Chapter 12
The Mechanism for Establishing the Binary Sex with Environmental Signals in the Crustacean *Daphnia magna*

Yasuhiko Kato and Hajime Watanabe

Abstract The cladoceran crustacean *Daphnia magna* produces females parthenogenetically under healthy conditions. In response to environmental stresses such as starvation and higher population density, it produces males that are genetically identical to their sisters. Environment-dependent male production in *D. magna* can be interpreted as the shift of the position of the sex on the spectrum from the female side to the male side by the environmental cues. In this review, we introduce how the environment-dependent male determination is mediated via the endocrine system and the evolutionary conserved sex-determining gene *Doublesex1* (*Dsx1*) in *D. magna*. We then describe how the binary sex can be achieved by regulation of *Dsx1* at transcriptional, epigenetic, and post-transcriptional levels to prevent intersex phenotype.

Keywords Doublesex1 · Environmental sex determination · *Daphnia magna* · Transcriptional regulation · Post-transcriptional regulation · Epigenetic regulation

12.1 Introduction

The healthy population of the cladoceran crustacean *Daphnia magna* is composed of females by parthenogenesis (Fig. 12.1). The female juvenile matures into an adult after several molting. It develops two ovaries aside from the gut and produces eggs without mating (Fig. 12.1, solid arrow). In contrast, under the stressed environment, such as shortened photoperiod, a lack of food, and/or their increased population density, the adult senses those environmental changes and parthenogenetically produces male offspring that are genetically identical with their sisters. Interestingly, the ovulated eggs are already destined to be males, meaning that the commitment of the sex of the offspring to males occurs in the ovaries (Fig. 12.1, dotted arrow). Under the stressed condition, females also switch their reproductive mode from

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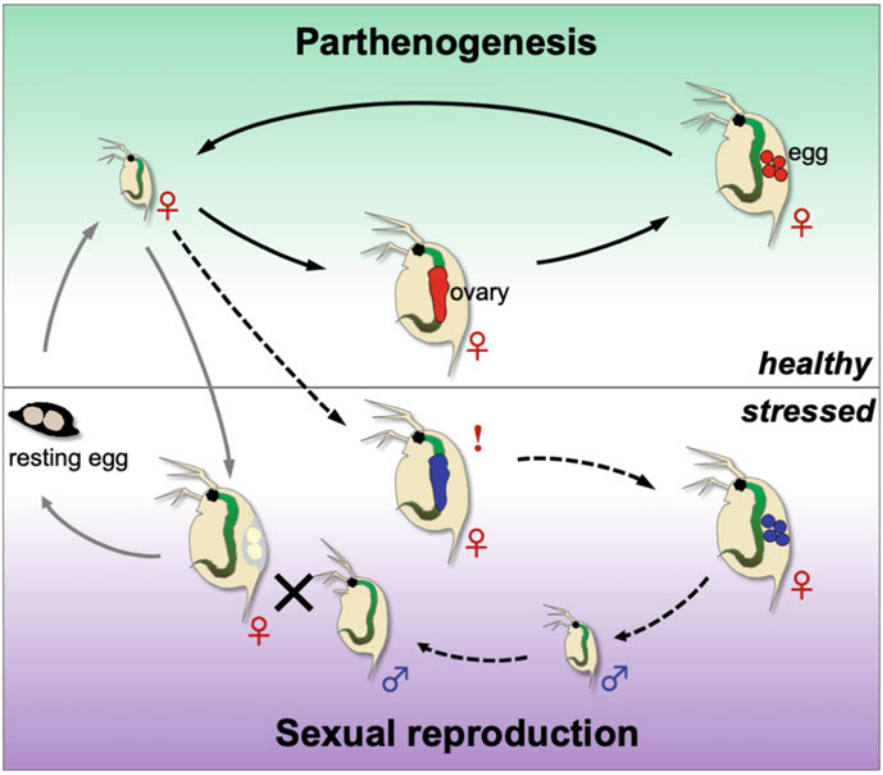

Fig. 12.1 Life cycle of *Daphnia*. This planktonic crustacean engages in cyclical parthenogenesis where it can switch the reproductive mode from parthenogenesis to sexual reproduction under stressed conditions. The ovaries harboring the oocytes that are destined to be developed as females or males are indicated with red or blue color, respectively. With the same color arrangement, the sex of the eggs is shown. In *Daphnia*, the same genotype can produce three different reproductive forms, parthenogenetic females, sexual males, and sexual females. The black arrows are used to show the cycle of female production by parthenogenesis. The dotted arrows indicate the process of male production by parthenogenesis under the stressed condition. The sexual males are necessary for the production of the resting eggs by sexual reproduction. The gray arrows show plastic change of female reproductive mode from parthenogenesis to sexual reproduction, which allows the females to mate with the males for making the resting eggs that can hatch when the surrounding environment is recovered. The exclamation mark refers to the sensing of the stressed environment and secretion of sesquiterpenoid necessary to destine the sex of the offspring to the male

parthenogenesis to sexual reproduction and mate with the males, leading to the production of resting eggs that can withstand desiccation and other adverse conditions. From the resting eggs, only females hatch and can increase their population by parthenogenesis in the subsequent growing seasons (Fig. 12.1, gray arrow) (Hebert 1978; Zaffagnini 1987). Intersex is extremely rare in *D. magna* (Mitchell 2001; Olmstead and LeBlanc 2007). From the concept of the spectrum of sex, environment-dependent male production can be interpreted as the shift of the

position of the sex on the spectrum from the female side to the male side by the environmental cues.

