1 **Title: *Dmy* initiates masculinity by altering *Gsdf/Sox9a2/Rspo1***
2 **expression in medaka (*Oryzias latipes*)**
3
4
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19 **Short Title:** Molecular mechanism of *Dmy* associated sexuality in medaka
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24
25 **Key words:** Dmy; sex reversal; loss-of-function; F3 progeny; GSDF; Sox9; Masculinity;
26 Medaka
27

Supporting Information:

1. **Supplementary materials and methods**

2. **Supplementary Table 1: Summary of GFP knockdown using *olvas-eGFP* transgenic medaka at F_0 generation.**

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10. **Supplementary Figure 3: Effect of *Dmy* knockdown on gonadal gene profile in adults.**

11. **Supplementary figure 4. *In vivo* evaluation of knockdown efficiency of F_2 transgenics.**

12. **Supplementary figure 5. *In vivo* chromatin immunoprecipitation analysis of candidate *Dmy* targets.**

13. **Supplementary Figure 6: Effect of *Gsdf* and *Sox9a2* overexpression on germ cell proliferation and reversal of *Dmy*-KD phenotype.**

14. **Supplementary figure 7. Schematic diagram showing the breeding and mating strategies of Dmy-KD transgenic line.**

69 **Supplementary Materials and Methods**

70 **Plasmid constructs**. The AS (Antisense) RNA expression constructs were designed
71 and evaluated using the online tool E-RNAi. Several constructs of different length (200-
72 320bp) were selected, amplified, evaluated *in vitro* before starting the actual experiment.
73 A selected primer pair carrying *Xba*I and *EcoR*V sites (Supplementary table 5) was used
74 to amplify a 220-bp region of the *Dmy* gene (Supplementary Fig. 1) from a plasmid
75 containing the complete *Dmy* ORF (a gift from Dr. M. Matsuda, Japan). A 359-bp
76 sequence of the *Gfp* gene was also amplified from the plasmid pCMV-GFP (Dr.
77 Krishnan, India) using specific primers with the same RE sites (Supplementary table 5).
78 The amplicons were cloned under the control of the CMV early promoter in pcDNA3.1
79 (+) (Invitrogen, USA) in the AS orientation. The constructs were transformed into *E. coli*
80 (XL1 blue) and the positive clones were confirmed by sequencing. The sequences were
81 also analysed for secondary structures *in silico* using RNA*fold* online software
82 (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.). The recombinant plasmids were designated
83 as pmDMY-AS and pEGFP-AS. Plasmid DNA used for downstream experiments was
84 purified using a plasmid purification kit (Qiagen, Germany). A schematic representation
85 of the AS RNA expressing construct is given in supplementary Fig 1.

86 The complete *Dmy* ORF (820 bp) was cloned into the pCDNA 3.1 histopo T-vector
87 using ORF-specific primers (Supplementary table 5). A clone that contained the insert in
88 the right orientation was selected after sequencing. The recombinant plasmid was named
89 pmCMV-DMY. Similarly, complete ORFs of *Wt1a/b*, *Ad4bp/Sf1, Sox9a2* and *Gsdf* were
90 also amplified (from previous constructs in the pGEMTeasy vector), cloned into pCDNA
91 3.1 histopo T-vector, sequenced to confirm the correct orientation and named as pCMV-
92 Wt1a/b, pCMV-Sf1, pCMV-Sox9a2 and pCMV-Gsdf, respectively. A 300 bp *Dmy*
93 promoter fragment was isolated using specific primer set and cloned in pGL3 basic vector
94 (Promega, USA) using Infusion-cloning kit (Clonetech, USA). Stop codon-less *Gsdf* and
95 *Sox9a2* ORFs were inframed with Cherry (from pmCherryN1 vector) and cyan (from

96 pamCyanN1 vector) ORF, respectively, and cloned into a pCS2 vector (a gift from Dr.
97 Ogino, NIBB, Japan) using Infusion cloning kit (Clonetech, USA). All the genes used for
98 real-time PCR and *in situ* analysis were cloned into pGEM T Easy vector.
99
100 **Gene knockdown in Cos7 cells.** Knockdown of *Dmy* expression by the pmDMY-AS
101 construct was tested in Cos-7 cells with no endogenous expression of *Dmy*, *Dmrt1,*
102 *Sox9a2, Ad4bp/Sf1* or *Gsdf*. Cos-7 cells were seeded in 24-well plates, at 5 X 10^5
103 cells/well, containing Dulbecco's modified Eagle's Medium (Sigma, USA) supplemented
104 with 10% charcoal/ dextran treated foetal bovine serum (Hyclone, USA). The cells were
105 incubated for 24 h at 37 ^{0}C with 5% CO_2 and co-transfected with 100 ng of pmCMV-
106 DMY and 5/ 10/ 100 ng of the pmDMY-AS construct using Fugene-6 transfection
107 reagent (Roche Diagnostics, Switzerland) following the manufacturer's instructions.
108 Cells transfected with only pmCMV-DMY served as positive controls while the negative
109 controls were treated with only the transfection reagent. The cells were grown for 96 h
110 after which total RNA was isolated and target transcripts quantified by real-time PCR as
111 detailed later. The transfections were performed in triplicate at one time point and then
112 repeated twice later. In separate experiments, cells were also transfected with pCMV-
113 DMRT1, pCMV-SOX9a2, pCMV-SF1 and pCMV-GSDF, and the effect of pmDMY-AS
114 on their expression was also quantified by real-time PCR. The transcript copy numbers
115 are shown as the mean ± SEM and the dose-response data was computed using Statview
116 v. 5 (SAS institute Inc, Cary NC).
117
118 **Experimental animals.** The QurtE strain of medaka was mostly used for this study.
119 This strain expresses a male-specific leucophore that allows easy sexing of fish. Another
120 strain called the *olvas-eGFP* transgenic medaka was also used. This strain carries the *Gfp*
121 gene under *vasa* promoter and is reported to express GFP fluorescence in germ cells in
122 both males and females[1]. All the fish were maintained at 26 ± 2 ^{0}C under a 14 h light and

123 10 h dark cycle. Eggs were collected within 30 min of fertilization and incubated in
124 distilled water (milli-Q) containing antifungal solution (Methylene blue, 0.0001%) at 26
125 ± 2^0C. Brooders and juveniles were fed with fresh artemia, while larvae were given
126 artificial feed. Classification of developmental stages was done according to the
127 descriptions of Iwamatsu[2]. All *in vivo* experiments were conducted following protocols
128 and procedures approved by Institutional Animal Care and Use Committee at the
129 National Institute for Basic Biology, Japan.
130

