Doctoral Dissertation

CELF1 represses *Doublesex1* expression via its 5' UTR in the crustacean *Daphnia magna*

(オオミジンコにおける **CELF1** による **5' UTR** を介した ***Doublesex1*** 遺伝子発現の抑制**)**

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Abbreviation list

GSD	: Genotypic sex determination
ESD	: Environmental sex determination
Dsx1	: Doublesex1
UTR	: Untranslated region
CUGBP1	: CUG-Binding Protein 1
CELF1	: CUGBP Elav-like Family Protein 1
siRNA	: Short interference RNA
DAPALR	: Doublesex1 alpha promoter associated long noncoding RNA
GFP	: Green fluorescent protein
EF1α1	: *elongation factor 1α-1* promotor/enhancer
GRE	: GU-Rich element

CHAPTER 1 GENERAL INTRODUCTION

1.1 Environmental sex determination

Sex determination can be broadly categorized into two groups according to the primary causal agent of sex determination. One is the most researched genotypic sex determination (GSD), in which sex chromosomes determine the sex of the individual and are diversely used in many species, such as mammals and *Drosophila melanogaster* (Bachtrog et al., 2014). In contrast, environmental sex determination (ESD) uses environmental factors to determine sex and favors when specific environments are more beneficial to one sex (J. J. Bull, 1981, 1987). One example of ESD is the European pond turtle, *Emys obicularis* (Figure 1.). *Emys* egg incubation at a temperature above 30°C produces female offspring. On the other hand, temperatures below 25°C will produce male offspring showing a temperature dependant sex determination (Pieau et al., 1994).

Fisherian model predicts the sex ratio is nearly 1:1 under GSD. However, there is a biased sex ratio under ESD depending on selective pressure caused by environmental effects such as temperature, daylength, nutrition, density and many others (Fisher, 1930; Korpelainen, 1990). Against these uncertain environmental conditions or having no control over which environment will occur, environmental sex determination is advantageous because it allows the embryo to adjust its sex based on environmental effects on fitness. However, there are also possible disadvantages of ESD (J. Bull, 1985). First, intersex can be produced because of late sex development and in response to environmental conditions. Second, ESD may allow climatic change to affect the population sex ratio. Despite the disadvantages, selective pressure has favored ESD over GSD in diverse taxa to initiate sex-specific molecular cascades. This is the possible reason why the environmental system has not always been replaced with the genetic system that determines sex at conception (Janzen & Phillips, 2006; Korpelainen, 1990).

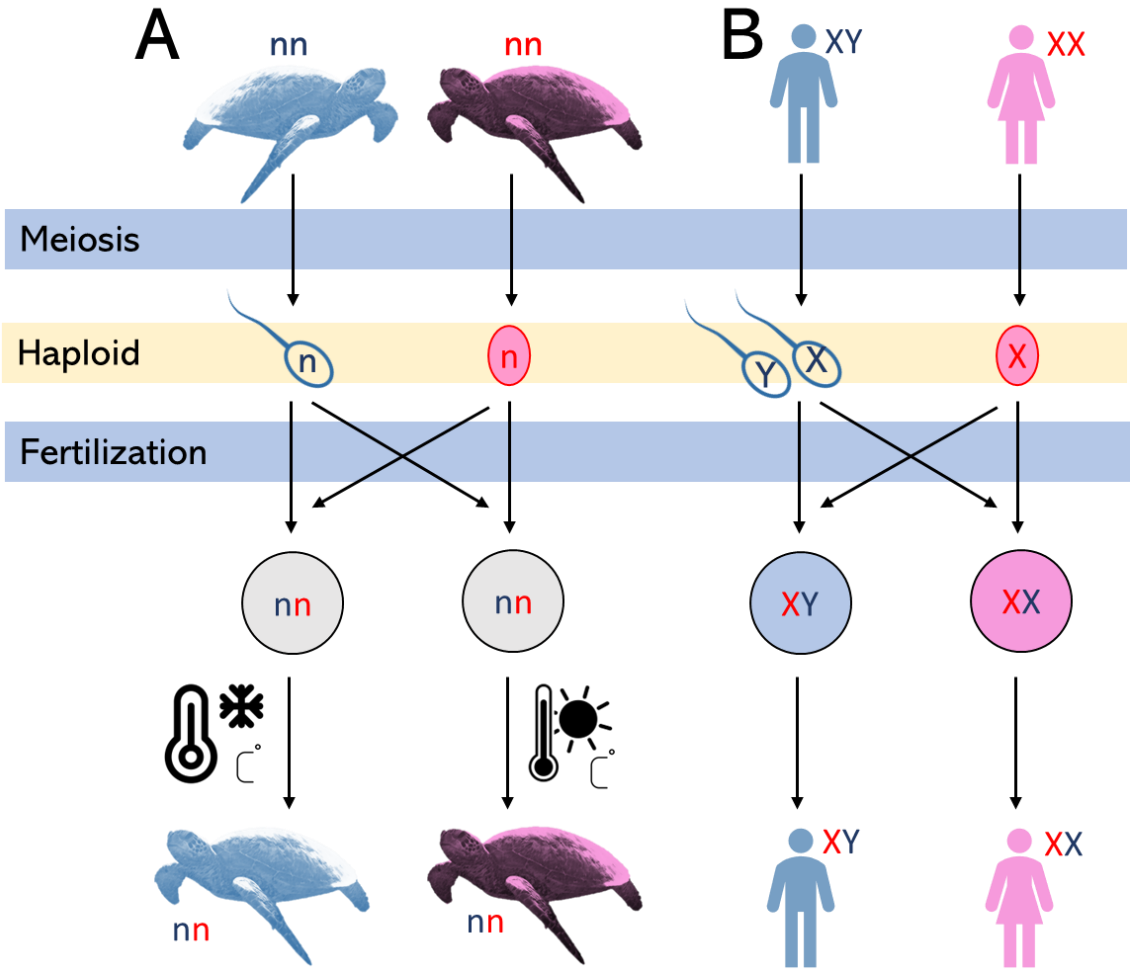


Figure 1. Environmental vs genotypic sex determination

Sex determination pathways are based on how it initiated. *Emys orbicularis* adopted temperature-dependant sex determination where the different temperatures will change the sex ratio between male and female offspring. On the other hand, *Homo sapiens'* sex determination is determined at conception with the genotypic signal from sex chromosomes.

Understanding the ESD is important because the evolutionary dynamics of genes and their mechanisms should be fundamentally different from the well-known GSD (Bachtrog et al., 2014; Valenzuela et al., 2012). Additionally, elucidation on ESD may help the biotechnology sector to develop genetic-based tools such as pest control (Siddall et al., 2022) and sex ratio control for aquaculture (Li et al., 2022). However, little is known about the specific mechanism behind the ESD despite having been reported to affect sex determination in diverse species to date (Weber & Capel, 2021)

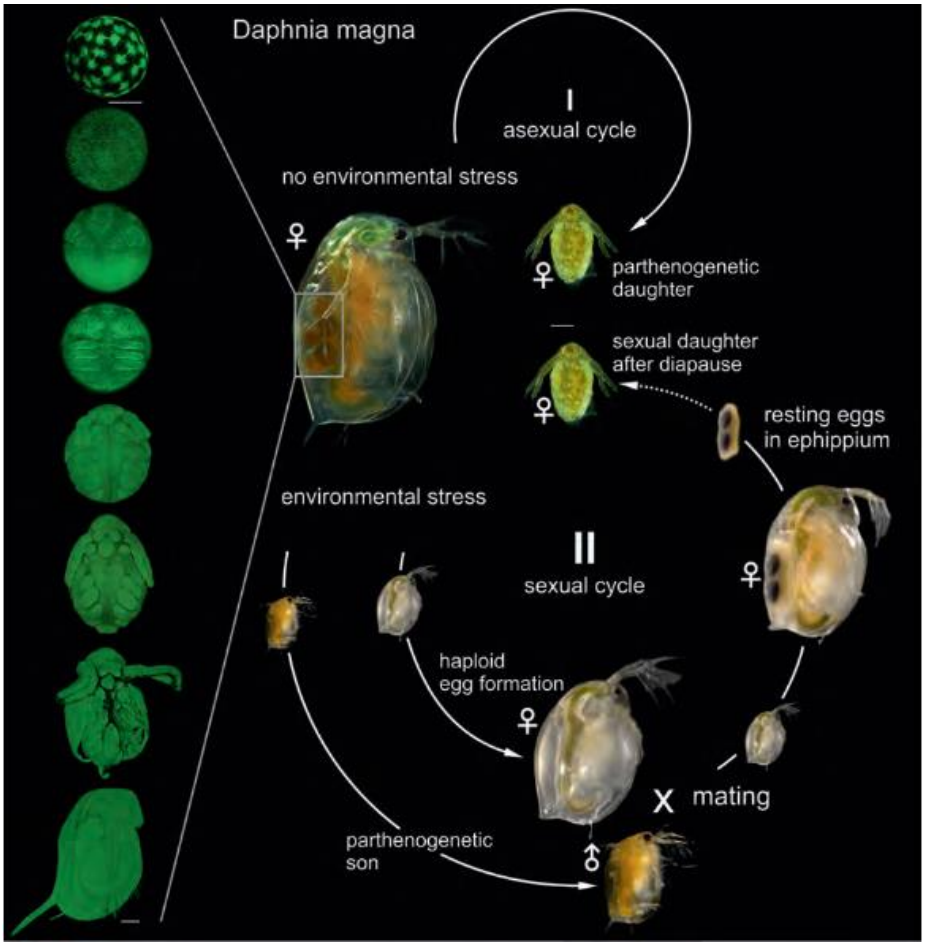


Figure 2. The life cycle of the water flea *Daphnia magna*

The branchiopod crustacean *Daphnia magna* undergoes parthenogenesis (asexual life cycle) under favorable conditions to produce female clones. The second reproductive strategy (sexual life cycle) is triggered under certain environmental stress. In this case, parthenogenetic females produce parthenogenetic sons (males) and parthenogenetic daughters with haploid eggs (females). The female will be fertilized by a male and produce sexual resting eggs that withstand harsh conditions. After diapause and a favorable condition is met, sexually produced daughters hatch and reproduce asexually again. The left column illustrates embryonic development inside the female brood chamber (Wanninger, 2015).

1.2 Model organism *Daphnia magna* and its molecular manipulation

The water flea crustacean *Daphnia magna* is a unique model for sex determination studies and is used extensively in many molecular studies as many molecular manipulation techniques are available. *Daphnia magna* sex determination relies on environmental cues such as photoperiod, nutrition, population density, and many substances disturbance in the environment to initiate further sex development cascades (Hebert, 1978; J. Lubbock, 1857).

Under favorable conditions, *D. magna* parthenogenetically produces females until a certain stress condition triggers the male development cascade (Figure 2). The environmental cues for male determination stimulate the neuroendocrine system and secrete sesquiterpenoid, which promotes the production of parthenogenetic eggs destined to develop into males (LeBlanc & Medlock, 2015; Toyota et al., 2021). This unique sex determination and its sensitivity towards environmental changes made *D. magna* attractive to be used in ecotoxicology studies for molecular investigation of ESD.

The recent development of molecular manipulation tools for deep functional analysis of genomic networking or certain gene characteristic was established, such as gene knockdown, overexpression, partial or total knockout, and gene knock-in (Kato, Shiga, et al., 2011; Kumagai, Matsuura, et al., 2017; Nakanishi et al., 2014, 2015; Törner et al., 2014). Moreover, the complete genomic sequence and transcriptomic data were publicly accessible (Byeon et al., 2022; Colbourne et al., 2011; Orsini et al., 2016; Watanabe et al., 2005). The short generation life and simple culture made knockout or knock-in mutant production far more efficient, faster, and easier (Ismail et al., 2018; Kumagai, Nakanishi et al., 2017; Nong et al., 2020) compared to other animal models like reptiles (Rasys et al., 2019). Therefore, *D. magna* is a suitable animal model for elucidating ESD mechanisms.

1.3 *Doublesex1*

Doublesex (Dsx) was first identified as an important transcription factor in *D. melanogaster* GSD and directed downstream genes responsible for sexually dimorphic development (Burtis & Baker, 1989). Doublesex contains two important domains, which are Dsx/Mab-3 (DM) domain near N-terminus and the oligomerization domain near C-terminus. The former DM domain is conserved in many genes related to sex determination in both GSD and ESD within the animal kingdom (Volff et al., 2003). The oligomerization domain that

enhances the Dsx DNA binding ability was also conserved among insect homologs (Bayrer et al., 2005). These important conserved domains have been named Dsx, the master sex determination among insects (Verhulst & Van de zande, 2015). Further reports have revealed that Dsx is not only an important factor in GSD but also in ESD, especially in *D. magna* (Kato, Kobayashi, et al., 2011).

Two paralog genes of Dsx have been characterized in *D. magna*, namely Doublesex1 (*Dsx1*) and Doublesex2 (Dsx2). Among these two genes, *Dsx1* specifically regulates the development of male traits and is exclusively expressed in male embryos (Kato, Kobayashi, et al., 2011). *Dsx1* transcription was controlled by two different promoters and transcribed into two different mRNA isoforms, *Dsx1α* and *Dsx1β* (Figure 3). The difference in the 5'UTR of both isoforms have different gene expressions in the gonads and, thus, further investigated.

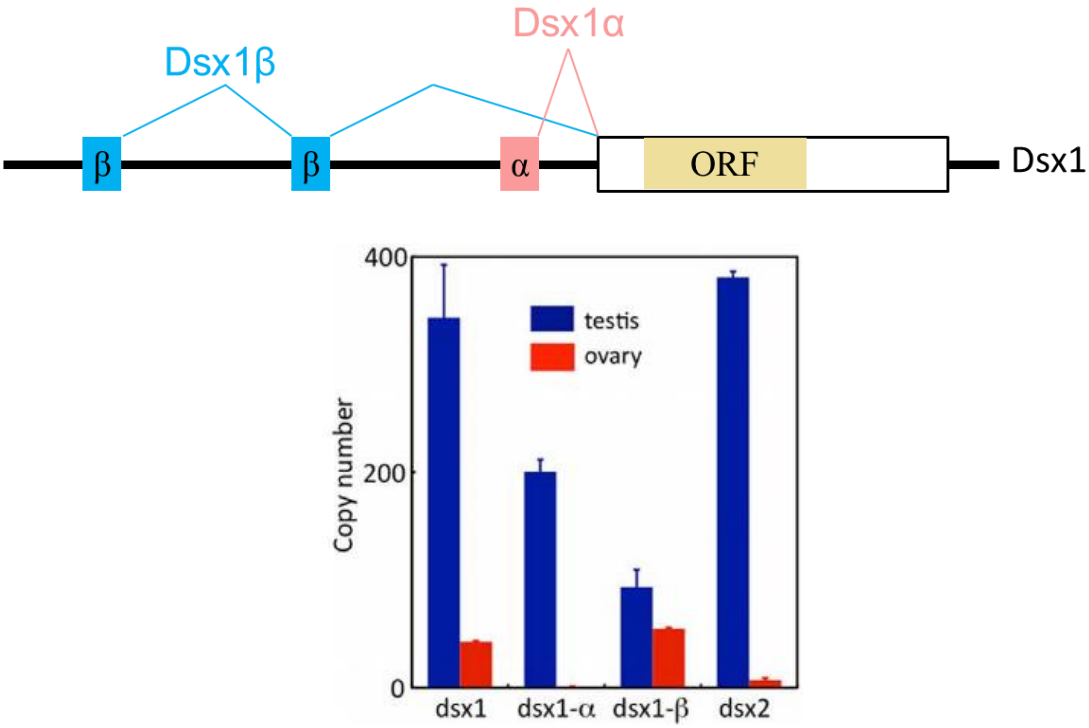


Figure 3. Genomic structure and expression of *Doublesex1* gene in *Daphnia magna*.

Exons are illustrated in boxes. The red boxes represent the *Dsx1α* isoform, while the blue box represents the *Dsx1β* isoform. The white box is the exon the two isoforms share, with the *Dsx1* coding sequence or open reading frame (ORF) represented in the yellow region. The bottom graph shows quantitative expressions of *D. magna* Dsx in the testis and ovary (Kato, Kobayashi, et al., 2011).

1.4 Doublesex1-alpha-promoter-associated-long noncoding -RNA (*DAPALR*)

Further investigation on the promoters of *Dsx1α* and *Dsx1β* leads to the discovery a non-coding element that regulates male development in *D. magna* (Kato et al., 2018). Elongation of the first antennae was one of the sexually dimorphic traits that only developed in male embryos. Injection of either chimeric DsRed2 mRNA with *Dsx1α* 5'UTR or only the 5'UTR was able to induce masculinization in female embryos with the elongation of the first antennae (Figure 4). Following this result, a non-coding region encoding the same *Dsx1α* 5'UTR was annotated and named the Doublesex1-alpha-promoter-associated-long noncoding -RNA (*DAPALR*) (Kato et al., 2018). Therefore, the molecular mechanisms between *DAPALR* and *Dsx1* were further investigated.

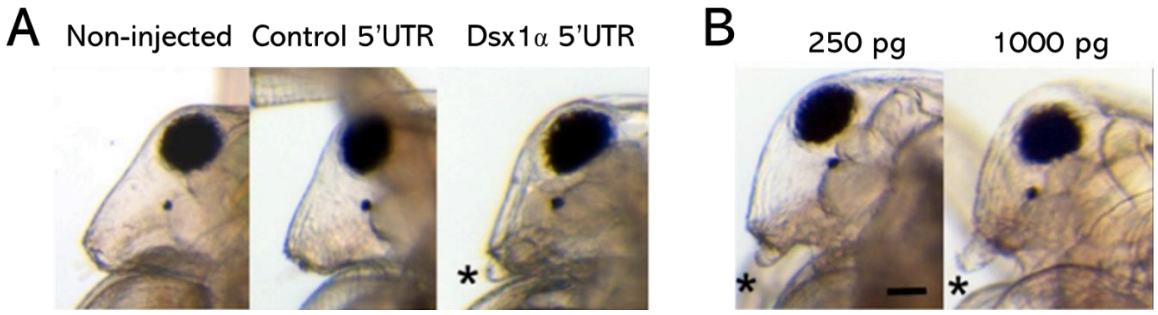


Figure 4. Male traits development of female *Daphnia* injected with *Dsx1α* 5´ UTRs.