Although the females and males are genetically identical, they show sex differences in morphology and behavior as they grow. At the first instar of the juvenile, males have more elongated first antennae, which allows us to distinguish their sex easily by microscopic observation. At this time, gonads develop on both sides of the intestinal tract in both males and females, and morphological sex differences can be already seen. As males grow, their swimming speed increases and they catch females for mating by hooks formed on the first thoracic leg, the most cephalic of the five pairs of thoracic legs. Recent progress of *D. magna* genomics (Orsini et al. 2017; Lee et al. 2019) and gene manipulation methods (Kato et al. 2011b; Törner et al. 2014; Nakanishi et al. 2014, 2016; Naitou et al. 2015) have enabled us to investigate the molecular mechanism underlying the sex differences in this organism. Here, we introduce how the environment-dependent male determination is mediated via the endocrine system and the evolutionary conserved sex-determining gene *Doublesex* (*Dsx*) in *D. magna*. We then introduce how the binary sex can be maintained to prevent intersex phenotype.

12.2 Male-Determining Hormone

In insects and crustaceans, ecdysteroids and sesquiterpenoids function as messengers that transmit information of the surrounding environment to cells in the body. For instance, in insects, both hormones control body size in response to nutrition by their coordinated production via the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR) signaling pathway (Koyama et al. 2013). Of those hormones, sesquiterpenoids are known to control species-specific responses to environments called phenotypic plasticity (Miura 2019). In termites, juvenile hormone III (JHIII) is the major sesquiterpenoid and regulates caste differentiation (Cornette et al. 2008). In aphids, JHIII is known to control not only wing-morph differentiation (Ishikawa et al. 2013) but also the reproductive mode (Corbitt and Hardie 1985). In crustacean *Daphnia*, methyl farnesoate (MF), a precursor of JHIII, is the candidate of physiologically active sesquiterpenoid (Toyota et al. 2015) and MF exposure leads to the formation of morphology for defense against its predator (Oda et al. 2011).

MF is also responsible for the male determination of *Daphnia*. Exposure of MF to *D. magna* can induce the production of male offspring (Olmstead and Leblanc 2002; Tatarazako et al. 2003). The juvenile hormone agonists Pyriproxyfen and Fenoxycarb stimulate male production more efficiently than MF in *D. magna* (Tatarazako et al. 2003) and this hormone-dependent sex determination is common in the cladoceran crustaceans (Oda et al. 2005). In *D. magna*, sesquiterpenoid-dependent control of sex is implemented in developing oocytes (Olmstead and Leblanc 2002; Kato et al. 2010). This sesquiterpenoid-sensitive period is consistent with the timing of the male determination trigged by the crowding environment in

the other cladoceran *Moina* sex determination (Banta and Brown 1929). Taken together, environmental signals would be processed in the mother's brain and be transmitted to the endocrine system that secrets the sesquiterpenoid, which in turn determines the sexual fate of the offspring before embryogenesis begins (Fig. 12.1, exclamation mark). The chemical structure of the sesquiterpenoid in *Daphnia* needs to be elucidated in the future.

The chemically induced males can be used for experiments to study the molecular mechanism underlying the sex difference because they have male-specific traits and fertility to produce the sexual eggs (Kato et al. 2011a; Nong et al. 2017). In the laboratory culture condition, the offspring of *D. magna* is 100% females while exposure of the Fenoxycarb or Pyriproxyfen leads to 100% male offspring. This chemical manipulation of sex is much more efficient and convenient than that by setting stressful conditions such as crowding, which has contributed to recent advances in the molecular biology of *D. magna* sex determination.

12.3 *Dsx1* Shifts the Position of the Sex Spectrum to the Male Side

12.3.1 Dsx1, *the Master Regulator of Male Development*

Dsx codes for the transcriptional factor harboring the DNA binding domain named the DM domain and was originally found in *Drosophila melanogaster* (Burtis and Baker 1989). Dsx regulates the expression of genes linked to the development of sexually dimorphic traits. The roles of the DM-domain transcription factors in sexual development are conserved not only in the other insects and *C. elegans* (Shen and Hodgkin 1988) but also in vertebrates such as fishes (Matsuda et al. 2002), amphibians (Yoshimoto et al. 2008), and mammals (Raymond et al. 2000).

On the *D. magna* genome, two *Dsx* orthologs, *Dsx1* and *Dsx2*, are tandemly located (Kato et al. 2011a). Dsx1 has a sequence homologous to the oligomerization domain that is conserved among insect Dsx proteins and is known to enhance specific DNA binding through its dimerization (Bayrer et al. 2005). In Dsx2, the oligomerization domain is more diverged and has substitutions of the amino acids on the extensive non-polar interface. Both *Dsx1* and *Dsx2* show strong male-biased expression during embryogenesis and the sexual maturation process (Kato et al. 2011a; Nong et al. 2017), suggesting their roles in the male differentiation. RNAi-mediated knockdown of *Dsx1* in male embryos resulted in sex reversal including not only feminization of organs such as the first antennae and the first thoracic legs but also the development of ovaries. Overexpression of *Dsx1* in females led to the development of the male-specific trait, the elongated first antennae. In contrast, in the case of *Dsx2*, neither gene silencing nor overexpression led to clear phenotypic changes of the sexually dimorphic traits. This less contribution of *Dsx2* to sexual development may attribute to the sequence divergence of the oligomerization

domain. These expression and functional analyses demonstrate that *Dsx1* is the master regulator of male development (Kato et al. 2011a).