131 **Knockdown of *Dmy* expression in medaka embryos.** One-two cell embryos of the
132 QurtE strain of medaka were electroporated with the pmDMY-AS plasmid construct (1
133 μg/ml) in 1X HBS buffer, pH 7.5[3]. Electroporation was carried out in a Cuy21 edit type
134 electroporator (Bex, Tokyo) at 24V, with 9 millisecond (ms) pulse, 900 ms pause for 3
135 times. Embryos electroporated with-HEPES buffered saline (HBS) alone and those
136 electroporated with pCMV-GFP constructs served as controls. peGFP-AS plasmids were
137 electroporated into one-two cell embryos of *olvas-eGFP* transgenic medaka to evaluate
138 the knockdown of GFP expression.
139

140 **Sampling.** Electroporated male embryos of the QurtE strain of medaka were
141 randomly sampled at 5 days after fertilization (daf) and 0, 5, 10, 20, 50 and 90 days after
142 hatching (dah) in order to check the gonadal morphological changes and further
143 confirming the gene knockdown effects at both genetic and phenotypic levels. Minimum
144 of 10 fish were sampled at each time point. The genetic sexes of all fish were confirmed
145 by *Dmy*-genomic PCR[4]. Electroporated *olvas-eGFP* transgenic medaka were periodically
146 observed for any fluorescence reduction and morphological defect until hatching, and all
147 the fish of 10 dah were culled and fixed for histological examination of the gonads. All
148 fish were also sexed accordingly[4]. 10 adults of control XY and DmyKD-XY (Dmy-AS
149 (+)) groups were dissected and different tissues were separately collected to prepare the

150 tissue distribution samples. The early stage samples (from Stage 20 to 39) were initially
151 separated based on leucophore expression and later the individuals were separately
152 screened using *Dmy* genomic PCR. The *Dmy* positive and negative samples (10
153 individual each) of the same stage were separately pooled together. Other earlier stage
154 RNA samples (from Stage 4 to 19) were collected from monogametic populations of XX
155 and XY. Three different pools of samples were prepared for reproducible results.
156
157 **Histology and *in situ* hybridisation.** Whole fish samples at 0, 5, 10 and 50 dah and
158 gonads of adults were fixed in Bouin' fixative, embedded in paraffin, sectioned at 5 µm
159 and subjected to standard haematoxylin-eosin (HE) staining. Paraformaldehyde (4%)
160 fixed, paraffin embedded samples (10 dah and adult) were used for *in situ* hybridisation.
161 Hybridisation was done using sense and anti-sense digoxigenin-labelled RNA probes
162 transcribed *in vitro* with a RNA labelling kit (Roche, Germany) from their respective
163 plasmids. Briefly, the sections were deparaffinised, hydrated and treated with proteinase
164 K (10 µg/ml, Roche) and then hybridised with sense or anti-sense DIG-labelled RNA
165 probes at 58 ^{0}C for 22 h. Hybridisation signals were then detected using alkaline
166 phosphatase-conjugated anti-DIG antibody (Roche) and NBT as described previously[5].
167 Whole mount *in situ* hybridisation was carried out using previously published protocols[6].
168
169 **Quantification of changes in gene expression by real-time PCR.** Changes in gene
170 expression were quantified using the ABI Prism 7000 sequence detection system
171 (Applied Biosystems, USA). Total RNA was isolated from cells, embryos, and gonads,
172 using RNeasy Mini kit (Qiagen, Germany). cDNA synthesis was carried out using
173 Quantitect RT PCR kit (Qiagen) from 100 ng of total RNA. The first strand cDNAs were
174 diluted to 100 µl for subsequent use. Gene-specific RT-PCR was performed using SYBR
175 green master mix (Applied Biosystem, USA) and 5 ng of cDNA, according to the
176 manufacturer's instructions. The real-time PCR primers are listed in Supplementary table

177 5. The PCR conditions included an initial denaturation at 94 ^{0}C (2 min) followed by 40
178 cycles at 94 ^{0}C (30 s) and 60 ^{0}C (1 min). *Ef1α* or *Rps18* was used as the internal control.
179 The absolute transcript copy number of each gene was determined with the help of
180 appropriate standard curves and normalized with the *Ef1α/ Rps18* copy numbers in each
181 sample. The reported values are average of sample triplicates.
182
183 **Assessment of the trans-generational knockdown effect.** The QurtE embryos
184 injected with pmDMY-AS were grown to adulthood. Based on secondary sexual
185 characters and the presence of leucophores, genetic males that sex reversed to females
186 were identified and mated with normal males to assess their breeding behaviour and
187 mating performance. Successfully mated XY females were used for the production of F_1
188 progeny (Supplementary figure 7). Caudal fin clips from XY females of the F_0 and F_1
189 generations were used for genomic DNA isolation[4]. The genome integration of the
190 pmDMY-AS construct was tested using a primer pair that amplified a portion of the
191 vector and *Dmy* AS sequence (Supplementary table 5) and then confirmed by sequencing.
192 Aliquots of the same genomic DNA samples were analysed by real-time PCR to estimate
193 the number of integrated copies. The breeding experiment was similarly performed with
194 F_1 and F_2 fish, and the offsprings were histologically examined at 10 and 20 dah.
195
196 **Characterisation of small RNAs.** To determine the AS plasmid associated
197 mechanism of RNA suppression *in vivo*, total RNA (enriched with small RNA) was
198 isolated from the adult gonads of F_1 progeny, using the miRNeasy mini kit (Qiagen),
199 following the manufacturer's protocol. Small RNAs were separated using 15%
200 denaturing polyacrylamide gel electrophoresis. A Northern blot analysis was performed
201 using a digoxigenin-labelled AS *Dmy* probe following the standard protocol. In a separate
202 experiment, RNA bands ranging from 18-28 nucleotides as well as 60-70 nucleotides
203 were excised from gels, and purified using RNA extraction buffer of the DynaExpress

