

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

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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 (*SFI*, *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 (haploinsufficiency). 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 program 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 referred 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 morphologically 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 continuously 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

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	<i>Xenopus laevis</i>	WZ	Dmrt1	Gene duplication	Transcription factor, key role in male sex determination and differentiation
Dmrt1bY	Medaka (<i>Oryzias latipes</i> , <i>O. curvinotus</i>)	XY	Dmrt1	Gene duplication	Transcription factor, key role in male sex determination and differentiation
SdY	Rainbow trout (<i>Oncorhynchus mykiss</i>)	XY	Irf9	Gene duplication	Interferon response factor, no gonadal function known
GsdfY	Luzon ricefish (<i>Oryzias luzonensis</i>)	XY	Gsdf	Allelic diversification	TGF- β factor, important role in fish gonad development
Sox3Y	Indian ricefish (<i>Oryzias dancena</i>)	XY	Sox3	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
amhY	Perjerrey (<i>Odontesthes hatcheri</i>)	XY	Amh	Gene duplication	Anti-Muellerian hormone, growth factor
amhr2Y	Fugu (<i>Takifugu rubripes</i>)	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 (<i>Cynoglossus semilaevis</i>)	WZ	Dmrt1	Allelic diversification	Transcription factor, key role in male sex determination and differentiation
GsdfY	Sablefish (<i>Anoplopoma fimbria</i>)	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