12.3.2 *Spatio-Temporal Expression of* Dsx1

In insects, *Dsx* is alternatively spliced in a sex-specific manner and the resulting isoforms control sex-specific development (Wexler et al. 2019). In contrast, *D. magna Dsx1* expression is not controlled by sex-specific splicing despite that this organism shares a common ancestor with insects (Schwentner et al. 2017), showing that the *Dsx1* is regulated at a transcriptional level (Kato et al. 2011a). To understand when and where the *Dsx1* expression is active throughout the life span, the transgenic *D. magna* recapitulating the *Dsx1* expression with the mCherry fluorescence was generated by TALEN-mediated knock-in (Fig. 12.2) (Nong et al. 2017). In this *Dsx1* reporter *Daphnia*, one of the two *Dsx1* alleles has the mCherry ORF sequence. This mCherry reporter was inserted at the position of the endogenous *Dsx1* start codon. Because another *Dsx1* allele is intact, this *Dsx1* hemizygous knock-out males have the reproductive ability and typical male-specific traits even though the slight feminization has been observed in the morphology and frequency of fertilization (Nong et al. 2017). This reporter strain was named Line B (Nong et al. 2020).

Using Line B, no clear mCherry signal was detected both in females and in males until the gastrulation stage. Thereafter, dynamic spatio-temporal expression of *Dsx1* was observed until the late embryonic stage (Nong et al. 2017). In male embryos, during stomodeal invagination (11 hpo), mCherry-positive cells are localized into

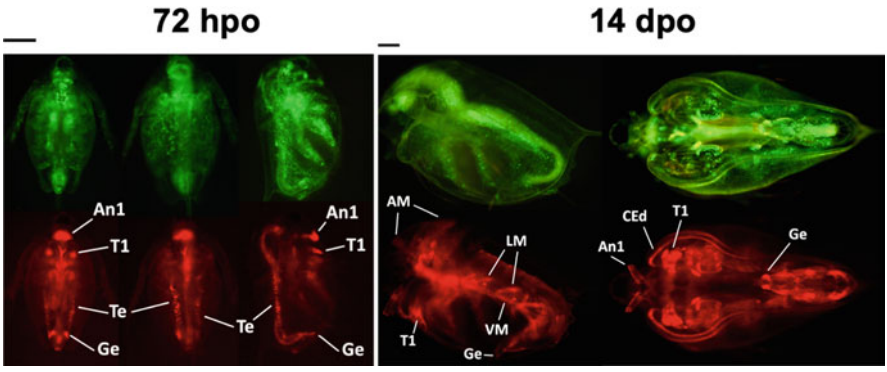

Fig. 12.2 Visualization of spatio-temporal activation of *Dsx1* using the *Dsx1* reporter strain named Line B. 72 hpo and 14 dpo indicate 72 h post-oviposition and 14 days post-oviposition, respectively. Upper panels show pictures of H2B-GFP expression with which cells and tissues are visualized. Lower panels show mCherry fluorescence that recapitulates Dsx1 expression. *A1* first antenna, *T1* first thoracic leg, *Ge* genital (i.e., penis in this case), *Te* testis, *AM* antennal muscle, *LM* lateral muscle, *VM* ventral muscle, *CEd* carapace edge. Scale bars = 200 μm. (The photos were reproduced from Nong et al. (2017))

the cell cumulus and around the blastopore. From 11 hpo to 16 hpo, these cell clusters move on the ventral region and reach the posterior region. The behavior of the mCherry-positive cells resembles that of the primary organizer in spider (Akiyama-Oda and Oda 2003) and chicken (Bénazéraf and Pourquié 2013), suggesting that the primary organizer is sexually plastic and controls sexual development of *D. manga*. When male embryos reach the late embryonic stage, more intense signals were detected. In more detail, at the naupliar segmentation stage (16–18 hpo), in addition to mCherry signals in the posterior region, the intense signals appear in the buds of the first antennae and along the border of the head and body segments. During the thoracic segmentation (18 hpo and later), strong mCherry fluorescence is observed in the first thoracic segment where a hook for capturing females is developed. In juveniles, mCherry signals are detected in organs showing male-specific characteristics such as first antennae, first thoracic legs, testes, and spermiduct-genitalia system (Fig. 12.2, 72 hpo). From the instar 5, consistent with the timing of transformation into mature males, the regions expressing mCherry are expanded into the other male-specific structures including the carapace edge, the tip of the penis, and the skeletal muscle (Fig. 12.2, 14 dpo). These demonstrate the spatio-temporal regulation of *dsx1* transcription for male development.

12.3.3 Dsx1 *Activity-Dependent Formation of the Sex Spectrum*

The Line B harbors a hemizygous *Dsx1* allele and exhibits slight feminization, which suggests that the *Dsx1* mutant can be a model for analyzing the molecular mechanism underlying the sex spectrum. Another *Dsx1* mutant showing the more feminized phenotypes was generated when knock-in of the *mCherry* reporter at the *Dsx1* locus was performed with the TALEN system (Nong et al. 2017, 2020). In this mutant, one *Dsx1* allele was disrupted by integration of the *mCherry,* and the other allele has a 6-bp deletion on the start codon of *Dsx1* ORF, possibly leading to the synthesis of the truncated Dsx1 protein that lacks the N-terminal region but still has intact DM domain and oligomerization domain. This compound heterozygous mutant was designated as Line A (Nong et al. 2020).

In males of Line A, sex-specific traits were more shifted to the female side throughout the life span compared to Line B (Fig. 12.3) (Nong et al. 2020). At the juvenile stages, the first antennae of the Line A male were less elongated than that of the Line B male and at the adult stages, their feminization of the morphology was more profound; larger body size, female-like shapes of the carapace, head region, and genital and anal region (Fig. 12.3). Line A male also developed an ovary-like structure although the eggs were not produced from this feminized gonad at least for several weeks. These phenotypic observations demonstrate that Line A and Line B males are sex intergrades with different degrees of feminization (Nong et al. 2020).

