

Regulatory *putsches* create new ways of determining sexual development

The development of multicellular organisms is controlled by gene-regulatory cascades. Generally, these cascades are headed by a master regulator that determines the development of a precursor cell lineage towards a given tissue or organ during embryogenesis. A notable feature is that the genes at the top of the cascade are more highly conserved than the downstream-acting factors. An outstanding example of this phenomenon is the homeobox-containing paired box 6 (*PAX6*) gene in its function as the master regulator of eye development. When, for example, the human gene is transgenically expressed in a *Drosophila* leg, it is able to induce the development of an eye at this ectopic and xenogenic position (Halder *et al.*, 1995). What develops is not a vertebrate-type eye but a fly eye because the genes downstream in the eye-development cascade are not conserved and are those that specify the development of the insect visual organ, which consists of the complex and very specific ommatidia.

However, the opposite is true for the genes of the sex-determining cascade. In this case, the genes at the top of the cascade are not conserved, whereas the downstream genes have homologues in a much broader spectrum of species (Graham *et al.*, 2003). The *SRY* gene—the master male sex-determining gene of mammals—has not been detected outside the eutherians. However, the other known members of the gene network involved in the development of the bipotential gonad anlage of the embryo towards either an ovary or testis—for example, the transcription factors Wilms tumour 1 (*WT1*) and *SOX9* (sex-determining region Y-type high-mobility group box 9), and the bone morphogenetic protein (BMP) growth factor family member anti-Müllerian hormone (AMH; for a review, see Wilhelm *et al.*, 2007)—are present in all vertebrates, including fish (Devlin & Nagahama, 2002). The most downstream component of this cascade, *DMRT1* (doublesex and male abnormal 3-related transcription factor 1), has homologues even in *Drosophila* (*doublesex*; *dsx*) and *Caenorhabditis elegans* (*male abnormal 3*; *Mab-3*) (Raymond *et al.*, 1998), whereas the top sex-regulatory genes of the fly and the worm are not conserved. This diversity at the molecular level seems to reflect the range of mechanisms by which sex is determined, including the X-chromosome-to-autosome ratio in *Drosophila* and *C. elegans*, and the presence of the Y chromosome in mammals. Notably, these are the only three groups of organisms in the animal kingdom for which we have a reasonably good knowledge about the molecular factors involved in sex determination and how they function. So far, we still do not know how sex determination is initiated in the WZ system of birds (although some candidate genes have been

named), or how the incubation temperature of the eggs of crocodiles and turtles is perceived and translated into the action of a—probably the same or highly similar—transcription factor cascade that initiates male or female gonad development in birds and mammals.

One of the few exceptions to our lack of knowledge about the master regulators of sex is the honeybee. Hasselmann and colleagues have recently reported exciting insights about how such a molecular diversity of regulatory pathways could have evolved (Hasselmann *et al.*, 2008). In the honeybee—as well as the more than 200,000 species of the insect order *Hymenoptera* (which includes bees, wasps and ants), many other insect groups and rotifers (which amount to an estimated 20% of all animal species)—sex determination follows a haplodiploid mode in which males are derived from unfertilized haploid eggs, whereas the fertilized diploid eggs generally develop into females. Sex determination in such haplodiploid species does not rely on a difference in heteromorphic sex-chromosome composition. The underlying genetic basis of haplodiploid sex determination was first elucidated in the *Hymenoptera* by the discovery that certain inbred crosses of wasps resulted in 50% of the fertilized eggs becoming diploid male offspring of biparental origin (Whiting, 1933). This finding suggested that the process of fertilization is not itself the signal of sexual regulation but, conversely, that the development into one sex or the other is controlled by an initial signal coming from either one allele or two different alleles of a single gene, the so called *complementary sex determiner* (*csd*). The biochemical nature of the initial signal of complementary sex determination remained unknown until the groups of Martin Beye and Robert Page jointly used fine-scale mapping and positional cloning approaches to identify and isolate the genomic region of the *csd* of the honeybee (Beye *et al.*, 2003). Sequence comparisons indicated that *csd* is a member of an Arg/Ser-rich (RS) protein family, and the highest similarity was found between its carboxy-terminal part and the *Drosophila* protein transformer (*Tra*), which is a downstream component of the genetic sex-determination cascade in the fruitfly (Beye *et al.*, 2003). No differences in the transcription or splicing of *csd* were detected between males and females that could act as sex-specific information. Thus, it is assumed that the *Csd* proteins act as dimers, and that heterodimers or homodimers differ in their ability to regulate the downstream factors of the cascade (Beye, 2004).