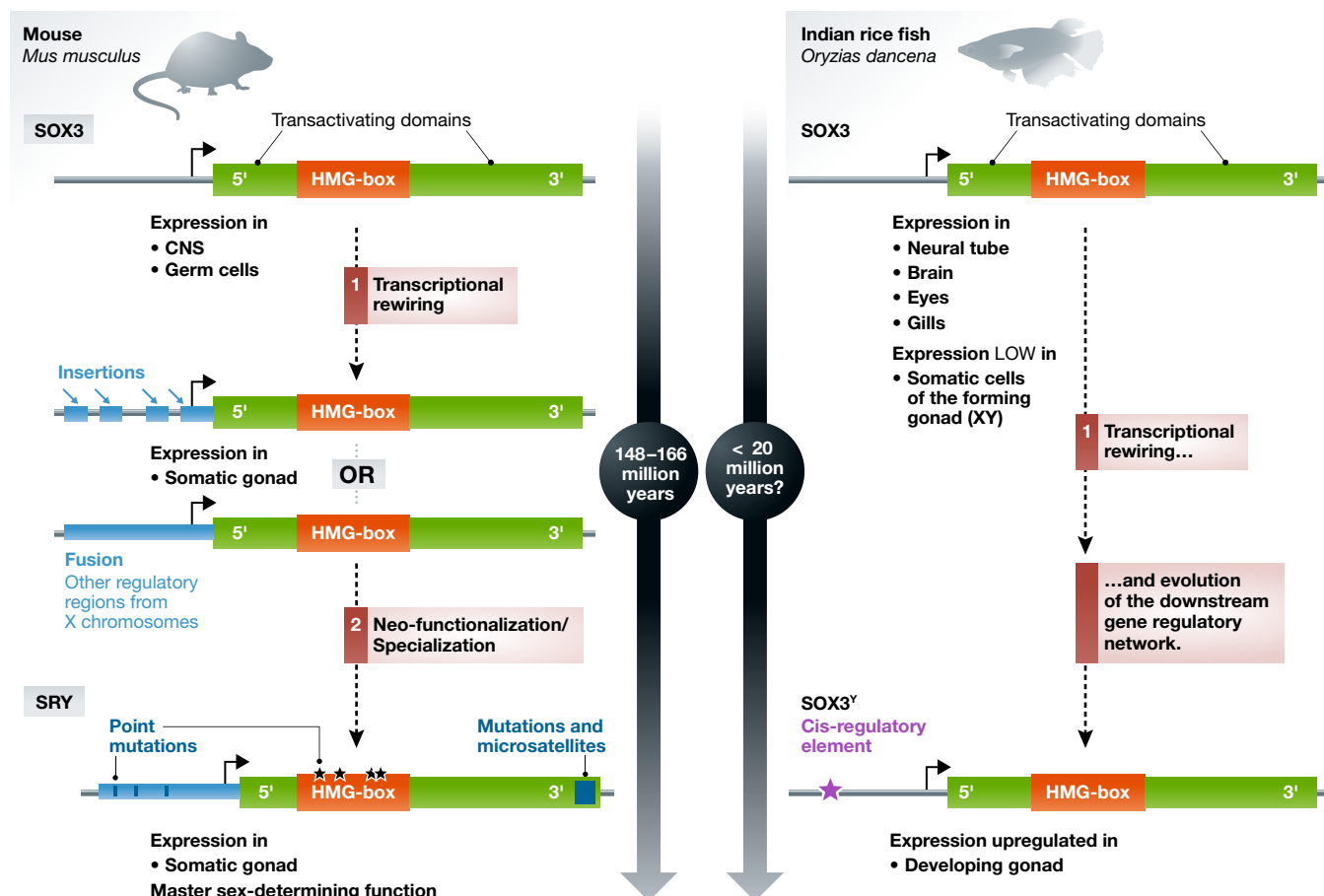


Figure 1. Independent evolution of *SOX3* genes toward a master sex-determining function in mice and Indian rice fish.

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

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 latipes*) (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. latipes* 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



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 (Gsd) 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