To reveal the gene network underlying the sex spectrum, wild-type females, Line A males, Line B males, and wild-type males were subjected to RNA-seq analysis

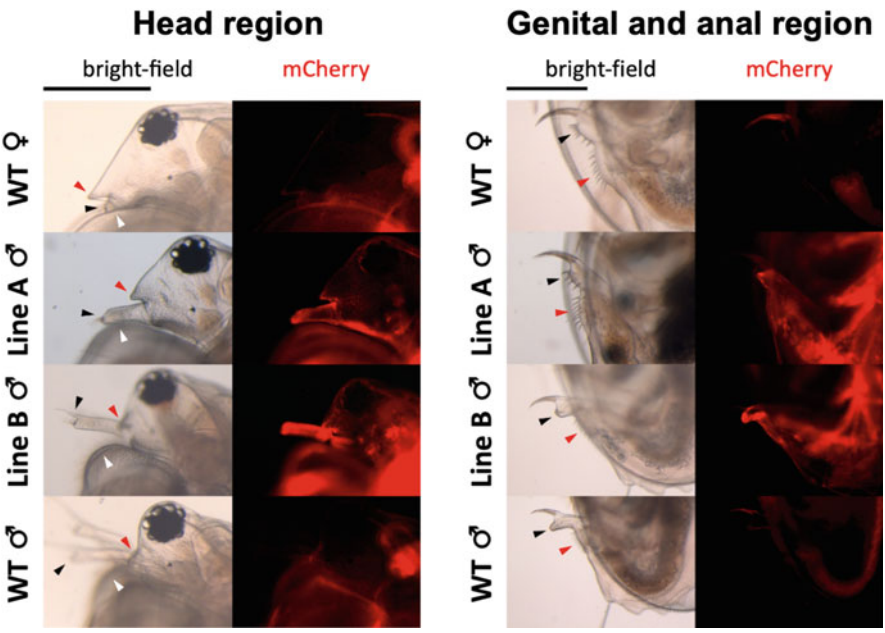

Fig. 12.3 Feminization in the *Dsx1* mutants that have the *mCherry* gene on the *Dsx1* locus. Dsx1 activity is strongest in wild-type males, followed by Line B males, Line A males, and wild-type females. Head region, side view of instar 8 daphniids. Black-arrow heads: first antennae. White arrowheads: carapace curve that reveals copulation hooks in males. Red arrowheads indicate the rostrum. The genital and anal region, side view of instar 8 daphniids. Black arrowheads: genital. Red arrowheads: anus. For each region, the bright-field and mCherry images are shown. Scale bars = 0.5 mm. (The photos were reproduced from Nong et al. (2020))

(Nong et al. 2020). Late embryos at 40 h post-oviposition that exhibit sexually dimorphic *Dsx1* expression were chosen for this transcriptome analysis. There were 1354 differentially expressed (DE) genes between wild-type females and wild-type males. Among those DE genes, 505 and 226 genes showed female- and male-biased expression. Interestingly, 78% and 22% of the female- and male-biased genes exhibited intermediate expression levels in the sex intergrades, Line A and Line B males. The genes linked with transportation and metabolism occupied 22% of the female-biased group. The genes functionally categorized into signaling pathways and embryonic development was more found in the male-biased groups (Nong et al. 2020). These findings indicate that altered Dsx1 expression or activity can change the position of the spectrum of the sex by modification of expression of its target genes.

12.4 Transcriptional Regulation of *Dsx1*

The *Dsx1* gene consists of four exons and occupies about 20 kb of the genome (Fig. 12.4a) (Kato et al. 2011a). The size of the first and second introns is approximately 9 kb and 8 kb, respectively, and is much larger than the average size of the intron (392 bp) in this species (Lee et al. 2019), suggesting the presence of important regulatory elements in these introns. From this locus, the two *Dsx1* mRNA isoforms, *Dsx1-α* and *Dsx1-β*, are produced by the alternative promoter usage. Transcription of *dsx1-α* isoform starts from exon 3 and continues until exon 4 harboring the CDS and 3′ UTR. *Dsx1-β* is encoded by the *β* isoform-specific exons 1 and 2 in addition to the protein-coding exon 4, meaning that both isoforms produce the same amino acid sequence of Dsx1 protein but their regulations are different.

The sesquiterpenoid signaling commits the sex of offspring to males from 4 to 10 h before ovulation (Kato et al. 2010). During this period, *Dsx1* does not show any