Lateral view of female *D. magna* head showing elongation of the first antennae represented by asterisks (*) following injection of chimeric DsRed2 mRNA with *Dsx1α* 5'UTR (A) and Dsxα 5'UTR mRNA only (B) in female embryos. The elongation of the first antennae, which exclusively develops in male embryos only induced by *Dsx1α* 5'UTR and not EF1α1 5'UTR (control) (Kato, Kobayashi, et al., 2011).

DAPALR is a capped, non-polyadenylated non-coding RNA (ncRNA) with a full length consisting of 3,650 bases (Kato et al., 2018). Non-coding RNA does not encode proteins but is transcribed without being translated into functional proteins. However, the recent explosion of knowledge in molecular biology has demonstrated ncRNAs' importance in regulating major biological processes. Its implications include development, differentiation, and metabolism throughout different species (Mercer et al., 2009; Ponting et al., 2009; K. C. Wang & Chang, 2011). The 205 bp of *DAPALR* overlaps with *Dsx1 α* 5´UTR (Figure 5), suggesting a similar

effect regulated by *Dsx1 α* 5´UTR on *Dsx1* expression may also be applied by *DAPALR* (Kato et al., 2018).

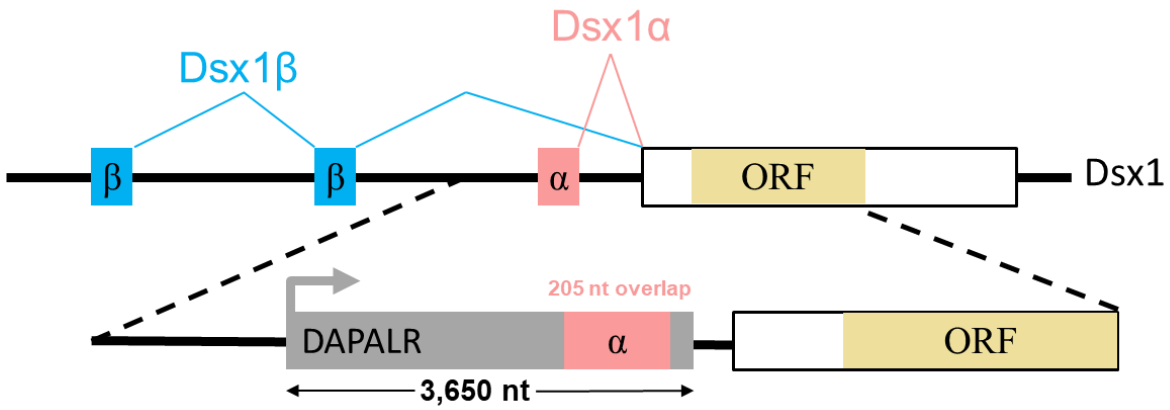


Figure 5. Genomic structure of *DAPALR* compared to the *Dsx1*

Exons are illustrated in boxes. The red boxes represent the *Dsx1α* isoform, while the blue box represents the *Dsx1β* isoform. The white box is the exon the two isoforms share, with the *Dsx1* coding sequence or open reading frame (ORF) represented in the yellow region. *A grey box and arrow indicate DAPALR position and transcription orientation*. The pink box within the *DAPALR* overlapped *Dsx1α* 5'UTR between the *Dsx1α* isoform and *DAPALR*.

Interestingly, the *DAPALR* expression pattern was highly similar to *Dsx1*, which is exclusively expressed in male embryos. This sexually dimorphic pattern with high expression in male embryos suggests a specific male-determining function in *Daphnia magna*. *DAPALR* function was further investigated with molecular manipulation to downregulate *DAPALR* expression in Daphnia embryos. *DAPALR* knockdown in male embryos resulted in feminization, such as egg production in the brood chamber (Figure 6). In addition, Prolonged *DAPALR* knockdown in male daphniids by stable stealth siRNA successfully produced viable G1 embryos. On the other hand, male-specific organs such as elongated first antennae were absent or significantly shortened compared to control male embryos. This result confirmed that the 205bp overlapped region in *DAPALR* is the transactivation element of the *Dsx1*-mediated male determining factor in *Daphnia magna* (Kato et al., 2018).

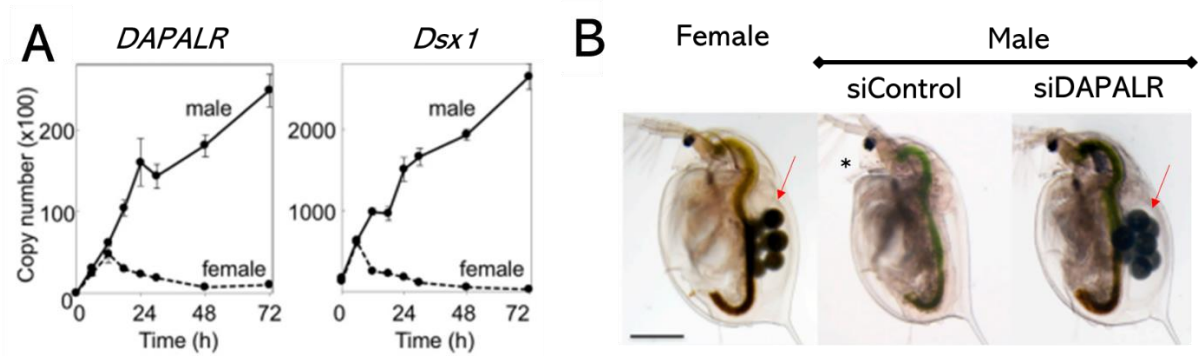


Figure 6. *DAPALR* expression pattern and feminization in *DAPALR* knocked down male embryo

(A) Temporal expression level of *DAPALR* during embryogenesis in the female and male embryo. Results are shown as expression level relative to the ribosomal protein L32. **(B)** Lateral view of adult wildtype female and male daphnia injected with control and *DAPALR* siRNAs. *DAPALR* siRNA-injected male embryos developed short first antennae represented by an asterisk (*) and produced eggs in their brood chamber after reaching adulthood.

1.5 *DAPALR*/*Dsx1α* 5'UTR associating proteins Shep

To elucidate the *DAPALR*-mediated *Dsx1* expression molecular mechanism, the identification of elements involved in the overlapped 205 bp of *Dsx1α* 5'UTR was investigated. The overlapped region of 205 bp *Dsx1α* 5'UTR tagged with FLAG peptide construct was used as a bait to pulldown associating protein in the *D. magna* lysate. Following the protein pulldown experiment, the pulled-down protein was detected by mass spectrometry (Adachi et al., 2014). Among the pulled-down proteins, Alan Shepard (Shep) and CUG binding protein 1 (CUGBP1) have the highest probability for binding to the overlapping sequence of *DAPALR*, and they did not associate with the negative bait samples like the *Dsx1ß* 5´ UTR (Perez et al., 2021).

One of the RNA binding proteins (RBP), Shep, was further investigated for its role in the *DAPALR-Dsx1* molecular network. Utilizing *the Dsx1-reporter strain (Nong et al., 2020) with mCherry mirroring the Dsx1 expression, the* functional analysis of Shep was performed. Knockdown of Shep significantly increased the mCherry fluorescence recapitulating *Dsx1* expression to 5-fold and 2-fold compared to control in females and males, respectively (Figure

8). In contrast, injection of Shep mRNA into male embryos significantly reduced the mCherry expression. Furthermore, the transcript level of *Dsx1* in both experiments did not reflect the mCherry expression observed with a fluorescence microscope. Altogether, these results suggested that Shep may repress *Dsx1* expression at the post-transcription level (Perez et al., 2021).

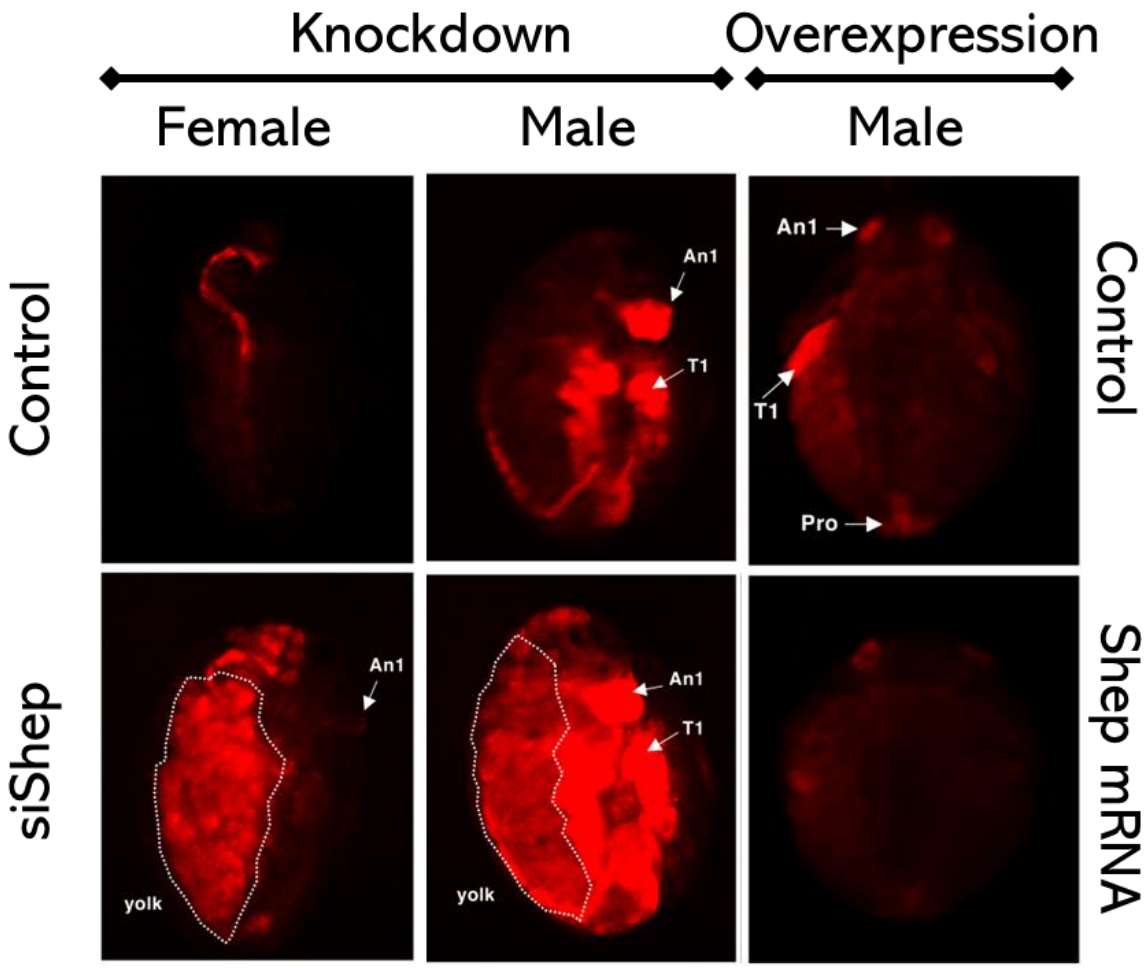


Figure 7. Shep protein represses *Dsx1* activity in male and female embryos

Functional analysis of Shep in *D.* magna. *Shep* knockdown enhances *Dsx1* activity, while overexpression of ectopic Shep represses the *Dsx1* activity. An1: first antennae, T1: first thoracic leg, dashed lines: yolk area.

Finally, the translational regulation of *Dsx1* by Shep and its transactivation element, *DAPALR*, was investigated (Perez et al., 2021). The luciferase gene was fused to intact *Dsx1* 5'UTR or *Dsx1* 5'UTR with deleted Shep binding site. The mRNA of both constructs was

synthesized *in vitro* and translated with or without Shep mRNA in Rabbit Reticulocyte Lysate. The luciferase activity was significantly repressed in intact *Dsx1* 5'UTR reporters with the presence of Shep. In contrast, deleted Shep binding site in *Dsx1* 5'UTR abolished the repression activity of Shep.

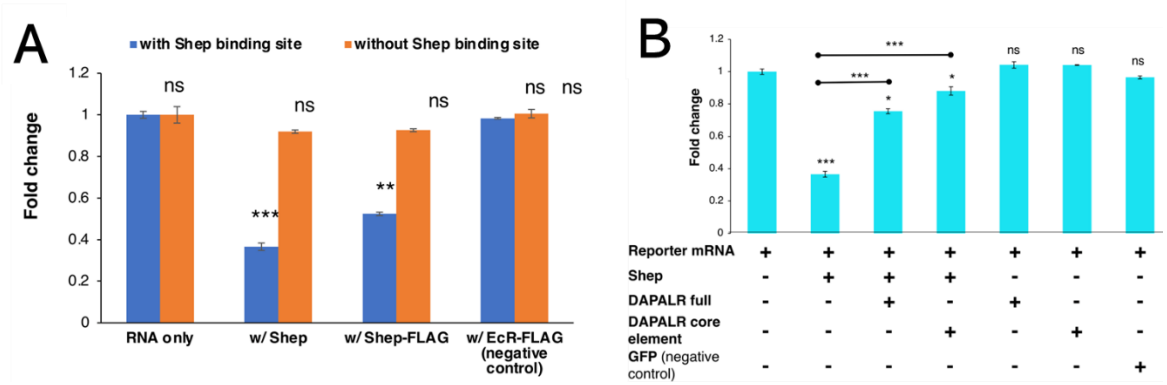


Figure 8. Shep represses *Dsx1* translation, and *DAPALR* alleviates the repression activity

(A) *In vitro* translation assay using Luciferase gene that fused with intact *Dsx1* 5'UTR or *Dsx1* 5 'UTR with deleted Shep binding site. Shep mRNA fails to repress luciferase activity in reporters with deleted Shep binding sites. **(B)** Shep mRNA significantly represses reporter translation, and *DAPALR* presence alleviates the repression activity (Perez et al., 2021).

Furthermore, adding *DAPALR* or the *Dsx1* transactivation element in *DAPALR* alleviates Shep repression efficiency in intact *Dsx1* 5'UTR reporter mRNA (Figure 8). Altogether, these results suggest Shep's role as a post-transcriptional repressor of *Dsx1* and *DAPALR* may sponge Shep to allow *Dsx1* expression in the male embryo. The noise cancelling model of *DAPALR* to Shep-*Dsx1* molecular mechanism was proposed in *D. magna* to avoid noise in *Dsx1* expression in female embryos (Figure 9).

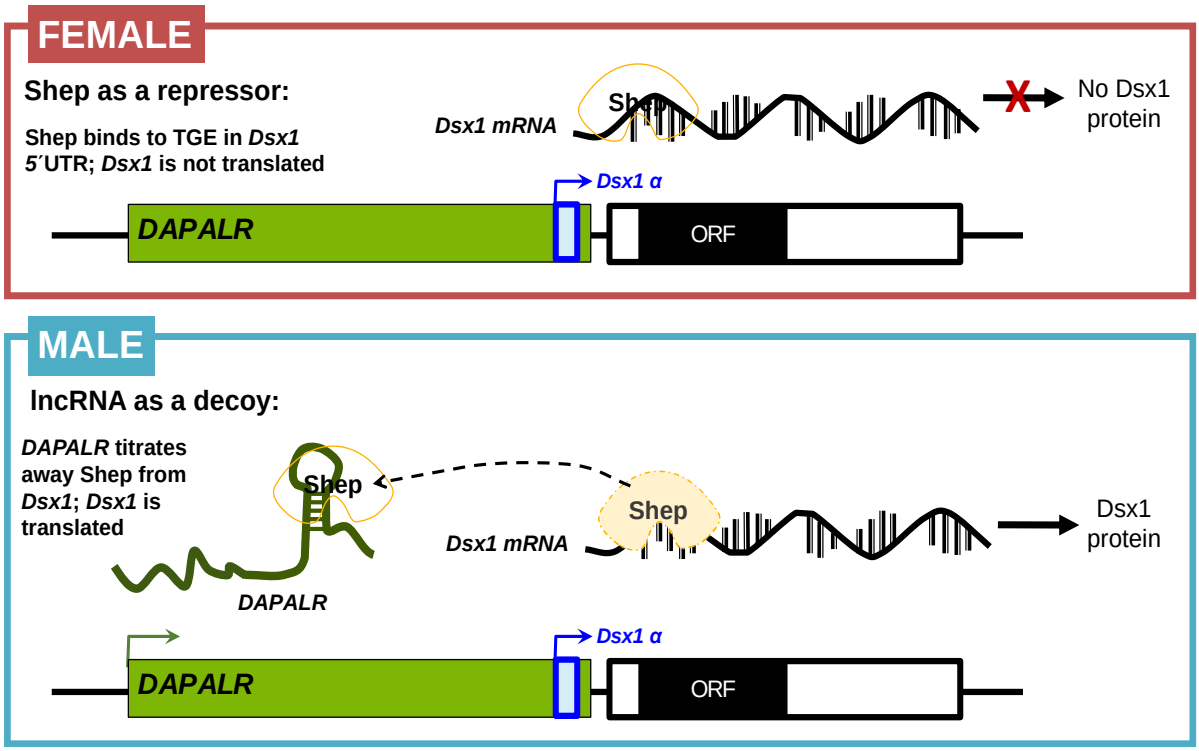


Figure 9. Noise-cancelling model of the current *Dsx1* regulation in *D. magna*

In females, RNA binding protein Shep represses *Dsx1* translation by binding to the *Dsx1α* 5' UTR. In the male, *DAPALR* is expressed as competing RNA that sequestered Shep and unlocks the Dsx1 expression (Perez et al., 2021).

