nature portfolio

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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	BZ X-710 fluorescence microscope (Keyence) was used for most phase contrast and fluorescence images from live cell cultures. Real time PCR results were collected by QuantStudio 7 Flex Real-Time PCR machine (Applied Biosystems). Live embryo images were captured by AxioZoom v16 (Zeiss) or SZX16 (Olympus) with Hamamatsu camera. Confocal images of immunostained tissue sections were collected by LSM800 (Zeiss). SDS-PAGE gal image was acquired by Gel Doc XR (Biorad). RNAseq was performed by BGISEQ-500. Mass-spectrometry analysis was performed using Q-Exactive Orbitrap (Thermo Fisher).
Data analysis	Immunofluoresence and live-cell images were analyzed by FIJI/ImageJ 1.53c. qPCR data was analyzed by QuantStudio Real-time PCR software (Applied Biosystems). Statistical analyses were done using Microsoft Excel v16.97.2 (Microsoft) and GraphPad Prism version 9.0 (GraphPad Software). RNA-seq analysis used the following tools: TopHat2 (2.1.1), Deseq2 (v1.22.2), R (v3.5.1), STAR aligner (v2.5.1b), ggplot2 (3.5.2).

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

All newly generated RNA-seq data were deposited in the Gene Expression Omnibus under accession number GSE254284 (chicken) and GSE298550 (quail, goose and ostrich).
Public data
mouse ES cells in naive, formative and primed states. GSE131555 (Kinoshita et al., Cell Stem Cell, 2021)
chicken embryos from EGK.I to EGK.VIII. GSE86592 (Hwang et al., FASEB J, 2018)
chicken embryos at HH4. SRX540095 (Weizmann Institute)
chicken embryonic fibroblasts. GSM2735363 (Fuet et al., Stem Cell Reports, 2018)
chicken reference genome (Ensembl GRCg6 106)
quail reference genome (Ensembl Coturnix_japonica.Coturnix_japonica_2.0.113)
goose reference genome (Ensembl Anser_cygnoides.GooseV1.0.113)
ostrich reference genome (Ensembl Struthio_camelus_australis.ASM69896v1.113)

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	Sample sizes were determined by magnitude and consistency of measurable differences. Preliminary experiments were performed when possible to determine requirements for sample size, taking into account resources available and ethical, reductionist animal use.
Data exclusions	No data was excluded.
Replication	All experiments were conducted with at least three biologically independent experiments. Exact number of experiments and samples are indicated in figure legends and the method sections.
Randomization	Samples were not randomized. Appropriate controls were included in each experiment. for culture condition development, same population of cells were split equally into different wells with or without drug treatment to exclude potential variation from cell condition and passage number. Effect of the drug treatment were imaged immediately after each other, to avoid confounding variables such as time-of-day on the drug effect.
Blinding	Investigators were not blinded to group allocation during sample processing since most experiments were conducted by the same few investigators. After sample processing, data collection and analysis were blinded.

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

Primary antibodies used: Myosin (MF-20, DSHB, 1:50), TuJ-1 (MAB1195, R&D Systems, 1:100), DAZL_EPR21028 (ab215718, Abcam, 1:750), P63_EPR5701 (ab124762, Abcam, 1:500), GFP_3E6 (A11120, Invitrogen, 1:500), SOX9 (AF3075, R&D Systems, 1:40), SSEA-1 (MC480, DSHB, 1:10), MITF (ab12039, Abcam, 1:800). GATA4 (sc-25310, Santa Cruz, 1:100), Brachyury/TBXT (AF2085, R&D systems, 1:300), SOX2 (ab5603, Sigma, 1:500), EMA-1 (EMA-1, DSHB, 1:10, RRID:AB_531885).
Secondary antibodies used: Goat Anti-Mouse IgM NorthernLights™ NL557 (NL019, R&D Systems, 1:200), Donkey Anti-Rabbit IgG (H+L)-Cy5-AffiniPure conjugated (711-175-152, Jackson ImmunoResearch, 1:200), Donkey Anti-Goat IgG (H+L)-Cy5-AffiniPure conjugated (705-175-147, Jackson ImmunoResearch, 1:200), Donkey Anti-Rabbit IgG (H+L) DyLight™ 549 conjugated (611-742-127, Rockland, 1:5000), Donkey Anti-Mouse IgG (H+L), Alexa Fluor 488 Conjugated (A-21202, Invitrogen, 1:1000), Donkey Anti-Mouse IgG (H+L) DyLight™ 549 conjugated (610-742-124, Rockland, 1:5000)

