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The replaceable master of sex determination: bottom-up hypothesis revisited

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Abstract

Different group of vertebrates and invertebrates demonstrate an amazing diversity of gene regulations not only at the top but also at the bottom of the sex determination genetic network. As early as 1995 based on emerging findings in *Drosophila melanogaster* and *C. elegans* Wilkins suggested that the evolution of the sex determination pathway evolved from the bottom to the top of the hierarchy. Based on our current knowledge, this review revisits the "bottom-up" hypothesis and applies its logic to vertebrates. The basic operation of the determination network is through the dynamics of the opposing male and female pathways together with a persistent need to maintain the sexual identity of the cells of the gonad up to the reproductive stage in adults. The sex-determining trigger circumstantially acts from outside the genetic network, but the regulatory network is not built around it as a main node, thus maintaining the genetic structure of the network. New sex promoting genes arise either through allelic diversification or gene duplication and act specially at the sex determination period, without integration into the complete network. Due to this peripheral position the new regulator is not an indispensable component of the sex determining network and can be easily replaced.

Introduction

Sex determination is defined as the process by which the undifferentiated bipotential gonad becomes committed to testis or ovary development. Classically this decision was described to be triggered by genetic factors, named genotypic sex determination (GSD), or environmental factors, defined as environmental sex determination (ESD) (1). The turning point to understand the genetic mechanism of sex determination came in 1905 with its correlation to the sex chromosomes in insects (2). In the early 80s, the understanding of the molecular processes of sex chromosome triggering a complex sex determination pathway was mainly stablished in the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* (3, 4). Only in 1991, the *Sry* gene, located on the Y chromosome, was shown to be necessary and sufficient to induce testis differentiation and subsequently male development, being the main regulator of sex in mammals (5). However, *Sry* was not found in other taxa outside marsupials and placental mammals, and several studies demonstrated that a great diversity of master sex determining genes evolved independently in different animal groups or even closely related species (1).

In 2002, based on two species of Diptera (*Drosophila melanogaster* and *Ceratitis capitata*), it was suggested that the "*top of the regulatory hierarchy can change dramatically as new species and genus evolve, while the slave genes at the bottom of the hierarchy remain the same, carrying out essentially identical functions from one species to the next*". This interpretation of the evolution of sex determination cascade became known as the "masters change, slaves remain" hypothesis (6). This was accepted by the scientific community almost without critique for the following 10 years, mainly due to the apparent conservation of the downstream regulator genes in different animal groups, and due to the lack of understanding of the role, expression and interaction between the genes inside the cascade.

During this period, with the accessibility of genome sequencing technologies, the quest for finding the sex chromosomes and the master sex determination genes in several GSD species started (7). At the same time, transcriptome and proteomics data challenged the idea of the highly conserved downstream regulators. In 2015, a broader comparison between different vertebrate species demonstrated that the downstream network presents subtle but functionally relevant differences in expression pattern and function. It was then hypothesized that "*those changes may be due to the impact of the new upstream regulator*". In parallel, some sex-related genes were identified as potential sex determination genes due to their recurrent appearance as master regulators in different species (e.g. TGF-β/Amh, Sox and DM domain factors) (8).

Interestingly, both reviews from 2002 and 2015 acknowledge the 1995 hypothesis by Adam Wilkins for the evolution of genetic sex determination pathways (6, 8). Wilkins compared the genetic pathways of *D. melanogaster* and *C. elegans* and suggested that "*the genetic pathway evolved in reverse order from the final step in the hierarchy up to the first*" (9). Even though the recent understanding about the sex determination pathway partially agree with the "bottom-to-top" evolution of the cascade, important statements used for the formulation of Wilkins' hypothesis were neglected. Here we revisit the "moving up the hierarchy" hypothesis in light of the current knowledge of the mechanisms of sex determination and evolution of genetic networks.

The "moving up the hierarchy" hypothesis.

Conceptually, there is a difference between sex determination and sex differentiation. The first is the initial event which makes the decision if the bipotential anlage goes into the male or female pathway. It is a determination of developmental fate without the determined cell showing a distinct phenotype different from the undetermined state. Sex differentiation describes the subsequent molecular, cellular and physiological changes that lead to the final stages of cellular and organ development and the realization of the developmental program for a functional testis or ovary (1, 10). Admittedly, it is often difficult to draw a clear border between both processes.

Comparing the sex determination genetic cascades of *C. elegans* and *Drosophila melanogaster*, Wilkins observed two common organizational features. The first is that the trigger of the cascade is the ratio between the X and the autosome set of chromosomes (X:A). In both species, when X:A is low, the gonad develops as testis, while a high X:A rate leads to female formation in *Drosophila melanogaster* and to hermaphrodite in *C. elegans*. The second feature is that the trigger "*activates one of two possible sequences of autosomal genetic switches, with alternative outcomes"*. The two different sexes have **opposite** "settings" of the switch at each step in both pathways, with male either having high activity and female low, or *vice versa* (9)(See in (8)).