204 miRNA cloning kit (Bio Dynamics, Japan). 5` and 3` linkers were added to the purified
205 small RNA and cDNA was produced, according to the manufacturer's instructions. The
206 cDNAs were PCR amplified and cloned into the pGEMTeasy vector (Promega, Japan).
207 Both colony PCR and dot blots (using dig-labelled AS *Dmy* probes) were performed to
208 screen the inserts and the positive clones were sequenced.
209
210 **Promoter analysis.** The promoter analysis was performed using a previously
211 described protocol[7]. Briefly, HEK293 cells were seeded in 24-well plates, at 5 X 10^5
212 cells/well, containing Dulbecco's modified Eagle's Medium (Sigma, USA). After 24
213 hours, the cells were transfected with pGL3-DMY-luciferase plasmid and either one of
214 pcDNA3.1-WT1a/b (both +/-KTS) plasmids, at different concentration (0-200 ng/well) in
215 triplicates. The luciferase assay was performed after 48 hours of transfection. The
216 experiment was repeated thrice for reproducibility. The Ol32 cells were similarly
217 prepared in L-15 medium (Sigma, USA) and co-transfected with pGL3-Gsdf or pGL3-
218 Sox9a2 and pcDNA3.1-Dmy to assess the *Dmy* associated *Gsdf* and *Sox9a2* promoter
219 activity.
220
221 ***In vivo* Chromatin Immunoprecipitation analysis of *Dmy***. Binding sites for *Dmy*
222 were identified using the matrix as provided[8], together with the Regulatory Sequence
223 Analysis Tools portal: RSat (http://rsat.ulb.ac.be/rsat/, last accessed February 15, 2015),
224 weight score $\geq$ 1, p-value $\leq$1. For *in vivo* chromatin immunoprecipitation (ChIP), the
225 ChIP expression-shearing kit (Active motif) was used according to the manufacturer's
226 instructions, using 20 mg of embryonic tissue samples from *Dmy-Gfp* mRNA injected
227 fish (50 embryos for each) and GFP monoclonal antibody (3 mg, AbCAM). The *Dmy-*
228 *Gfp* plasmid was constructed by sequentially fusing *Dmy*-5`UTR, *Dmy*-ORF (stop codon
229 less), *eGFP*, and *Dmy*- 3`UTR into a pCS2 vector using infusing cloning kit (Clonetech)
230 and RNA was synthesized with mMESSAGE mMECHINE SP6 kit (Ambion, USA)

231 following polyA addition with Poly-A tailing kit (Ambion) After tissue disaggregation
232 and cell re-suspension, DNA was sheared according to the manufacturer's protocols. The
233 ChIP procedure using *Dmy-Gfp* mRNA embryos was validated as described earlier[8].
234

235 **Rescue of *Dmy* knockdown effects.** pmDMY-KD XY adult females (F_3 generation)
236 were mated with *olvas-eGFP* XY males. The resulted progenies were screened for
237 integration, grown to maturity and backcrossed with *olvas-eGFP* XY fish for 3
238 consecutive generations and *olvas-eGFP-Dmy*-KD transgenic line was created. The F_3
239 generation fish developed from this line were used for rescue analysis. Briefly, the *Gsdf*-
240 mcherry and *Sox9a2*-cyan mRNA, flanked by 5' and 3' untranslated regions of respective
241 genes, were synthesized with mMESSAGE mMECHINE SP6 kit (Ambion, USA)
242 following polyA addition with Poly-A tailing kit (Ambion). Control RNAs (only mcherry
243 and cyan, flanked by both 5' and 3' UTRs of *Gsdf* and *Sox9a2,* respectively) were
244 similarly prepared. The purified RNA was injected in one-two cell stage embryos of
245 *Dmy*-KD fish @ 1ng/ul. Both reporter gene expression (mcherry and cyan, whatever
246 applicable) and gonad development were monitored using Axioplan2 imagining (Zeiss,
247 USA) confocal microscope for each embryo separately. The embryos showing reporter
248 gene expression were then grown separately until 10 dah in a 24 well dish @ 1
249 embryo/well and either fixed/ preserved for histological/ real-time PCR analysis or grown
250 further to assess morphological sex change. Few of the manipulated fish caudal fin bud
251 clips were used for genomic integration and genomic sex determination. Only the XY
252 embryos having *Dmy*-KD insert (confirmed by integration PCR) were included in the
253 analysis.
254

255 **Data analysis.** Statistical differences in relative mRNA expression between
256 experimental groups were assessed by One Way ANOVA, followed by Tukey's test. All
257 statistical analyses were performed using SPSS, version 18 software. All experimental

258 data are shown as the mean ± SEM. Differences were considered statistically significant
259 at $p<0.05$, if not otherwise mentioned. Specially used data analysis methods are described
260 in the respective sections.
261
262
263
264 **References**
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286 medaka shows incomplete conservation of the downstream regulatory network of
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288

289
290 **Supplementary tables:**
291
292 **Supplementary table 1: Summary of GFP knockdown using *olvas-eGFP* transgenic**
293 **medaka at F_0 generation.**
294

Stage and type of sampling	Analysis method	Number of fish sampled	Number of fish changed
0 dah	Microscopic GFP fluorescence observation[1]	10	6
10 dah	Real-time PCR[2]	10	10
	Microscopic GFP fluorescence observation[1]	10	7
Adult (4 months)	Secondary sexual character [3]	20	0

295
296 Note: Numbers in superscript indicate the biological end points analysed using the samples. 1. Change in
297 *olvas-eGFP* expression pattern; 2. Change in germ cell number and occurrence of meiosis/ meiotic arrest;
298 3. Occurrence of fan like anal fin and forked dorsal fin in XX, and tapering anal fin and fused dorsal fin in
299 XY fish.
300
301
302

303 **Supplementary table 2: Summary of shDNA dependent *Dmy* knockdown in QurtE**
304 **medaka.**
305

Sampling group	Analysis method	Number of fish sampled	Mitotic cell number ± SE	Meiotic cell number ± SE	Number of fish changed	% change
pmCMV-DMYsh1	QPCR[1]	10			10	100
	HE[2]	10	101±27.6	0	2[3]	20
pmCMV-DMYsh2	QPCR[1]	10			9	90
	HE[2]	10	104± 14.4	0	4[3]	40
pmCMV-DMYsh3	QPCR[1]	10			10	100
	HE[2]	10	119 ± 11.8	1 ± 0.8	2[3], 1[4]	20
pmCMV-DMYsh1 +sh2	QPCR[1]	10			8	80
	HE[2]	10	210 ± 31.7	3 ± 2.9	5[3]	50
pmCMV-DMYsh1 +sh3	QPCR[1]	10			10	100
	HE[2]	10	230 ± 42.1	6 ± 4.2	5[3], 3[4]	50
pmCMV-DMYsh1+sh2+sh3	QPCR[1]	10			10	100
	HE[2]	10	390 ± 21.2	19 ± 9.2	8[3], 6[4]	80

306
307 Note: Numbers in superscript indicate the biological end points analyzed using the samples. 1. Change in
308 gene expression; 2. Change in germ cell number and occurrence of meiosis/ meiotic arrest; 3. Mitotic germ
309 cell population; 4. Meiotic germ cell population.

