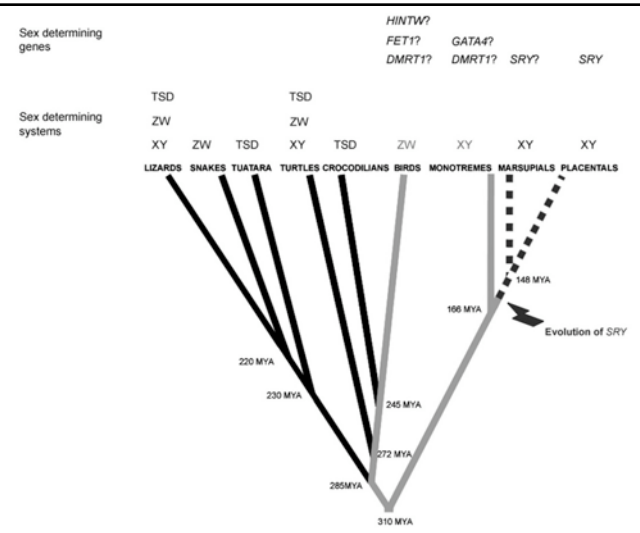
Sex Determination in Other Species



Sex chromosome differentiation.
A. Reconstructed evolutionary path of sex chromosome differentiation in humans. Sex chromosomes originate from autosomes that acquired a sex-determining function (the Sry gene) after their split from monotremes. Suppression of recombination between the sex chromosomes, associated with degeneration of the non-recombining region of the Y chromosome, results in the morphological and genetic differentiation of sex chromosomes. Recombination suppression occurred in multiple episodes along the human X and Y chromosome, forming so-called evolutionary strata. The oldest stratum is shared between eutherian mammals and marsupials, while the youngest stratum of humans is primate-specific. **B.** The degree of sex chromosome differentiation ranges widely across species, spanning the entire spectrum of homomorphic to heteromorphic sex chromosomes, from a single sex-determining locus, as seen in pufferfish, a small differentiated region (strawberry and emu), most of the sex chromosomes apart from short recombining regions (humans), to the entire sex chromosome pair, as seen in *Drosophila*. Note that the sex chromosomes are not drawn to scale.



Diversity of sex determination systems for representative plant and animal clades.
The bubble insert graph for the plant clades represents the relative proportion of species with documented sex chromosomes within plants with separate sexes. Vertebrates: Mammalia (placental, marsupial, and monotreme mammals), Aves (birds), Reptilia (turtles, snakes, crocodiles, lizards), Amphibia (frogs,



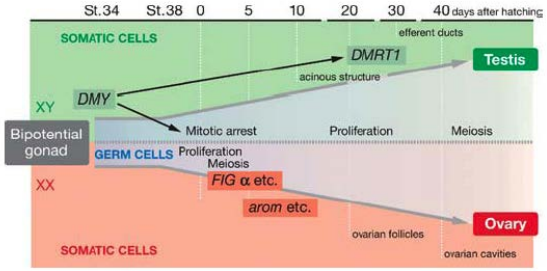
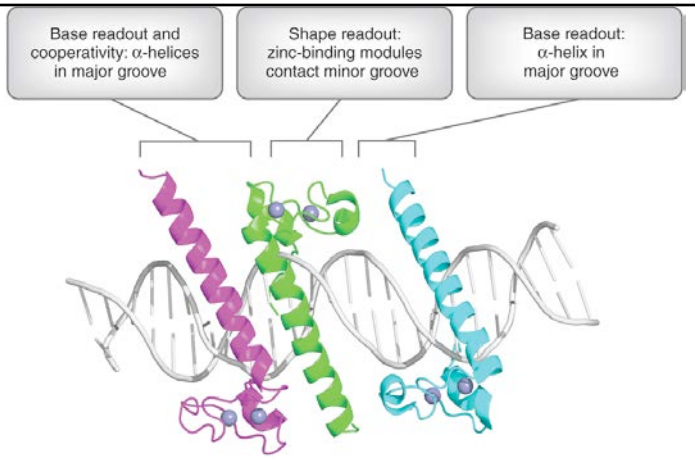
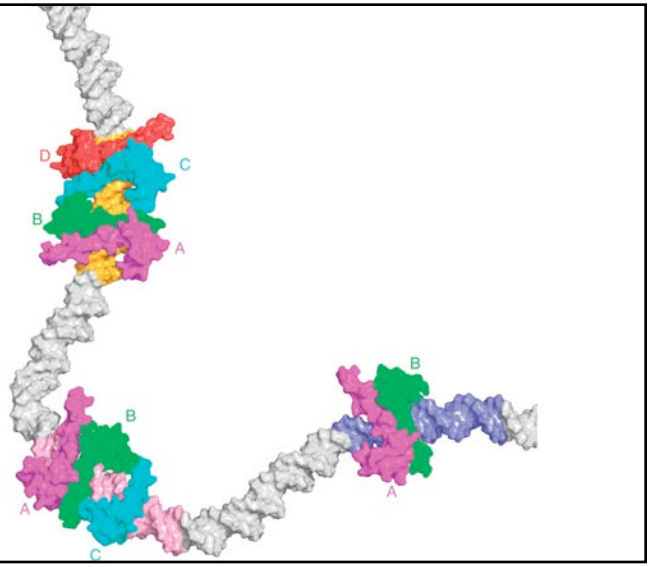