In such situations, the molecular mechanics of the switches is easy to imagine, due to an apparent linear structure of the sex cascades. In *Drosophila melanogaster*, the high X:A ratio (female) enables the transcription of the *Sex lethal* gene (*Sxl*). Its encoded protein (SXL) promotes the female-specific splicing of *Transformer* (*Tra*). TRA in female (TRA^F) is a functional protein and forms a complex with TRA-2, which acts by favoring the female-specific splicing of the *Doublesex* (*Dsx*) gene, which then activates the downstream female network. In male (low X:A ratio), the male-specific transcript of *Tra* is expressed by "default" and translates

to a non-functional splice variant (TRA^M). Consequently, the absence of active TRA leads to the expression of the male-specific splicoform of *Dsx* gene and the production of male-type DSX (DSX^M), which in turn leads the bipotential gonad to develop as testis (11). *C. elegans*, has a different system of switches that, unlike Drosophila *melanogaster*, represents a negative regulatory cascade. Here, "*the high activity at any one step reflects the inhibition of the activity of the previous step in the hierarchy*". These sequences of intervening flip-flop switches lead to the activation of important downstream genes such as *Mab-3*, the homolog of *Drosophila melanogaster Dsx*, which is crucial for testis development (12) (See in (8)). It has to be highlighted that the pathway in both *Drosophila melanogaster* and *C. elegans*, has a dual outcome: to trigger important downstream activators and at the same time to repress the opposite pathway.

Based on this knowledge Wilkins was not only trying to explain the rise of a new sex determination gene, but also to describe the probable evolutionary history of the sex determination pathways. Wilkins proposed that sex determination pathways "*evolved by successive selective steps, in which each step had, at some point, a positive selective value of its own*". He discussed that there is an optimal range of sex ratios for any given population, and an event that skews that range towards any sex will promote the selection of a gene for the opposite sex to act at the top of the hierarchy. Hence, after successive steps of selection, the most upstream gene, leading to the most stable 1:1 sex ratio, would be fixed on the sex chromosome according to Fisher's postulate (13). Thus, the sex determination pathways would have evolved *backwards*, from the most downstream gene to the most upstream (e.g. sex determining genes on the sex chromosome) (9). In the case of *C. elegans* and *Drosophila melanogaster*, due to the linear structure of the pathways, this hypothesis seemed plausible. However, vertebrates have a much more complex and diverse genetic structure of activation and repression, making it difficult to track the connections of the downstream genes of the cascade. Yet, the background information that permitted Wilkins to draw his hypothesis can be useful to explain the diversity in gene expression of sex-related genes in vertebrates, and the mechanisms through which a new sex-determining trigger can arise and fix in the population.

What is the sex determination trigger controlling?

The bipotential gonad. The initial and crucial step necessary to discuss the evolution of the sex determination pathway is to define what exactly the genes in the top of the hierarchy are controlling. It is important to note that the activation of the master sex determination gene in all known cases starts after the genital ridge is formed, but its expression timing varies between

or even within species, called the "sex determination window" (14). We can take mice as example to describe the bipotential gonad prior to sex determination. The master activator, *Sry* is expressed in XY individuals at around embryonic day (E) 11.0, reaches its peak of expression at E11.5, and vanishes shortly after E.12.5. However, the formation of the genital ridge begins on the ventral surface of the mesonephros (intermediate mesoderm) as paired thickenings of the celomic epithelial layer already at around E9.5. The critical molecule responsible for the development of gonadal precursor cells in both males and females is the nuclear receptor *Nr5a1* (also known as *Ad4BP*/*Sf1*) (15). The precise mechanism underlying the restricted upregulation of *Nr5a1* in the progenitor cells is not completely elucidated. Genes like *Gata4, Six1* and *Six4* are known to be important for initiation and/or maintenance of high *Nr5a1* expression in the progenitor cells, but the upstream regulators of those genes remain unknown (16). *Gata*4-deficient mice fail to form the genital ridge in both XX and XY fetuses prior to sex determination (17). Nr5a1 was suggested to be important in the transactivation of *Sry* and other important testis regulators as *Sox9* and *Amh*. At later stages (E12.5), after the gonad determination period, the expression of *Nr5a1* persists in the testes but diminishes in ovaries. Together with *Nr5a1*, the genes *Lhx9*, *Wt1* and *Emx2* have been demonstrated to be required for growth and maintenance of the genital ridge. Full knockout mice for *Lhx9*, *Nr5a1*, *Wt1*, and *Emx2* genes develop the genital ridge. However, it later degenerates (17).

Hence, prior to the sex determination decision, the bipotential gonad is already formed and expresses the machinery necessary to respond to genetic or environmental factors to induce the female or male pathway.

The opposing pathways allow plasticity. While the fruit fly and *C. elegans* have the X:A ratio as a common feature, the genetic trigger is extremely diverse in other animals, varying from dominant or dosage sensitive male or female sex determiners on heterogametic chromosomes, with or without autosomal influence, to complex systems without a major sex chromosome, or with a diverse number of chromosomes that harbor several loci participating in the process (18-20). Despite this diversity, Wilkins pointed out as a common feature that the sex determination pathway is a constant opposition between male and female determining regulatory cascades, and if any of those steps fails to activate one sex, the bipotential gonad still has the capacity to trigger the pathway of the opposite sex, which will be discussed below. This may be the main principle to understand the evolution of the sex determination system.

