Supporting Information

Fujimoto et al. 10.1073/pnas.1007032107

SI Materials and Methods

Molecular Cloning of *dnd*, *vasa*, *dmrt1*, *foxl2*, and *p450arom*. Unfertilized eggs were used to clone *dnd* and *vasa*, testes for *dmrt1*, and ovaries for *foxl2* and *p450arom.* Poly(A) RNA was extracted from unfertilized eggs and ovaries using QuickPrep micro mRNA purification kit (GE Healthcare), and total RNA was extracted from testes using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. 3′-RACE cDNA and 5′-RACE cDNA were produced with 1 μg poly(A) RNA and total RNA using the SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. PCR amplification of each gene was performed using the 3′-RACE cDNA as the template and the degenerate primer sets listed in Table S4. The DNA fragments from each PCR were cloned using the TOPO TA cloning kit for sequencing (Invitrogen) and eight clones from each gene were amplified by PCR using the following vector-specific primers: M13 forward (−20), 5′-GTAAAACGACGGCCA-3′ and M13 reverse, 5′-CAGGAAACAGCTATGAC-3′. Subsequently, partial DNA sequences of each gene were determined by the dideoxy chain termination method using BigDye Terminator v3.1 cycle sequencing kits (Applied Biosystems) and bidirectional sequencing on a DNA analyzer 3130xl (Applied Biosystems). Using the information obtained from partial sequencing, 5′- and 3′-RACE were then performed according to the manufacturer's instructions.

Sequence Information of *dnd*, *vasa*, *dmrt1*, *foxl2*, and *p450arom*. A 2,741-bp *vasa* cDNA was isolated; this had an ORF of 1932 bp encoding a putative 644-aa protein with characteristic 215-aa DEAD-box helicases and a 130-aa helicase superfamily C-terminal domain (AB531493). It was similar to Vasa orthologs of zebrafish (86%, NP_571132.1), goldfish (75%, AAX22125.1), tilapia (76%, BAB19807.1), and medaka (74%, NP_001098146.1).

A 1671-bp *dnd* cDNA was isolated; this had an ORF of 1116 bp encoding a putative 372-aa protein containing a 74-aa RNA recognition motif (AB531494). It was similar to Dead end orthologs of zebrafish (57%, NP_998960.1), rainbow trout (47%, NP_001118133.1), and medaka (46%, NP_001157988.1).

A 639-bp partial sequence of *dmrt1* was obtained (AB531495). This partial sequence encoded a putative 188-aa protein and had 93% homology to the ortholog of large-scale loach (*Paramisgurnus dabryanus)* (ABK88911). The DM domain was not included in the fragment.

A 1,555-bp *foxl2* cDNA was isolated; this had an ORF of 882 bp encoding a putative 294-aa protein with a characteristic 111-aa forkhead domain (AB531497). It was similar to Foxl2 orthologs in zebrafish (79%, NP_001038717), tilapia (76%, AAT36328), medaka (76%, NP_001098358), and rainbow trout (76%, AAS87040).

A partial *p450arom* cDNA of 1861 bp was isolated; this had an ORF of 1572 bp encoding a putative 524-aa protein (AB531496). It was similar to P450arom orthologs in the common carp (80%, ACB13197), zebrafish (79%, AAK00643), goldfish (79%, AAC14013), and wrasse (69%, AAR37048). The aromatase also contained conserved regions: helical region, Ozol's peptide, aromatic region, and heme-binding region.

RT-PCR Analysis. Gonads were dissected from 2-y-old wild-type and *dnd* morphants and used for extraction of total RNA by a NucleoSpin RNA II kit (Macherey-Nagel). Each tissue sample included a gonad from a single fish. Three hundred nanograms of total RNA was reverse-transcribed by PrimeScript RTase (Takara) to synthesize cDNA. RT-PCR primers are shown in Table S4. PCR conditions were as follows: 94 °C (1 min), then 30 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (45 s), and ending with a 5-min extension phase at 72 °C. PCR products were electrophoresed on a 1.2% agarose gel and visualized after ethidium bromide staining using a UV-transilluminator. RT-PCR was performed using two independent samples from each gonadal type.

Table S1. Survival rates and the number of PGCs in larvae injected with different doses of *dnd*-MO

	No. of eggs used	No. of normal larvae	No. of abnormal larvae	Survival rates	No. of observed larvae	Mean no. of PGCs	SD	No. of PGCs observed in each larva
Intact control	48	46	1	0.958	12	48.5	7.06	60, 57, 54, 54, 53, 53, 48, 44, 41, 40, 40, 38
Control MO (1,000–2,000 pg/embryo)	48	44	3	0.917	11	50.5	13.00	68, 62, 59, 58, 58, 52, 49, 46, 42, 40, 21
dnd-MO (250–500 pg/embryo)	48	46	0	0.958	12	10.2	8.07	28, 23, 17, 14, 8, 7, 7, 7, 7, 4, 0, 0
dnd-MO (500–1,000 pg/embryo)	48	45	2	0.938	12	7.8	14.05	44, 31, 9, 7, 1, 1, 0, 0, 0, 0, 0, 0
dnd-MO (1,000–2,000 pg/embryo)	48	46	0	0.958	12	0.0	0.00	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
dnd-MO (2,000–4,000 pg/embryo)	48	42	2	0.875	12	0.0	0.00	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0

Table S2. Classification of gonads in 13-mo-old loach following injection of *dnd*-MO at the one- to two-cell stage

		Morphological feature of gonads			
	Sample size	Ovary	Two testes	One testis	Atypical
Intact control	11	5	6	0	0
Control MO (1,000–2,000 pg/embryo)	11	1	10	0	0
dnd-MO (250–500 pg/embryo)	8	5	2	0	1
dnd-MO (500–1,000 pg/embryo)	27	3	9	5	10
dnd-MO (1,000–2,000 pg/embryo)	22	0	2	2	18
dnd-MO (2,000–4,000 pg/embryo)	13	0	0	0	13