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

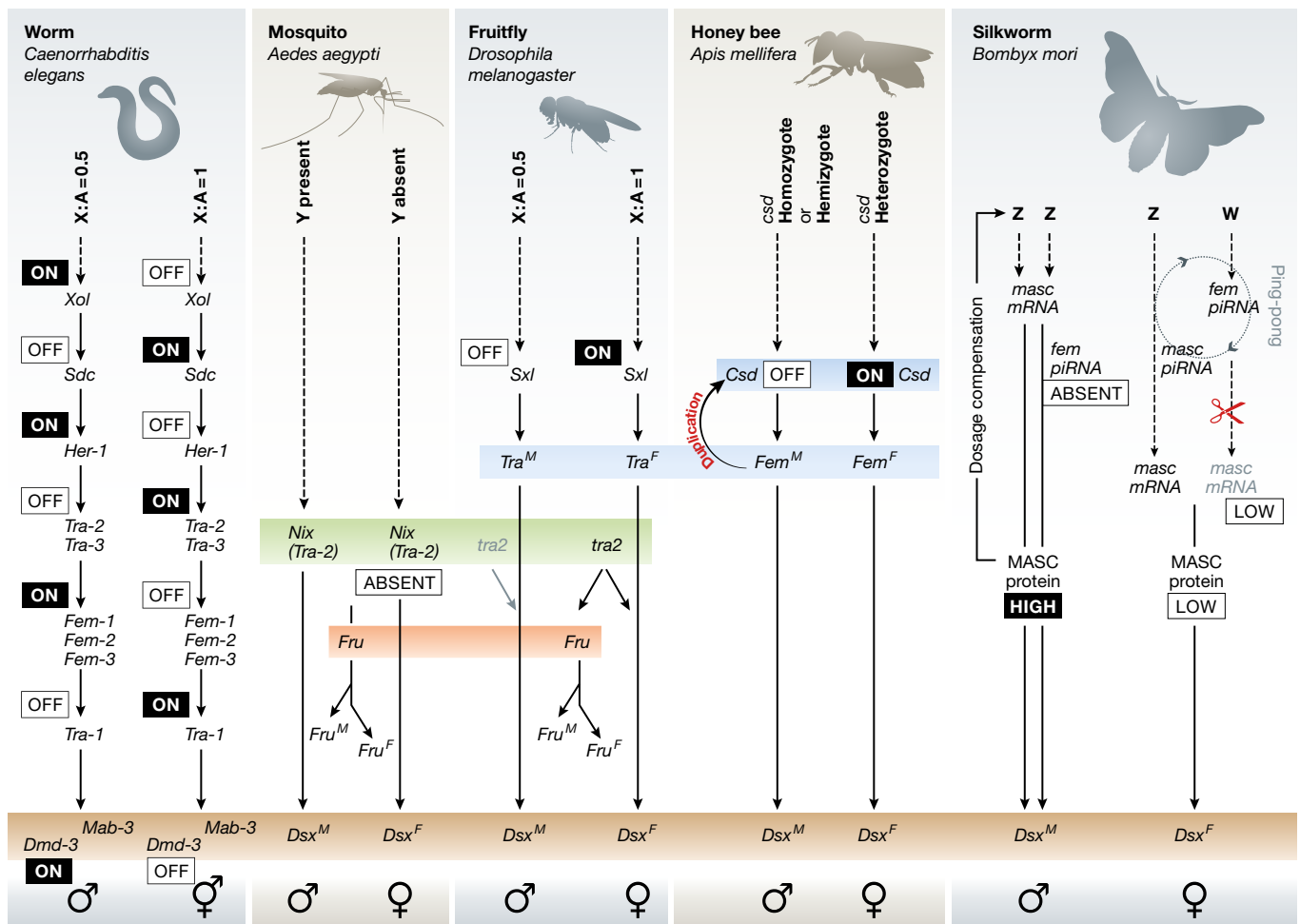


Figure 3. Sex-determining cascades in *C. elegans* and some insects.

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 mellifera*, leading to female development, and hemizyosity 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.

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, *Drosophila*, 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

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

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 lifelong 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.

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 lifelong 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 *lrf9* 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.