In vertebrates, the sex determination pathway is a complex network of multiple regulatory interactions and less linear when compared to well-investigated invertebrates. The

identification of upstream regulators and the understanding of the molecular downstream network(s) revealed the outstanding plasticity of the system for new master sex regulators to evolve. In addition, despite an apparent conservation of the downstream regulators as components of the network, the genes described as sex-related factors present a great diversity in expression pattern in different vertebrate lineages (8). For instance in XY mammals, SRY binds directly to the TESCO sequence in the promoter of the *Sox9* gene, and thus coordinates testis development (21). In the absence of SOX9, the early gonad activates the female pathways and develops towards ovaries (22) (See in (8)). However, data from medaka fish, *Oryzias latipes*, and chicken suggest that *Sox9* does not have the same crucial role in early male development as in mammals (23, 24).

The *Dmrt1* gene is an extensively studied transcription factor, which is considered to be one of the main downstream switches in sex determination of metazoans (25). The DMRT (Doublesex and Mab-3 related transcription factor) proteins are characterized by a highly conserved zinc finger-like DNA-binding motif, named DM domain, and are homologous to the DSX and MAB-3 proteins of *Drosophila melanogaster* and *C. elegans* respectively. In mammals and most other vertebrates studied so far, *Dmrt1*, like its fly and worm homologues, has its crucial role at a downstream position, being required for male gonadal differentiation of somatic cells and germ cells (26). In birds *Dmrt1* is located on the Z chromosome and studies suggest that the dosage effect of *Dmrt1* regulates the sex determination, with males containing two copies (ZZ), leading to testis development and females (ZW) having only one (27). In medaka, a gene duplication of *dmrt1* on the Y chromosome (*dmrt1bY*) became the master sex regulator in *Oryzias latipes* and *Oryzias curvinotus*. In both species of medaka, the autosomal copy of *dmrt1*, designated *dmrt1a*, is still in a downstream position (28, 29). Some other members of the DMRT gene family are also involved in gonad development in males and females (30). In ovarian development, the *Foxl2* gene was suggested to be one of the main regulators in female development. *Foxl2* gene is predominantly expressed in ovaries of metazoans, and *Foxl2* was shown to be crucial in folliculogenesis in several vertebrates. It was demonstrated that Foxl2 directly activates the promoter of the aromatase gene (*Cyp19a1*), which is involved in the synthesis of estrogen (31). However, as for *Dmrt1*, the exact upstream mechanism which activates *Foxl2* is still unknown (See in (8)).

Here, we assume that Wilkins correctly concluded that a conserved feature between species is that the genes of the cascade are not only regulating the final downstream male or female switches. At the same time the genes of the cascade repress the opposite pathway. Currently, strong evidences indicate that the same can be extrapolated to vertebrates. Loss of *Dmrt1* in

several species led to reprogramming of the sexual fate of the somatic cells of the gonad, whereby Sertoli cells trans-differentiate to granulosa cells and *Foxl2* was upregulated (32, 33). The opposite was observed, when ectopic *Dmrt1* expression in genotypic females silenced *Foxl2* and the formation of testis structure was observed (34). Hence, it was suggested that some sex-related genes can have multiple roles in gonad development. Male-related genes as *Dmrt1*, *Amh*, *Sox9* for instance, are important for testis formation, but at the same time represses female factors, like *Foxl2*, and are also related to germ cell proliferation and survival (35). Most importantly, repression of female fate seems to be a life-long status, meaning that the adult gonad maintains the feature of the bipotential gonad to activate the genetic pathway of opposite sex when the cascade of the primary sex is absent or repressed. Hence, This suggests that repression or activation of sex determination pathways can be triggered by other factors than the original sex determination gene, e.g. by temperature, stress, hormones and pollutants (Fig. 1).

Making a new sex determining trigger.

The molecular evolution of the sex determination cascade in vertebrate resulted in a tremendous diversity of sex-determining triggers (genetic or environmental) in different taxa and species. There is a critical window of time when the master trigger can induce the sex determination network. As consequence, any genetic or environmental alteration that occurs during this period can disturb the process and increase the chance of sex reversal. The existence of this window was demonstrated in different group of vertebrates with ESD and GSD, and it was demonstrated the sex determining trigger must act in that crucial time window (1). Here we describe several mechanisms by which a master sex initiator can act on the sex determination pathways.

The dosage-effect. The molecular mechanism of sex determination can be described as a system having low resilience or high instability. At a short period of development (sex-determining window), the bipotential gonad can receive signals from both male and female pathways, but a small difference between them can be determinant in the decision to form testis or ovary (1). Consequently, any molecular process that leads to differential expression of male and female promoting genes could be the origin of a new sex determination trigger.

The best-known example for expression level regulation of sex determination are birds, where the presence of two *Dmrt1* alleles on the Z chromosomes of the males leads to testis formation, while in the ZW constitution, due to the absence of *Dmrt1* from W, one allele of this gene is insufficient to trigger the male cascade, which then leads to ovary formation. The presence of a putative W-specific gene responsible to activate the female pathway and/or to block the male

cascade in birds was proposed, but no evidence of its existence has been provided to date (36). An analogous situation was reported in a fish, the Chinese half-smooth tongue sole, where the two copies of *dmrt1* on the Z are linked to testis development. The W has no functional copy of *dmrt1* and the lower transcript levels are connected to female development (37, 38). Interestingly, in humans, deletions in the p arm of chromosome 9 affecting only one copy of the *DMRT1* gene resulted in a completely feminized external genitalia and no palpable gonads in XY humans (39). This result suggested that *DMRT1* is haploinsufficient for testis formation, confirming its dosage-effect observed in other vertebrates.