Figure 3

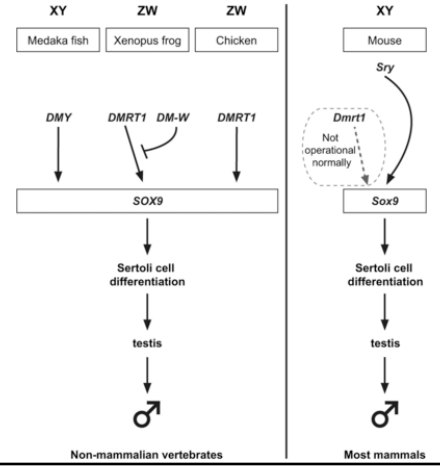
Conceptual illustration of normal sexual development of gonads in the medaka. Morphological events in germ cells are shown in the inner area of gray arrows, whereas those in somatic cells are shown in the outer area of gray arrows. Sex-specific genes expressed in the XY gonad and in the XX gonad are shown in green and pink boxes with black characters, respectively. Black arrows represent putative functions of DMY.

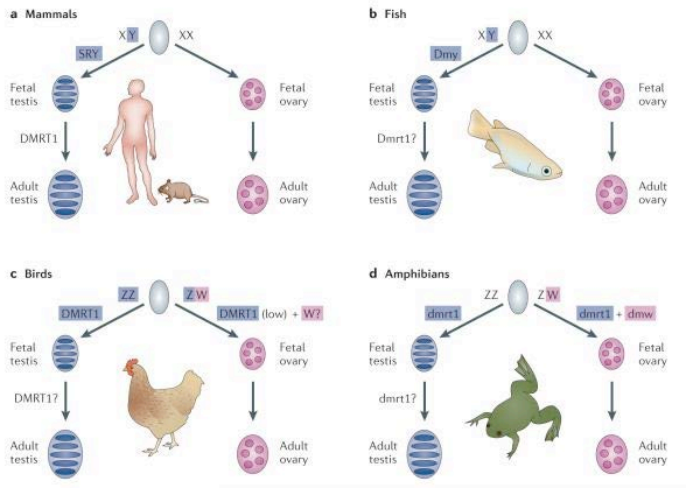


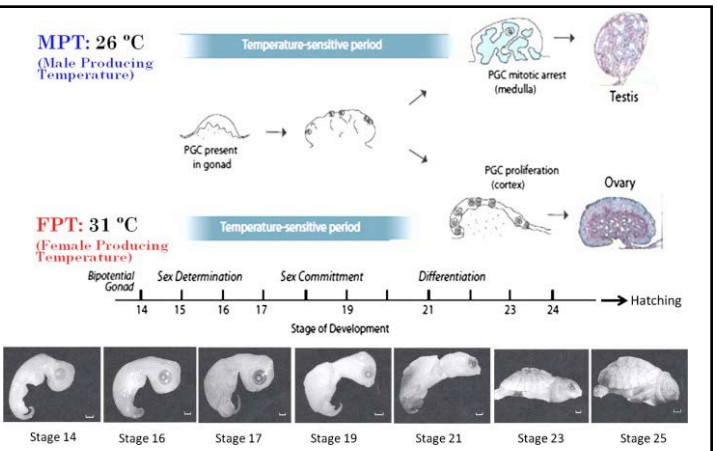
Cocrystal structure of the quaternary DMRT1–DNA complex.
Three α-helices intrude into the major groove (base readout), two of them (purple and green) in the same region and the third α-helix (cyan) a helical turn away. The zinc-binding modules bind the minor groove (shape readout). In addition, protein-protein contacts are formed between the three DM domains (cooperativity).