310 **Supplementary table 3: Summary of *Dmy* knockdown in medaka.**
311

Stage of Sampling	Analysis method	Number of sample (N)	Number of fish with abnormal characters
F_0 generation			
0 dah	Real-time PCR[1]	10	8
	Histology[2]	30	7
10 dah	Real-time PCR[1]	10	10
	Histology[2]	10	9
	ISH[3]	10	6
50 dah	Histology[2]	10	2*, 5[#]
Adult	Secondary sexual character[4]	10	7
	Breeding behaviour[5]	5	5
	Gene integration[6]	5	2
F_1 generation[$]			
10 dah	Histology[2]	361	60
	ISH[3]	10	3
Adult	Secondary Sexual Characters[4]	50	15
	Genome integration[6]	12	3
	Breeding behaviour[5]	3	3
F_2 generation			
10 dah	Histology[2]	10	3
	ISH[3]	10	4
Adult	Secondary sexual character[4]	30	12
	Breeding behaviour[5]	12	4
F_3 generation			
2 daf	Histology[2, 7]	10	4
0 dah	Rescue with *Sox9a2*[2,8]	20	0
	Rescue with *Gsdf*[2,8]	20	0
	Rescue with *Sox9a2* and *Gsdf* coexpression[,2,8]	10	10
Adult	Secondary sexual character[4]	42	41
	Breeding behaviour[5]	5	5

312
313 Note: Numbers in superscript indicate the biological end points analysed using the samples. 1. Change in
314 gene expression; 2. Change in gonadal structure, germ cell number and occurrence of meiosis/ meiotic
315 arrest; 3. Changes in cellular localisation of different gene; 4. Occurrence of tapering anal fin and fused
316 dorsal fin; 5. Mating Characteristics i.e. chasing, dancing, coiling, and pressing; 6. Positive PCR
317 amplification of AS probe using a vector and gene specific primer pair; 7. Differences in *Wt1* expression; 8.
318 Gonadal structure at 50dah; '*' Partial sex change and occurrence of testis ova; '#' Complete sex change

319 and occurrence of complete ovary; '[$]' combined profiles of two founder. The breeding behaviour and gene
320 integration checks were performed with XY female fish (depending on secondary sexual characters).
321

322 **Supplementary table 4: Generation and evaluation of Dmy-AS founder transgenics**
323

Crossing method:	Control XY ♂ **X** Dmy-KD-XY♀	
Characters	Founder 1	Founder 2
Number of eggs laid in first three week from 5 month of age	211	187
Hatched embryos after 7 days of fertilization	189	172
Dmy-AS(-) XX ♀ embryo at 10 dah*	53	45
Dmy-AS(+) XX ♀ embryo at 10 dah*	47	48
Dmy-AS(-) XX ♂ embryo at 10dah*	0	1
Dmy-AS(+) XX ♂ embryo at 10dah*	0	0
Dmy-AS(-) XY ♀ embryo at 10 dah*	**0**	**0**
Dmy-AS(+) XY ♀ embryo at 10 dah*	**32**	**28**
Dmy-AS(-) XY ♂ embryo at 10 dah*	38	36
Dmy-AS(+) XY ♂ embryo at 10 dah*	19	14

324
325 The sex reversals are indicated in bold. '*' Genotyping, integration specific PCR and histology of
326 individual embryo was carried out to determine the gonadal sex.

327
328

Supplementary table 5: Egg production characteristics of *Dmy* knockdown fish.

Groups	Age (month)	Progeny status			
		F_2	**F_3**	**F_4**	**F_5**
Control					
	4	125.7±5.0	134.0±5.3	121.3±5.4	139.0±4.2
	5	289.0±4.7	274.0±6.2	274.0±5.9	260.0±4.2
	6	331.0±4.0	347.3±4.7	340.7±4.3	355.0±6.7
	7	374.0±14.8	369.7±2.2	336.0±5.9	362.3±3.9
	8	359.7±8.5	334.0±4.4	389.0±4.5	328.7±3.3
	Total	**1479.3±22.2**	**1459.0±20.0**	**1461.0±22.5**	**1445.0±19.4**
DMY- KD	4	119.7±4.8	115.7±4.8	125.7±4.5	119.7±1.9
	5	235.0±4.5	254.3±6.0	223.3±6.6	230.7±6.6
	6	321.0±4.0	364.7±4.5	364.0±5.5	341.0±3.5
	7	365.0±13.5	359.0±5.7	378.0±19.8	385.7±2.9
	8	386.3±6.0	341.3±5.0	318.0±5.3	354.0±10.1
	Total	**1427.0±6.0**	**1435.0±14.6**	**1409.0±16.3**	**1431.0±8.7**
Significanc (p<0.05)	**NS**	**NS**	**NS**	**NS**	**NS**

329

330 **Supplementary table 6: List of major primers used in this study.**
331

Primer name	Sequence	Purpose
Dmy 24 F	TCCTATTATGGAAACCTGCACAACTAC	*Dmy* real-time PCR
DMY 26R	GAAGGAGTGCATGCGGTACTG	*Dmy* and *Dmrt1* real-time PCR
Dmrt1 25 F	TCCTCCTACTATGGAAACCTGTACCA	*Dmrt1* real-time PCR
Gsdf realtime F	GGGCTGGACACTATTCGAGA	
Gsdf realtime R	CATGACACAGAGGAGCTGGA	
Sf1 realtime F	AGCTGCTACTCTGGAAACGA	
Sf1 realtime R	ACTGGCAATCTTCTTGGCAGC	
GFP realtime F	CGACAACCACTACCTGAGCA	
GFP realtime R	GAACTCCAGCAGGACCATGT	
Olvas realtime F	CCCAAAGTGACCTACATC	
Olvas realtime R	AAGTTGATGCCCATCTTG	Measuring Gene expression by real-time PCR
Foxl2 realtime F	AAACCTGCTACTCTGGACGC	
Foxl2 realtime R	AGTCAAATCTTCTTGATTC	
Fig1a realtime F	TGTACTGCTGCATCGAGAAGTA	
Fig1a realtime R	ATGCTGCAACACCAGTCTAGT	
Spo11 realtime F	TCGATTCTGGTGCCGTCTTCT	
Spo11 realtime R	ATGCTGAAGGTTTCTCGCAGG	
Rspo1 realtime F	TGCAACACCAGTCTAATG	
Rspo1 realtime R	TTCTGGTGCCGTCTTCTAGG	
Cyp19a1 RT F	AGCTTATTTTGCCCAAGGCC	
Cyp19a1RT R	TTGAGCAGCAGGAGCATGAAA	
Gsdf ORF F	ATGTCTTTGGCACTCATT	ORF amplification, *in situ* probe preparation, synthetic RNA preparation
Gsdf ORF R	CTACTTTTTGCAGGGCTGCT	
Sf1 ORF F	AGCAAGGGTGTGAGGAG	
Sf1 ORF R	TTTTTGCAGGGCTGCT	
Dmy ORF F	CCGCGGGAGCTCATGAGCAAGGAGAAGCAGTGC	ORF amplification, *in situ* probe preparation
Dmy ORF R	GGATCCGAATTCTGGAGTTGGCCGGGAAGACG	
Spo11 F	GATGCAAGGAGAGAGTT	
Spo11 R	ATACTCAGCTGTTTGGGTCACA	
Foxl2 F	TGCACCTGACACCAGTCT	
Foxl2 R	TAGACAACACCGAGTCTG	
Olvas F	AAGAGCTCCCAGCAAGGC	
Olvas R	TCGGAGCTCATGAGCAAGG	
Rspo1 F	TGCAACACCAGTCTAATG	*In situ* probe preparation
Rspo1 R	TAGACTGCCCGTCATG	
Fig1α F	ATGAAGGTGCCAGAGGCGGAAT	
Fig1α R	TTAATCCCTCGAAGCTTGATCG	
Dmy-as-Xba1 F1	TCTAGAGGAAACCTGTACAACTACCA	
Dmy-as-Eco RV R1	GATATCCATGGGTGGAGGTGAGGCT	Amplification of *Dmy* antisense region, integration check
M13 F	GATATCCGAGCATCTCCAGTAGGAGG	
M13 R	TCTAGATCTACAGCATGAAGTGCAA	Colony PCR,