1.6 CUG-binding protein elav-like family proteins (CELF)

The human ortholog of CUG-Binding Protein 1 (CUGBP1) has been identified to be associated with *Dsx1α* 5 'UTR and has become the focus of this study. CUGBP1 was known as CUG-BP Elav-like family protein 1 (CELF1), a CUG-BP Elav-like family protein member and regulates alternative splicing, mRNA stability, and mRNA translation (Chekulaeva et al., 2006; Dold et al., 2020; Lee et al., 2010; Moraes et al., 2006; Webster et al., 1997). In humans, CELF family members have many aliases, such as CUG binding protein and Elav-type RNA binding protein 3. However, the recent consensus in the field agreed on a standard nomenclature as CELF1, CELF2, CELF3, CELF4, CELF5, and CELF6 (Dasgupta & Ladd, 2012). Moreover, all six proteins share the same structure with three RNA recognition motifs (RRMs) that conserved in all CELF family proteins. These RRMs have been identified to bind

to CUG repeats at first and was refined later on as GU-rich element in the target mRNA (Faustino & Cooper, 2005; N. A. Timchenko, Iakova, et al., 2001). The diverse role of CELF1 might be related to the regulation of many mRNAs, especially the *Dsx1* mRNA in *D. magna*.

1.7 Objective of this study

In this study, I aim to reveal how the element interacting with *Dsx1* 5'UTR and its transactivation element in *DAPALR* regulates *Dsx1* expression. Recently, the molecular network between a sense-overlapping lncRNA (*DAPALR*) and RNA-binding proteins (Shep) involved the intricate sex determination of *D. magna*. Despite the proposed molecular model involving *DAPALR*-Shep-*Dsx1*, many factors are needed to clarify and elucidate this model. One of the factors to elucidate is the other RBP, the CUG binding protein 1 (CUGBP1), which may also involve the proposed model. In this study, I aim to elucidate CUGBP1 role in regulating *Dsx1* in *D. magna*. Altogether, this study may deepen the understanding of the CELF1-*Dsx1* molecular network and bring more light to the robust sex-determination system in *D. magna*.

In eucaryote cell nuclei, messenger RNAs (mRNAs) are created by post-transcriptional regulation of the pre-mRNA transcript before getting translated into the cytoplasm (Darnell, 1982; Dreyfuss et al., 1993). The post-transcriptional modification includes capping, pre-mRNA splicing, and polyadenylation. Then, the mRNAs will be transferred to the cytoplasm, where protein synthesis happens in which the mRNA stability and translation are also subject to regulation. These post-transcription regulations were mostly mediated by RNA binding protein (RBP) which makes the ribonucleoprotein (RNP) complex to its target RNA. In RNP complexes, RBP and RNA regulate each other. RBP regulates the modification, stability, translation, and localization of RNAs. On the other hand, RNAs like lncRNAs can influence the stability, specificity, function, and localization of RBPs (Thelen & Kye, 2020). Despite

their key roles in post-transcription RNA control, RBP regulation on sex-determining genes, especially in *Daphnia magna,* is poorly understood.

Hence, this research aims to elucidate the important role of RNA binding protein in biological processes like sex determination. At the same time, this study may bring more clarity to the proposed model of decoy lncRNAs in regulating *Dsx1* expression. To achieve my goals, I conducted my research in two parts:

1. The functional analysis of *DAPALR*/*Dsx1α* 5'UTR associating protein, CGUBP1. I annotated and re-classify CUGBP1 to CELF1 based on the phylogenetic analysis. The functional analysis was done by observation of temporal expression, knockdown, and overexpression in female and male embryos. I revealed that CELF1 represses *Dsx1* expression at a post-transcriptional level. This part will be described in Chapter 2.
2. Finally, the molecular mechanism of *Dsx1* regulation by CELF1 was investigated using post-transcription assays. Using the predictive model, I investigated the putative binding site of CELF1 in *Dsx1α* 5'UTR and challenged the CELF1 function with *Dsx1α* 5'UTR lacking the putative binding site. The post-transcription assay showed that the putative binding site is needed for *Dsx1* repression by CELF1 *in vivo*. This will be described in Chapter 3.

CHAPTER 2 CELF1 conserved in *D. magna* and represses *Dsx1* post-transcriptionally

2.1 Introduction

RNA-binding proteins (RBPs) play key roles in the post-transcriptional control of RNAs and subsequently regulate gene expression in many biological processes such as development, differentiation, metabolism, or specific regulation like sex determination (Dassi, 2017; Milne & Hodgkin, 1999; Salz & Erickson, 2010). In the last decades, many RBPs have been identified as an essential factor in the development of many organisms. Moreover, dysregulation of RBP has been linked to cancers (King et al., 2011; Yang et al., 2010) and human genetic diseases (Gebauer et al., 2021). Therefore, elucidating RBPs' molecular mechanism in many biological processes is important to gain an understanding of RNA-RBP interaction and its implication in various organisms.

Our previous study has identified lncRNA *DAPALR* and its associating RBP Shep harmonically regulates *Dsx1* expression in *D. magna*. The noise-cancelling model was proposed in terms of the *DAPALR*-Shep-*Dsx1* molecular network, which regulates *Dsx1* expression at the post-transcriptional level (Perez et al., 2021). To avoid transcriptional noise of *Dsx1* in female embryos, Shep bind to *Dsx1* mRNA, subsequently silencing its expression. In male embryos, *DAPALR* acts as a sponge for Shep and unlocks the *Dsx1* repression to trigger the male development cascades. This model was thought to avoid producing intersex and maintain sexual dimorphism in *D. magna*. However, since sex reversal was not observed in Shep downregulation or upregulation alone, another factor was assumed to partake in this molecular network utilizing the transactivation element in *DAPALR*.

In this chapter, to further elucidate the proposed noise cancelling model, I investigated CUGBP1, the other RBP that has been identified to bind to the overlapped region of *DAPALR* and *Dsx1α* 5'UTR. Targeting this protein, I performed deep functional analysis to clarify its

role in the sex determination of *D. magna*. I annotated and re-classify CUGBP1 as CELF1 in *D. magna* based on its phylogenetic analysis with its gene orthologs in multiple species. I analyzed the CELF1 expression pattern, downregulation, and upregulation experiment *in vivo* to interpret CELF1 function in regulating *Dsx1* expression.

2.2 Materials and Methods

2.2.1 Wildtype *Daphnia magna* and *Doublesex1* reporter strain culture condition

Wildtype *Daphnia magna* strain (NIES) was used as the base for the established transgenic strains used in this study. This strain was obtained from the National Institute of Environmental Studies (NIES, Tsukuba, Japan) and cultured for many generations in our lab. Using this strain, the genomic extract was used in investigating CELF1 temporal expression. Transgenic *Daphnia magna* strains that served as *Dsx1* reporters have been established in our lab (Nong et al., 2017, 2020) and used in knockdown and overexpression. This Daphnia strain functioned as a reporter of the *Dsx1* expression and was named Line-B.

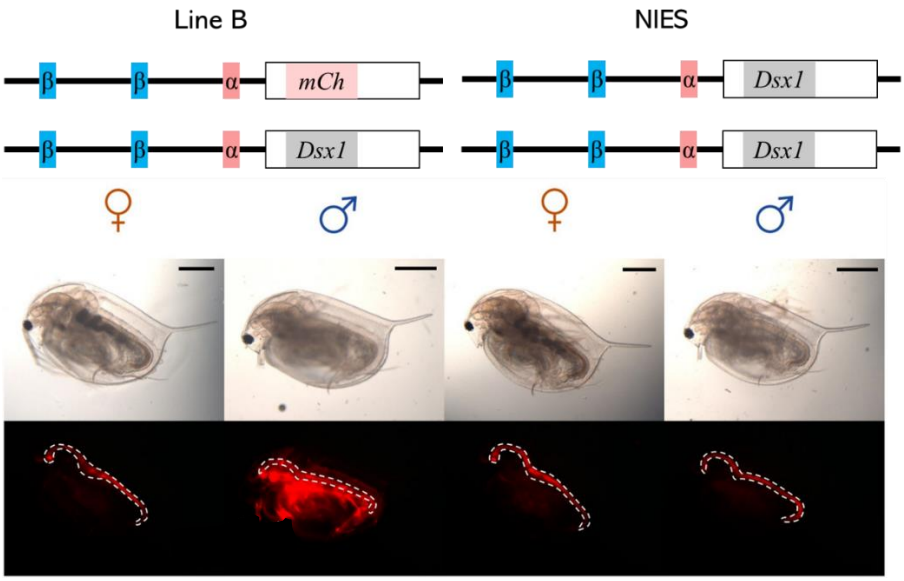


Figure 10. *Dsx1* expression mirrored by mCherry fluorescence in Line-B.

Comparison of Dsx1 alleles in wildtype and Dsx1 strain reporter mutant. Male observation showed a prominent signal of *Dsx1* in male traits

One of Line-B's *Dsx1* alleles contains the inserted mCherry gene using TALEN knock-in system (Figure 10). As a result, mCherry fluorescence represents the *Dsx1* expression in this strain and allows the observation of the male-specific organs where *Dsx1* is highly localized. In addition, Line-B has eGFP fused to histone H2B gene under the control of the *elongation factor 1α-1* promotor/enhancer (EF1α1), allowing visualization of embryonic development. Utilizing these advantages, Line-B was used in the knockdown experiment to allow observation of *Dsx1* expression under CELF1 downregulation by fluorescence microscope. A variant of Line-B strains without H2B-GFP was used for overexpression and called Line-B minus.

Daphnia strains were cultured in artificial freshwater Aachener Daphnien Medium (ADaM) (Klüttgen et al., 1994) under the following conditions: culturing temperature of 22-24°C in a constant light/dark photoperiod of 16h/8h. Furthermore, the daphniids were fed once a day with 120 µl of $8x10^9$ cells/mL Chlorella Vulgaris (Nikkai Center, Tokyo, Japan) and ten µl 0.15 g/mL baker's yeast (Lesaffre, Marcq-en-Barœul, France).

2.2.2 Induced male production by Juvenile Hormon analogue exposure

Male embryos were produced using Juvenile Hormones analogue exposure to investigate the Dsx1 regulation by CELF1 in both sexes. To induce male production, 2-3 weeks old female daphniids with 42 h old embryos in their brood chamber were exposed to the synthetic juvenile hormone analogue, fenoxycarb (Wako Pure Chemical, Osaka, Japan) during a critical stage of oocyte development (Tatarazako et al., 2003). Fenoxycarb was diluted in N-N-Dimethyl Formamide (Nacalai teque Inc, Kyoto, Japan) to 1 mg/L concentration stock. Then, the fenoxicarb stock was added to ADaM until the final concentration was 1µg/L. The female daphniids were exposed to this medium for 16-18h and fed 10 µl of $8x10^8$ cells/mL per

Daphnia. After exposure, the daphniids were moved to normal ADaM, and freshly ovulated male eggs were collected for injection.

2.2.3 Phylogenetic analysis

Amino acid sequences of CELF family proteins were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/), as shown in Table 4. Each protein's whole amino acid or RRMs sequences were subjected to multiple sequence alignment and used to construct the phylogenetic tree. Based on the amino acid sequences, multiple sequence alignments were constructed using the Clustal W in MEGA version 10.0.5 (Kumar et al., 2018). The following settings were used for the analysis: pairwise alignment parameters: gap opening penalty = 10.00, gap extension penalty = 0.1, and identity protein weight; matrix multiple alignment parameters: gap opening penalty = 10.00, gap extension penalty = 0.20, and delay divergent cut-off = 30%. The phylogenetic reconstruction was performed using the p-distance algorithm and the neighbour-joining method implemented in MEGA.

Table 1. Accession numbers of CELF family orthologs and their nomenclature.

Species	CELF family member(s)	Gene	Uniprot Accession number
H. sapiens	CELF1	CELF1	Q92879
Human	CELF2	CELF2	O95319
	CELF3	CELF3	Q5SZQ8
	CELF4	CELF4	Q9BZC1
	CELF5	CELF5	Q8N6W0
	CELF6	CELF6	Q96J87
M. musculus	CELF1	celf1	P28659
Mouse	CELF2	celf2	Q9Z0H4
	CELF3	celf3	Q8CIN6
	CELF4	celf4	Q7TSY6
	CELF5	celf5	A0A5F8MPH2
	CELF6	celf6	Q7TN33
D. rerio	CELF1	celf1	Q9IBD0
Zebrafish	CELF2	celf2	Q6P0B1
	CELF3	celf3	Q9IBD1

	CELF4	celf4	Q6DGV1
	CELF5	celf5	B3DJA7
	CELF6	celf6	A0A0R4ID04
D. melanogaster Fruit fly	CELF1-2	bru	Q960Z4
		bru-2	Q0E8R3
	CELF3-6	bru-3	Q9VU91
Outgroup *D. magna*	-	ELAV protein 2	APZ42_028252

Species	CELF family member(s)	Gene	Genebank Accession number
A. melifera Honey Bee	CELF1-2	CELF2*	XP_026294967.1
	CELF3-6	CELF4*	XP_006557659.1
T. castaneum Red Flour Beetle	CELF1-2	CELF2*	XP_015838769.1
	CELF3-6	CELF4*	KYB25053.1
P. promelas Bonefish	CELF1	*celf1*	XP_039541988.1
C. elegans Roundworm	CELF1-2	*etr-1*	NP_001367867.1
	CELF3-6	*unc-75*	NP_492958.3
D. magna Water flea	CELF1-2	CELF1*	XP_032788705 (JAL60823.1)
	CELF3-6	CELF4*	JAM69815.1
D. Pulex Water flea	CELF1-2	CELF1*	EFX71524
	CELF3-6	CELF3*	XP_046453064.1

2.2.4 Temporal expressions pattern of CELF1

The temporal changes in *CELF1* expression level during embryogenesis were analyzed using total RNA extract from male and female *Daphnia* embryos, collected at 0, 6, 12, 18, 24, 30, 48, and 72 h after ovulation. RNA extract of each embryo stage was subjected to cDNA synthesis and then used in RT-qPCR using a CELF1 specific primer set.

2.2.5 Microinjection

Foreign material injection into *Daphnia* eggs was performed using an established protocol (Kato, Shiga, et al., 2011). Freshly ovulated eggs from 2-3 weeks old *Daphnia* mothers were obtained using microdissection and transferred into an ice-chilled M4 medium

(Elendt & Bias, 1990) containing 80 mM sucrose (M4-sucrose). The injection capillary needles (Narishige, Tokyo, Japan) were prepared using a capillary puller (Sutter Instrument, Novato, USA). Injection cocktails were inserted into the capillary needles, and the pointy end of the needle was cut using insect pin (Shiga, Tokyo, Japan) under microscope. The injection volume was confirmed as liquid droplet size using mineral oil under certain gas pressure. Optimum needles hole for injection and injection volume were adjusted for the best injection condition. For the injection solution, 2 mM Lucifer Yellow (Invitrogen, Carlsbad CA, USA) was mixed as an injection marker for each experiment. Following 1 hour after microinjection, survived eggs were transferred into each well of 96-well plates with 100 µL of M4-sucrose medium and were kept in an incubator at 23°C.

2.2.6 CELF1 knockdown by RNAi

Small interference RNAs were designed using the website Block-iT RNAi Designer at https://rnaidesigner.thermofisher.com/rnaiexpress/. The sequence of this siRNA is as follows: siCELF1 (5'- GCAATGAGCGTAAACTCTT -3'). As a negative control, siRNA targeting a random sequence that does not affect the *Daphnia* development was used: siControl (5'-GGUUAAGCCGCCUCACAUTT-3') (Asada et al., 2014). The siRNA oligonucleotides were dissolved in DNase/RNase-free water (Life Technologies Inc.; Grand Island, NY, USA). Two nucleotides dTdT were added to each 3′ ends of the siRNAs. The siRNAs were diluted with the injection marker 2 mM Lucifer Yellow dye (Invitrogen, Carlsbad CA, USA) to have the final concentration of 100 µM or 300 µM. The cocktails were injected into female or male eggs of the *Dsx1*-reporter strain *Daphnia*. Samples were then observed at 24 h after injection and collected at 48 h for RNA extraction and cDNA synthesis, as previously described (Kato et al., 2018). To confirm CELF1 transcript level changes between control and siRNA injected-embryo, both samples were subjected to RT-qPCR.

2.2.7 Ectopic CELF1 overexpression by mRNAs delivery

To create chimeric CELF1 mRNA, CELF1 CDS was amplified by PCR using a CELF1-specific primer set (Table 2) and synthesized cDNA derived from NIES strain total RNA extraction. Then, CELF1 CDS was subcloned into a pCS2 vector harbouring the T7 polymerase promoter, *EF1α1 5′* UTR, and 3' UTR derived from the chimeric DsRed2 mRNA expression plasmid construct (Törner et al., 2014) using GeneArt Seamless Cloning and Assembly Enzyme Mix (Invitrogen, Carlsbad CA, USA). The plasmid construct was named pEF1α1-CELF1. For control mRNA preparation, the *CELF1* CDS of pEF1α1-CELF1was then replaced with the CDS of *GFP* (Figure 11.) using seamless cloning. The GFP region was amplified from the 4xEcRE-H2B-GFP plasmid (Asada et al., 2014).