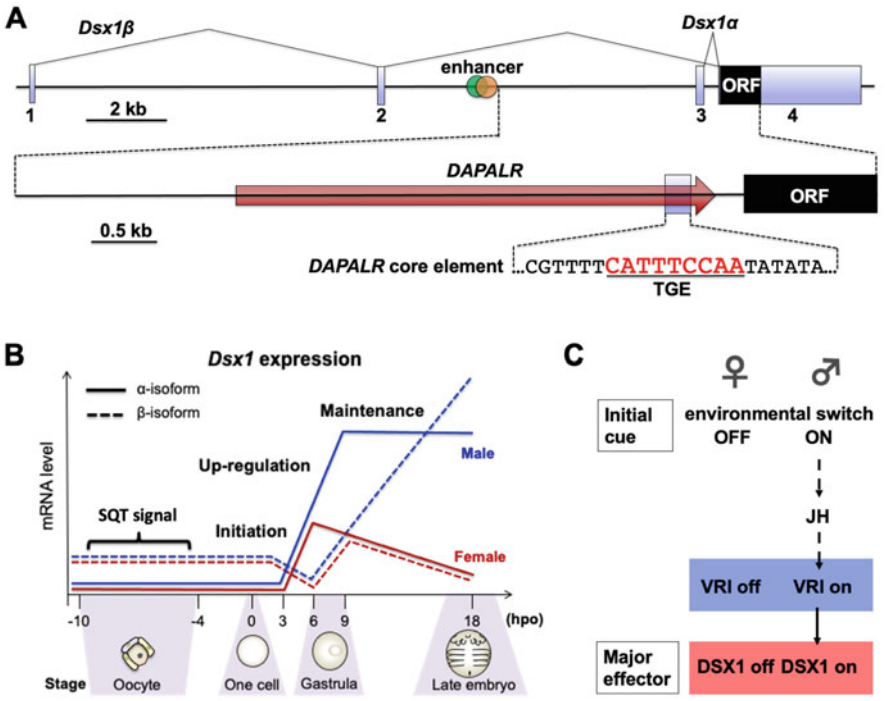

Fig. 12.4 Structure and expression of *Dsx1*. (**a**) The exon-intron structure of Dsx1. Exons of *Dsx1* are indicated with light blue boxes. The red arrow shows the transcribed region of *DAPALR*. *TGE* TRA-2/GLI element. The potential Vri and Dsx1 binding sites are shown in the enhancer with green and orange circles, respectively. (**b**) Temporal expression patterns of Dsx1 isoforms during late oogenesis and early embryogenesis. *hpo* hour post-ovulation, *SQT* sesquiterpenoid. (**c**) A potential sex-determining cascade in *D. magna*. (The figures (**b** and **c**) were reproduced from Mohamad Ishak et al. (2017))

sexually dimorphic expression (Fig. 12.4b). The male-specific expression of *Dsx1* is established via the three processes during embryogenesis: initiation, upregulation, and maintenance (Mohamad Ishak et al. 2017). Non-sex-specific transcription of *Dsx1-α* mRNA occurs before the early gastrulation stage at 6 h post-ovulation (hpo) (initiation). Male-specific activation of *Dsx1-α* expression is implemented during the gastrulation stage from 6 hpo to 9 hpo, and its expression level is maintained in late embryos (18 hpo). *Dsx1-β* isoform is maternally deposited in the ovulated eggs and its male-specific expression begins 3 h later than *Dsx1-α*. These expression patterns of the *Dsx1* isoforms in early embryos explain why male-specific mCherry signals are detected in the *Dsx1* reporter strain Line B after the gastrulation stage. Transcription of *Dsx1-α* isoform is more exclusive in males than that of *Dsx1-β*, suggesting its predominant role in male development (Kato et al. 2011a; Nong et al. 2017).

The male-specific upregulation of the *Dsx1-α* isoform is controlled by the bZIP transcription factor Vrille (Vri) (Mohamad Ishak et al. 2017). The consensus binding element of the mammalian Vrille ortholog, E4BP/NFIL3, is present in intron 2 (Fig. 12.4a) and overlapped with the consensus Dsx binding site. The combinatorial binding of the bZIP protein and Dsx has been reported to control the *Drosophila* yolk protein gene 1, which might suggest conservation of mode of Dsx action between *Daphnia* and *Drosophila*. Importantly, at the early gastrulation stage (6 hpo), just before male-specific *Dsx1* activation occurs, *Vri* transcript is significantly more abundant in male embryos although, in late embryos (18 hpo), it exhibits no sexually dimorphic expression. Knockdown of *Vri* in male embryos reduced *Dsx1* expression whereas its overexpression in female embryos increased the *Dsx1* transcript level. *Dsx1* upregulation was also diminished by the introduction of a mutation into the enhancer harboring the potential Vri and Dsx1 binding sites (Mohamad Ishak et al. 2017). The candidate of the binding site for sesquiterpenoid-dependent transcription factor MET is located in the *Vri* promoter, suggesting the possibility that the sesquiterpenoid-bound MET activates *Vri* expression in early gastrula (6 hpo), and the resulting Vri protein controls upregulation of *Dsx1* in late gastrula (9 hpo) (Fig. 12.4c). Because loss- and gain-of- *Vri* function led to embryonic lethality, it remains unknown whether the Vri is the sole factor for *Dsx1* upregulation. Co-option of Vri as a regulator of *Dsx* expression in the sex-determining pathway has not been reported in other species, supporting the idea for plasticity of the regulatory mechanism of *Dsx* in animals (Herpin and Schartl 2015).

12.5 Epigenetic Regulation of *Dsx1*

Since male and female *Daphnia* are genetically identical, it may be possible that the epigenetic factors are involved in male-specific *dsx1* expression. The result indicating the sex difference of the chromatin structure at the *dsx1* locus has been obtained when the *dsx1* enhancer sequence harboring the potential Vri and Dsx1 binding site

in the intron 2 was mutated by CRISPR/Cas in *D. magna* (Fig. 12.4b) (Mohamad Ishak et al. 2017). In this experiment, the two separate gRNAs, gRNA-1 and gRNA-2 that recognize the upstream and downstream sequence of the enhancer respectively, were designed, synthesized, and co-injected with Cas9 protein into the eggs that are destined to develop into females. However, no mutation was introduced both in somatic and in germ cells. In contrast, when the same Cas9 RNP was injected into eggs that develop as males, indel mutations were successfully introduced into the enhancer region, which resulted in the silencing of the *dsx1* gene (Mohamad Ishak et al. 2017). The efficiency of the double-strand break by the CRISPR/Cas in the heterochromatin region has been known to be significantly lower than that in the euchromatin region (Chen et al. 2016; Daer et al. 2016; Jensen et al. 2017). Less DNA accessibility of the CRISPR/Cas was also demonstrated quantitatively by using the genome-wide sequencing approaches (Chung et al. 2020). Thus, the inability of the double-strand break in the enhancer region may suggest heterochromatinization at the *dsx1* locus in the female.