T7	GATATCGACCCTCCATACTGAAGGA	Sequencing, *in situ* probe preparation
T3	TCTAGACTCTTTGTTCTGGCAAAGCC	Sequencing, insert check
Sp6	GATATCGGTTGCAGGGCAGATGTAGT	
Bgh R	TCTAGAATGGGAACCACTTTGGACTC	
pcDNA 3.1 807 F	GATATCAGCTGAAGATGGTTGGGTTG	
PCDNA 3.1 1127 R	ATGAAGGTGCCAGAGGCGGAAT	
Dmy genomic F	TTAATCCCTCGAAGCTTGATCG	Genomic PCR for sorting of sex
Dmy genomic R	TCTAGAAGGAGGAGCTTGGGATTTGT	
EF1α F	GATATCCGTCCCTCCACAGAGAAGAG	Internal control for Real Time PCR
EF1α R	TCTAGACTCTTCTCTGTGGAGGGACG	
Dmy5utrF	TCTTTTTGCAGGATCGAAAGACTGTTCTCCGGTAAATTGACG	
DmyORFR	CTCACCATTTTGGTTTCACTGCTCATGGAGTTGG	Construction of *Dmy* overexpressing plasmid
EGFP-F	AACCAAAATGGTGAGCAAGGGCGAG	
EGFP-R	GCTTTTCCTTACTTGTACAGCTCGTCCATGCC	
Dmy3utrF	CAAGTAAGGAAAAGCTGCGAGGC	
Dmy3utrR	CGAATCGATGGGATCGACCATCTCATTTTTTATTCTTGATTTTTCACTTTCATCT	
Gsdf geno-F1	CTGCCGGATCATTCTCCTTG	Real-time analysis of ChIP DNA
Gsdf-geno-R1	GGGCCTGGGAACCTGAGT	
Sox9a2geno-F1	GCGCACGAGGTTTTATTGAGT	
Sox9a2geno-R1	TCTCTTTCCAGGCTCCTTAATG	
Rspo1 geno-F1	ATGGTGTGCAGTCTGGTTTAAT	
Rspo1 geno-R1	ATAAACTTTGCAATCATAACTGTT	

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336 **Supplementary figures:**

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338 **Supplementary figure 1. Construction of *Dmy* knockdown plasmid and *in silico*
339 analysis of efficacy.** The *Dmy*-knockdown plasmid was constructed on a pcDNA3.1 (+)
340 vector backbone (A). The AS fragment/probe, predicted to have the best secondary AS
341 RNA structure (B), was inserted in a reverse orientation into the pcDNA 3.1(+) vector to
342 generate the knockdown plasmid.

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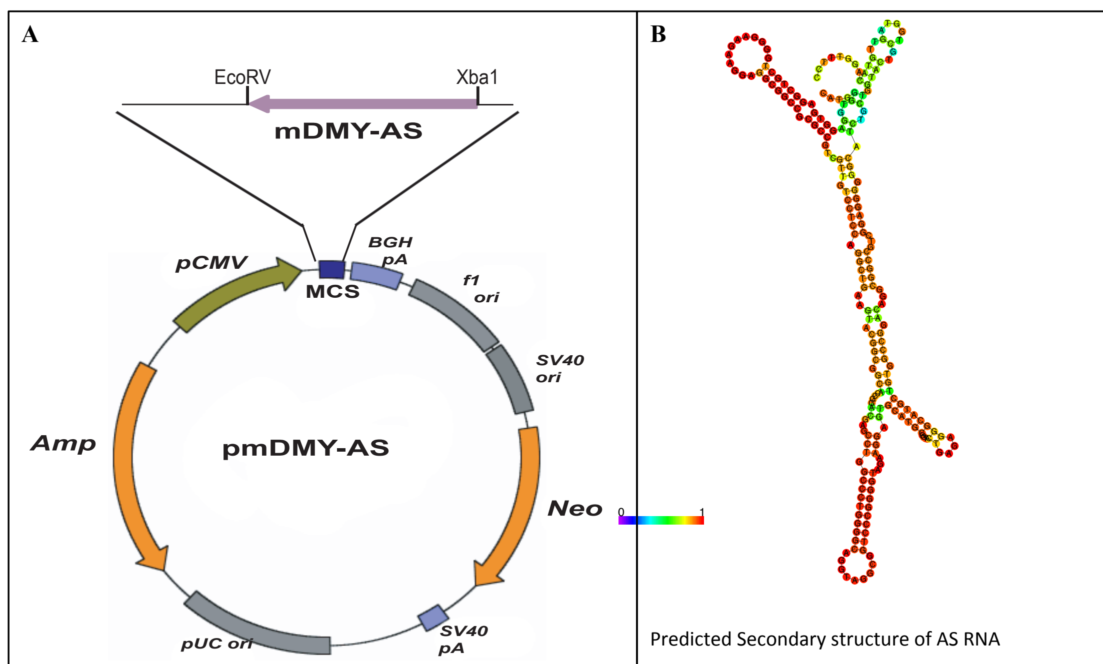