Table 2. Primer sets for seamless cloning

Target fragment	Primer sequence (5’ to 3’)
CELF1 CDS	Forward: 5’-ATGGAGATGCTCAATTCGT-3’ Reverse : 5’-CTAGTAAGGTTTAGAGGCGTCTTT-3’
Vector plasmid	Forward: 5’-TCTAAACCTTACTAGATGGAGGCTACTATTCCATCC-3’ Reverse : 5’-ATTGAGCATCTCCATGGTGGCGACCGGTGGAT-3’

In vitro transcription and poly(A) tail addition were performed with mMESSAGE mMACHINE T7 RNA Polymerase and Poly(A) Tailing kits, respectively (both Ambion, Foster City, CA, USA). The synthesized RNA size and the attached poly(A) tail length were analyzed by denaturing formaldehyde gel electrophoresis.

CELF1 ectopic expression was performed by injecting 3200 ng/ul CELF1 mRNA mixed with 2 mM Luciferase Yellow dye into Line-B variant eggs without endogenous GFP fluorescence called Line-B minus (Perez et al., 2021). These eggs were induced to be male before injection, as mentioned above. The sample was observed under a fluorescent microscope to measure the mCherry fluorescence and the phenotype of male organs at 24 h and 48 h. To

confirm the *Dsx1* transcript level, samples were collected for total RNA extraction at 48 h and subjected to RT-qPCR.

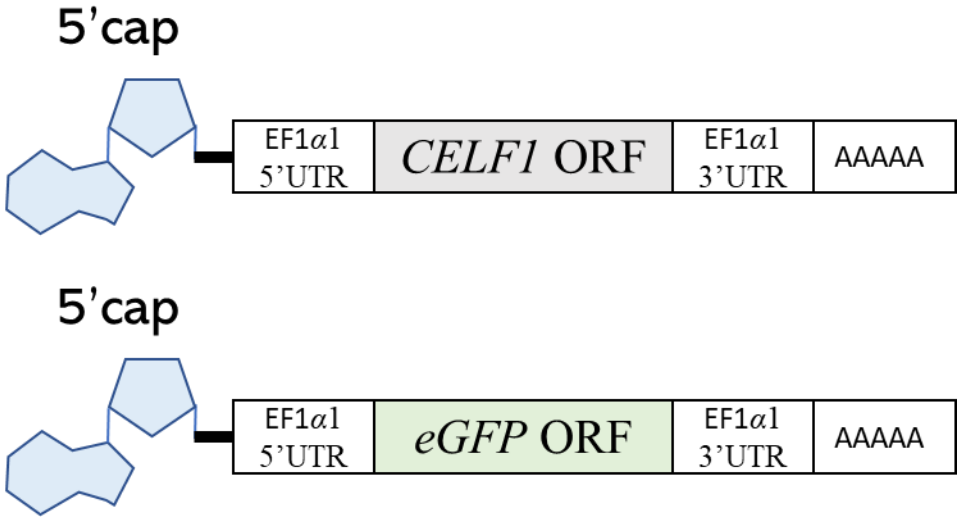


Figure 11. Chimeric mRNA structure for CELF1 overexpression.

2.2.8 Fluorescence photography and quantification

Injected sample photos were taken using Leica DC500 CCD Digital Camera mounted on a Leica M165FC fluorescence microscope (Leica Microsystem, Mannheim, Germany). Fluorescence photography was done using GFP2 and mCherry filters under the following conditions in Table 3.

Table 3. Leica microscope parameters for fluorescence observation.

Conditions	Exposure time	Gain	Saturation	Gamma
GFP	1.0 s	3.0x	1.0	1.0
mCherry	2.0 s	8.0x	1.0	1.6

The fluorescence intensities were calculated using ImageJ software, following the calculation protocol from a previous study (Törner et al., 2014). The measurement of background fluorescence normalized the total embryo fluorescence of each sample. In addition, Relative

Fluorescence Intensity (RFI) was calculated following the protocol of a previous study (Perez et al., 2021). The total fluorescence of injected-embryos was divided by uninjected-embryos fluorescence in the same clutch to avoid the basal fluorescence intensity variance between different clutches. Relative fluorescence intensity was then calculated as a fold-change of sample embryos compared to control embryos.

2.2.9 Total RNA extraction and cDNA synthesis

Fresh samples for each experiment were collected in 2 mL tube and were frozen quickly in liquid nitrogen. Frozen zirconia beads of 1.0 ⌀ and 3.0 ⌀ sizes were added to each tube, and samples were smashed using Microsmash at 3000rpm for 90 s. Total RNA was extracted using Sepasol-RNAi solution (Nacalai Tesque; Kyoto, Japan) according to the manufacturer's protocol and followed by phenol/chloroform purification. Finally, the precipitated RNA pellet was washed using 70% alcohol and dissolved in 18 µL of ultrapure RNAse-free water. The purified total RNA was measured by Nanodrop 2000 (Thermofisher Scientific) and subjected to cDNA synthesis using random primers (Invitrogen; Carlsbad, CA, USA) and the SuperScriptIII Reverse Transcriptase (Invitrogen) according to the manufacturer's recommended protocol.

2.2.10 Quantitative real-time PCR

The temporal changes in *CELF1* expression level during embryogenesis were analyzed using previously synthesized cDNA (Mohamad Ishak et al., 2017) from male and female *Daphnia* at different time points (0, 6, 12, 18, 24, 30, 48, and 72 h after ovulation). Each cDNA was subjected to RT-qPCR using the CELF1-specific primer set shown in Table 4. To check the expression level changes of the genes of interest (*CELF1* and *Dsx1*) between the control-

and siCELF1-injected samples or GFP mRNA- and CELF1 mRNA-injected samples, cDNA from 48-hour embryos were subjected to RT-qPCR.

The expression level of *CELF1* and *Dsx1* in RNAi or overexpression experiments were prepared as three replicates for RT-qPCR. mRNA transcripts were measured using StepOnePlus™ Real-Time PCR System (Agilent Technologies), Power SYBR Green qPCR Mastermix (Invitrogen, Carlsbad CA, USA), and a specific primer designed to amplify <150 bp PCR products under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, and last amplification round of 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec. *Dsx1*-specific primer set sequences were designed, as shown in Table 4. Expressions based on the Ct value during amplification were calculated and normalized by quantitating the expression level of the ribosomal protein gene *L32* (Kato et al., 2008). Finally, dissociation curve analysis and gel electrophoresis were performed to confirm the correct amplicon size and the absence of non-specific bands.

Table 4. Primer sequences for RT-qPCR for the temporal expression profile, RNAi, mutagenesis, and overexpression experiments.

Gene target	Primer sequence (5′ to 3′)
CELF1	Forward: 5'- CGGCATCCAGCAATTCACTAC-3' Reverse: 5'- CGTCACACTTCCACCACCAC-3'
Dsx1	Forward: 5'- AAGTTTGGTGTAGGGGAGGATGAG-3' Reverse: 5'- CCATTCATCATTACCAAATCCCTTC-3'
L32	Forward: 5'- GACCAAAGGGTATTGACAACAGA-3' Reverse: 5'- CCAACTTTTGGCATAAGGTACTG-3'

2.3 Results

2.3.1 Sequence conservation of *D. magna* CELF1 and its orthologs

We previously identified an ortholog of human CUG binding protein 1 (CUGBP1) as a candidate protein that binds to *Dsx1α* 5' UTR (Perez et al., 2021). I focused on CUGBP1 to elucidate the role of another RBP that binds to the 205bp overlapped region in *Dsx1*. I performed the blast search for the protein sequence on the *D. magna* genome assembly in NCBI

(ASM2063170v1.1) and renamed *D. magna* CUGBP1 ortholog as CELF1 following the recent nomenclature. I found that *D. magna* CELF1 consists of 593 amino acid residues and harbours three RNA recognition motifs (Figure 12). The RRM1, RRM2, and RRM3 were highly conserved across diverse taxa. However, the divergent domains between species were distinctively different to one another.

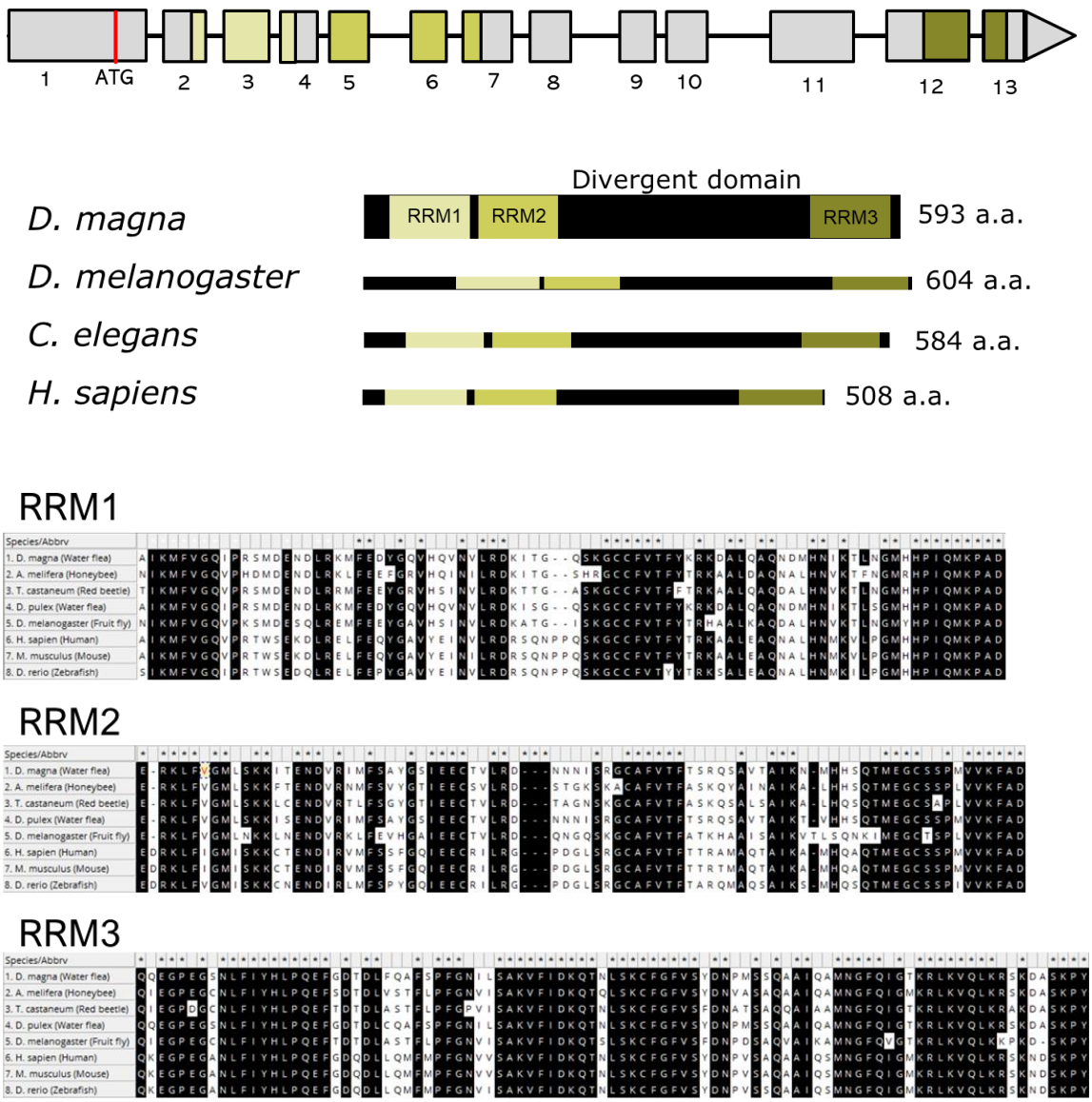


Figure 12. Genomic annotation of *D. magna CELF1* and protein structure of CELF1 orthologs in various species

The genomic structure of *D. magna CELF1* shows exons in a black box and RRM domains of RRM1 (Light yellow), RRM2 (Gold), and RRM3 (Dark yellow). Three conserved RNA Recognition motifs (RRMs) of CELF1 in various species. A unique sequence of the divergent domain is located between RRM2 and RRM3.

I analyzed the phylogenetic relationship of the *D. magna* CELF1 ortholog using amino acid sequences of CELF proteins from various animal species (Table 1). Multiple alignments of CELF family proteins amino acid sequence clustered the CELF family protein into two major subfamilies, which are the CELF1-2 subfamily and the CELF3-6 subfamily. The CELF family proteins in most invertebrates, including primate chordates, are only represented by two proteins (Table 1). While in the vertebrates, it expanded to six proteins and even more, as predicted in zebrafish (Brimacombe & Ladd, 2007). My result suggests that the CELF protein underwent duplication into two genes, and additional duplication did not occur until the emergence of vertebrates. This result is in agreement with the previous reports of large-scale paralogous duplication after the divergence of vertebrates (Mazet & M. Shimeld, 2002). I confirmed that *D. magna* CELF1 belongs to the CELF1-2 subfamily (Figure 13).

The topology of the phylogenetic relationship between CELF protein orthologs was in good agreement with the taxonomic relationship between insects and crustaceans. *Daphnia magna* CELF1 represented both vertebrate orthologs CELF1 and CELF2. Therefore, to avoid confusion, I re-classify *D. magna* CELF1 as representative of CELF1 and CELF2 in the CELF1-2 subfamily. My result supports the hypothesis that hexapods, including insects, originated from crustaceans (Glenner et al., 2006). Altogether, the sequence conservation of *D. magna* CELF1 suggests that this protein is indeed CELF1 in *D. magna* with high similarity to other species CELF1 and may also function as an RNA regulator in *D. magna*.

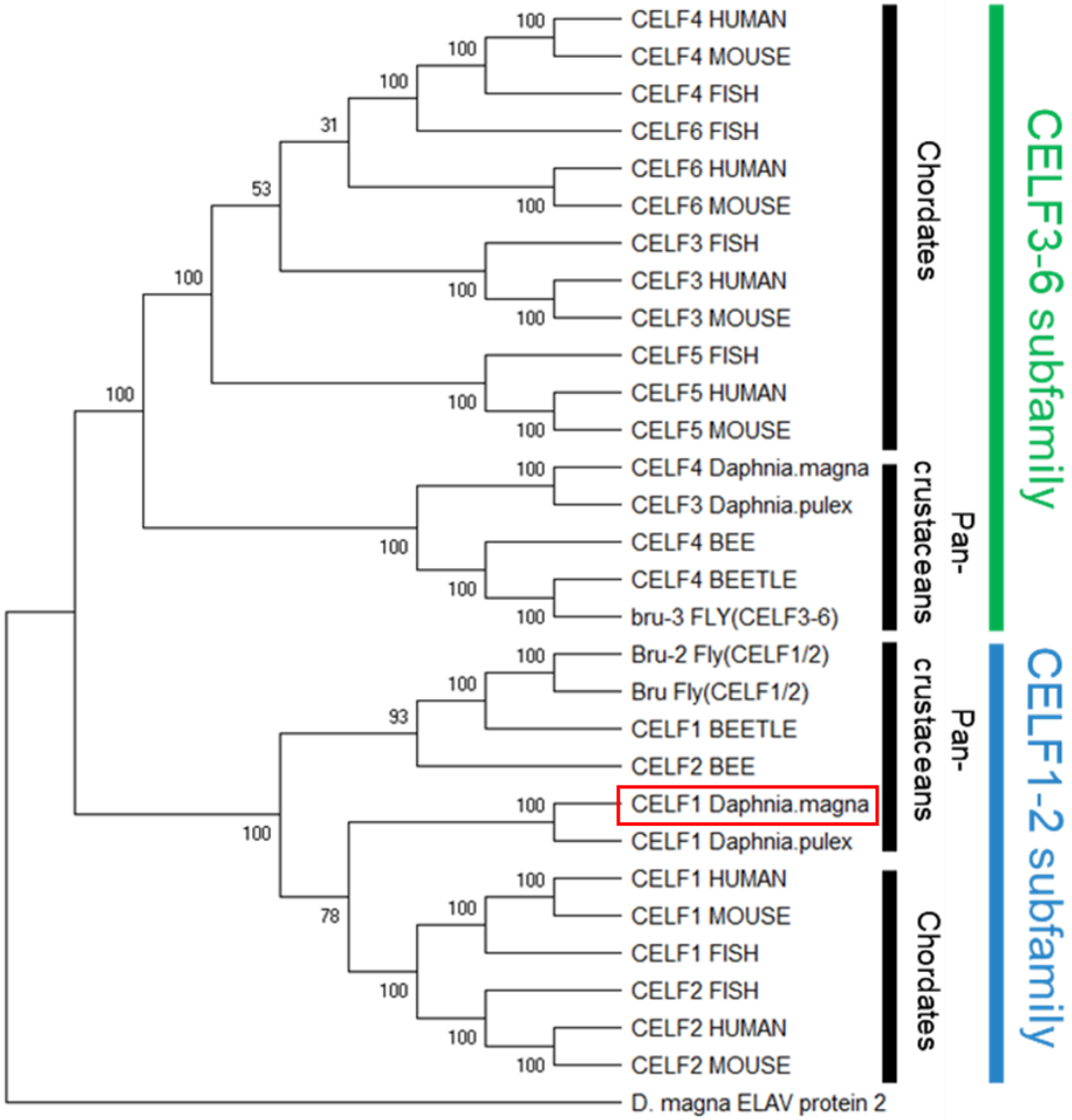


Figure 13. Phylogenetic analysis of CELF1 orthologs in various species

Phylogenetic tree of CELF family protein separates species group; Red square: *D. magna* CELF1. The bootstrap values of 1000 replicates were shown next to the branches. The bar indicates branch length and corresponds to the mean number of the differences (P<0.05) per residue along each branch. Evolutionary distances were computed using the p-distance method.

2.3.2 *CELF1* expression profile during embryogenesis

We found that CELF1 binds to the 5'UTR of the Dsx1 gene, which is exclusively expressed in males. Therefore, I asked whether CELF1 expression is also sexually dimorphic following the *Dsx1* expression pattern. I investigate the expression pattern of CELF1 during

embryogenesis using RT-qPCR. Unlike *Dsx1*, the expression is not sexually dimorphic. CELF1 was expressed in males and females with similar patterns (Figure 14).