In *D. pulex*, the sex differences of epigenetic modifications including DNA methylation and histone modification have been comprehensively investigated using samples from a mixture of juveniles and adults with different ages (3, 8, and 15 days old) (Kvist et al. 2020). DNA methylation was analyzed by whole-genome bisulfite sequencing. Despite the extremely low methylation level, the differentially methylated CpGs were predominantly found within gene bodies. At the *dsx1* locus, CpG methylation frequency was higher in the female genome in contrast to the overall tendency of higher methylation levels in the male genome (Kvist et al. 2020). For the histone modifications, active and repressive histone marks, histone H3 trimethylation at lysine 4 (H3K4me3) and lysine 27 (H3K27me3), were analyzed by chromatin immunoprecipitation. Among the sex-specific H3K4me3 peaks, 78% and 22% were detected in males and females respectively, suggesting its role for male-specific gene activation. On the other hand, H3K27me3 peaks were predominantly detected in females and the female-specific peaks occupied 84% of the sex-specific peaks. At the *Dsx1* locus on the male genome, the H3K4me3 level was around 300-fold higher but the H3K27me3 level was around 5000-fold lower compared to the female genome (Kvist et al. 2020). These demonstrate that the sex-specific *Dsx1* expression is epigenetically controlled.

12.6 Post-transcriptional Regulation of *Dsx1*

12.6.1 5′ UTR-Overlapping lncRNA DAPALR *Activates* Dsx1 *Expression*

In the process of analyzing the role of the *Dsx1* 5′ UTR in the post-transcriptional regulation, a long noncoding RNA that overlaps the 5′ UTR of the *Dsx1-α* isoform in the sense orientation and activates *dsx1* expression has been identified (Kato et al.

2018). In this analysis, the chimeric *DsRed2* reporter mRNA harboring either *Dsx1-α* or *Dsx1-β* 5′ UTR was generated and injected into the eggs that develop into female embryos. Unexpectedly, the reporter RNA with the *Dsx1-α* 5′ UTR led to the development of the male-specific trait, the elongation of the first antennae, although it lacked the *Dsx1* ORF. The RNAs that only coded for the *Dsx1-α* 5′ UTR also induced male differentiation. When those RNAs harboring the *Dsx1-α* 5′ UTR were injected into eggs of the *Dsx1* reporter line (Line B), the mCherry reporter fluorescence was increased, suggesting a potential role of the *Dsx1* 5′ noncoding sequence in the regulation of its own expression (Kato et al. 2018). Interestingly, in the database of the genomic tiling path microarrays from *D. pulex*, there was the long noncoding RNA (lncRNA) that is transcribed from the transcription start site of the *Dsx1-α* and overlaps with the exon 3 encoding its 5′ UTR. The orthologous RNA in *D. magna* was identified, characterized, and named *doublesex1 alpha promoter-associated long RNA* (*DAPALR*). *DAPALR* is 3650 nt of a capped and non-polyadenylated RNA that does not include the *Dsx1* ORF (Fig. 12.4a) (Kato et al. 2018).

Similar to *Dsx1*, *DAPALR* expression increases over 72 h in male embryos until the juvenile stage but its expression level is ten times lower than that of *dsx1* (Kato et al. 2018). The silencing of *Vri*, a gene encoding the transcriptional activator of *dsx1*, resulted in the decrease of the *DAPALR* expression while its overexpression increased the *DAPALR* levels significantly. Disruption of the Vri binding site in the enhancer region reduced *DAPALR* expression, indicating co-regulation of expression of *Dsx1* and *DAPALR* by the Vri protein (Kato et al. 2018). To investigate the role of *DAPALR* in the regulation of male development and *Dsx1* expression, siRNAs specific to *DAPALR* were injected into eggs destined to become males, which resulted in the reduction of *Dsx1* expression and in turn led to the feminization of somatic tissues such as the first antennae and development of ovarian tissues. In addition, more potent and stable stealth siRNAs enabled the injected *Daphnia* to produce eggs that developed normally into females (Kato et al. 2018). Since the RNAi has been known to induce heterochromatin formation at the targeted locus (Martienssen and Moazed 2015), there might be the possibility that DAPALR RNAi induced reduction of *Dsx1* transcript levels indirectly via spreading of heterochromatin region at the *Dsx1* locus. Thus, the effect of overexpression of *DAPALR* on sexual development was also tested. Injection of the *DAPALR* expression plasmid into female embryos of the *Dsx1* reporter strain led to an increase of mCherry fluorescence intensity and masculinization. These demonstrate the role of DAPALR in regulating male-specific *Dsx1* activation (Kato et al. 2018).

12.6.2 DAPALR *Functions as a Decoy of Shep*

The core region of *DAPALR* overlapping with the *Dsx1-α* 5′ UTR (Fig. 12.4a) is sufficient to activate *dsx1* expression (Kato et al. 2018). To identify proteins that bind to the core region, RNAs that code for 205 nt of the overlapping region were

synthesized in vitro, conjugated with a Flag-peptide, and used for an immunoprecipitation experiment using a *D. magna* lysate (Perez et al. 2021). Analysis of the co-immunoprecipitated proteins by MS revealed significant binding of the two RNA binding proteins: Alan Shepard (Shep) and CUG binding protein 1 (CUGBP1) to the core element. Shep function was further analyzed because its ortholog Sup-26 in *C. elegans* has been known to regulate the sex-determining gene *Tra-2* at the translation level (Mapes et al. 2010).