In the examples above, the dosage-effect relies on the absence of one allele of the sex regulator due to the sex chromosome constitution. In other cases, the sex-related gene is present on both sex chromosomes, but allelic variation between them leads to a differential expression and, consequently, determination of sex. In the fish *Oryzias luzonensis*, for instance, the *gsdf* (gonadal soma derived growth factor) gene is located on the X chromosome ($gsdf^X$), and the allelic version on the Y is the male sex-determining gene $gsdf^Y$. In this species, the amino acid sequences of $gsdf^X$ and $gsdf^Y$ are the same, but the expression of $gsdf^Y$ is higher in the early gonad of XY animals than in XX fish (40). In the case of the killifish *Nothobranchius furzeri*, allelic variation on the sex chromosomes was shown for the *gdf6* (growth differentiation factor 6) gene. It was suggested that the allele on the Y, *gdf6Y*, is the male sex determiner of this species. Gdf6Y contains 15 amino acid changes and a 3 amino acid deletion when compared to the Gdf6 allele on the X chromosome. The mRNA expression analyses showed that *gdf6* and *gdf6Y* are similarly expressed in both sexes prior to sex determination, but around hatching *gdf6Y* is significantly increased in males (41). Similarly, in the tiger pufferfish *Takifugu rubripes*, two alleles of the anti-Müllerian hormone receptor type II (*amhr2*) gene are present, but one allele contains a missense SNP change in the kinase domain of the protein, which is predicted to lead to a less active receptor. In this case, the male is heterozygous, presenting at least one fully functional version of the receptor, but the female is homozygous for the defective receptor (42).

Anti-male and anti-female sex determination genes. There are natural examples of sex determining genes where the mechanism works by strictly repressing the male or the female pathway. In the frog *Xenopus laevis*, the sex chromosomal system is ZZ/ZW and a duplicated copy of *dmrt1* on the W, named *dm-w*, has a dominant-negative effect by interfering with the transcriptional activation of target genes of *dmrt1*, acting as an anti-male factor and leading to ovary formation (43). The *dm-w* gene is expressed only in female, and it is transiently expressed in the primordial bipotential gonad, although *dmrt1* shows continued expression after sex

261 determination (44). It is important to note that Dm-W is not completely blocking the action of
262 Dmrt1 but decreasing its activity in females. This is conceptually similar to the dosage effect in
263 birds and tongue sole.

264 In salmonids the sex chromosomal system is XX/XY, and the male sex-determiner, *sdY*, is a
265 duplication of the immune-related gene *irf9* on the Y chromosome. SdY has been shown by *in*
266 *vitro* experiments to repress the female pathway through direct interaction with Foxl2 with the
267 consequence that the male pathway is active (45) (Bertho et al. in this series). In both cases, the
268 frog and the trout, the sex determining genes seem to have a specific role in repressing the
269 opposite pathway.

270 **ESD and GSD are entangled.** Another very important observation from Wilkins is that "*the*
271 *distinction between GSD and ESD systems is not always absolute. Systems that are essentially*
272 *ESD systems can possess a degree of genetic determinism while in a few GSD systems, variant*
273 *populations with some degree of environmental determinism can arise"* (9). In vertebrates,
274 several examples of GSD species with a well-known sex chromosomal system and sex-
275 determining gene are still able to respond to environmental factors have been studied. In fish,
276 especially teleost, this feature is more frequently observed, mainly due to the fact that most
277 species are oviparous and the embryos are easily exposed to environmental cues during the
278 critical sex determining time window (46). Cultivation of eggs at high temperatures leads to
279 female-to-male sex reversal in GSD species with robust sex-determining genes, as medaka
280 (*Oryzias latipes*) (47), tilapia (*Oreochromis niloticus*) (48) and pejerrey (*Odontesthes*
281 *bonariensis*) (49). Experiments showed that temperature increases the cortisol levels in the
282 embryos in several fish species, and, in medaka, it was suggested that cortisol could directly
283 activate the promoter of *dmrt1a* in XX animals during the sex determination period, activating
284 the male cascade (50-53).

285 Reptiles are the group of vertebrates classically known to have temperature as the main sex
286 determination trigger in some taxa, e.g. crocodiles and some turtles (1). The exact molecular
287 mechanism to explain how temperature impacts the sex determination cascade is not known.
288 However, recent studies in the red-eared slider turtle (*Trachemys scripta elegans*) provided the
289 first evidences how the environmental trigger can act on the intrinsic genetic pathway. In *T.*
290 *scripta*, higher temperatures lead to female development, and transcriptome analyses
291 comparison between gonads of embroys reared at 26°C (male) and 32°C (female), uncovered
292 high expression of *Kdm6b* at 26°C. KDM6B is a histone demethylase and data support that it
293 induces the transcription of *Dmrt1* by eliminating the trimethylation of the H3K27 histone near

to the promoter of this important male factor (54). Despite being the only known mechanism through which ESD works, the example of *T. scripta* together with the temperature influence on GSD species, bring us back to the main feature of sex determination, in which the bipotential gonad has the ability to respond to any signal that would repress or activate the downstream pathway of one of both sexes. In case of ESD, the trigger selected is environmental, while in GSD it is genetic (Fig. 1). Hence, the constant presence of the both opposing male and female networks together with the preserved ability to respond to environmental cues explain how ESD could switch to GSD and vice-versa. In fact, the group of reptiles present several examples of ESD going to GSD, especially in turtles (55) and geckos (56), and the transition from GSD to ESD seems to be rare, present mostly in fish (57).