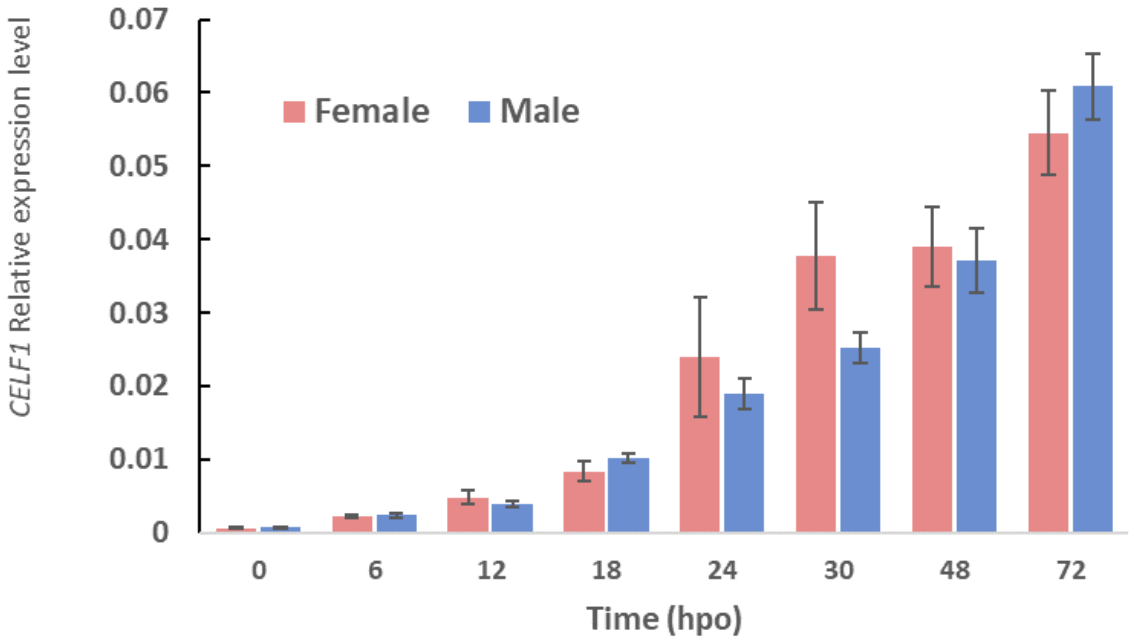


Figure 14. *CELF1* temporal expression pattern in embryogenesis stages of *D. magna*.

D. magna CELF1 expression level in embryonic developmental stages. Results were shown in relative expression levels normalized with the ribosomal protein L32. hpo, hours post-ovulation. Error bars indicate the standard error of the mean, and the Student's T-test between both sexes shows no significant difference. N=3

2.3.3 CELF1 knockdown strengthen *Dsx1* expression signal

To investigate the role of the *CELF1* ortholog in *D. magna*, I silenced *CELF1* expression via RNA interference (RNAi) (Kato, Shiga, et al., 2011). First, I injected 300 µM of *CELF1*-specific siRNA (siCELF1) into female eggs from the wild-type of *D. magna*. Of the ten injected eggs, 90% (9/10) stopped development before 48 hours post-ovulation, a timing when clear sexual dimorphism in *Dsx1* expression and organ formation appears. On the other hand, injection of 100 µM siCELF1 decreased the ratio of the non-viable samples down to 25% (9/36).

Based on this result, I decided to use 100 μM siCELF1 to investigate the CELF1 function on the sexual development of *D. magna*. To examine the role of *CELF1* in *Dsx1* regulation, I used the *Dsx1* reporter strain (also named Line-B) (Nong et al., 2017). This transgenic line has the *mCherry* gene inserted at the position of the *Dsx1* start codon in one allele resulting in a mCherry expression under the control of endogenous *Dsx1* promotor/enhancer. Line-B develops male-specific traits similar to the wild-type because another *Dsx1* allele is intact. This transgenic line also harbors the *H2B-eGFP* gene under the control of the *elongation factor 1α-1* (*EF1α-1*) promotor/enhancer. It allows us to visualize the localization of each cell and map the internal structure of *D. magna* (Kato et al., 2012).

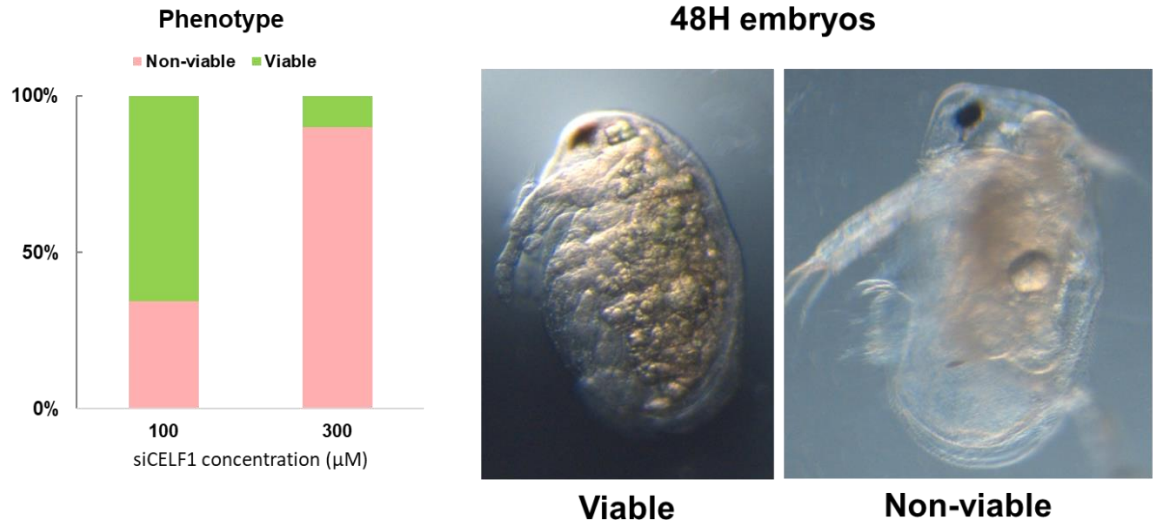


Figure 15. High concentration of siCELF1 injection produces non-viable embryos

Injection of 300 μM siCELF1 into daphnia eggs causes abnormal development and non-viable embryos. siCELF1 concentration was optimized to 100 μM to observe clear differences in sexual development and *Dsx1* activity.

CELF1 siRNA injection enhances the mCherry fluorescence intensity up to 2.6-fold and 1.5-fold compared to control in female and male embryos, respectively (Figures 16 and 17). In female embryos, *CELF1* downregulation led to mCherry fluorescence except for the sexually

dimorphic organs (Figure 17) and did not induce sex reversal. *Dsx1* expression mirrored by mCherry fluorescent was measured and observed by fluorescence microscope following siCELF1 injection. The mCherry fluorescence was increased in the female body and most visible in the yolk region of female embryos (Figure 17-yellow dashed line).

To investigate the effects of *CELF1* downregulation in male embryos, I collected eggs committed to males by exposing the Line-B mother to fenoxycarb during a critical stage of oocyte development (Kato, Kobayashi, et al., 2011; Tatarazako et al., 2003). siCELF1 injection into male eggs increased mCherry fluorescence up to 1.5-fold ubiquitously (Figure 16). Increased mCherry expression was observed in the whole body, including male-specific tissues (Figure 13), such as the first antennae and the first thoracic appendage (Ebert, 2005).

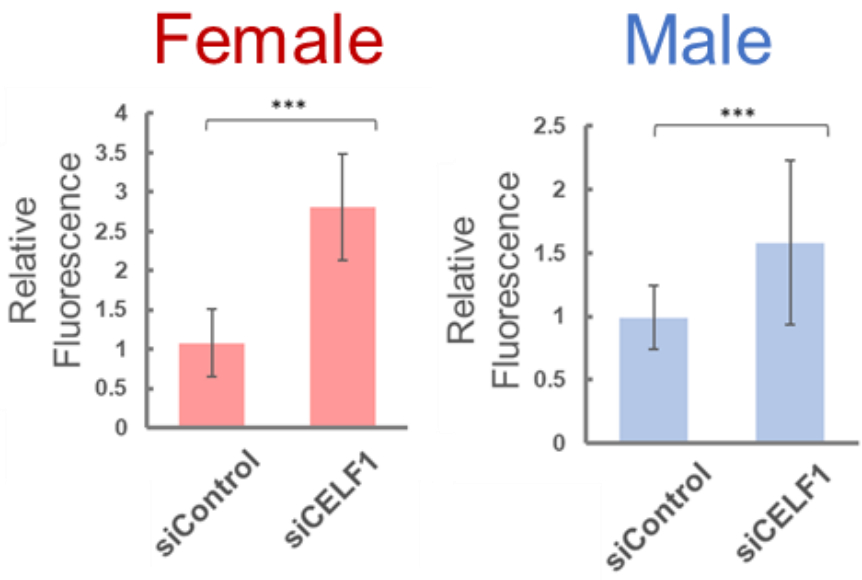


Figure 16. siCELF-injected embryos mCherry fluorescence intensity relative to the siControl-injected embryos

Relative fluorescence intensity was calculated as fold-change compared to siControl injected embryos in female (red) and male (blue) embryos. Error bars indicate the standard error of the mean, and the significance was tested using Student's T-test ***p<0.001. N=12

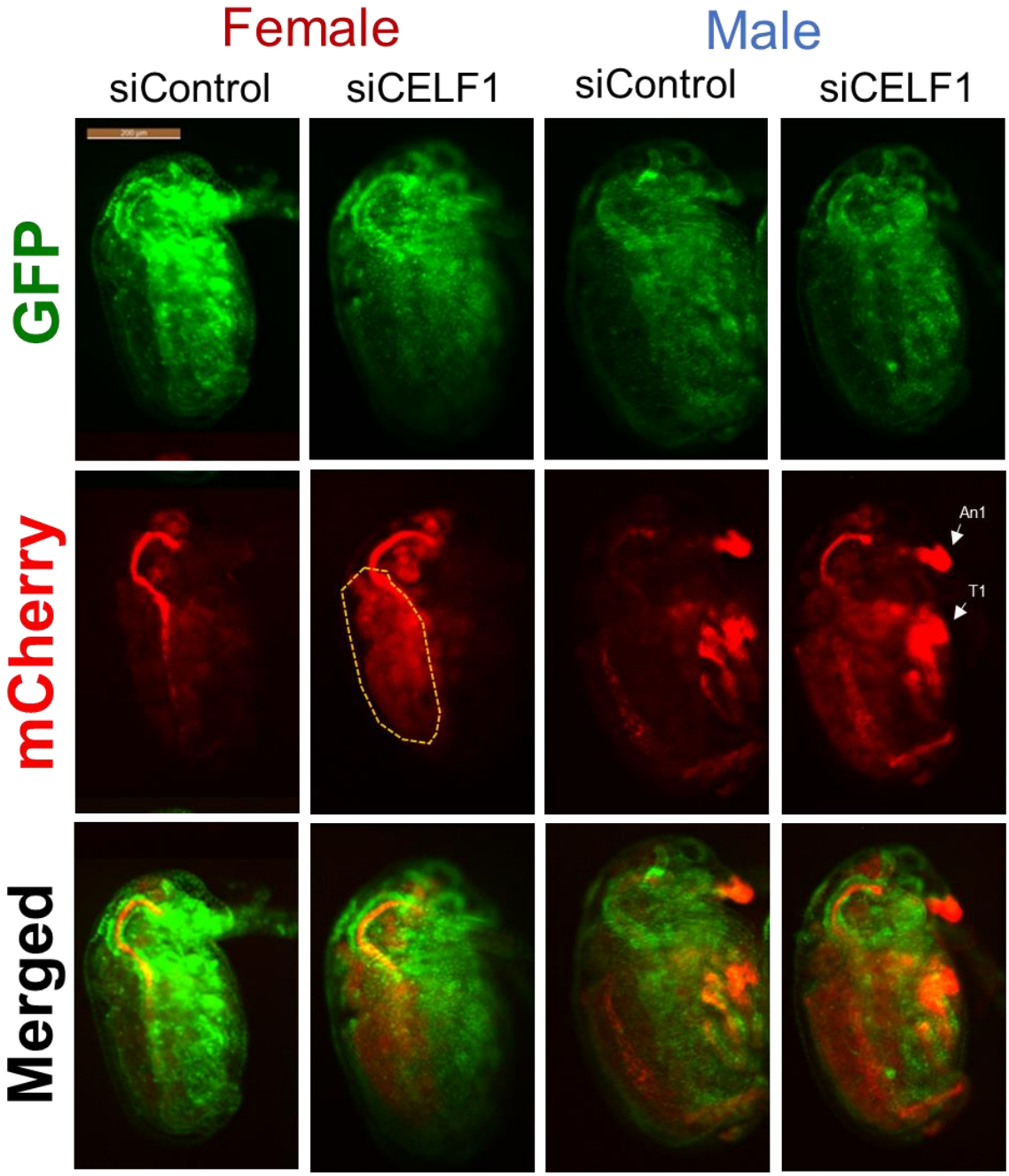


Figure 17. *CELF1* knockdown in *Dsx1* reporter strain observation.

Lateral views of female and male embryos of the *Dsx1* reporter strain injected with control siRNA and *CELF1* siRNA were observed 48 h after injection. mCherry fluorescence mirrored *Dsx1* expression, and GFP fluorescence in the nucleus allows visualization of Daphnia body structure. The merged mCherry and GFP images were used to highlight the localization pattern of mCherry expression. An1: first antennae, T1: first thoracic leg, dashed yellow lines: yolk area.

Because silencing the *CELF1* changed the *Dsx1* expression as observed in mCherry fluorescence, I further confirmed whether the *Dsx1* mRNA level was affected. I examined the *Dsx1* expression in the siCELF1-injected embryos by the RT-qPCR. siCELF1 injection

reduced the target *CELF1* mRNA level (Figure 17B) indicating CELF1 knockdown was successful. However, in contrast to the mCherry fluorescence (Figures 16 and 17), the *Dsx1* transcript levels showed no significant difference between *CELF1* RNAi and control embryos (Figure 18A). This result suggests a possibility of *Dsx1* post-transcription regulation by CELF1.

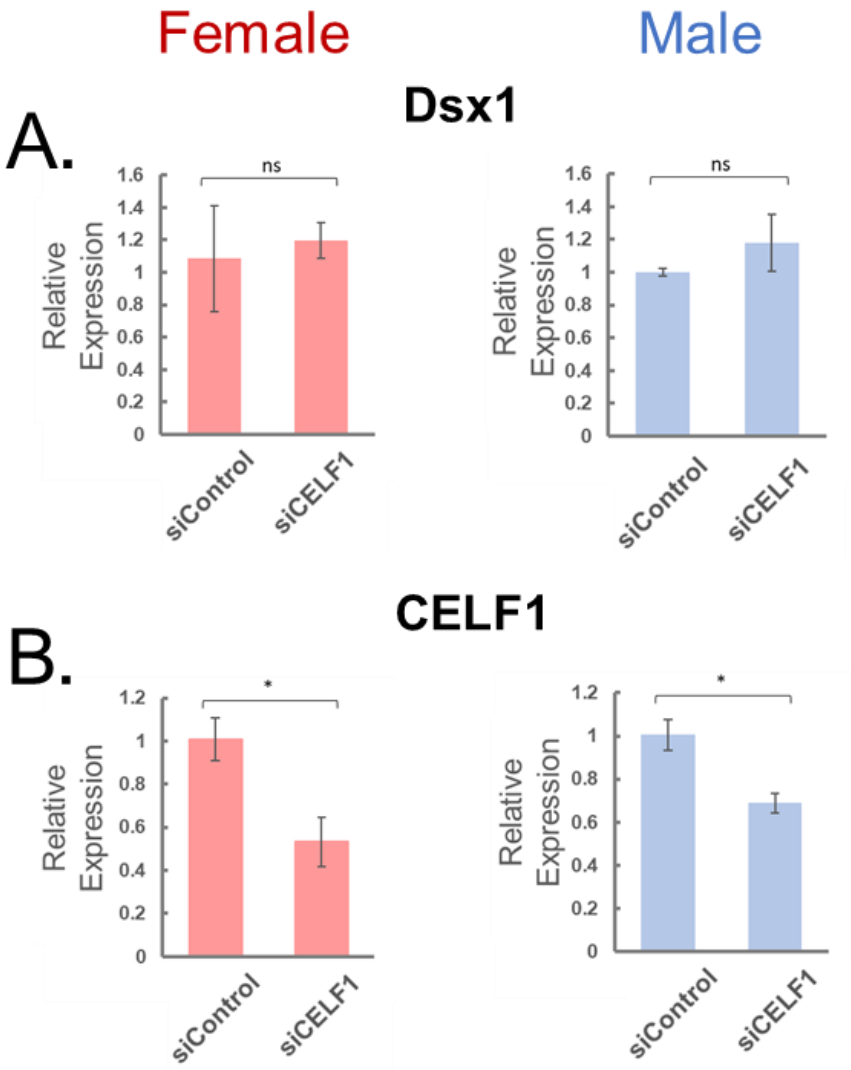


Figure 18. Gene transcript level in female and male embryos 48 h after siCELF1 injection.

Gene transcript level of siCELF1 and siControl injected samples in female (red) and male (blue) 48 h post-injection for **(A)** *Dsx1* and **(B)** *CELF1*. RT-qPCR results were shown as relative expression levels to control normalized with the expression level of the ribosomal protein *L32*. Error bars indicate the standard error of the mean. *p<0.05, ***p<0.001, ns: not significant (Student's T-test). N=3

2.3.4 Overexpression of CELF1 suppressed *Dsx1* expression in *D. magna* male embryos

Because the CELF1 knockdown experiment increased mCherry expression, possibly at the post-transcription level, I further investigate the suppression activity of CELF1 in *Dsx1* expression. I overexpressed CELF1 by injecting *in vitro* transcribed *CELF1* mRNA into Line-B eggs that are destined to develop into males. Following injection, 24 h and 48 h observation of mCherry fluorescence were done by fluorescence microscope.