References

1. Graham P, Penn JK, Schedl P (2003) Masters change, slaves remain. *BioEssays* 25: 1–4
2. Haag ES, Doty AV (2005) Sex determination across evolution: connecting the dots. *PLoS Biol* 3: e21
3. Herpin A, Schartl M (2008) Regulatory putsches create new ways of determining sexual development. *EMBO Rep* 9: 966–968
4. Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* 351: 117–121
5. Lovell-Badge R, Robertson E (1990) XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development* 109: 635–646
6. Just W, Rau W, Vogel W, Akhverdian M, Fredga K, Graves JA, Lyapunova E (1995) Absence of Sry in species of the vole *Ellobius*. *Nat Genet* 11: 117–118
7. Soullier S, Hanni C, Catzeffis F, Berta P, Laudet V (1998) Male sex determination in the spiny rat *Tokudaia osimensis* (Rodentia: Muridae) is not Sry dependent. *Mamm Genome* 9: 590–592
8. Waters PD, Wallis MC, Marshall Graves JA (2007) Mammalian sex—Origin and evolution of the Y chromosome and SRY. *Semin Cell Dev Biol* 18: 389–400
9. Sato Y, Shinka T, Sakamoto K, Ewis AA, Nakahori Y (2010) The male-determining gene SRY is a hybrid of DGC8 and SOX3, and is regulated by the transcription factor CP2. *Mol Cell Biochem* 337: 267–275
10. Foster JW, Graves JA (1994) An SRY-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene. *Proc Natl Acad Sci USA* 91: 1927–1931
11. Graves JA (2006) Sex chromosome specialization and degeneration in mammals. *Cell* 124: 901–914
12. Stevanovic M, Lovell-Badge R, Collignon J, Goodfellow PN (1993) SOX3 is an X-linked gene related to SRY. *Hum Mol Genet* 2: 2013–2018
13. Caetano LC, Gennaro FG, Coelho K, Araujo FM, Vila RA, Araujo A, de Melo Bernardo A, Marcondes CR, Chuva de Sousa Lopes SM, Ramos ES (2014) Differential expression of the MHM region and of sex-determining-related genes during gonadal development in chicken embryos. *Genet Mol Res* 13: 838–849
14. Takehana Y, Matsuda M, Myosho T, Suster ML, Kawakami K, Shin IT, Kohara Y, Kuroki Y, Toyoda A, Fujiyama A et al (2014) Co-option of Sox3 as the male-determining factor on the Y chromosome in the fish *Oryzias latipes*. *Nat Commun* 5: 4157
15. Oshima Y, Naruse K, Nakamura Y, Nakamura M (2009) Sox3: a transcription factor for Cyp19 expression in the frog *Rana rugosa*. *Gene* 445: 38–48
16. Bowles J, Schepers G, Koopman P (2000) Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* 227: 239–255
17. Pask AJ, Harry JL, Renfree MB, Marshall Graves JA (2000) Absence of SOX3 in the developing marsupial gonad is not consistent with a conserved role in mammalian sex determination. *Genesis* 27: 145–152
18. Rizzoti K, Brunelli S, Carmignac D, Thomas PQ, Robinson IC, Lovell-Badge R (2004) SOX3 is required during the formation of the hypothalamo-pituitary axis. *Nat Genet* 36: 247–255
19. Sutton E, Hughes J, White S, Sekido R, Tan J, Arboleda V, Rogers N, Knowler K, Rowley L, Eyre H et al (2011) Identification of SOX3 as an XX male sex reversal gene in mice and humans. *J Clin Invest* 121: 328–341
20. Sekido R, Lovell-Badge R (2008) Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* 453: 930–934
21. Koyano S, Ito M, Takamatsu N, Takiguchi S, Shiba T (1997) The Xenopus Sox3 gene expressed in oocytes of early stages. *Gene* 188: 101–107
22. Miura I, Ohtani H, Nakamura M, Ichikawa Y, Saitoh K (1998) The origin and differentiation of the heteromorphic sex chromosomes Z, W, X, and Y in the frog *Rana rugosa*, inferred from the sequences of a sex-linked gene, ADP/ATP translocase. *Mol Biol Evol* 15: 1612–1619
23. Graves JA (2013) How to evolve new vertebrate sex determining genes. *Dev Dyn* 242: 354–359
24. Miura IET, Ohtani H, Uno Y, Nishida C, Matsuda Y, Graves JAM (2009) The W chromosome evolution and sex-linked gene expression in the frog *Rana rugosa*. In *Sex Chromosomes: Genetics, Abnormalities and Disorders*, Weingarten CN, Jefferson SE (eds), pp 123–140. Scottsdale, AZ: Nova Science Inc
25. Wilhelm D, Palmer S, Koopman P (2007) Sex determination and gonadal development in mammals. *Physiol Rev* 87: 1–28
26. Kashimada K, Koopman P (2010) Sry: the master switch in mammalian sex determination. *Development* 137: 3921–3930
27. Zhao L, Ng ET, Davidson TL, Longmuss E, Urschitz J, Elston M, Moisyadi S, Bowles J, Koopman P (2014) Structure-function analysis of mouse Sry reveals dual essential roles of the C-terminal polyglutamine tract in sex determination. *Proc Natl Acad Sci USA* 111: 11768–11773