In August 2008, Hasselmann and co-workers from the Beye laboratory published the identification of a second gene involved in the sexual development of the honeybee that also localizes to the sex-determination locus (Hasselmann *et al.*, 2008); this gene was called *feminizer* (*fem*). Interestingly, the *fem* gene encodes a protein that has a C-terminal RS-rich and Pro-rich domain with a high degree of sequence identity (>70%) to the *Csd* protein. Therefore, *Fem* is also a member of the Ser/Arg (SR)-type proteins, which are commonly thought to be involved in regulation of RNA splicing. Molecular phylogenetic analyses showed that *csd* is a duplicate of *fem* that arose between 10 and 70 million years ago during the evolution of the honeybee lineage. Furthermore, RNA interference (RNAi)-induced

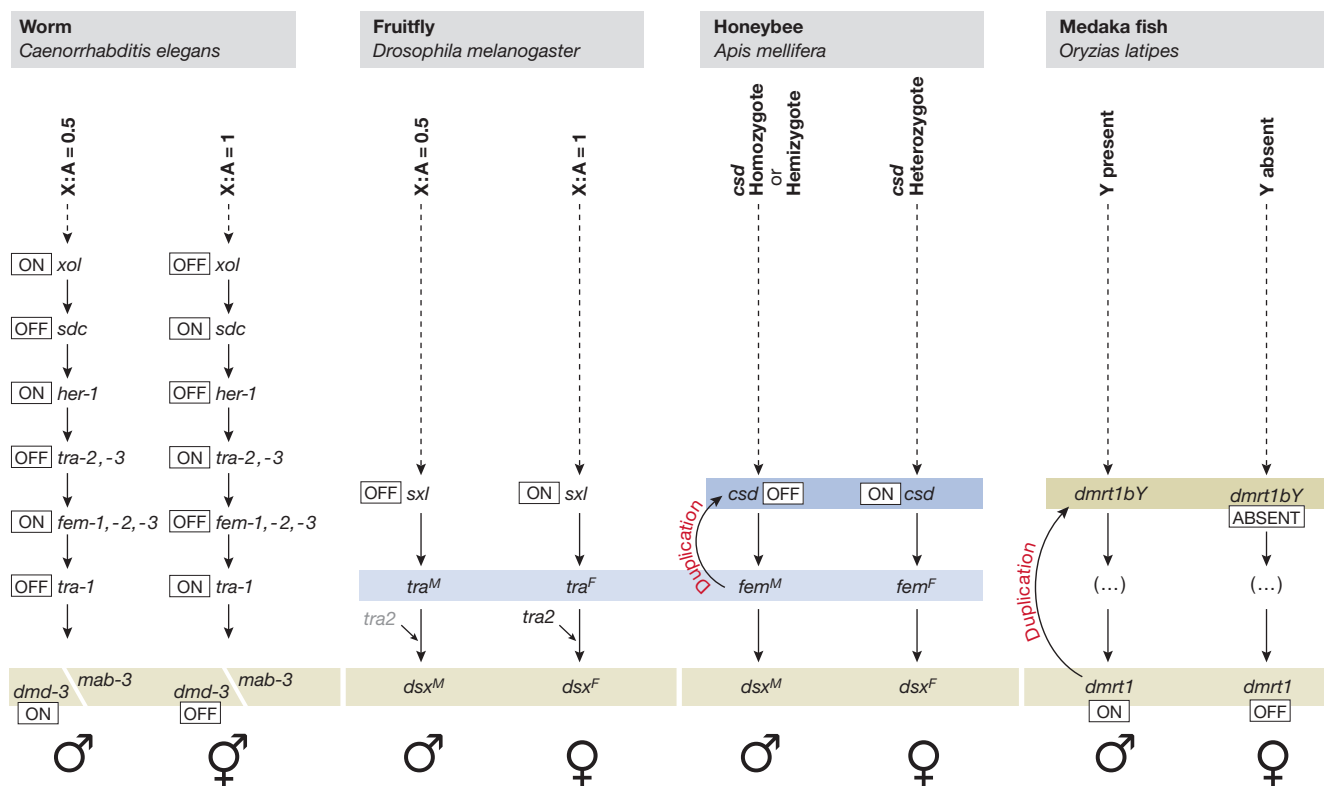


Fig 1 | Sex-determining cascades in the worm, insects and medaka fish. Molecular pathways leading to the formation of the gonad in *Caenorhabditis elegans*, *Drosophila melanogaster*, *Apis mellifera* and *Oryzias latipes*. Conserved doublesex/male abnormal 3/*dsx* and *mab-3* related transcription factor 1 (*dmrt1*) or *Drosophila transformer* (*tra*)-like homologues are indicated with light-brown and light-blue boxes, respectively; duplicated factors in the cascades are also highlighted (brown and blue boxes, respectively). Note that *tra* of *C. elegans* is not related to *tra* of *Drosophila*. *csd*, complementary sex determiner; *dmd3*, doublesex/*mab-3* domain family member 3; *dsx*, doublesex; *fem*, feminizer; *her-1*, hermaphroditization of XO-1; *sdc*, sex determination and dosage compensation defective; *sxl*, sex lethal; *xol*, XO lethal.

knockdown of *fem* resulted in a developmental switch that changed females into a male phenotype, whereas it had no effect in males. As identical results were observed for *csd* RNAi-knockdown experiments (Beye *et al*, 2003), these findings convincingly define *fem* as a second binary switch gene of the honeybee sex-determination pathway, in addition to *csd*.

Hasselmann and colleagues also analysed *fem* splicing. They could identify sex-specific *fem* transcripts with the same 5' untranslated region (UTR) sequence, but with differences in their downstream exon composition that resulted in prematurely terminated male-specific *fem* transcripts. Notably, the occurrence of sex-specific splice variants is an important regulatory mechanism already described for the worm and fly sex-determination pathways (Fig 1). Finally, by showing *csd*-induced sex-specific splicing of *fem*, the authors could conclude that the binary switch of the sex-determination pathway is implemented by alternative splicing of the *fem* transcript in response to heterozygosity at *csd*.