Shep mRNA expression did not show any sexual dimorphism in contrast to *Dsx1* and *DAPALR*, suggesting its role in both genders (Perez et al. 2021). To investigate the function of *Shep*, using the *Dsx1* reporter strain Line B, generation of *Shep* mutant has been attempted by introducing indel mutations in the regions that code for the RNA recognition motif (RRM). Although biallelic disruption of *Shep* was likely to be lethal, one monoallelic mutant harboring 15 bp insertion in frame was established. Both males and females of this *Shep* mutant showed significantly higher mCherry fluorescence than the wild-type, suggesting that Shep may suppress *Dsx1* both in males and females. In contrast, *Dsx1* mRNA expression levels were not significantly different between the *Shep* mutant and wild-type in either male or female, suggesting that Shep likely inhibits *Dsx1* translation (Perez et al. 2021).

In *C. elegans*, the Shep ortholog Sup-26 represses translation of *Tra-2* via its binding to the TRA-2/GLI element (TGE) that is located in the *Tra-2* 3′ UTR (Mapes et al. 2010). A highly conserved sequence with TGE was found in the *Dsx1-α* 5′ UTR (Fig. 4a). To investigate this element, either the GFP reporter mRNA with the intact *Dsx1-α* 5′ UTR or the one lacking the potential TGE was injected into female eggs, resulting in higher GFP fluorescence of the embryos injected with the mutated mRNAs than those with the RNAs with the intact TGE. Co-injection of *Shep* mRNA with the intact *Dsx1-α* 5′ UTR:: GFP reporter mRNA led to reduction of the GFP fluorescence. Shep-dependent repression of translation through the potential TGE element was confirmed in vitro by co-incubation of the Luciferase (Luc) reporter mRNA harboring the intact *Dsx1-α* 5′ UTR with the Shep mRNA in the rabbit reticulocyte lysate (Fig. 12.5a). Interaction between the TGE-like element and Shep was further proved to be direct by the pull-down assay. These demonstrate that Shep suppresses the translation of the reporter mRNA through the TGE-like motif (Perez et al. 2021).

DAPALR has the TGE-like motif in the region overlapping with the *Dsx1-α* 5′ UTR (Fig. 12.4a). To examine how *DAPALR* controls *Dsx1* expression, *DAPALR* and *Shep* mRNA were co-incubated with the Luc reporter mRNA harboring the intact *Dsx1-α* 5′ UTR in the reticulocyte lysate. In this reaction, *DAPALR* canceled translational repression by Shep via the TGE element. Importantly, the core element of *DAPALR* harboring the TGE motif had the same ability as the full-length of *DAPALR* (Fig. 12.5a). These rescue efficiencies of *DAPALR* or its core element were dose-dependent (Fig. 12.5b). These results show the function of *DAPALR* as a decoy of Shep (Perez et al. 2021).

For activation and repression of *Dsx1*, the chromatin of this locus is possibly open and closed in males and females, respectively. However, expression noise of *Dsx1* in females might occur due to the nature of stochasticity in gene expression (Kærn et al.