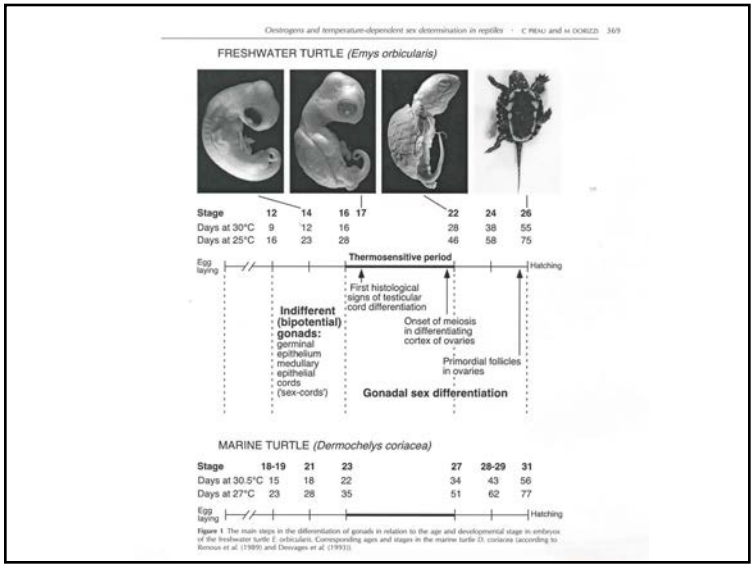
Female-to-male sex reversal in mice caused by transgenic overexpression of Dmrt1. Zhao L, Svingen T, Ng ET, Koopman P.Development. 2015 Mar 15;142(6):1083-8.

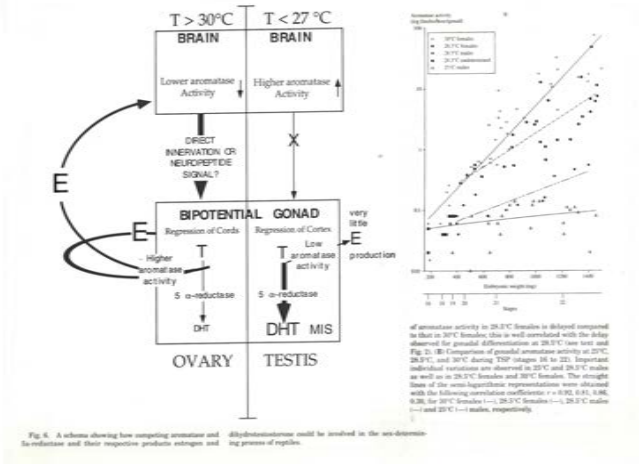




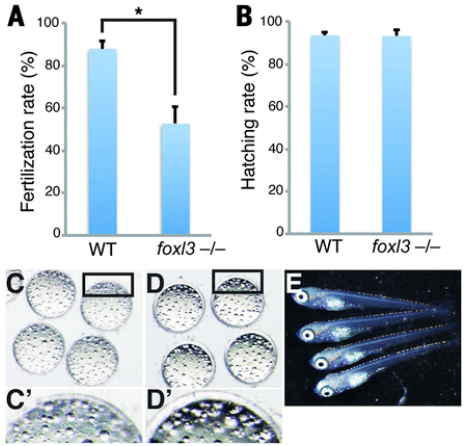


Top: temperature-dependent sex determination and corresponding embryonic developmental stages in the red-eared slider turtle (Trachemys scripta). Bipotential embryonic gonads contain primordial germ cells (PGC) and differentiate to either ovary (at 31 ˚ C, FPT) or testis (at 26 ˚ C, MPT) (From Ramsey et al., 2007). Bottom: embryonic development from stage 14 to stage 25 in the red-eared slider turtle (adopted from Greenbaum, 2002).