Applying the "bottom up" hypothesis in vertebrates: The selective advantage of the recurrent sex-determining genes.

Looking at the genes identified as master sex-determining triggers, some recurring regulators were observed in multiple species and designated as "usual suspects" (Table). Most sex-determining genes originated from allelic variation or gene duplication of genes long known to be involved in the regulatory network of gonad development (8). What gives the sex determination precursors the potential to become a master new sex-determining gene? A way of answering this question is analyzing the function of those "usual suspect" genes in gonad formation and reproduction for both male and female (Table). Interestingly, amongst those genes, *dmrt1*, *gsdf* and *amh* are classically described as "male-related" genes (58). This way of classifying the sex determination network can lead to the conclusion that those genes have an exclusive role for the development of one sex only. Nevertheless, this strict conclusion is not supported by a closer look at the full spectrum of their functions.

Dmrt1. In mammals, *Dmrt1* is expressed in the primordial gonad of both sexes and subsequently decreases in expression in the ovary and increases in the testis. *Dmrt1*, in males, is expressed in the Sertoli cells and in spermatogonia. However, Dmrt1 was also demonstrated to be important for the formation of follicles in juvenile ovaries. *Dmrt1*$^{-/-}$ mutants not only had an impact on testis development, but also showed dysregulation of oocyte development in females (59).

TGF-ß family. The *Amh*, *Gsdf* and *Gdf6* are male-promoting genes and encode ligands of the TGF-ß signaling pathways. Amh (anti-Müllerian hormone) binds to Amh-receptor2 (Amhr2) and was originally correlated only to the regression of the Müllerian duct during the development of the urogenital system in amniotes (46). *Gsdf* is important in testis development,

and and was lost in the vast majority of tetrapods (60). Both the Amh/Amhr2 system and *Gsdf* have similar effects when knocked out. Experiments in different fish demonstrated that the mutants exhibited uncontrolled germ cell proliferation at early stages of development and in the adults of both sexes. In addition, male-to-female sex reversals were observed and these females had reduced fertility suggesting additional roles in the ovary (40, 61, 62). *Gdf6* has not been described so far as being involved in gonad development. However, GDF6 and BMP4, via SMAD factors, influence *Id* (inhibitors of differentiation) genes, and thereby suppress differentiation of mouse embryonic stem cells (63). BMP4 has an important role in primordial germ cell differentiation, and treatments of epiblast-derived stem cells with this factor resulted in specification into germ cells (64). In addition, other closely related genes like *Gdf9* and *Bmp15* are important in ovarian development of mammals and fish (65, 66) (Pan et al. in this series) (Table).

Sox3 and the *SOX* family. The *SOX* (Sry-type HMG box) gene family contains the HMG box DNA binding domain closely related to the one of Sry (67). It is commonly accepted that the *Sox3* gene is the ancestor of *Sry*, being located on the X chromosome of mammals. This gene is not a primary sex determiner, but it is highly expressed in testis and ovary of mammals, and *Sox3* KO mice display disrupted gametogenesis with gonad dysgenesis in both sexes (68). In *Oryzias dancena*, *sox3* is the Y-linked male sex determination gene, pointing to the potential of this gene to be a male sex promoting factor (69) (Table). Other members of the SOX family have also been shown to be important in gonad development in vertebrates. *Sox8* was shown to be involved in reinforcing *Sox9* function and even substituting its role (70). *Sox10*, a close relative from *Sox9*, is expressed at low levels in primordial gonads of both sexes in mice, and transgenic expression of this gene in gonads of XX mice resulted in transcriptional activation of targets of SOX9 and development of testes and male physiology (71). In Japanese medaka, *Oryzias latipes*, *sox5* has a role in regulating germ cell number and disruption of this gene leads to XX female-to-male sex reversal (72).

Overall, there are two common features of the "usual suspects" genes that should be highlighted. First, they do not have a strict sex-specific role, being necessary for both functional ovary and testis. In some species, the bipotential gonad already expresses those sex-related genes prior to sex determination, and the expression bias occurs only after the master trigger is activated (1). Hence, genes classically defined as "male-related" are in fact genes that are needed at high expression levels during and after the critical developmental window to lead the gonad towards testis, and the same can be inferred for the "female-related" genes. Indeed, transgenic experiments in different vertebrates demonstrate that at the critical window

overexpression of "male-related" genes (e.g. *Dmrt1*, *Sox3*, and *gsdf*) (34, 73, 74), as well as "female-related" genes (e.g. R-spondin) (75) takes over the role on sex determination and produces sex-reversal. Interestingly, the ectopic expression of *Sox3* in the bipotential gonad of mice not only generated XX males, but the mechanism of testis differentiation was through upregulation of *Sox9*, similar to the mechanism downstream of SRY (73). The second feature of the "usual suspects" is that disruption of some of these genes leads not only to sex-reversal in most species, but also to disruption of gametogenesis in both sexes, as discussed above.

Currently, the crosstalk between germ cells and soma is not fully understood. In vertebrates, one morphological difference between male and female is that female germ cells proliferate and/or enter meiosis earlier than in males (76, 77). In addition, "male-related" genes as *Dmrt1*, *Amh* and *Gsdf* are known to be important in controlling germ cell proliferation and survival and keep the germ cells quiescent in the early and adult gonads (46, 53, 74).