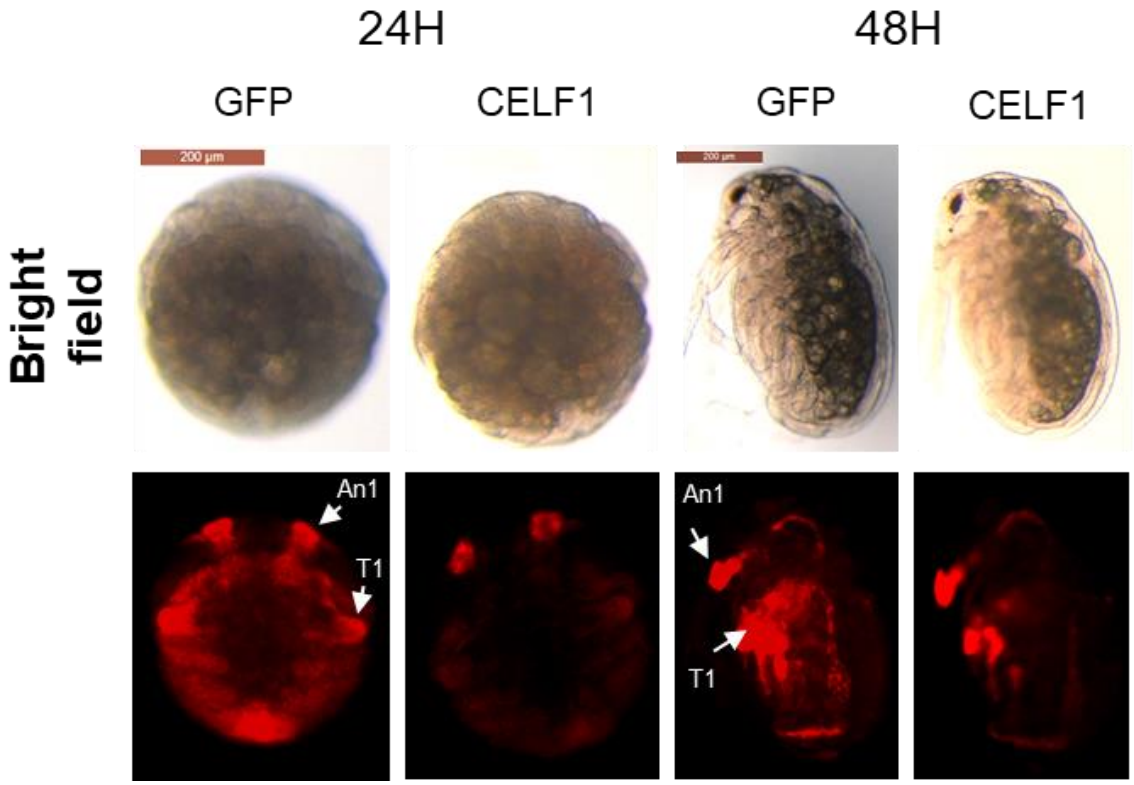


Figure 19. Ectopic expression of CELF1 in male *Dsx1* reporter strain.

Ventral view of 24 h male embryos and lateral view of 48 h male embryos of *Dsx1* reporter strain following injection with GFP mRNA only or coinjection of GFP and *CELF1* mRNA. mCherry fluorescence mirroring *Dsx1* expression and Bright field images helps understand the localization of mCherry expression. An1: first antennae, T1: first thoracic appendage.

The mCherry fluorescence recapitulating *Dsx1* expression was reduced significantly not only in the male specific organs such as the first antennae and the first thoracic appendage, but also in the whole body (Figures 19) at both 24 h and 48 h observation. mCherry fluorescence

intensity was significantly reduced to 0.6-fold in *CELF1* mRNA and *GFP* mRNA coinjected embryos compared to *GFP* mRNA-only injected embryos at 48 h post-injection (Figure 20). No visible defect was observed at 48 h after *CELF1* mRNA injection. In contrast to the significant change of mCherry fluorescence intensity by CELF1 overexpression, there were no significant differences in *Dsx1* transcript levels between *CELF1*-mRNA and *GFP*-mRNA-injected embryos (Figure 21). Altogether, these results supported the suppressing activity of CELF1 in *Dsx1* expression, most likely at a post-transcription level.

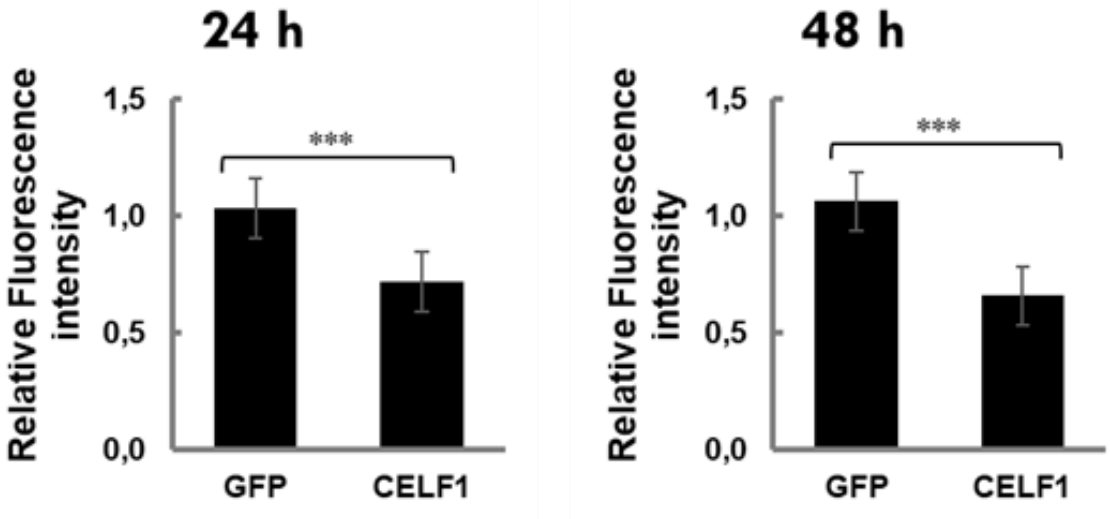


Figure 20. Relative mCherry fluorescence intensity following CELF1 overexpression in males.

mCherry fluorescence of male embryos injected with any of *CELF1* mRNA and *GFP* mRNA at 24 h and 48 h post-injection. Error bars indicate the standard error of the mean. N=12 ***p<0.001 (Student's T-test).

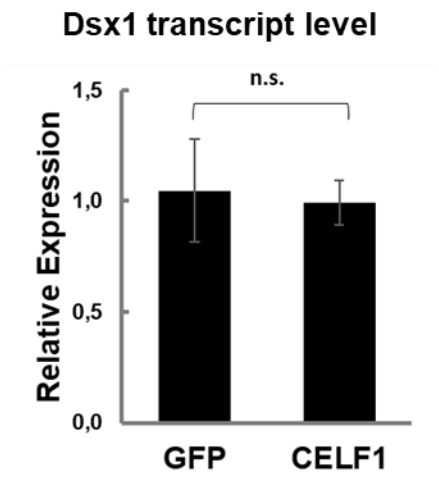


Figure 21. Gene transcript level in male embryos 48 h after CELF1 overexpression.

The *dsx1* transcript level of male embryos injected with *CELF1* mRNA and *GFP* mRNA at 48 h post-injection. RT-qPCR results were shown as relative expression levels to control normalized with the expression of the ribosomal protein *L32*. Error bars indicate the standard error of the mean. ns: not significant (Student's T-test). N=3

2.4 Discussion

CELF1 has been identified as one of the associating proteins in the transactivation element of *DAPALR* together with the shep protein. Alan Shepard or shep is an RNA-binding protein discovered in Drosophila and known to have an important role in nervous system development. Shep regulates the target RNA gene expression at both transcriptional and post-transcriptional levels, such as translational regulators (Olesnicky et al., 2018; Schachtner et al., 2015). On the other hand, CUGBP Elav-like Family protein 1 or CELF1 is also reported to have an essential role in regulating DNA and RNA by alternative splicing, mRNA stability, and mRNA translation (Chekulaeva et al., 2006; Dold et al., 2020; Lee et al., 2010; Moraes et al., 2006; Webster et al., 1997). The CELF1-2 subfamily was known as a gene regulator and its implication in somatic (Milne & Hodgkin, 1999) and gonadal development (Kress et al., 2007). In addition to gonad development, modulation of CELF1/2 ortholog in medaka fish impacted the expression of sex-determining related genes *dmrt1*, *Tra2*, and *Sox10* (Herpin et al., 2019), suggesting that CELF1 potentially have sex determination function in *D. magna*. Therefore, elucidating CELF1's implication on the sex determination

of *D. magna* may help us to understand the dynamic of environmental sex determination mechanism in many species, especially *D. magna*. In this chapter, I investigate the CELF1 role in regulating *Dsx1* as it was found to associate with the *Dsx1* 5'UTR and the transactivation element in *DAPALR*

In the past, CELF family members have been called many aliases, such as CUG binding protein (CUGBP, now called CELF1) and Elav-type RNA binding protein 3 (ETR-3, now CELF2). However, the recent consensus in the field agreed on a standard nomenclature as CELF1, CELF2, CELF3, CELF4, CELF5, and CELF6 (Dasgupta & Ladd, 2012). All six CELF family members have three RNA recognition motifs (RRMs) and a unique linker sequence between RRM2 and RRM3 named divergent domain, which categorized them into two subfamilies: CELF1-2 and CELF3-6 (Ladd et al., 2001). I discovered two CELF family orthologs in *D. magna*, identified as CELF1 and CELF4 (Table 1). Based on the phylogenetic analysis, CELF1 belong to the CELF1-2 subfamily and represents both human proteins in *D. magna* (Figure 13). Three RRMs amino acids sequence in CELF1 was highly conserved among diverse species, as observed in the multiple sequence's alignments (Figures 12) and phylogenetic tree (Figures 13).

Interestingly, even though CELF1 was identified to associate with the *Dsx1* 5'UTR, the CELF1 temporal expression showed a similar expression pattern between males and females. Moreover, the CELF1 expression increased proportionally with age (Figure 14). The CELF1 expression pattern suggests that it has essential functions in both males and females.

To investigate the CELF1 function to *Dsx1* expression, I knockdown CELF1 expression in the *Dsx1* reporter strain. Following CELF1 knockdown, *Dsx1* activity was increased in both sexes. In females, mCherry signal mirroring Dsx1 expression was significantly increased, especially in the yolk region, compared to the control. Similar to females, CELF1 knockdown in males increases the *Dsx1* activity ubiquitously. In addition to

the male-specific organ, such as the first antennae and the first thoracic appendage, the mCherry signal can be observed in the whole embryo (Figure 17). On the contrary, *Dsx1* mRNA transcript level measurements by RT-qPCR showed no significant change between CELF1 knocked-down embryos and control embryos.

Following CELF1 knockdown in female embryos, an increased activity of *Dsx1* was observed, especially in the yolk region. This phenomenon occurs probably because *Dsx1* was tightly regulated and suppressed in female embryos. On the other hand, the independent RNA synthesis and regulation of yolk cells from the embryo somatic cell (Park & Yoshitake, 1970) might differentially regulate the *Dsx1* expression in that particular area so that the expression increase was more prominent compared to other areas. In addition, mCherry signal intensity in male embryos showed a similar increase in yolk cells like female embryos in the addition of the prominent signal in the male-specific organ. These results suggest that CELF1 represses *Dsx1* expression in female and male embryos.

The *Dsx1* expression mirrored by the mCherry signal was significantly increased in the CELF1 knocked-down embryos showcasing CELF1 suppression activity on *Dsx1*. However, despite the notable increase in the mCherry signal, the *Dsx1* mRNA transcript level measurements showed no significant change following the CELF1 knockdown. Moreover, knockdown in females did not lead to sex reversal. These results imply that (1) CELF1 suppression to *Dsx1* alone was insufficient for the male sexual development and (2) the suppression activity might occur at a post-transcription level

To validate this result, I overexpressed CELF1 in male embryos using a chimeric mRNA, showing a significantly lower mCherry signal in the whole embryo. This result confirmed the suppression activity of *Dsx1* by CELF1. Similar to the knockdown experiment, the *Dsx1* mRNA transcript level is also not affected by CELF1 overexpression, even though the *Dsx1* expression mirrored by mCherry fluorescent is significantly reduced. This strongly

suggests that CELF1 did not suppress the transcription of *Dsx1* but rather affected the expression in a later stage.

After transcription, RNA from a specific gene may go through several regulations by RBP before finally getting expressed, such as adapting mRNA susceptibility to RNases and controlling the accessibility of the ribosome binding site of mRNAs (Van Assche et al., 2015). The post-transcription regulation will determine the mRNA stability and translation efficiency, ultimately leading to its final gene expression. CELF1 has been known to control mRNA stability (Lee et al., 2010) by binding to the target mRNA 3'UTR and is long known as a translation repressor by binding to the CUG repeat in Oskar protein (Dold et al., 2020; Webster et al., 1997). As I did not observe modulation of mRNA level following CELF1 knockdown or overexpression, it is attractive to speculate that CELF1 did not target *Dsx1* mRNA stability and rather its translation efficiency. However, further investigations are needed to support this hypothesis.

To follow the current hypothesis, I further investigate the post-transcription regulation of *Dsx1* mRNA by CELF1 *in vivo* using mRNA reporter and confirmed the CELF1 binding sequence in *Dsx1* mRNA.

CHAPTER 3 CELF1 repressed *Dsx1* expression via the GU-rich element of the *Dsx1α* 5' UTR in embryos

3.1 Introduction

RNA binding proteins (RBPs) are the important post-transcriptional regulator of gene expression and are implicated in many cellular processes. Many RBP can shuttle between different cell compartments to regulate mRNA metabolism from mRNA splicing, localization, stability, and translation by binding to its target mRNA (Chekulaeva et al., 2006; Dold et al., 2020; Lee et al., 2010; Moraes et al., 2006; Webster et al., 1997). This post-transcription regulation of mRNA is crucial for proper gene expression in many cellular functions because aberrant gene expression will lead to cell death or cancer.

The previous result suggests CELF1 as a post-transcriptional regulator of *Dsx1α* mRNA. Until now, many studies have reported CELF1's essential role in post-transcription regulation, such as mRNA decay and translation repressor. CELF1 can bind to Signal Recognition Particle Proteins (SRPs) mRNA with GU-rich sequence in its 3'UTR and induces mRNA decay (Russo et al., 2017). Utilizing the similar binding site, CELF1 ortholog, Bruno can bind to the 3'UTR of the oskar (*osk*) mRNA in *D. melanogaster,* leading to its translation repression (Chekulaeva et al., 2006). Moreover, CELF1 works antagonistically with *Ol-BSF* to reduce Medaka fish sex-determining gene *dmrt1bY* expression by binding to CUG repeat in the 3'UTR and hindering male gonad development (Herpin et al., 2019). CELF1 binding to target mRNA is crucial to activating its post-transcription regulation.

To regulate the target RNA, RBP must bind to the specific binding site element in the target mRNA. The binding specificity of RBP was mediated by the RNA recognition motifs (RRMs) domain which is conserved among specific RBP families. As a post-transcriptional regulator, CELF1 was first discovered in *D. melanogaster* as Bruno protein which functions as a translator repressor of oskar protein. CELF1 was identified to prefer CUG repeat as its

binding site on the target mRNA. Hence its first name is CUG-Binding Protein (CUGBP) (Webster et al., 1997). However, a recent discovery reported CELF family could control gene expression at a post-transcriptional level by binding not only to the CUG repeat element (Webster et al., 1997) but also to the GU-rich element (GRE) in the target mRNA (Faustino & Cooper, 2005; Takahashi et al., 2000; Xia et al., 2017). The GRE diversely enriched in many mRNAs and GRE locations in the RNA usually determined CELF1-specific regulation. Target mRNA with GRE in introns induces alternative splicing of many mRNA transcripts by CELF1 and can trigger certain isoforms for a specific protein function or regulation (Dasgupta & Ladd, 2012). On the other hand, GRE in the 3'UTR is commonly found to be a stabilization/degradation factor to many target mRNAs by CELF1 (Vlasova-St. Louis et al., 2013). Lastly, CELF1 binding to GRE in the 5'UTR is very rare (Beisang et al., 2012). The translational regulation of CELF1 by utilizing 5' UTR has been reported only in three mRNAs. CELF1 binding to the 5'UTR of p21 & C/EBPB mRNA showed enhanced translation (N. A. Timchenko, Cai, et al., 2001; N. A. Timchenko, Iakova, et al., 2001). In contrast, CELF1 binding to the 5'UTR of p27 mRNA showed repressed translation by CELF1 binding in the 5'UTR (Y. Zheng & Miskimins, 2011). The CELF1 function in this binding pattern might be specific to a certain regulation which is not yet understood.

In this chapter, I aim to elucidate if *Dsx1α* 5'UTR mRNA also possesses the GRE as a CELF1 binding site or if other factors facilitated CELF1 suppression because CELF1 was identified to associate with the *Dsx1α* 5'UTR at first. First, I gathered CELF1 binding site sequence information from the previous report and searched for the same motif in *Dsx1α* 5'UTR. Second, I confirmed the CELF1 binding to the confirmed GRE by *in vivo* assay using GFP reporter mRNA. Altogether, I revealed whether the suppression of *Dsx1* expression by CELF1 is strictly dependent on the presence of the GRE or not.