Sex determination. foxl3 is a germ cell-intrinsic factor involved in sperm-egg fate decision in medaka.Nishimura T, et al. Science. 2015 Jul 17;349(6245):328-31.



***foxl3*$^{-/-}$ XX gonads produce functional sperm.**

(A) Fertilization rate of artificial insemination by using sperm from wild-type testes (n = 3) and *foxl3*$^{-/-}$ ovaries (n = 8). In each artificial insemination, one gonad was used. (B) Hatching rate of the fertilized eggs in (A). (C) Unfertilized eggs. (D) Eggs fertilized by sperm derived from *foxl3*$^{-/-}$ ovaries. The activated egg membrane is shown in (D'). (E) Embryos hatched from the eggs in (D). Statistics by two-tailed Student's t test; *P < 0.05.

The evolutionary dynamics of major regulators for sexual development among Hymenoptera species. Biewer M, Schlesinger F, Hasselmann M. Front Genet. 2015 Apr 10;6:124

Overview of the evolutionary relationship of the fem gene and copies (fem1, csd, tra) in social insect species.

Gene duplication-coevolutionary model for sex determining key regulators in bees.

Table 1. Known master sex-determining genes in vertebrates and insects, and their paralogs.

Species	Master sex determining gene	Sex-determining mechanisms	Gene paralog	Paralog function	Reference
mammals	*Sry*	sex-determining Y	*Sox3*	HMG-box transcription factor	[77]
chicken (*Gallus gallus*)	*dmrt1*	dose-dependent Z	-	SD pathway transcription factor	[12]
African clawed frog (*Xenopus laevis*)	*dmW*	sex-determining W	*dmrt1*	SD pathway transcription factor	[13]
medaka (*Oryzias latipes*)	*dmrt1Y*	sex-determining Y	*dmrt1*	SD pathway transcription factor	[78,79]
(*Oryzias luzonensis*)	*gsdfY*	sex-determining Y	*gsdf*	secretory protein in SD pathway	[80]
Patagonian pejerrey (*Odontesthes hatcheri*)	*amhY*	sex-determining Y	*amh*	anti-Mullerian hormone	[155]
rainbow trout (*Oncorhynchus mykiss*)	*sdY*	sex-determining Y	*Irf9*	interferon regulatory factor	[82]
tiger pufferfish (*Takifugu rubripes*)	*amhr2*	dose-dependent X	*amhr*	anti-Mullerian hormone receptor	[156]
smooth tongue sole (*Cynoglossus semilaevis*)	*dmrt1*	dose-dependent Z	-	SD pathway	[14]
fruit flies (*Drosophila*)	*Sxl*	dose-dependent X	*CG3056*	mRNA splicing, non-sex specific	[83,84]
housefly (*Musca domestica*)	*F*	sex-determining W	*tra*	SD pathway switch splice factor	[17]
silkworm (*Bombyx mori*)	*Fem*	sex-determining W	-	piRNA	[85]
honeybee (*Apis mellifera*)	*csd*	haplodiploid	*tra*	SD pathway switch splice factor	[16]
wasp (*Nasonia vitripennis*)	*Nvtra*	haplodiploid	*tra*	SD pathway switch splice factor	[15]