Wilkins proposed that, if a given condition produces a change far from the optimal sex ratio in a population, there will be a frequency-dependent selection favoring the minority sex. Thus, the rise of a dominant gene, favoring the minority sex, might spread rapidly in the population. Then, he reasoned that the new determinant might need an additional selective advantage to increase its frequency in the population in order to be fixed. Two general possibilities were suggested. The first is that the "*the newly arising dominant might be tightly linked to an allele of another gene which is under positive selection*". The other possibility is that "*a newly arisen sex determining allele would have a direct selective value of its own, independent of its effect on sexual development*". Wilkins realized already at that time that genes known to be part of the sex determination pathways could have this additional selective advantage. In addition, he suggested that, "*genes that are functionally redundant at some extent would be expected to be the primary source of new sex-determining alleles*. *In principle, mutation of a regulatory gene to a new function, if associated with a favorable selection coefficient for viability or fertility, and whose original function could be supplied by members of its own or other gene families, might help to spread the neomorph, independently of its effect on sex determination"* (9). As demonstrated above, the "usual suspects" in vertebrates belong to the TGF-ß, SOX and DMRT families, which are known to act on the viability and fertility of the gametes. These families are composed by many structurally and functionally closely related members, which may display some levels of redundancy (Table).

The co-evolution of the sex-determining trigger and the downstream pathway

A replaceable master. Nicolas Perrin in 2016 raised the question of whether there is a necessity for an initial trigger of sex determination. He suggested that any random fluctuation in the expression of key genes should be enough to launch the process. This theory was named Random Sex Determination (RSD) (78).

The only conclusion that can be made from the current knowledge is a non-intuitive statement: the whole evolutionary process selects a replaceable sex-determining trigger. One can argue that such an unstable system would not last long and that evolution would stabilize the sex determination process, locking the downstream network to the master trigger (genetic or environmental), which is for instance the case in therian mammals, birds and some reptiles (79). However, it can be difficult to understand the advantage of a system that selects a trigger that can be so easily replaced, as it is the case in fish. One answer may be that the replacement of the master may allow the trigger to get better. Another answer may come from the fact that the master trigger is not really a "master" in the sense that it acts at the very start or "top" of the gonadal development process. As already mentioned above, the bipotential gonad expresses the complete machinery to respond to a male or female sex promoting signal, and the genes that were classically called male and female genes are also important for reproductive roles in both sexes.

Biologically and conceptually it is impossible to have the male and the female sexes as independent from each other. The gonadal sex determination and sexual differences can be understood as a polymorphic state, which makes sense, since both male and female belong to the same species. Through this perspective, the role of the master trigger is not to develop a brand-new organ, but to make the decision what kind of reproductive role one organism will have within the same species. Throughout the evolution of animals, the capacity of the somatic gonad to become either testis or ovary became extremely advantageous, for instance in case of an environmental event that skews the sex ratio to one of the sexes, the appearance of a new sex determiner can arise within few generations.

The sex-determining trigger is on the one hand necessary to insure that the decision will be made in the right developmental time, and on the other hand the trigger cannot play a key, dominant role in the gene regulatory network. In other words, the master trigger can act from outside on the genetic network of sex determination, but the regulatory network is not necessarily built around it as a main node, which thus maintains its genetic structure (Fig. 2). In this way, the sex-determining trigger does not work as a master, but more as a sex-promoting signal. This logic can be even more obvious in genetic sex-determining system, since only part

of the population contains the master gene. In this case, to reach the optimal 1:1 sex ratio, the gonad of all individuals must be able to respond to the polymorphic signal, which is the sex-determining gene linked to the sex chromosome. Which evolutionary and molecular processes select a trigger to act on a network while maintaining its structure?

Acquiring a new sex determination function but keeping the old reproductive role. Based on current knowledge, evidence shows that the new sex-determiner acts during the sensitive window and can be easily replaced by another sex-related gene. The crosstalk between the downstream genes and the sex determiner should be to restrict the action of the new determiner to the sex determination window and not disturb the sex differentiation and reproductive processes. The genes from the TGF-ß, SOX and DMRT families following allelic diversification or gene duplication, have the potential to become the sex determination trigger, but the new determiner does not change the original role of the ancestral gene. This implies a co-evolution of the downstream network and the sex determination trigger. The *sdY* gene in salmonids (*irf9*) is perhaps the only known example where the determiner arose outside from the classic sex-related genes, since, to date, no connection between *irf9* and gonad differentiation has been shown.

Fish of the genus *Oryzias* provide powerful information to understand the evolution of a new upstream regulator. The *dmrt1bY* gene is the sex-determining gene of *O. latipes* and *O. curvinotus*, and $gsdf^{Y}$ is the male-determiner in *O. luzonensis* (40, 80). Despite sharing *dmrt1bY*, *O. curvinotus* is phylogenetically closer to *O. luzonesis* than to *O. latipes*. Extensive studies on *dmrt1bY* from *O latipes* showed that transposable elements inserted in the promoter of this gene brought *cis*-regulatory elements which led to expression within the sex determination window (81). In this species, the autosomal ancestral *dmrt1* (*dmrt1a*) and *dmrt1bY* co-evolved a regulatory system where Dmrt1a blocks the expression of *dmrt1bY* through direct binding to the promoter after Dmrt1bY has fulfilled its novel function during the sex determination period (82, 83). Genome analyzes suggest that *O. luzonensis* lost *dmrt1bY* and $gsdf^{Y}$ became the new sex-determiner. Transgenic experiments showed that $gsdf^{Y}$ can take over the role of *dmrt1bY* as sex-determiner when introduced into *O. latipes* (74). It was inferred that $gsdf^{Y}$ could appear and persist in the population independently from the presence of *dmrt1bY* and then moved from the bottom to the top of the hierarchy, as suggested by Wilkins in 1990.