3.2 Materials and Methods

3.2.1 Wildtype *Daphnia magna* culture condition

Wildtype *Daphnia magna* strain (NIES) was used in this study. This strain was obtained from the National Institute of Environmental Studies (NIES, Tsukuba, Japan) and cultured under the same laboratory condition mentioned in Chapter 2 (see 2.2.1 Wildtype *Daphnia magna* and Doublesex1 reporter strain culture condition).

3.2.2 *Dsx1α* 5'UTR-GFP with CELF1 putative binding site mutant reporter construct

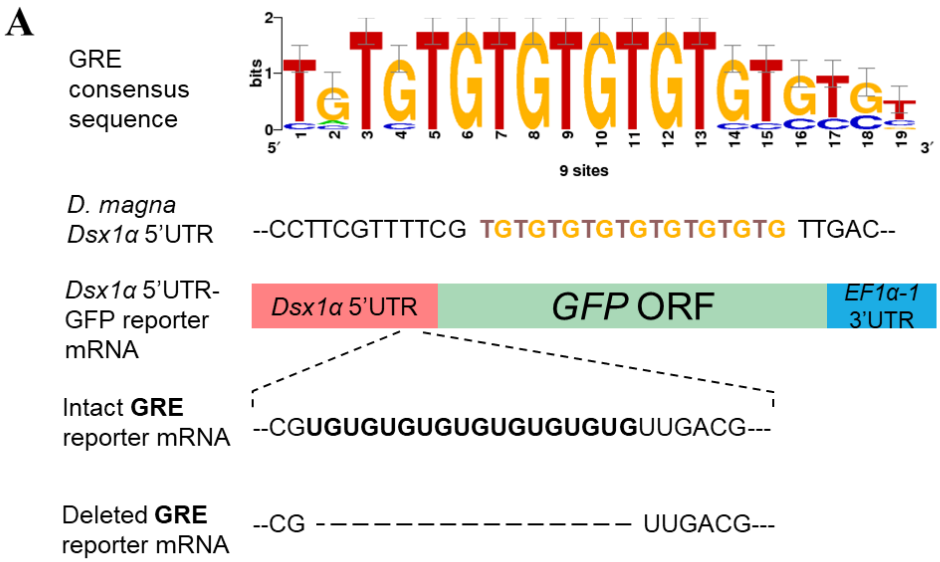
To identify the CELF1 putative binding site in *D. magna Dsx1α* 5'UTR, I look for a consensus sequence by using CELF1 binding site sequences from the previous reports, as listed in Table 5. Among these sequences, I searched enriched motifs using online web tools, Regulatory Sequence Analysis Tools (RSAT), at http://rsat.sb-roscoff.fr/. The enriched motifs were then used as CELF1 putative binding site sequence in *D. magna*

Table 5. Target sequences of CELF1 binding in CELF1 orthologs.

Gene	Organism	Sequence	Method	Reference
nrx-1 *unc-75*	*C. elegans*	GAGGCGGCTTTTGAATGAAA AAAAACCC TACACATCTGTGTGTGTGTGT GTGTGTGTGTGTCCCCCGCC	CLIP-Seq	(Chen et al., 2016)
cTnT Mse	*G. gallus*	AATAAATCGCGGGTCGGTGTG TCCTGTGCCTTTCCCTGCTTGG GAAA	CLIP	(Ladd et al., 2001)
osk	*D. melanogaster*	GATCCAATGTATGTTAATTGT ATGTATTA	CLIP	(Webster et al., 1997)

To prepare the GFP reporter driven by *Dsx1α* 5'UTR, pEX-*Dsx1* 5′ UTR::GFP (Perez et al., 2021) was used as a template for mRNA synthesis. This plasmid was used as a template to delete the potential *CELF1* binding site using the primer set as follows: Forward (5'-TCCCCTTCGTTTTCGTTGACGTTTTCATTTCCA-3') and Reverse (5'-

AAATGAAAACGTCAACGAAAACGAAGGGGAAAT-3') resulting in the generation of pEX-*Dsx1* 5′ UTR GRE mutant::GFP (Figure 22). For internal control, the *CELF1* CDS of pEF1α1-CELF1 was then replaced with the CDS of *mCherry* using seamless cloning to produce pRCS21-EF1α-1-mCherry, which was used to synthesize EF1α-1-mCherry mRNA as a template. The *mCherry* region was amplified from the bicistronic reporter plasmid in the previous study (Kumagai, Matsuura, et al., 2017).



B

AGTGCATGATCCCCGCGCGAAGGGAAAGCTTCGTACCGAAAAATCGTAGAACC
ACTGTTTAGTTCCCTTTTGGTGCCTTACTGAGTAAGTTGGCTCGTTCTCGATCTC
GATTTTTTGACTCTTCGTGCTTTTGTCGCACAGTTATTTCCCCTTCGTTTTCG**TGT**
GTGTGTGTGTGTGTGTGTTGACGTTTTCATTTCCAATATATAG

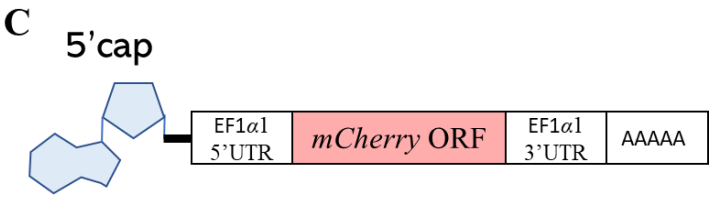


Figure 22. GRE consensus sequence in *D. magna* and *Dsx1α* 5'UTR-GFP reporter construct

(A) GRE consensus sequence as CELF1 putative binding site is present in the *D. magna Dsx1α* 5'UTR. **(B)** CELF1 putative binding site position in the 205bp core sequence of *Dsx1α* 5'UTR. **(C)** EF1α-1-mCherry mRNA construct for internal control.

In vitro transcription and poly(A) tail addition were performed with mMESSAGE mMACHINE T7 RNA Polymerase and Poly(A) Tailing kits, respectively (both Ambion, Foster City, CA, USA). The synthesized RNA size and the attached poly(A) tail length were analyzed by denaturing formaldehyde gel electrophoresis.

In vivo post-transcription assay was performed by coinjecting 40 ng/µl reporter mRNA and mCherry mRNA into NIES female eggs. The sample was observed under a fluorescent microscope to measure the GFP fluorescence at 24 h. An additional 40 ng/µl CELF1 mRNA was coinjected into the eggs for the overexpression experiment.

3.2.3 Fluorescence photography and quantification

Injected sample photos were taken using Leica DC500 CCD Digital Camera mounted on a Leica M165FC fluorescence microscope (Leica Microsystem, Mannheim, Germany). Fluorescence photography was done using GFP2 and mCherry filters, as mentioned in Chapter 2 (see 2.2.5 Fluorescence photography and quantification).

3.3 Results

3.3.1 GU-rich elements also conserved in *Daphnia magna Dsx1α* 5'UTR mRNA

As the previous chapter suggests, CELF1 acts as a post-transcription repressor in *D. magna*. Previous reports stated two main post-transcription regulation by CELF1 were utilizing GU-rich element (GRE) in the target mRNA. CELF1 binding site consensus has been extensively researched. My literature research found that the CELF1 ortholog in *D. melanogaster* binds to CUG repeat in the *osk* mRNA (Webster et al., 1997). Another report in *C. elegans* using CLIP-Seq showed overrepresented motifs for CELF1 orthologs binding as UGUGUGUG in its mRNA target (Chen et al., 2016). The higher taxa, like *G. gallus* CELF1 orthologs, showed

preferential binding to UG-rich elements in muscle-specific splicing enhancers (MSEs) that were also conserved in humans (Ladd et al., 2001).

Using RSAT, I found an enriched motif of CELF1 putative binding site with CELF1 orthologs binding sites as references. The consensus sequence of the CELF1 binding site was UGUGUGUGUGUGUGUGUGU in mRNA (Figure 22A). Interestingly, this motif was present in the Dsx1α 5'UTR, especially in the 205 bp core sequences and near the start codon (Figure 22B). This finding suggests that CELF1 may utilise the GRE in the *Dsx1α* 5'UTR to regulate *Dsx1* expression.

3.3.2 CELF1 repressed *Dsx1* expression via the GU-rich element of the *Dsx1α* 5' UTR in embryos

To validate that *Dsx1* expression suppression by CELF1 strictly depends on the GRE in the *Dsx1α* 5'UTR, I further investigate the function of GRE presence in CELF1-dependant *Dsx1* repression. I designed and constructed *Dsx1*-driven GFP reporter mRNA and observed *in vivo* translation efficiency of the target GFP as fluorescence intensity. Based on the previous GRE consensus sequence result, I tested GFP reporter with or without GRE presence. To contrast the result of the CELF1 de-repression with GRE deletion, I also measure the reporter mRNA with GRE fluorescence with overexpression of CELF1. These reporter mRNAs were injected into NIES female eggs.

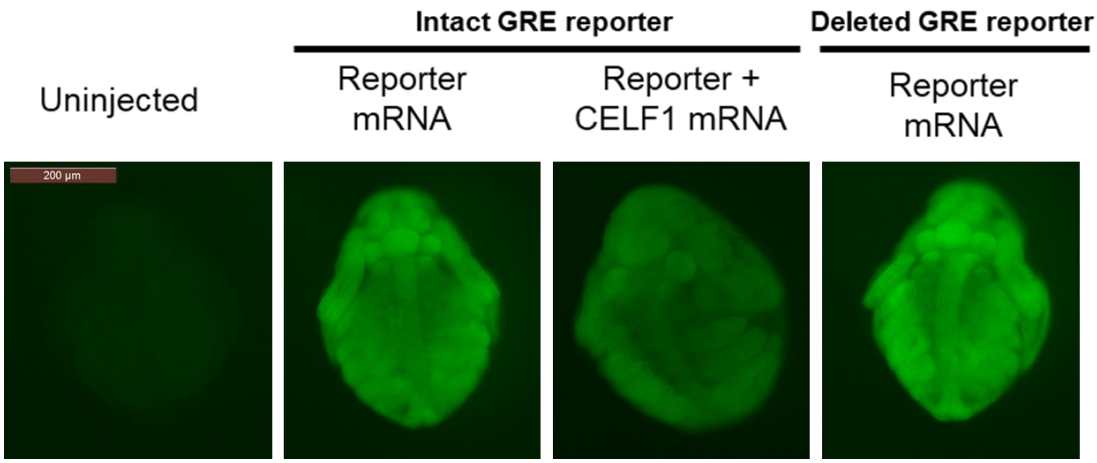


Figure 23. Translation efficiency of *Dsx1* 5′ UTR-GFP reporter mRNA variants with and without the GRE/CELF1 binding site *in vivo*.

Female embryos of wild-type *D. magna* injected with GFP reporter mRNA with or without mutated *Dsx1α* 5' UTR were observed at 24h (ventral view). In addition, the translation efficiency of GFP reporter mRNA was also observed in the presence of *D. magna* CELF1 overexpression. GFP fluorescence mirroring the ectopic *Dsx1* translation.

The results showed that embryos injected with the intact GRE reporter mRNA had lower GFP translation efficiency than embryos injected with the GRE mutant reporter mRNA (Figures 23 and 24). Quantification of relative GFP intensity using ImageJ showed that Intact GRE reporter mRNA had 15-fold GFP fluorescence intensity compared to uninjected. In contrast, GRE mutant mRNA has 23-fold GFP fluorescence intensity compared to uninjected. This result suggests that reporter mRNA's higher translation efficiency was due to the lack of GRE in the *Dsx1α* 5'UTR.

On the contrary, CELF1 overexpressed embryos have lower fluorescence intensity than embryos without CELF1 overexpression. Coinjection with CELF1 mRNA lowers the reporter GFP intensity from 15-fold to 9-fold if compared to uninjected. This further indicates that CELF1 utilizes GRE in the *Dsx1α* 5'UTR for repressing *Dsx1* expression.

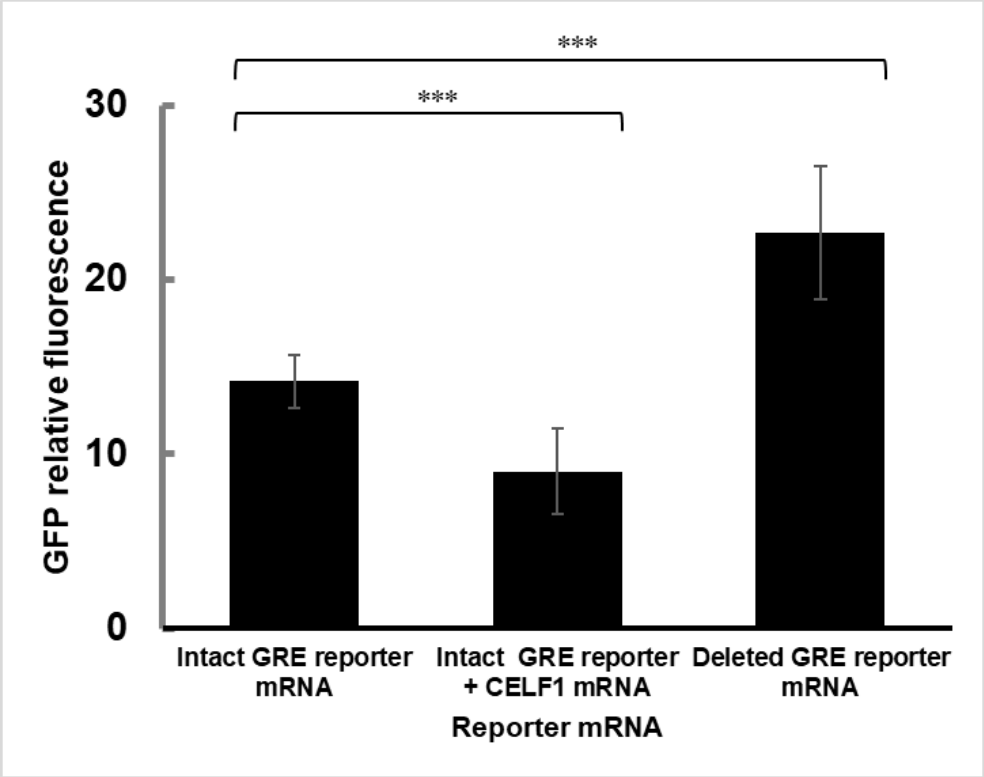


Figure 24. Relative GFP fluorescence intensity of *Dsx1* 5′ UTR-GFP reporter mRNA variants with and without the GRE/CELF1 binding site

Relative GFP fluorescence increase of GFP reporter mRNA injected samples in female embryos at 24h post-injection. Error bars indicate the standard error of the mean. ***p<0.001 (Student's T-test). N=12

3.4 Discussion

In the previous chapter, CELF1 can repress *Dsx1* expression at the post-transcription level. CELF1 orthologs in the previous report showed that GRE in the target mRNAs acts as a CELF1 binding site and is crucial to triggering CELF1 activity. Using the known binding site sequences of CELF1 orthologs, I searched for a GRE consensus sequence from all target mRNAs. The result suggests that the dinucleotide (UG)9 repeat motifs are a consensus sequence for the CELF1 binding site. Consistent with my hypothesis, I found the GRE consensus sequence conserved in the *Dsx1α* 5'UTR, especially in the 205 bp core sequence that also overlapped with the transactivation element of *DAPALR* (Kato et al., 2018).

To understand the importance of this GRE in the CELF1-dependent *Dsx1* suppression, I performed *in vivo* translation assay to compare the translation efficiency of GFP reporter

mRNA driven by *Dsx1α* 5'UTR in female NIES strain with GRE mutant reporter mRNA. The fluorescence microscope observation after 24 h showed that the GFP translation of embryos injected with reporter mRNA lacking GRE has around 60% higher translation compared to embryos injected with intact GRE reporter mRNA. This result indicates that the endogenous CELF1 in embryo interacts with the GRE in the reporter mRNA, repressing its translation. In addition, translation efficiency was further decreased with the coinjection of ectopic CELF1 mRNA. Altogether, these results suggest that CELF1 repressed *Dsx1* expression via the GU-rich element of the *Dsx1α* 5' UTR in embryos.

This hypothesis was supported by the previous report that discovered CELF1 ortholog in *D. melanogaster*, also known as Bruno, associated with the Bruno-response element (BRE, which is identical with GRE at last findings) and repressed *sex-lethal* (*sxl*) translation in oogenesis (Z. Wang & Lin, 2007). This regulation eventually controls the downstream sex determination processes in *D. melanogaster* as sxl is known to be important for oocyte differentiation, and unregulated sxl will ultimately cause aberrant meiotic processes that are indispensable in male and female development (Salz & Erickson, 2010). In the higher taxa like medaka fish, CELF1 bind to the master regulator of male sex determination, dmrt1bY 3'UTR (D3U-box motif), and modulate its mRNA stability. CELF1 work antagonistically with bsf to control dmrt1bY translation expression in which CELF1 will destabilize the mRNA and promotes degradation, while bsf will act as the mRNA stabilization factor (Herpin et al., 2019). Based on these previous reports and current experiments, it is natural to conclude that CELF1 also controls the sex-determining gene, *Dsx1* expression in *D. magna*.