Validation

Myosin (MF-20, DSHB): https://dshb.biology.uiowa.edu/MF-20
TuJ-1 (MAB1195, R&D Systems): https://www.rndsystems.com/products/neuron-specific-beta-iii-tubulin-antibody-tuj-1_mab1195
DAZL (ab215718, Abcam): https://www.abcam.com/products/primary-antibodies/dazl-antibody-epr21028-ab215718.html usage in avian species was previously shown in D Huss et al., Front Cell Dev Biol. 2019, HJ Choi et al., J Anim Sci Biotechnol. 2022, and YC Chen et al., Development, 2023.
P63 (ab124762, Abcam): https://www.abcam.com/products/primary-antibodies/p63-antibody-epr5701-ab124762.html usage in chicken species was previously shown in S Guioli et al., Development, 2020.
GFP (A11120, Invitrogen): https://www.thermofisher.com/antibody/product/GFP-Antibody-clone-3E6-Monoclonal/A-11120
SOX9 (AF3075, R&D Systems): https://www.rndsystems.com/products/human-sox9-antibody_af3075 usage in chicken species was previously shown in S Guioli et al., Development, 2020.
SSEA-1 (MC480, DSHB): https://dshb.biology.uiowa.edu/MC-480-SSEA-1
MITF (ab12039, Abcam): https://www.abcam.com/products/primary-antibodies/mitf-antibody-c5-ab12039.html
GATA4 (sc-25310, Santa Cruz): https://www.scbt.com/p/gata-4-antibody-g-4?srsltid=AfmBOooND5LSuBxI0845LMuSQfbHLUobEE-PEmoAjlyvM8QHSZxGCVD8
Brachyury/TBXT (AF2085, R&D systems): https://www.rndsystems.com/products/human-mouse-brachyury-antibody_af2085
SOX2 (ab5603, Sigma): https://www.sigmaaldrich.com/US/en/product/mm/ab5603?srsltid=AfmBOoolQf8_9sqLf4x4mKxVv29urzWhuI9kSu0sfgLAinlAKiFFHEGI
EMA-1 (DSHB): https://dshb.biology.uiowa.edu/EMA-1

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

Avian ESCs derivation used fertile eggs from the following sources. Both male and female avian cell lines were derived in this study. Chicken breeds includes Rhode Island Red (AA lab eggs, Westminster, CA), White Leghorn, Black Plymouth Rock, Frizzle Polish (all from Meyer Hatchery, OH). Pheasant and Goose eggs were from Sunstate Ranch, Sylmar, CA. Ostrich eggs were from OstrichLand, Solvang, CA. Japanese quail and turkey eggs were from AA lab eggs, Westminster, CA. Duck eggs were from Metzer Farms, Gonzales, CA. Peafowl eggs were from Miyazaki City Phoenix Zoo, Miyazaki, Japan. Lenti-X 293T cell line was acquired from Takara (cat#632180).

Authentication

Chicken cell lines were validated by genomic PCR and qRT-PCR using specific primers. Chicken and quail cell lines were validated by karyotype analysis.

Mycoplasma contamination

All cell lines were regularly checked with mycoplasma test and negative results were obtained.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified cell lines were used.

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	Fertile chicken eggs (Rhode Island Red from AA lab egg and Specific-Pathogen-Free White Leghorn eggs from Charles River) were used as the recipient embryos for chimera production. Fertile Japanese quail eggs from AA lab egg were also used as recipient embryos for chimera production. Once hatched, chimeric chickens were housed in animal facility at University of Southern California. Fertile avian eggs used for ESC derivation were purchased from the following sources: chicken breeds includes Rhode Island Red (AA lab eggs, Westminster, CA), White Leghorn, Black Plymouth Rock, and Frizzle Polish (all from Meyer Hatchery, OH), pheasant and goose eggs (Sunstate Ranch, Sylmar, CA), ostrich eggs (OstrichLand, Solvang, CA), Japanese quail and turkey eggs (AA Lab Eggs, Westminister, CA), duck eggs (Metzer Farms, Gonzales, CA), peafowl eggs (Miyazaki City Phoenix Zoo, Miyazaki, Japan).
Wild animals	No wild animals were used in the study.
Reporting on sex	For experiments to determine the germline contribution of chicken ESCs, we were focusing on examining the contribution of male chicken ESCs inside male chicken recipients.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	All animal procedures were carried out in accordance with approved guidelines from the University of Southern California Institutional Animal Care and Use Committees (protocol 20823).

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

Plants

Seed stocks	*Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.*
Novel plant genotypes	*Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.*
Authentication	*Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.*

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	Genetically-engineered chicken ESCs grown in OT/3i/chLIF/CP67 medium were dissociated using 0.05% trypsin and washed with PBS and resuspended in PBS+1% albumax and passed through a 35 µm cell strainer (Falcon).
Instrument	Cytoflex SRT Cell Sorter
Software	CytExpert was used to collect/record the data. Flow cytometry data was analyzed with Floreada.io.
Cell population abundance	About 90% of the cells grown in OT/3i/chLIF/CP67 medium were positive for either GFP or BFP expression. And the remaining of the non-fluorescent cells include GFP knockout chicken ESCs and irradiated fibroblast cells which served as feeder layer.
Gating strategy	Debris and doublets were removed from the main population of cells using FSC vs SSC gating. To determine the population of genetically engineered chicken ESCs, a GFP+ chicken ESC line was used as control to draw the gate for the GFP and BFP channel.

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