28. Dubin RA, Ostrer H (1994) Sry is a transcriptional activator. *Mol Endocrinol* 8: 1182–1192
29. Lovell-Badge R, Canning C, Sekido R (2002) Sex-determining genes in mice: building pathways. *Novartis Found Symp* 244: 4–18; discussion 18–22, 35–42, 253–257
30. Pannetier M, Tilly G, Kocer A, Hudrisier M, Renault L, Chesnais N, Costa J, Le Provost F, Vaiman D, Vilotte JL *et al* (2006) Goat SRY induces testis development in XX transgenic mice. *FEBS Lett* 580: 3715–3720
31. Chen YS, Racca JD, Sequeira PW, Phillips NB, Weiss MA (2013) Microsatellite-encoded domain in rodent Sry functions as a genetic capacitor to enable the rapid evolution of biological novelty. *Proc Natl Acad Sci USA* 110: E3061–E3070
32. Zhao L, Koopman P (2012) SRY protein function in sex determination: thinking outside the box. *Chromosome Res* 20: 153–162
33. Herpin A, Schartl M (2011) Vertebrate sex determination: questioning the hierarchy. *FEBS J* 278: 1001
34. Herpin A, Schartl M (2011) Dmrt1 genes at the crossroads: a wide-spread and central class of sexual development factors in fish. *FEBS J* 278: 1010–1019
35. Thoma EC, Wagner TU, Weber IP, Herpin A, Fischer A, Schartl M (2011) Ectopic expression of single transcription factors directs differentiation of a medaka spermatogonial cell line. *Stem Cells Dev* 20: 1425–1438
36. Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D (2000) Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14: 2587–2595
37. Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hirsch B, Hodgkin J, Zarkower D (1998) Evidence for evolutionary conservation of sex-determining genes. *Nature* 391: 691–695
38. Miller SW, Hayward DC, Bunch TA, Miller DJ, Ball EE, Bardwell VJ, Zarkower D, Brower DL (2003) A DM domain protein from a coral, *Acropora millepora*, homologous to proteins important for sex determination. *Evol Dev* 5: 251–258
39. Chong T, Collins JJ III, Brubacher JL, Zarkower D, Newmark PA (2013) A sex-specific transcription factor controls male identity in a simultaneous hermaphrodite. *Nat Commun* 4: 1814
40. Kato Y, Kobayashi K, Watanabe H, Iguchi T (2011) Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a Doublesex gene in the sex-determining pathway. *PLoS Genet* 7: e1001345
41. Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A *et al* (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99: 11778–11783
42. Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N *et al* (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* 417: 559–563
43. Veitia R, Nunes M, Brauner R, Doco-Fenzy M, Joanny-Flinois O, Jaubert F, Lortat-Jacob S, Fellous M, McElreavey K (1997) Deletions of distal 9p associated with 46, XY male to female sex reversal: definition of the breakpoints at 9p23.3–p24.1. *Genomics* 41: 271–274
44. Chen S, Zhang G, Shao C, Huang Q, Liu G, Zhang P, Song W, An N, Chalopin D, Volff JN *et al* (2014) Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nat Genet* 46: 253–260
45. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH (2009) The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature* 461: 267–271
46. Yoshimoto S, Okada E, Umemoto H, Tamura K, Uno Y, Nishida-Umehara C, Matsuda Y, Takamatsu N, Shiba T, Ito M (2008) A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proc Natl Acad Sci USA* 105: 2469–2474
47. Kim S, Bardwell VJ, Zarkower D (2007) Cell type-autonomous and non-autonomous requirements for Dmrt1 in postnatal testis differentiation. *Dev Biol* 307: 314–327
48. Matson CK, Murphy MW, Griswold MD, Yoshida S, Bardwell VJ, Zarkower D (2010) The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. *Dev Cell* 19: 612–624
49. Matson CK, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D (2011) DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* 476: 101–104
50. Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier AC, Klugmann C, Klasen C, Holter NI *et al* (2009) Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 139: 1130–1142
51. Boulanger L, Pannetier M, Gall L, Allais-Bonnet A, Elzaïat M, Le Bourhis D, Daniel N, Richard C, Cotinot C, Ghyselinck NB *et al* (2014) FOXL2 is a female sex-determining gene in the goat. *Curr Biol* 24: 404–408
52. Georges A, Auguste A, Bessiere L, Vanet A, Todeschini AL, Veitia RA (2014) FOXL2: a central transcription factor of the ovary. *J Mol Endocrinol* 52: R17–R33
53. Lindeman RE, Gearhart MD, Minkina A, Krentz AD, Bardwell VJ, Zarkower D (2015) Sexual cell-fate reprogramming in the ovary by DMRT1. *Curr Biol* 25: 764–771
54. Masuyama H, Yamada M, Kamei Y, Fujiwara-Ishikawa T, Todo T, Nagahama Y, Matsuda M (2012) Dmrt1 mutation causes a male-to-female sex reversal after the sex determination by Dmy in the medaka. *Chromosome Res* 20: 163–176
55. Krentz AD, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D (2011) DMRT1 promotes oogenesis by transcriptional activation of Stra8 in the mammalian fetal ovary. *Dev Biol* 356: 63–70
56. Herpin A, Braasch I, Kraeussling M, Schmidt C, Thoma EC, Nakamura S, Tanaka M, Schartl M (2010) Transcriptional rewiring of the sex determining dmrt1 gene duplicate by transposable elements. *PLoS Genet* 6: e1000844
57. Herpin A, Schindler D, Kraiss A, Hornung U, Winkler C, Schartl M (2007) Inhibition of primordial germ cell proliferation by the medaka male determining gene Dmrt I bY. *BMC Dev Biol* 7: 99
58. Herpin A, Schartl M (2011) Sex determination: switch and suppress. *Curr Biol* 21: R656–R659
59. Matson CK, Zarkower D (2012) Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. *Nat Rev Genet* 13: 163–174
60. Cutting A, Chue J, Smith CA (2013) Just how conserved is vertebrate sex determination? *Dev Dyn* 242: 380–387
61. Nakamura S, Watakabe I, Nishimura T, Picard JY, Toyoda A, Taniguchi Y, di Clemente N, Tanaka M (2012) Hyperproliferation of mitotically active germ cells due to defective anti-Müllerian hormone signaling mediates sex reversal in medaka. *Development* 139: 2283–2287
62. Hattori RS, Murai Y, Oura M, Masuda S, Majhi SK, Sakamoto T, Fernandino JJ, Somoza GM, Yokota M, Strussmann CA (2012) A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. *Proc Natl Acad Sci USA* 109: 2955–2959