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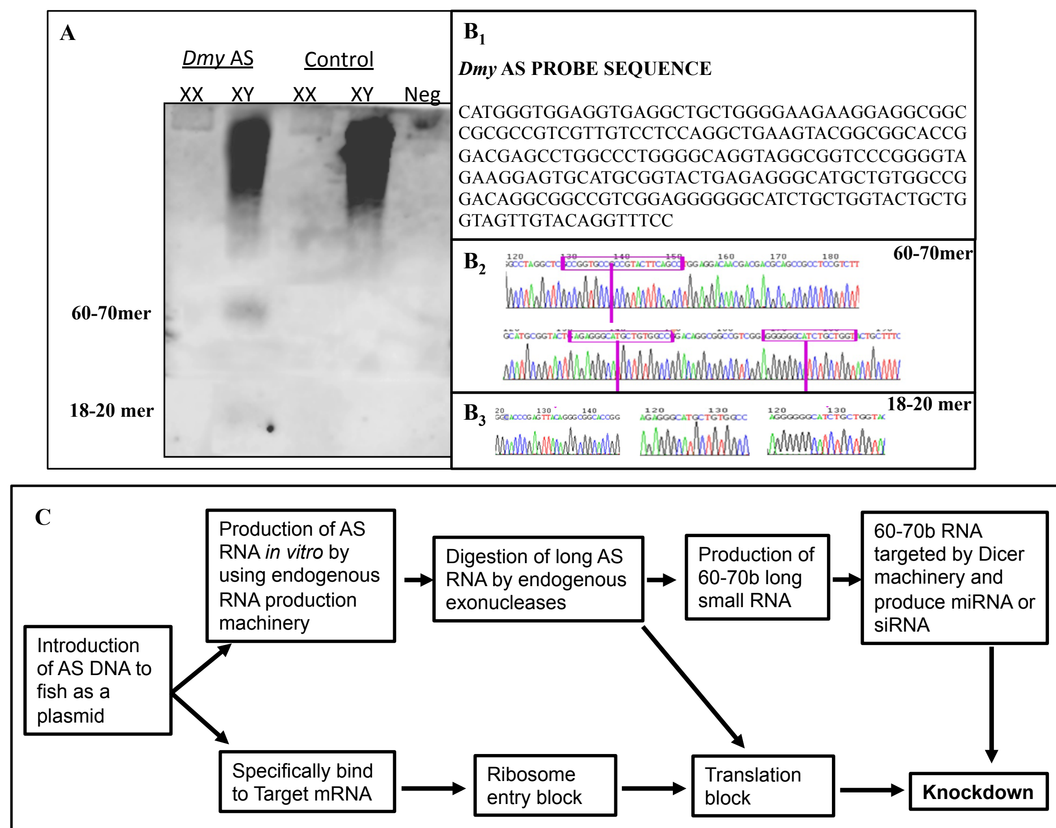
Supplementary figure 2. Possible mechanism of vector based AS DNA dependent AS RNA machinery *in vivo*. Northern blotting was used to identify the *Dmy* specific RNA degradation (A). Lane 1, 2, 3, 4, 5 in northern blot represents small RNAs from adults of *Dmy-KD* F_1 XX, *Dmy-KD* F_1 XY, wild QurtE medaka XX, wild QurtE medaka XY and negative control (only RNA loading buffer), respectively (A). The maximally expressed lower molecular weight RNAs, marked in the chemo-luminescent graph as per their sizes i.e. 60 -70 mer and 18-28 mer, were used for further isolation and small-RNA library construction. The original *Dmy* AS sequence (B_1) was used as a reference to find out the *Dmy* specific small AS RNAs in the small-RNA libraries constructed using 60-70 (total clones= 4261) and 18-28 (total clones = 6176) mer purified RNAs. Chromatograms of two different 70 mer (out of total 4261 clones) and three different 18-28 mer (out of total 6176 clones) are presented in B_2 and B_3, respectively. The pink boxes in B_2 panel represents the sequences of 18-28 mer RNAs obtained from the 18-28 mer library. (C) Schematic representation of hypothetical pathway of knockdown *in vivo*.



362 **Supplementary figure 3. Effect of *Dmy* knockdown on gonadal gene profile in**
363 **adults.** Data are presented as both individual values (with dots and triangles) and mean of
364 15 XY individuals (white and grey columns for control and knockdown groups,
365 respectively). Inset: representative figure for genotyping of the fish used for real-time
366 PCR, where the upper and lower band represents *Dmrt1a* and *Dmy* specific amplification,
367 respectively; M-marker.

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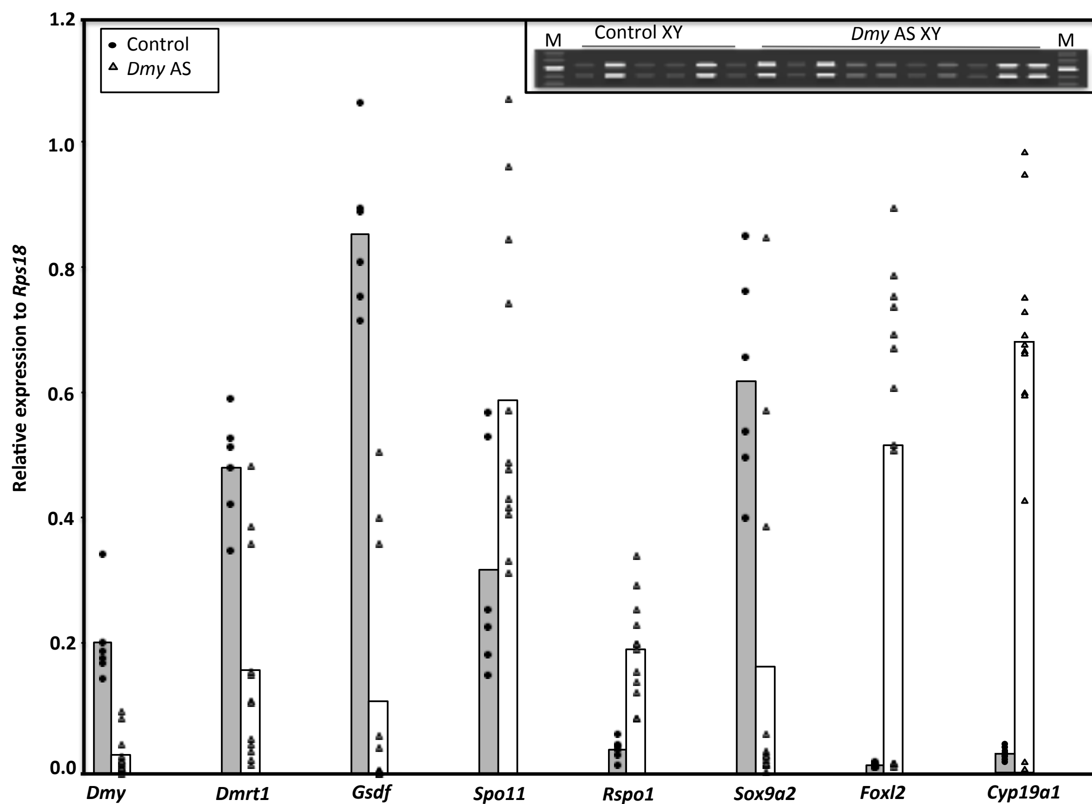