The current result showed that (1) CELF1 modulation did not affect the *Dsx1* transcript level and (2) translation efficiency of GFP reporter mRNA modulated by the presence of GRE in *Dsx1α* 5'UTR suggesting that the *Dsx1* repression is achieved through translational control rather than mRNA decay. First, the CELF1 function in alternative splicing commonly exploits

the GRE in introns like in *G. gallus* cTNT-MSE, promoting the exon 5 inclusion. Utilizing the similar GRE in introns, CELF1 works synergistically with Lark protein orthologs to promote male-specific splicing of *B. mori Dsx* (Z. Z. Zheng et al., 2019). Second, as a regulator of mRNA stability, CELF1 utilizes GRE in target mRNA 3'UTR by competing with mRNA stabilization agents (e.g. HuR) or recruiting mRNA degradation factors (e.g. PolyA Ribonuclease) (Moraes et al., 2006; Russo et al., 2017). CELF1 is also associated with short-lived mRNA with GRE in their 3'UTR (Bohjanen et al., 2015). While most of the mRNA decay mechanism by CELF1 exploits GRE in the target mRNA 3'UTR, no report found the same mechanism exploiting the GRE in the 5'UTR. Lastly, the translational control of CELF1 to the target mRNA with GRE has been reported mostly in the 5'UTR. This protein binds to the 5' UTR of *p21* and *C/EBPβ* mRNA in human cancer cells, enhancing their translation efficiency (Timchenko, Cai, et al., 2001; Timchenko, Isakova, et al., 2001). On the contrary, CELF1 functions as a negative translation regulator via binding to the 5' UTR of *p21* mRNA in the mouse lens cell line (Siddam et al., 2018) and *p27* mRNA in the breast cancer cell (Y. Zheng & Miskimins, 2011). Therefore, it is typical to assume that CELF1 suppress *Dsx1* expression via translational regulation in *Dsx1α* 5'UTR.

As CELF1 is expressed in both males and females, I hypothesized that the default state in which CELF1 is associated with *Dsx1α* mRNA is the repressed state of *Dsx1* expression by the CELF1 protein. Then, specific elements expressed only in male embryo de-repressed Dsx1 translation by competing with CELF1 in binding the Dsx1α 5'UTR or becoming a decoy substrate for CELF1, exacerbated CELF1 function in suppressing *Dsx1* expression. In this premise, CELF1 acts as a safekeeping mechanism to keep the *Dsx1* expression checked every time to maintain the default asexual life cycle and keep in the leaky expression of *Dsx1*, avoiding sexual ambiguity (Figure 25).

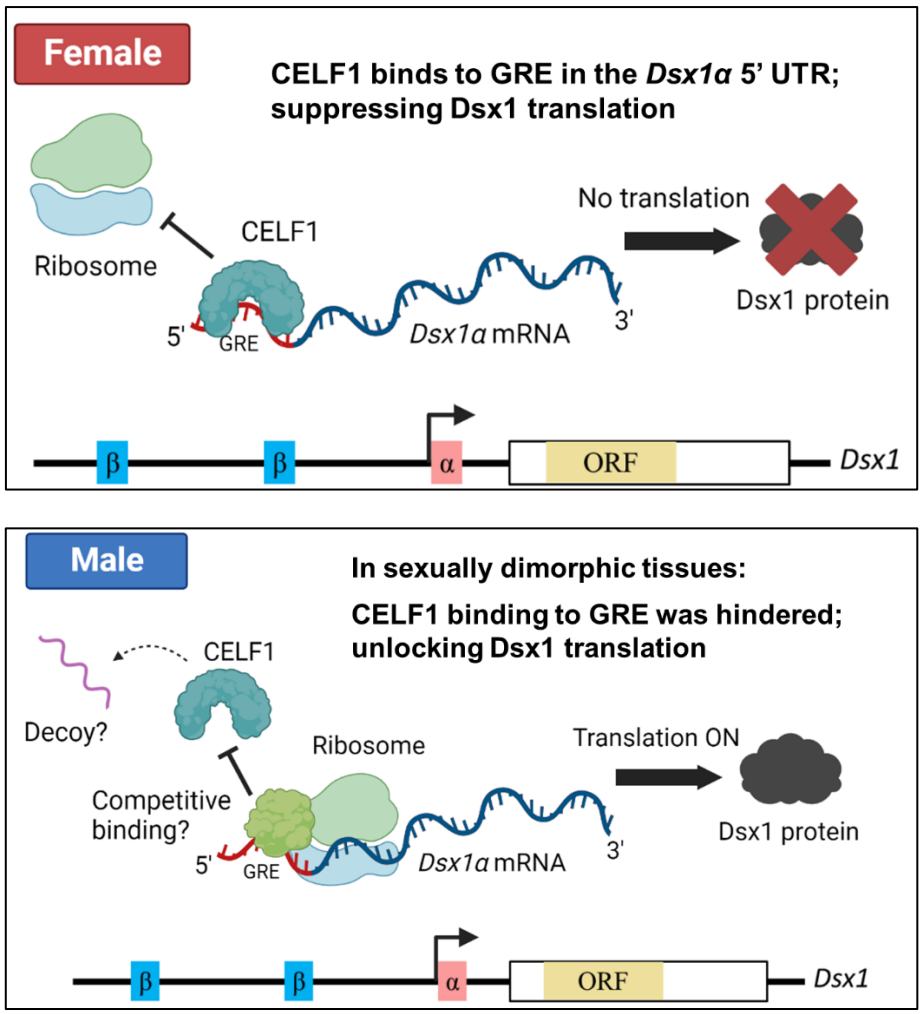


Figure 25. The potential role of CELF1 in female and male embryos

This hypothesis is supported by the noise-cancelling model composing RBP shep and lncRNA *DAPALR*. In this model, the shep protein acts as a translational repressor by binding to the core sequence of the *Dsx1α* 5'UTR in males and females, suppressing the noisy expression of *Dsx1* (Perez et al., 2021). However, as *DAPALR* is exclusively expressed in male (Kato et al., 2018) and possess the same core sequence as *Dsx1α* 5'UTR, *DAPALR* can act as a molecular sink to sponge shep protein from *Dsx1α* 5'UTR. This mechanism allows *D. magna* to keep *Dsx1* from accidental expressions and control the expression exclusively in males. It is natural to assume that both shares the same mechanism as GRE were in the proximity of the shep binding site in *Dsx1α* mRNA and *DAPALR* transactivation element. Moreover, both

orthologs have been known to target sex-determining gene and regulates their translation (Mapes et al., 2010; Z. Wang & Lin, 2007).

This finding shed new light on the sex-determination molecular network in *D. magna*. CELF1, as the new player discovered in the *Dsx1* safekeeping mechanism, answers the question of why previously shep de-repression alone cannot induce sex reversal in *D. magna*. This finding also opens another possibility that CELF1 and shep protein may work in synergy to keep the *Dsx1* from abnormal expression. The fine-tuning of both proteins may be crucial to the proper spatiotemporal expression of *Dsx1* as both proteins expressed ubiquitously in the whole tissues. However, it must be noted that shep is mainly expressed in neurons (Olesnicky et al., 2018) and CELF1 mainly in brain and muscle tissues (Blech-Hermoni et al., 2013), suggesting they might regulate *Dsx1* expression in different tissues independently.

Altogether, I revealed one of the missing links in the intricate mechanism of sex determination in *D. magna*. While this mechanism may be specific in *D. magna*, this study also highlighted the potential for CELF1 mechanisms in many cellular processes as CELF1 is involved in the translation regulation of GRE-containing mRNAs and expressed ubiquitously in somatic and germline cells (Kress et al., 2007; Milne & Hodgkin, 1999).

CHAPTER 4 GENERAL DISCUSSION AND CONCLUSION

4.1 General discussion

The crustacean *D. magna* lacks sex chromosomes and utilizes environmental cues for sex determination. *D. magna* produces only female offspring in favorable conditions. In contrast, environmental cues stimulate male production (Hebert, 1978; J. Lubbock, 1857). Previous studies revealed that environmental cues are converted into sesquiterpenoid signaling and activate the *Dsx1* gene (LeBlanc & Medlock, 2015; Toyota et al., 2021). This gene codes for the DM-domain transcription factor and orchestrates the male-developmental program (Kato, Kobayashi, et al., 2011). Manipulation of *Dsx1* expression and activity in females leads to the generation of intersex phenotype (Kato, Kobayashi, et al., 2011), demonstrating that the *Dsx1* gene must be tightly silenced in females throughout development, and it would be upregulated precisely in a spatio-temporal manner for male production. Thus, unraveling the regulatory mechanism of *Dsx1* expression is essential for understanding how sex is determined in this species. In this study, I investigated the function of the RNA-binding protein CELF1, which was identified as one of the proteins associated with the 5' UTR of the *Dsx1α* isoform (Perez et al., 2021).

CELF1 has been reported to target sex-determining and development genes across many species. First, as an alternative splicing factor, CELF1 works synergistically with Lark protein orthologs to promote male-specific splicing of *In chapter 2* Dsx (Z. Z. Zheng et al., 2019). Second, as an mRNA destabilizing factor, it works antagonistically with Ol-bsf to reduce Medaka fish sex-determining gene dmrt1bY expression and hinder male gonad development (Herpin et al., 2019). Third, as a translational repressor, CELF1 (Bru) represses the master sex-determining gene Sxl in Drosophila, promoting the male dosage compensation and somatic differentiation cascade (Salz & Erickson, 2010; Z. Wang & Lin, 2007). In addition, CELF1 has a critical role in controlling gonadal development in the fruit fly, mice, and nematode (Boateng

et al., 2017; Boulanger et al., 2015; Flora et al., 2018; Kress et al., 2007). During evolution, CELF1 might be repeatedly used in the animal sex-determining pathways.

I found that CELF1 functions as a post-transcriptional repressor of the *D. magna Dsx1* gene. My result showed that CELF1 repressed *Dsx1* expression, possibly via binding to the GU-rich element (GRE) of the *Dsx1α* 5' UTR. GRE is a predominant binding site of CELF1 (Faustino & Cooper, 2005; Takahashi et al., 2000; Xia et al., 2017). CELF1 bound to the 5' UTR of p21 and C/EBPβ mRNA in human cancer cells and enhanced their translation efficiency (N. A. Timchenko, Cai, et al., 2001; N. A. Timchenko, Iakova, et al., 2001). On the contrary, CELF1 functions as a negative translation regulator via binding to the 5' UTR of p21 mRNA in the mouse lens cell line (Siddam et al., 2018) and p27 mRNA in the breast cancer cell (Y. Zheng & Miskimins, 2011). Since (1) the *Dsx1* transcript level did not change by CELF1 silencing and overexpression and (2) deletion of GRE from the *Dsx1α* 5' UTR increased its GFP reporter expression, it may be possible that CELF1 may suppress *Dsx1* translation via its 5' UTR.

During embryogenesis, CELF expression did not show any sexual dimorphism. In addition, in both sexes, CELF1 silencing de-repressed *Dsx1* expression. The *Dsx1* expression increase only in male 6 hours after ovulation and is localized in the sexually dimorphic organs such as the first antennae, first thoracic appendage, and gonads (Kato, Kobayashi, et al., 2011). In the knockdown females, *Dsx1* expression was observed in the yolk region and was not detected in the sexually dimorphic traits such as the first antennae suggesting CELF1 is important but insufficient. This result may explain the absence of sex reversal in females. Further loss-of-function experiment to observe the sex reversal was not possible due to CELF1 affecting the embryo's viability. This is probably because CELF1 downregulation may affect the global change of mRNAs alternative splicing, stability, and translation (Blech-Hermoni et al., 2016). This is also supported by the previous study that reported CELF1 ortholog, ETR-1

downregulation results in the development arrest and lethality in *C. elegans.* This phenotype might be related to the important ETR-1 role in muscle tissues and embryogenesis (Milne & Hodgkin, 1999). Moreover, CELF1 knockout in mice showed significant growth retardation and infertility, suggesting that CELF1 knockout or strong downregulation may harm *D. magna* and lead to abnormal embryogenesis in somatic or germ cells (Kress et al., 2007). Overexpression of CELF1 in male embryos reduced mCherry fluorescence, and the importance of the GRE element for repression of *Dsx1α* expression was successfully evaluated in female embryos. Based on these data, CELF1 possibly has the ability to repress *Dsx1* expression both in females and males

The non-sex-specific role of CELF1 on *Dsx1* repression could provide insight into the CELF1 function to set the threshold of *Dsx1* expression. In females, *Dsx1* protein from noisy expression may bind to the potential *Dsx1* binding site upstream of the *Dsx1α* transcription start site (Mohamad Ishak et al., 2017) and self-activate its expression via a positive feedback loop. CELF1 might avoid unintended *Dsx1* translation and subsequently eliminates the generation of sexual ambiguity in females. Since the *Dsx1* protein is expressed in a tissue and time-specific manner in males (Nong et al., 2017), it would be possible that CELF1 contributes precise control of *Dsx1* activation in males.

In this premise, I must include the RNA binding protein Shep and long noncoding RNA (lncRNA) named *DAPALR* (Kato et al., 2018; Perez et al., 2021). Shep also represses *Dsx1* translation by binding to the *Dsx1α* 5' UTR, and *DAPALR* is an endogenous competing RNA that sequestered Shep. CELF1 joined this model as a translation repressor of *Dsx1*. In females, both CELF1 and Shep will bound to *Dsx1α* 5'UTR to keep the noisy expression of *Dsx1*, avoiding sex ambiguity. In males, DAPALR presence titrates both proteins and allows the translation machinery to translate the *Dsx1* protein (Figure 26). Further studies to prove this model and the synergy between CELF1 and Shep are needed in the future.

This model was supported by the previous reports of the decoy mechanism involving CELF1 and lncRNA or microRNA. LncBATE10 functions as a decoy to titrate Celf1 way from Pgc1α mRNA, which otherwise will be repressed by Celf1 in brown adipose tissues (Bai et al., 2017). In lung cancer cells, miR-574-5p sponges CELF1 and prevent binding to mPGES-1 3'UTR, enhancing specific isoform splicing (Emmerich et al., 2020). Moreover, CELF1 protein-protein interaction has been reported. CELF1 interacts with the initiation factor, eIF2, which is thought to promote the use of the downstream AUG in the target mRNA (L. T. Timchenko et al., 2006). Altogether this supports the hypothesized model of CELF1 with the noise-cancelling model, which may allow us to recognize an elegant control of sex determination in *D. magna*.

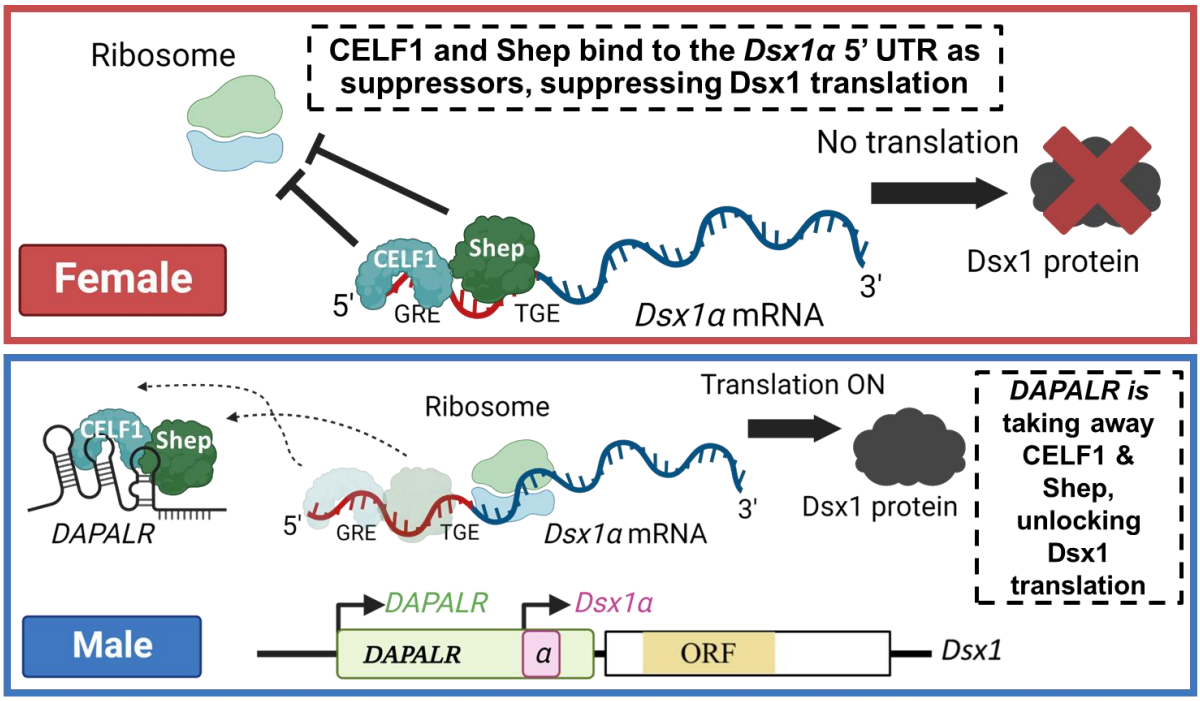


Figure 26. The updated hypothesis of the "noise-canceling" mechanism of the *DAPALR*-Shep-CELF1 to regulate *Dsx1* and the sex determination in *D. magna*.

4.2 Conclusion

CELF1, an RNA-binding protein, has been identified to be associated with the male-determining gene, *Dsx1,* in *Daphnia magna*. I performed a deep functional analysis *in vivo* in this study, and I discovered that

1) CELF1 has conserved three RRMs that conserved within its orthologs in diverse species, and it suppresses *Dsx1* expression at the post-transcription level; and

2) CELF1 suppresses *Dsx1* expression via binding to its GRE in the *Dsx1* 5'UTR by regulating *Dsx1* mRNA translation. Moreover, this GRE might also involve in the decoy mechanism by *DAPALR* to sequester CELF1 from *Dsx1* mRNA in male embryos.

This study shed new light on the sex-determination molecular network in *D. magna*. CELF1, as the new player, has an important role in the *Dsx1* expression safekeeping to avoid sexual ambiguity in *D. magna*. In addition, this study serves as a missing link in the previously proposed model composing *DAPALR* and Shep to control *Dsx1* translation. This study showed that it might be crucial to find the synergy between CELF1 and Shep in regulating *Dsx1* translation. Altogether, this brings vital information to elucidate the dynamic regulation not only in ESD but also in many cellular processes.

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LIST OF PUBLICATION

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"Always pass on what you have learned." -Yoda-