63. Kamiya T, Kai W, Tasumi S, Oka A, Matsunaga T, Mizuno N, Fujita M, Suetake H, Suzuki S, Hosoya S *et al* (2012) A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (fugu). *PLoS Genet* 8: e1002798
64. Crespo B, Gomez A, Mazon MJ, Carrillo M, Zanuy S (2013) Isolation and characterization of Ff1 and Gsdf family genes in European sea bass and identification of early gonadal markers of precocious puberty in males. *Gen Comp Endocrinol* 191: 155–167
65. Gautier A, Le Gac F, Lareyre JJ (2011) The gsdf gene locus harbors evolutionary conserved and clustered genes preferentially expressed in fish previtellogenic oocytes. *Gene* 472: 7–17
66. Horiguchi R, Nozu R, Hirai T, Kobayashi Y, Nagahama Y, Nakamura M (2013) Characterization of gonadal soma-derived factor expression during sex change in the protogynous wrasse, *Halichoeres trimaculatus*. *Dev Dyn* 242: 388–399
67. Sawatari E, Shikina S, Takeuchi T, Yoshizaki G (2007) A novel transforming growth factor-beta superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). *Dev Biol* 301: 266–275
68. Shibata Y, Paul-Prasanth B, Suzuki A, Usami T, Nakamoto M, Matsuda M, Nagahama Y (2010) Expression of gonadal soma derived factor (GSDF) is spatially and temporally correlated with early testicular differentiation in medaka. *Gene Expr Patterns* 10: 283–289
69. Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, Fujiyama A, Naruse K, Hamaguchi S, Sakaizumi M (2012) Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. *Genetics* 191: 163–170
70. Rondeau EB, Messmer AM, Sanderson DS, Jantzen SG, von Schalburg KR, Minkley DR, Leong JS, Macdonald GM, Davidsen AE, Parker WA *et al* (2013) Genomics of sablefish (*Anoplopoma fimbria*): expressed genes, mitochondrial phylogeny, linkage map and identification of a putative sex gene. *BMC Genom* 14: 452
71. Cho S, Huang ZY, Zhang J (2007) Sex-specific splicing of the honeybee doublesex gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. *Genetics* 177: 1733–1741
72. Bopp D, Saccone G, Beye M (2014) Sex determination in insects: variations on a common theme. *Sex Dev* 8: 20–28
73. Williams TM, Carroll SB (2009) Genetic and molecular insights into the development and evolution of sexual dimorphism. *Nat Rev Genet* 10: 797–804
74. Cline TW, Meyer BJ (1996) Vive la difference: males versus females in flies versus worms. *Annu Rev Genet* 30: 637–702
75. Scali C, Catteruccia F, Li Q, Crisanti A (2005) Identification of sex-specific transcripts of the *Anopheles gambiae* doublesex gene. *J Exp Biol* 208: 3701–3709
76. Lagos D, Koukidou M, Savakis C, Komitopoulou K (2007) The transformer gene in *Bactrocera oleae*: the genetic switch that determines its sex fate. *Insect Mol Biol* 16: 221–230
77. Geuverink E, Beukeboom LW (2014) Phylogenetic distribution and evolutionary dynamics of the sex determination genes doublesex and transformer in insects. *Sex Dev* 8: 38–49
78. Suzuki MG, Ohbayashi F, Mita K, Shimada T (2001) The mechanism of sex-specific splicing at the doublesex gene is different between *Drosophila melanogaster* and *Bombyx mori*. *Insect Biochem Mol Biol* 31: 1201–1211
79. Verhulst EC, Beukeboom LW, van de Zande L (2010) Maternal control of haplodiploid sex determination in the wasp *Nasonia*. *Science* 328: 620–623