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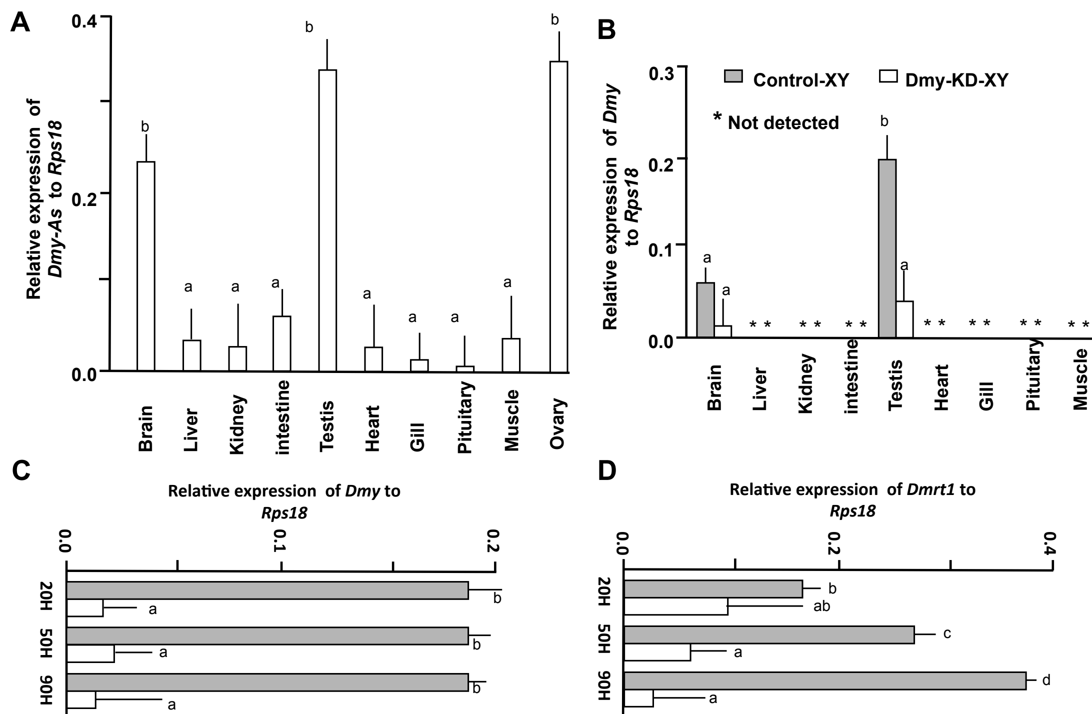
371 **Supplementary figure 4. *In vivo* evaluation of knockdown efficiency of F2**
372 **transgenics.** The distribution of antisense RNA in different tissues were assessed by AS-
373 RNA specific primers to evaluate the germ line transmission in Dmy-KD (Dmy-AS(+)) 5
374 month adults (A). The same samples were further checked for *Dmy* transcription,
375 compared with control XY and plotted in graphs as relative abundance to internal control
376 (B). 20, 50 and 90 dah Dmy-AS(+) XY individuals were evaluated for *Dmy* (C) and
377 *Dmrt1* (D) transcriptional silencing. Note: Each column in the graph represents mean ±
378 SEM of 10 individuals and letters (a, b, c, etc.) above the bars indicate that these groups
379 differ significantly ($p<0.05$) from each other.
380



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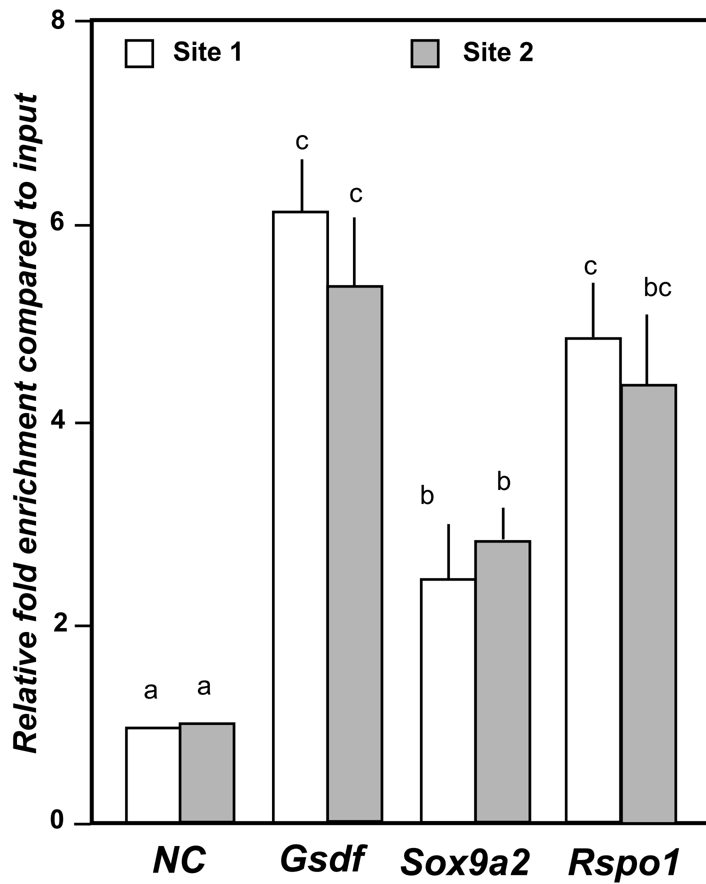
383 **Supplementary figure 5. *In vivo* chromatin immunoprecipitation analysis of**
384 **candidate *Dmy* targets.** Relative expression to control input was calculated using
385 realtime PCR and plotted on the graph. Data are presented as means ± SEM of 4 separate
386 experiments and significances ($p<0.05$) are denoted by different letters.
387



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389 **Supplementary figure 6. Effect of *Gsdf* and *Sox9a2* overexpression on germ cell**
390 **proliferation and reversal of *Dmy*-KD phenotype.** Germ cell (green colour)
391 proliferation and clustering patterns of *olvas-eGFP* transgenic medaka (both control and
392 *Dmy*-KD) gonads at S39 (1 day before hatching) were assessed using confocal
393 microscope. Isolated (type-I) and clustered (type- II) germ cells are marked with * and #,
394 respectively. (A-D) *Olvas-eGFP*-control-XX and (E-H) *olvas-eGFP-Dmy*-KD-XY
395 embryos. Note: N= 9 per group and control RNAs were prepared by mcherry/cyan fusion
396 with *Gsdf* or *Sox9a2* 3` UTR.

