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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	All micrographs were taken using a SZX12 stereoscopic microscope with DP-70 camera system (Olympus, Tokyo, Japan), a BX51N-34FL fluorescent microscope with DP-73 camera system (Olympus), and FV 10i confocal laser-scanning microscope (Olympus) . Ploidy analysis: Guava PCA-96 (Millipore, Billerica, MA) Genome-wide genotyping analysis: cutadapt (ver. 4.0), Sickle (ver. 1.33), fastx_trimmer (ver. 0.0.14), Stacks (ver. 2.62).
Data analysis	GraphPad Prism version 5.0 (GraphPad Software, CA, USA), Image J software (ver. 1.54i), Cytosoft 5.3 in GUAVA.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The main data supporting the results of this study are available within the paper and supplementary information. The raw sequence data generated by GRAS-Di

have been deposited in the the DDBJ BioProject database under BioProject accession number PRJDB18157 [https://ddbj.nig.ac.jp/search/entry/bioproject/PRJDB18157]. The GenBank accession numbers of reference sequences for generating primers are listed in Supplementary Table 5. All unique materials used are available from the authors on request. Source data are provided with this paper.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	n/a
Reporting on race, ethnicity, or other socially relevant groupings	n/a
Population characteristics	n/a
Recruitment	n/a
Ethics oversight	n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to predetermine sample size. The sample size for our animal experiments in this study was based on our experience with experimental models, anticipated biological variables, and previous literatures. Sample numbers were described in the Methods and Figure legends.
Data exclusions	No data were excluded from the analyses.
Replication	All biological experiments were repeated, at least, twice and reproduced. Interspecific hybridization of embryonic lethal hybrids was performed in duplicate because the number of ELT broodstocks was limited. Conversely, viable hybrids were produced in triplicate or more replicates. Survival and growth tests of hybrid little tuna were performed in triplicate using hatched larvae from different batches of in vitro fertilization. Transplantation experiments were performed in triplicate, and each result was described in supplementary files.
Randomization	Analysis of donor-derived gametes production was performed using all transplanted recipients. No randomization was used.
Blinding	Genome sequencing and analysis were performed by technical staffs who were blinded to the experimental groups. Blinding was not relevant to the other experiments in fish because fish had to be genotyped by PCR.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

The primary antibodies used: Anti-Ddx4 antibody used as germ cell marker is a rabbit polyclonal antibody against the synthetic peptide immunogen derived from the Pacific bluefin tuna Ddx4 amino acid sequence. Antibody No. 149, which can specifically recognize Pacific bluefin tuna germ cells, is a mouse monoclonal antibody developed in our previous study (Yazawa et al., 2021). Antibody No.102B, which can specifically recognize bluefin tuna sperm, is a mouse monoclonal antibody developed in this study. Antibody No. 152, which can specifically recognize type A spermatogonia in Pacific bluefin tuna germ cells, is a mouse monoclonal antibody developed in our previous study (Ichida et al., 2019).
The secondary antibody used: Goat anti-Mouse IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific), Cat# A21042; Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (Thermo Fisher Scientific), Cat# A21428; Goat anti-mouse IgG (H+L), Alexa Fluor 488 (Thermo Fisher Scientific), Cat# A28175; anti-DIG-AP, Fab fragments (Roche), Cat# 11093274910; Anti-IgG (H+L chain) (Mouse) pAb-HRP (MBL), Cat# 330.
Dilution ratio of antibodies used: Anti-Ddx4, 100-fold; No.149, 1000-fold in IHC and 5000-fold in ICC; No.102B conjugated with Alexa594, 10-fold; No.152, 200-fold; Anti-DIG-AP antibody, 500-fold, Anti-IgG (H+L chain) (Mouse) pAb-HRP, 10000-fold; Secondary antibodies, 200-fold.