80. Hediger M, Henggeler C, Meier N, Perez R, Saccone G, Bopp D (2010) Molecular characterization of the key switch F provides a basis for understanding the rapid divergence of the sex-determining pathway in the housefly. *Genetics* 184: 155–170
81. Beye M, Hasselmann M, Fondrk MK, Page RE, Omholt SW (2003) The gene csd is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* 114: 419–429
82. Hasselmann M, Gempe T, Schiott M, Nunes-Silva CG, Otte M, Beye M (2008) Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. *Nature* 454: 519–522
83. Beye M (2004) The dice of fate: the csd gene and how its allelic composition regulates sexual development in the honey bee, *Apis mellifera*. *BioEssays* 26: 1131–1139
84. Hall AB, Basu S, Jiang X, Qi Y, Timoshevskiy VA, Biedler JK, Sharakhova MV, Elahi R, Anderson MA, Chen XG *et al* (2015) A male-determining factor in the mosquito *Aedes aegypti*. *Science* 348: 1268–1270
85. Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp C, Cabau C, Bouchez O, Fostier A, Guiguen Y (2012) An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. *Curr Biol* 22: 1423–1428
86. Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H, Arai Y, Ishihara G, Kawaoka S, Sugano S, Shimada T *et al* (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* 509: 633–636
87. Herpin A, Adolphi MC, Nicol B, Hinzmann M, Schmidt C, Klughammer J, Engel M, Tanaka M, Guiguen Y, Schartl M (2013) Divergent expression regulation of gonad development genes in medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination. *Mol Biol Evol* 30: 2328–2346
88. Canto P, Soderlund D, Reyes E, Mendez JP (2004) Mutations in the desert hedgehog (DHH) gene in patients with 46, XY complete pure gonadal dysgenesis. *J Clin Endocrinol Metab* 89: 4480–4483
89. Clark AM, Garland KK, Russell LD (2000) Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. *Biol Reprod* 63: 1825–1838
90. Pierucci-Alves F, Clark AM, Russell LD (2001) A developmental study of the Desert hedgehog-null mouse testis. *Biol Reprod* 65: 1392–1402
91. Zhang Y, Li F, Sun D, Liu J, Liu N, Yu Q (2011) Molecular analysis shows differential expression of R-spondin1 in zebrafish (*Danio rerio*) gonads. *Mol Biol Rep* 38: 275–282
92. Chassot AA, Bradford ST, Auguste A, Gregoire EP, Pailhoux E, de Rooij DG, Schedl A, Chaboissier MC (2012) WNT4 and RSP01 together are required for cell proliferation in the early mouse gonad. *Development* 139: 4461–4472
93. Chadi S, Buscara L, Pechoux C, Costa J, Laubier J, Chaboissier MC, Pailhoux E, Vilotte JL, Chanut E, Le Provost F (2009) R-spondin1 is required for normal epithelial morphogenesis during mammary gland development. *Biochem Biophys Res Commun* 390: 1040–1043
94. Kashimada K, Pelosi E, Chen H, Schlessinger D, Wilhelm D, Koopman P (2011) FOXL2 and BMP2 act cooperatively to regulate follistatin gene expression during ovarian development. *Endocrinology* 152: 272–280
95. Meinhardt A, O'Bryan MK, McFarlane JR, Loveland KL, Mallidis C, Foulds LM, Phillips DJ, de Kretser DM (1998) Localization of follistatin in the rat testis. *J Reprod Fertil* 112: 233–241
96. Nakamura S, Watakabe I, Nishimura T, Toyoda A, Taniguchi Y, Tanaka M (2012) Analysis of medaka *sox9* orthologue reveals a conserved role in germ cell maintenance. *PLoS ONE* 7: e29982

97. Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu Y, Takeda H, Araki K, Morohashi K, Ozato K (2002) Sox9 in a teleost fish, medaka (*Oryzias latipes*): evidence for diversified function of Sox9 in gonad differentiation. *Mol Reprod Dev* 63: 5–16
98. Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM (2001) Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* 104: 875–889
99. Forconi M, Canapa A, Barucca M, Biscotti MA, Capriglione T, Buonocore F, Fausto AM, Makapedua DM, Pallavicini A, Gerdol M *et al* (2013) Characterization of sex determination and sex differentiation genes in *Latimeria*. *PLoS ONE* 8: e56006
100. Halder G, Callaerts P, Gehring WJ (1995) Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* 267: 1788–1792
101. Wilkins AS (1995) Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. *BioEssays* 17: 71–77
102. Gempe T, Beye M (2011) Function and evolution of sex determination mechanisms, genes and pathways in insects. *BioEssays* 33: 52–60
103. Wilson CA, High SK, McCluskey BM, Amores A, Yan YL, Titus TA, Anderson JL, Batzel P, Carvan MJ III, Schartl M *et al* (2014) Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. *Genetics* 198: 1291–1308
104. Nanda I, Hornung U, Kondo M, Schmid M, Schartl M (2003) Common spontaneous sex-reversed XX males of the medaka *Oryzias latipes*. *Genetics* 163: 245–251
105. Beukeboom LW, Perrin N (2014) *The Evolution of Sex Determination*. New York: Oxford University Press
106. Bull JJ, Charnov EL (1977) Changes in the heterogametic mechanism of sex determination. *Heredity* 39: 1–14
107. Charlesworth B, Charlesworth D (2000) The degeneration of Y chromosomes. *Philos Trans R Soc Lond B Biol Sci* 355: 1563–1572
108. Blaser O, Grossen C, Neuenschwander S, Perrin N (2013) Sex-chromosome turnovers induced by deleterious mutation load. *Evolution* 67: 635–645
109. van Doorn GS, Kirkpatrick M (2007) Turnover of sex chromosomes induced by sexual conflict. *Nature* 449: 909–912
110. Lynch M (2007) The evolution of genetic networks by non-adaptive processes. *Nat Rev Genet* 8: 803–813
111. DeFalco T, Capel B (2009) Gonad morphogenesis in vertebrates: divergent means to a convergent end. *Annu Rev Cell Dev Biol* 25: 457–482