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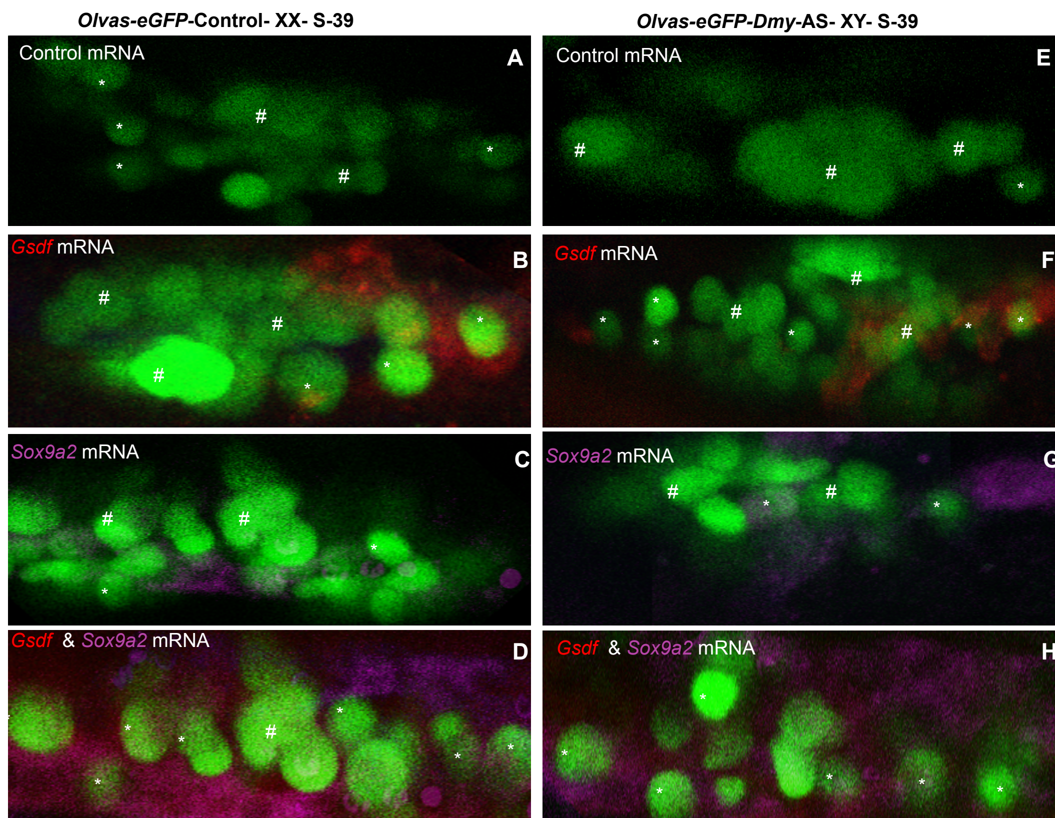

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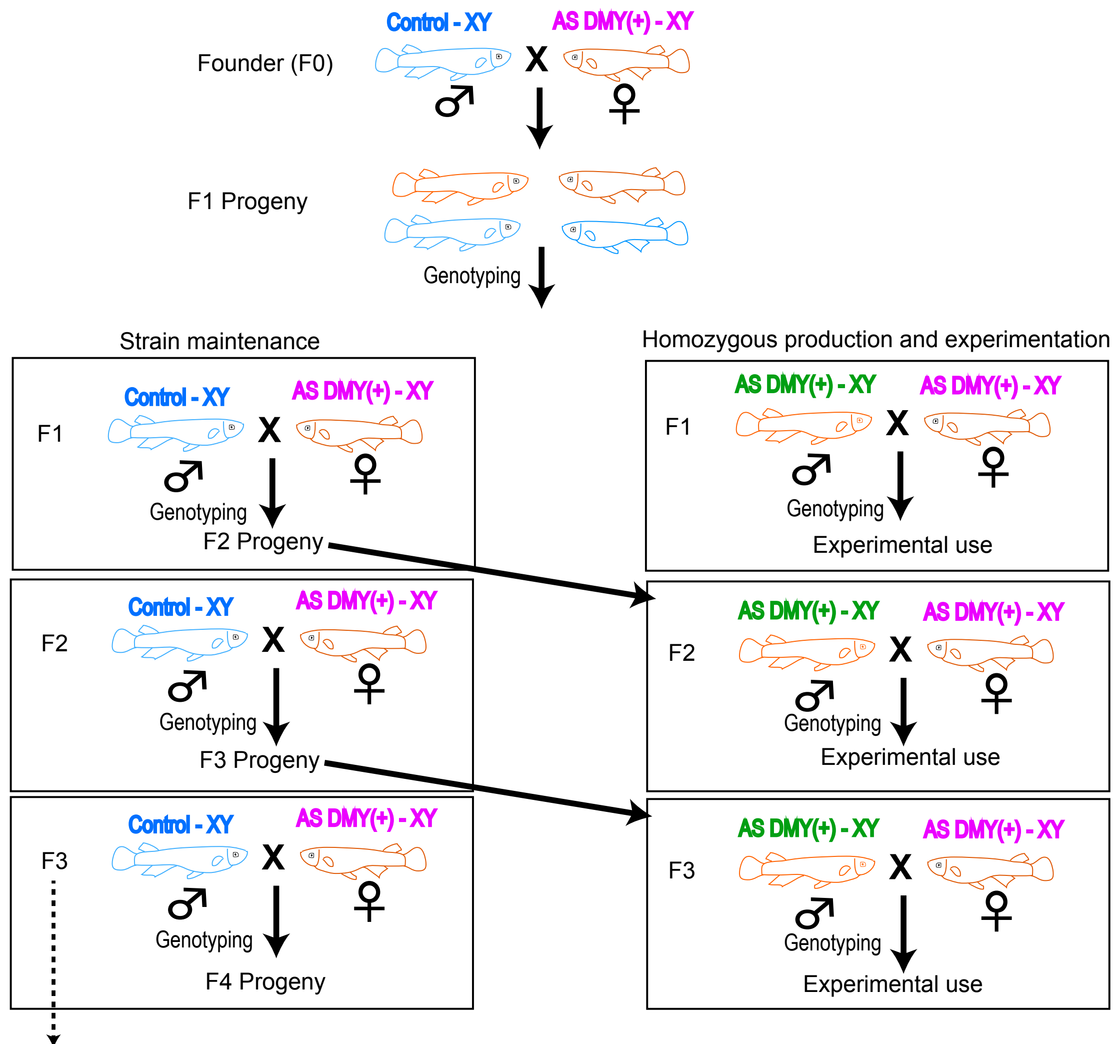
399 **Supplementary figure 7. Schematics of breeding and mating strategies of Dmy-KD**
400 **transgenic line.** The adult individuals were analysed for genome integration and genetic
401 sex before mating in each generation.
402



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