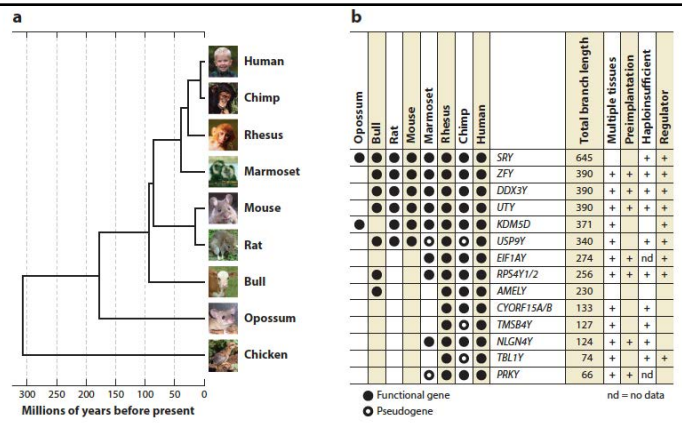
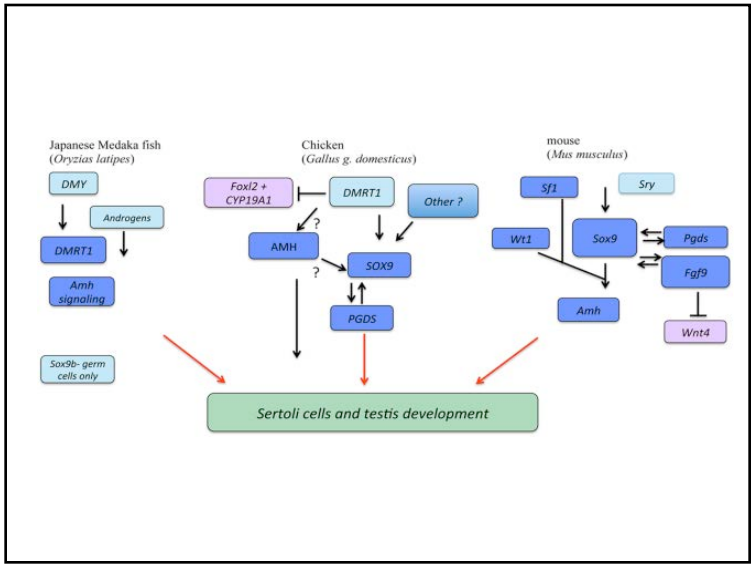
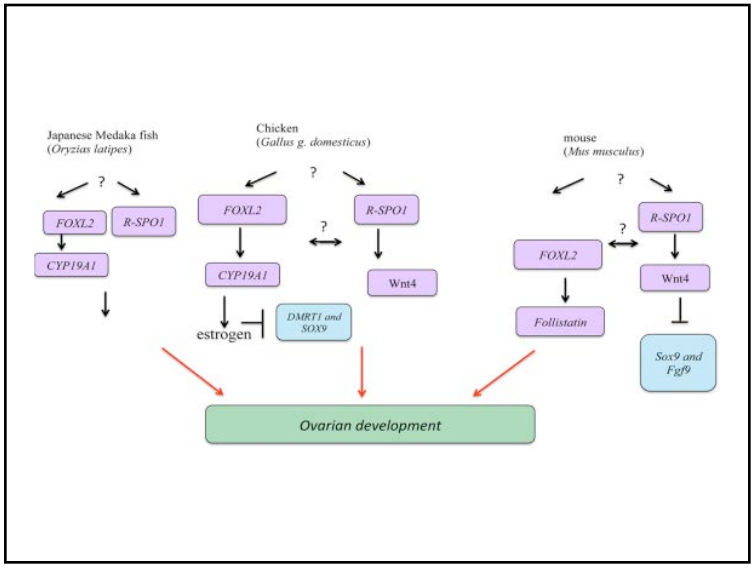
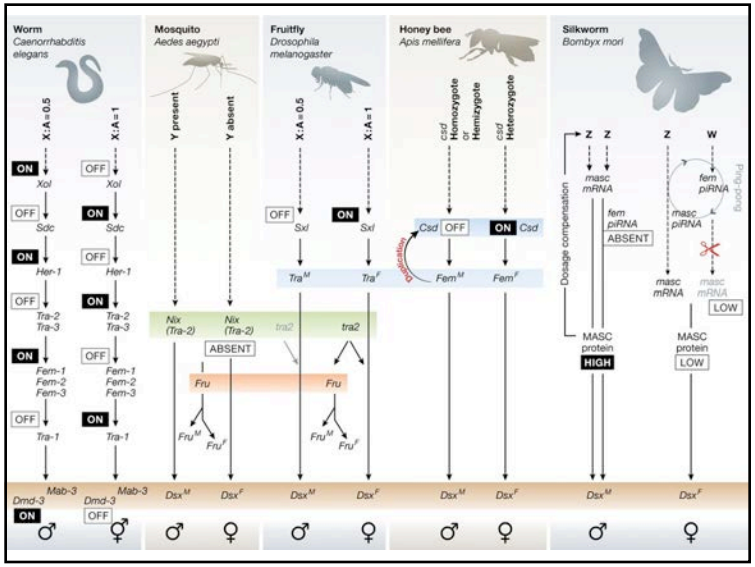