Interestingly, *csd* itself is not alternatively spliced. As it is a duplicated version of *fem*, this poses the question of whether the ability to be sex-specifically alternatively spliced has been lost by *csd* or gained by *fem*. As *tra*, the *fem* homologue in *Drosophila* (Fig 1), is analogously spliced, one can reasonably assume that sex-specific splicing was indeed lost for the duplicated copy that

is now *csd*. Understandably, as it is at the top of the cascade, there is no possibility of splicing regulation for *csd*.

A homologue of *dsx/mab-3*, which is the most downstream factor of the fly and worm sex-determination cascades, has also been identified in the honeybee (Cho *et al*, 2007). In *Drosophila*, the Fem-like factor Tra regulates the differential splicing of *dsx* homologues. Therefore, it is reasonable to assume the same regulation from *fem* towards the *dsx* homologue in the honeybee, although this has not yet been confirmed experimentally (Beye, 2004).

The evolution of gene interactions in the sex-determining cascades characterized by conservation at the bottom and diversity at the top could be explained convincingly by an evolutionary scenario in which these hierarchies evolve from a common downstream component that acquires new upstream regulators—which will naturally vary in different evolutionary lineages (Wilkins, 1995). The case uncovered by Hanselmann and colleagues in the honeybee might seem to be an exception; here, in an extension of the Wilkins 'bottom-up hypothesis', the master regulator is not elected and recruited from the outside to perform a new sex-determining function, but rather a member of the existing cascade usurps the position at the top. However, on closer inspection, this situation is not so exceptional. A similar situation exists in the medaka fish, which is the only non-mammalian vertebrate in

which the master sex-determination gene is known (Matsuda *et al*, 2002; Nanda *et al*, 2002). In this case, the *Dmrt1* gene—the most downstream gene in all sex-determining cascades known so far—underwent gene duplication approximately 5–10 million years ago. The new copy was inserted into another chromosome, which became the Y chromosome (Schartl, 2004). There, the duplicated *Dmrt1* gene became the primary determinant of male sexual development and testes determination. In conclusion, it seems as if gene duplication of downstream components—as described for the medaka fish and now also for the honeybee—is more widespread than previously thought and provides an efficient evolutionary mechanism to create new sex-determination mechanisms.

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