Table S3. Distribution patterns of *vasa*-positive cells of wild-type and *dnd* morphants at each stage

				Distribution of *vasa*-positive cells		
	Stage	*N*	No. of *vasa*-positive individuals	Normal	Normal and ectopic	Ectopic
Wild-type	Embryonic shield	18	18	18	0	0
	2–4 somite	27	27	27	0	0
	20 somite	27	27	27	0	0
	30 somite	28	28	24	4*	0
	Larvae	14	14	14	0	0
dnd morphants	Embryonic shield	15	15	15	0	0
	2–4 somite	14	14	0	14†	0
	20 somite	15	15	1‡	7†	7
	30 somite	18	17	1‡	6†	10
	Larvae	18	0	0	0	0

*A small number of *vasa*-positive cells were distributed in ectopic position, although most of *vasa*-positive cells were normal position.
†Most of *vasa*-positive cells were distributed in ectopic position.
‡These embryos had a small number of *vasa*-positive cells distributed at normal position.

Table S4. Sequences of primers used in the present study

Primer	Purpose	Sequence (5′–3′)
dead end-degenerate-F	Partial cloning	ACGGCGGCCCTCC(GATC)CC(GATC)GG(GATC)TGG
dead end-degenerate-R	Partial cloning	CCGGTCGATGTACTTGGC(AG)TA(GATC)GC(AG)AA
dead end-5′ RACE-R1	RACE cloning	TCCGGTCGATGTACTTGGCGTAGGCGA
dead end-5′ RACE-R2	RACE cloning	CTGGAAGAGCGGGATTAGGGTGTCCTCA
dead end-3′ RACE-F1	RACE cloning	CTGCTACAGCTTTTCAACCCGCAGGCAC
dead end-3′ RACE-F2	RACE cloning	TCCCGCTCTTCCAGAGAGTCGGCA
vasa-degenerate-F	Partial cloning	GGACGAGACCTGATGGCTTG(CT)GC(GATC)CA(AG)AC
vasa-degenerate-R	Partial cloning	TGTTCGTCCGATTCGGTG(GATC)AC(AG)TA(GATC)TC
vasa-5′ RACE-R	RACE cloning	GTGCGTTCATTTCCTGTGGTTCTC
vasa-3′ RACE-F	RACE cloning	GGTGGTTTATGGCGGGACAAACACTGGA
vasa-RT-F	RT-PCR	CTGAACCTGCCATGGATGAC
vasa-RT-R	RT-PCR	CTTCACCTCCTTTATAACCCTCAC
dmrt1-degenerate-F	Partial cloning	CGACGACAGCAGGCTCA(AG)GA(AG)GA(AG)GA
dmrt1-degenerate-R	Partial cloning	CGGTACTGAG(AG)(AG)GACA(CT)GTTGTGG
dmrt1-3′ RACE-F1	RACE cloning	GAGGAGATGGGCATCTGCAATCCA
dmrt1-3′ RACE-F2	RACE cloning	AGCTCCATCACCCACCACCACAAGT
dmrt1-3′ RACE-F3	RACE cloning	TGAAAGCAGCTCAGAGTCCGGCACCTT
dmrt1-RT-F	RT-PCR	GAGATGGGCATCTGCAATCC
dmrt1-RT-R	RT-PCR	GCTGCTTTCACACTCCAGTC
foxl2-degenerate-F	Partial cloning	CCCTTTCTACGAGAAGAACAAGAA(AG)GG(GATC)TGGCA
foxl2-degenerate-R	Partial cloning	AGGCTGTCGGGAGCA(GATC)GC(AG)AA(CT)TG
foxl2-5′ RACE-R1	RACE cloning	GCGCTGTTGGACGGTACCGGTGAG
foxl2-5′ RACE-R2	RACE cloning	GGAGCCGAGAGGTAGCTGCCGTATCC
foxl2-3′ RACE-F1	RACE cloning	GGATGAAGCGGCCGTTCAGACCGTCA
foxl2-3′ RACE-F2	RACE cloning	AACGGCATGGGTCACCATCAGCACC
foxl2-3′ RACE-F3	RACE cloning	TGTCTGAGGTTTCACCCGGTAGTGGA
foxl2-RT-F	RT-PCR	AAGCCGCCGTACTCTTATGTGG
foxl2-RT-R	RT-PCR	TCCCATTTGACAGGACGCGTAG
p450arom-degenerate-F	Partial cloning	ACATCGTGCGAGTGTGGAT(AT)AA(CT)GG
p450arom-degenerate-R	Partial cloning	GCTCGAGGTCCGCATCC(AG)AA(GATC)GG(CT)TG
p450arom-5′ RACE-R1	RACE cloning	TCTGGACCAGCGATCACCATCTCC
p450arom-5′ RACE-R2	RACE cloning	GGCGTCCTGCAACTCCTGAGCTTCT
p450arom-3′ RACE-F1	RACE cloning	TCGGGATGCATGAACAGGGCATTA
p450arom-3′ RACE-F2	RACE cloning	CAGTTCACCGATAGCCAGCGACAGG
p450arom-3′ RACE-F3	RACE cloning	GGAATGTTAACGACGTGACATCGGAGTG
p450arom-RT-F	RT-PCR	CTGCAGGGAGTCACAGCTAC
p450arom-RT-R	RT-PCR	CAGTTCTGCAGCGAAGTCAAG
β-Actin-RT-F	RT-PCR	TTACCCACACCGTGCCCATCTAC
β-Actin-RT-R	RT-PCR	TACCGCAAGACTCCATACCCA