Conclusions and perspective

In 1995, Wilkins provided in his seminal paper the basic arguments to understand the evolution of the sex determination pathway. He reasoned based on the knowledge from *Drosophila*

melanogaster and *C. elegans*, that the sex determination cascade evolved from the bottom to the top. Since then, quite a number of experimental studies on sex determination in various species have increased our knowledge base and allowed us to revisit long-standing hypotheses.

Led by the diversity of master sex-determining genes found in different lineages of vertebrates, attention was directed to the top of the cascade. However, a closer look discloses the importance of the downstream regulatory network and the sex differentiation period. First, we know now that the process of constant mutual repression between the sexes maybe is the universal feature in animals, or at least in vertebrates, which creates an unstable status that is known as gonadal developmental plasticity. The bipotential gonad expresses the basic machinery to respond to the sexual triggers, which can be genetic and/or environmental. Most of the sex determination genes arose from allelic variance or gene duplication of the downstream regulators, and disruption of those genes lead to sex reversal and/or gonad dysgenesis.. The new sex-determiner acts during the sex determination period through transcriptional rewiring and/or mutation of the ancestor gene, regulating the expression levels of genes expressed in the bipotential gonad. This regulation can also be controlled by other factors than a sex-determining gene, like temperature, hormones, and pollutants.

The difference of our global analyses to the classical "masters change, slaves remain" hypothesis is that the effect of the co-evolution between a new sex-determining gene and the downstream pathway is functionally restricted to the sex determination window, meaning that other changes in the downstream network for sex differentiation and reproductive roles may occur independently from the sex trigger. Those changes could be through selective processes or even by genetic drift. Here we suggest that these changes create the pre-condition for the emergence of a new sex-determining trigger (preferentially from the sex determination cascade), by allelic variation or gene duplication. The new sex-determiner would then be fixed in the population to act only in the sex determination window and not affecting the whole sex differentiation network and the original role of sex-related genes. If the new sex determiner became an integrative part of the network, it would be much harder if not impossible to replace.

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Authors' Contributions

MCA and MS developed the concept and drafted the article. AH revised the manuscript and contributed to the concept and evidence presentation.

Competing Interests

The authors declare no potential conflict of interests.

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719 **Table: Role of autosomal sex-related genes that became sex-determining gene.**

720

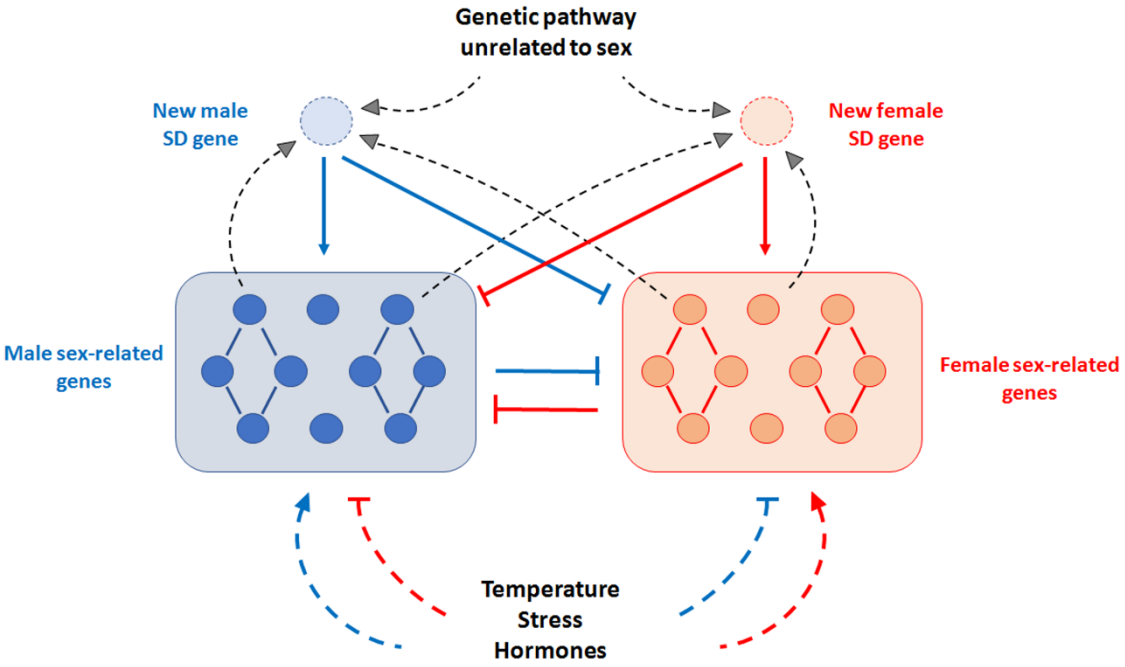
Gene	Sex-determing versions	Function in reproduction	Gene disruption in the gonad
Sox3	*Sry, sox3Y*	Transcription factor, required in formation of the hypothalamus–pituitary axis, expressed in developing gonads	Disrupted gametogenesis with gonad dysgenesis
Gsdf	*gsdf^{Y}*	TGF-ß factor, important role in fish gonad development; expressed close to the spermatogonias of adult testis	Hyperproliferation of germ cells. Male-to-female sex reverse of XY animals.
Amh	*amhbY*	Anti-Muellerian hormone, growth factor expressed in the early gonad and close to the spermatogonias of adult testis	Hyperproliferation of mitotic active germ cells. Male-to-female sex reverse of XY animals.
Amhr2	*amhr2-SNP*	Type II receptor for Amh, important function in gonad development and germ cell proliferation.	Hyperproliferation of mitotic active germ cells. Male-to-female sex reverse in 50% of XY animals.
Gdf6	*gdf6^{Y}*	Important in controlling cell differentiation, no gonadal function known	Not Described
Dmrt1	*dmrt1bY, dmW*	Transcription factor, key role in male sex determination and differentiation	Dysregulation of oocyte development. Male-to-female sex reverse.
Irf9	*sdY*	Interferon response factor, no gonadal function known	Not Described