Validation

Antibodies were validated based on the specificity/selectivity using samples and reproducibility of result. The specificity of the No. 149 and No.152 antibody was confirmed in our previous reports (Ichida et al., 2019, Yazawa et al., 2021). Reference: Ichida, K. et al. Specific visualization of live type A spermatogonia of Pacific bluefin tuna using fluorescent dye-conjugated antibodies. Biol. Reprod. 100, 1637–1647 (2019). Yazawa, R. et al. Establishment of a tracing technique for transplanted bluefin tuna germ cells in recipient's gonads using monoclonal antibodies specifically recognizing bluefin tuna spermatogenic cells. Fish. Sci. 87, 105–112 (2021). The specificity of the #102B was confirmed in this study (Figure 4a). The specificity of anti-Ddx4 was confirmed in this study (Figure 3d and Supplementary Figure 5).

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	Mouse myeloma cells of the P3U1 line (JCRB0708, JCRB Cell Bank, Osaka, Japan)
Authentication	P3U1 cell line distributed from the JCRB Cell Bank had been confirmed as contaminant free and authentificated.
Mycoplasma contamination	P3U1 cell line was confirmed to be negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	n/a

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals

Six adult female BALB/c mouse aged 4-24 weeks were used all experiment. All the mice had free access to food and water, and housed under 12 hour light/dark cycle, at 22◦C, and 45% humidity in average. After the experiment, these fish were killed by breaking medulla oblongata after anesthesia.

Wild animals

All recipient fish used in this study were F1 generation of wild-caught broodstocks. Chub mackerel caught by hook and line and transferred by live fish car. Chub mackerel was then maintained in 10-m3 fibre-reinforced plastic (FRP) tanks with flowthrough seawater (80 l/min) under a natural photoperiod and water temperature. Yearling ELTs were caught by sea net or hook and line and were held until they were two years old in a sea net pen located in Kushimoto, Wakayama, Japan. ELT broodstock (BW 2.0–2.5 kg, FL 40–50 cm) were transferred by live fish car to Tateyama station and maintained in a 70-m3 FRP tank with flowthrough seawater (160 l/min) under a long-day photoperiod (24 h of light) and natural water temperature. Fertilized eggs of striped bonito were obtained from spontaneous spawning of broodstock maintained in a Tokyo Sea Life Park, Tokyo, Japan. Donor testis of Pacific bluefin tuna were obtained from fish farmers. After the experiment, these fish were killed by breaking medulla oblongata after anesthesia.

Reporting on sex

The recipient's sex was clearly described in the manuscripts. Sex identification was performed by observation of the gonad. No sex identification was used in survival and growth tests during the larval stage because little tuna are still sexually undifferentiated during

	this stage.
Field-collected samples	The sperm of five Scombridae species (bullet tuna, skipjack tuna, longtail tuna, Pacific bluefin tuna, and Atlantic little tuna) were cryopreserved in the field and transported to our laboratory using a dry shipper. After the sperm collection, these fish were killed by breaking medulla oblongata after anesthesia. These cryopreserved sperm were stored in a liquid nitrogen storage tank. More detailed information is provided in our previous report (Kawamura et al., 2020). Reference: Kawamura, W. et al. Development of a simple method for sperm cryopreservation of Scombridae fishes in outdoor environments. Aquac. Res. 51, 3376–3383 (2020).
Ethics oversight	Institutional Animal Care and Use Committee of Tokyo University of Marine Science and Technology (Approval number, R5-54).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Samples for ploidy analysis were prepared with CyStain PI Absolute T kits (Partec, Munster, Germany) according to the manufacturer's instructions. Hatched larvae and sperm were dissociated with nuclei extraction solution in CyStain PI Absolute T kits for 30 min at room temperature and then fixed in 70% ethanol for at least 30 min. The cell suspension was filtered through a nylon screen with a pore size of 42 µm to eliminate non-dissociated cell clumps. Approximately 1 × 106 of these fixed cells were stained with staining solution containing 60 µg/ml propidium iodide (PI) and 300 µg/ml RNase A in PBS for 1 h at room temperature.
Instrument	Guava PCA-96 (Millipore, Billerica, MA) for analysis of ploidy.
Software	Cytosoft 5.3 in GUAVA
Cell population abundance	The DNA contents of all cells obtained from whole larvae were analyzed. No sorting was used.
Gating strategy	No gating was used.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.