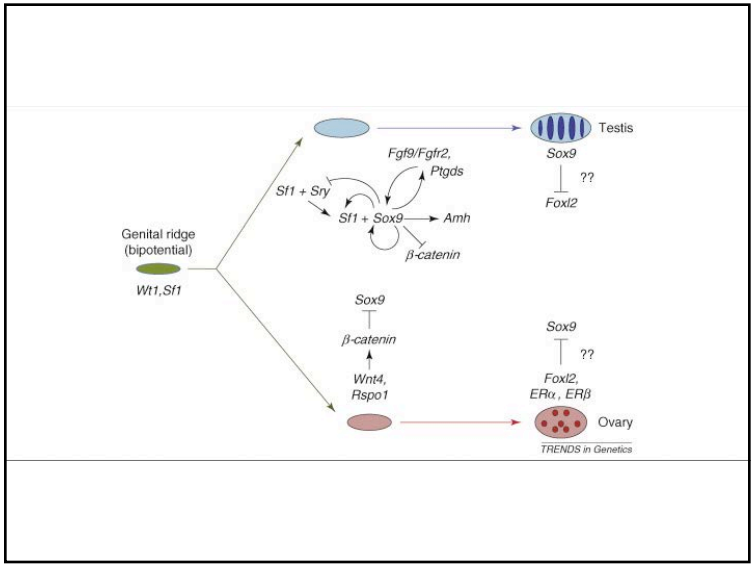
Figure 6

Comparative Y-chromosome sequencing reveals long life spans and functional coherence of human MSY (male-specific region of the Y chromosome) single-copy genes. (*a*) Species tree indicating evolutionary relationships between the eight mammals with SHIMS (single-haplotype iterative mapping and sequencing)-sequenced ancestral MSY sequences. Chicken is shown as an outgroup. Branch lengths are drawn to scale. (*b*) Species distribution and features (expression breadth across tissues, expression in preimplantation embryos, haploinsufficiency probability, and predicted regulatory function) of human MSY single-copy ancestral genes, which are ranked according to evolutionary longevity. Total branch length for a given gene is the sum of branch lengths for each species possessing an intact homolog of that gene.


Japanese Medaka fish
(Oryzias latipes)
DMY
Androgens
DMRT1
Amh signaling
Sox9b- germ cells only
Chicken
(Gallus g. domesticus)
Foxl2 + CYP19A1
DMRT1
Other ?
AMH
SOX9
PGDS
mouse
(Mus musculus)
Sf1
Sry
Wt1
Sox9
Pgds
Fgf9
Amh
Wnt4
Sertoli cells and testis development


Japanese Medaka fish
(Oryzias latipes)
FOXL2
R-SPO1
CYP19A1
Chicken
(Gallus g. domesticus)
FOXL2
R-SPO1
CYP19A1
Wnt4
estrogen
DMRT1 and SOX9
mouse
(Mus musculus)
FOXL2
R-SPO1
Wnt4
Follistatin
Sox9 and Fgf9
Ovarian development


Worm
Caenorrhabditis elegans
Mosquito
Aedes aegypti
Fruitfly
Drosophila melanogaster
Honey bee
Apis mellifera
Silkworm
Bombyx mori
X:A = 0.5
X:A = 1
Y present
Y absent
csd Hemizygote or Hemizygote
csd Heterozygote
Z Z
Z W
masc mRNA
fem piRNA
ABSENT
MASC protein
HIGH
LOW
Dosage compensation
Xol
Sdc
Her-1
Tra-2
Tra-3
Fem-1
Fem-2
Fem-3
Tra-1
Mab-3
Dmd-3
Nix (Tra-2)
Sxl
tra2
Fru
Csd
Dsx
Fem
Tra


Genital ridge
(bipotential)
Wt1,Sf1
Sf1 + Sry
Sf1 + Sox9
Fgf9/Fgfr2,
Ptgds
Amh
β-catenin
Testis
Sox9
??
Foxl2
Sox9
β-catenin
Wnt4,
Rspo1
Sox9
??
Foxl2,
ERα , ERβ
Ovary
TRENDS in Genetics