Figure Legends:

Figure 1. Hypothetical source and interaction between the sex-determining trigger and the sex determination pathway. Genes involved in the development of one of the sexes also perform a role in repressing the opposite pathway. The sex-determining trigger can be genetic (GSD) or environmental (ESD), and it acts in promoting or repressing the male (blue) or the female (red) pathway. The genetic sex-determining trigger originates (black arrows) from genes of the already stablished sex determination pathway, or even from genetic pathways unrelated to sex.

Figure 2. Model for a spatial-temporal influence of the sex-promoting signal on the sex determination genetic network. Main genetic factors are expressed in the bipotential gonad and the sex-promoting signal acts outside of the network, leading the gonad to differentiate towards testis or ovary. The pool of genes involved in the network is maintained in the differentiated gonad, but their relative importance changes depending on the outcome of the determination phase. Genes higher expressed in testis are classically named "male-related genes" (dark blue) and genes with higher expression in ovaries are called "female-related" genes (dark red), but their role is not restricted only to one sex. In the example, a male sex-determining (SD) gene increases the activity (blue arrow) of *amh* disbalancing the network to develop towards male. The same principle can be observed for environmental cues, that could, for instance, induce the female pathway by blocking important male factors as *dmrt1*, or by activating female drivers as estrogen receptor (Esr1).

742 **Figure 1**



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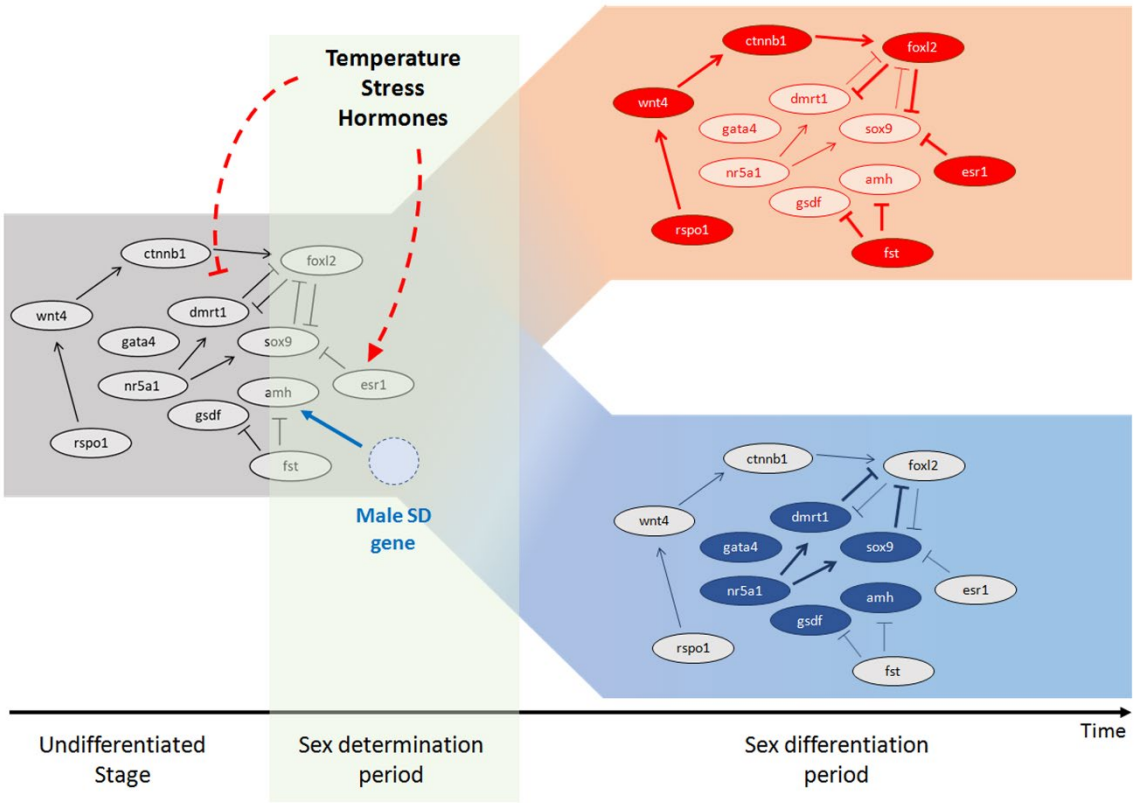
745 **Figure 2**



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