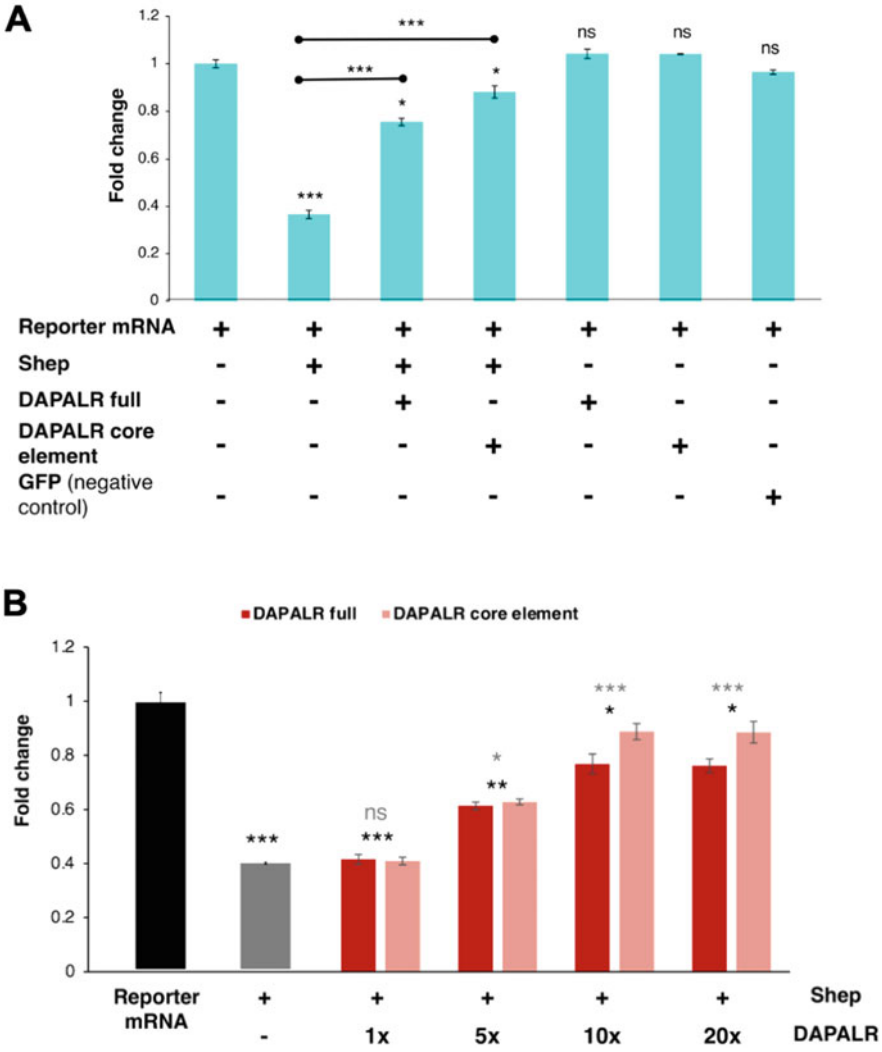

Fig. 12.5 Post-transcriptional regulation of *Dsx1* by *DAPALR* and Shep. (**a**) Relative luciferase activity after in vitro translation assay of *Dsx1* 5′ UTR-*Luc* reporter mRNA with intact TGE upon addition of *Shep* mRNA, *DAPALR* full RNA, *DAPALR* core element, and *GFP* mRNA (negative control). Samples were compared against the expression of the *Dsx1* 5′ UTR-*Luc* reporter mRNA without the addition of any other mRNAs. The endpoints of the line above the bars show which samples were additionally compared statistically. (**b**) Relative luciferase activity after in vitro translation assay of *Dsx1* 5′ UTR-*Luc* reporter mRNA with *Shep* mRNA and different concentrations of full region of *DAPALR* and its core element. Error bars indicate the standard error of the mean, $n = 3$. Black asterisks show significant statistics compared with the expression of the Dsx1 5′ UTR-Luc reporter mRNA. Gray asterisks show significant statistics compared with the Reporter mRNA with Shep. Error bars indicate the standard error of the mean, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant (Student's *t*-test). (The figures were reproduced from Perez et al. (2021))

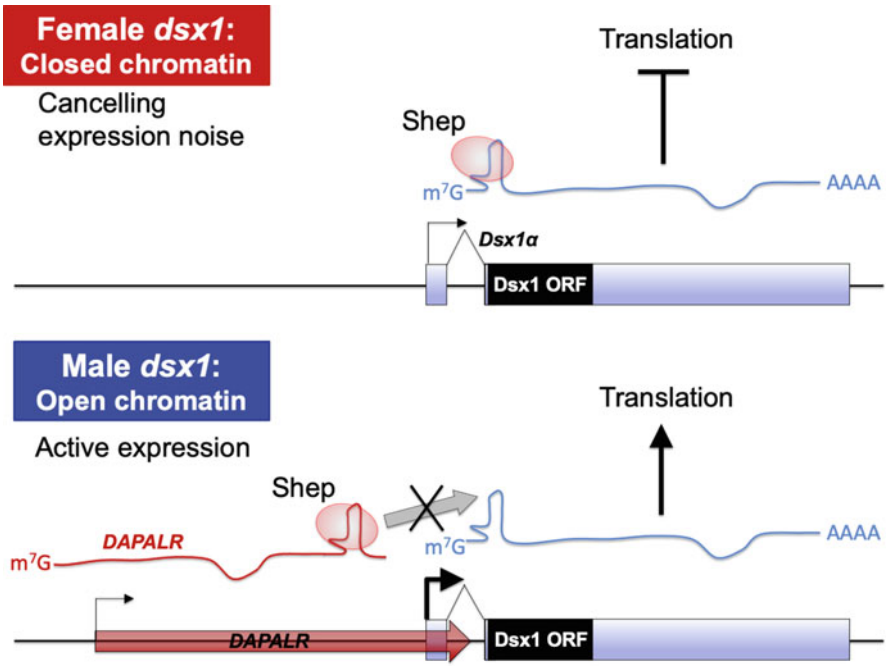

Fig. 12.6 The potential mechanism for establishing the sex-specific *Dsx1* expression. Transcriptional, epigenetic, and post-transcriptional regulations are integrated to achieve male-specific *Dsx1* expression for avoiding sexual ambiguity

2005; Ozbudak et al. 2002). To ensure the inactive state of *Dsx1*, there would be the noise-canceling system where Shep represses unintended expression of *Dsx1* (Fig. 12.6). In males, to unlock the safe lock on *Dsx1* expression, *DAPALR* can counteract this repression by sequestering Shep (Fig. 12.6). To unravel this post-transcriptional regulation by *DAPALR* and Shep, the mode of RNA recognition by Shep and stoichiometry of *DAPALR*, Shep, and *Dsx1* mRNA needs to be elucidated.

12.7 Concluding Remarks and Future Perspectives

In *D. magna*, environmental signals shift the position of the sex on the spectrum from the female side to the male side. Mothers sense severe environments and destine the sex of offspring to males via sesquiterpenoid signaling. This signaling occurs at the late stage of developing oocytes and leads to their commitment to male development possibly by changing the *Dsx1* locus from the closed to open chromatin state, which allows Vri for binding to the *Dsx1* enhancer and leads to upregulation of *DAPALR* and *Dsx1* for initiating the male developmental program. In contrast, in

females, *Dsx1* is silenced in the whole body and Shep represses translation of the unintended expression of *Dsx1* derived from noise to prevent intersex phenotype.

Collectively, transcriptional, epigenetic, and post-transcriptional regulation of *Dsx1* could contribute to the establishment of the binary sex in *D. magna*. Factors involving each regulation began to be identified in the last few years. Further studies are needed to address how each regulation is mutually linked. To achieve it, an important step is to analyze the gene regulatory mechanism in the cellular contexts with help of cell sorting and single-cell analysis. It would also be important to investigate the connection between the sesquiterpenoid signaling and *Dsx1* activation. These comprehensive analyses of *Dsx1* regulation will contribute to understanding molecular mechanisms underlying both establishment of the binary sex and the formation of sex spectrum in *D. magna*.

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