

**Mutations in *TUBA4A* lead to human zygotic arrest
and early developmental failure**

Abbreviations

E284G	c.851G>A;p.E284G mutation of TUBA4A
E284K	c.850G>A;p.E284K mutation of TUBA4A
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HGMD	Human Gene Mutation Database
IVF	<i>In vitro</i> fertilization
MI	Metaphase I
MII	Metaphase II
TUBA4A	Tubulin alpha 4a
TUBB8	Tubulin beta 8 class VIII
WT	Wild type

Supplemental Methods

Patients

The patients with zygotic arrest and female infertility were recruited from the First Affiliated Hospital of Anhui Medical University. The human sample collection and analysis were approved by the Ethics Committee for Clinical Medical Research of the First Affiliated Hospital of Anhui Medical University (No. PJ2020-13-10). Each patient signed the informed consent, and 5 mL of peripheral blood was collected.

Whole-exome sequencing (WES) analysis

WES was carried out as described (Chen, Guo et al. 2022). The process for variants filtering was as follows. Variants including missense, nonsense, frameshift, and splice-site with minor allele frequency < 1% in public databases, including Genome Aggregation Database, 1000 Genomes, and the NHLBI Exome Sequencing Project were retained. Then variants in genes

associated with oocyte maturation and early embryonic development or expressed highly during the GV, MI, MII, zygote, and 2-cell stage were mainly focused on.

Transcriptome and translome analysis

Transcriptome analysis of TUBA4A in human and mouse GV oocyte to blastocyst used RNA-sequencing data from GSE36552, and GSE71434 (Yan, Yang et al. 2013, Zhang, Zheng et al. 2016). The translome analysis was based on Ribo-lite data from GSE197265 and GSE165782 (Xiong, Xu et al. 2022, Zou, Zhang et al. 2022). Translational efficiency (TE) analysis was calculated by the ratio of Ribo-lite and mRNA-seq (FPKM+1/FPKM+1). TE > 3 was considered a high TE, and TE < 0.33 was considered a low TE.

Mouse oocyte collection, embryo culture, and microinjection

All animal experiments were conducted following the Guide for the Care and Use of Animals for Research Purposes. The protocol for mouse oocyte collection and embryo isolation was approved by the Institutional Animal Care and Use Committee and Internal Review Board of Tsinghua University. GV oocytes were collected from the ovary of 3 – 4 weeks old C57BL/6 females 48h after PMSG injection and then were incubated in M2 medium (Sigma M7167) supplemented with 10 μ M Cilostamide (TargetMol 68550) at 37°C. Embryos were collected from wild-type C57BL/6 females (Charles River). Zygotes for mRNA microinjection were collected from mated female mice 20 h post-HCG, then cultured in KSOM medium (Merck Millipore MR-106-D) in a 37.5°C 5% CO₂ incubator. Microinjection of wild-type and mutant TUBA4A mRNAs at a concentration of 200 ng/ μ L into the mouse GV oocytes or zygotes was performed using Eppendorf FemtoJet and a Leica microscope micromanipulator. In the oocyte rescue experiment, WT and mutant TUBA4A mRNAs were co-injected at concentrations of 400 ng/ μ L and 200 ng/ μ L. In the zygote rescue experiment, WT and mutant TUBA4A mRNAs were co-injected at concentrations of 200 ng/ μ L and

200 ng/ μ L.

Plasmid cloning and RNA synthesis

Human and mouse *TUBA4A* cDNA was cloned into the RN3P vector for *in vitro* transcription of mRNA. mRNAs were generated using the T3 mMESSAGE mMACHINE Kit according to the manufactures instructions (Ambion).

Immunostaining and confocal microscopy

For oocyte immunostaining, mouse oocytes were fixed in 1% PFA at 4°C overnight, then permeabilized with 0.25% Triton X-100 for 20 min. Embryos were then blocked with 5% BSA at room temperature for 2 h and incubated with primary antibody overnight at 4°C, followed by washing in PBST and incubating with secondary antibody for 1 h at room temperature. DNA was stained by DAPI (Sigma-Aldrich, 28718-90-3). For sperm immunostaining, selected spermatozoa were washed with phosphate-buffered saline (PBS) and were fixed with 4% paraformaldehyde for 30 min at room temperature. Then, fixed spermatozoa were smeared on slides pre-dripped with poly-lysine, followed by permeabilization with 0.5% Triton X-100 for 15 min and blocking with 5% BSA for 1 h at room temperature. Then the slides were incubated with primary antibody overnight at 4°C. The slides were sequentially washed with PBS and incubated with a secondary antibody for 1 h at room temperature. Finally, the slides were counterstained with DAPI to label DNA for 10 min at room temperature. Antibodies used in this study are: TUBA4A (Abcam, ab228701), α -tubulin (SCBT, sc53029). Secondary antibodies are Dylight 549-Goat Anti-Rat (Earthox, E032340) and Dylight 488-Goat Anti-Rabbit (Earthox, E032220). Immunostaining images were acquired with a Nikon A1R HD25 confocal microscope using an oil immersion 60 \times objective. Raw data were processed using open-source image analysis software Fiji ImageJ.

Ethics approval

The human sample collection and analysis were approved by the Ethics Committee for Clinical Medical Research of the First Affiliated Hospital of Anhui Medical University (No. PJ2020-13-10). All animal experiments were conducted following the Guide for the Care and Use of Animals for Research Purposes. The protocol for mouse embryo isolation was approved by the Institutional Animal Care and Use Committee and Internal Review Board of Tsinghua University.

Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Statistical significance was determined by Student's *t*-test (two-tail) for two groups; one-way or two-way Analysis of Variance (ANOVA) for multiple groups using Graphpad prism8 software. $P < 0.05$ was considered significant.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Consent to participate

The human sample collection and analysis were approved by the Ethics Committee for Clinical Medical Research of the First Affiliated Hospital of Anhui Medical University and carried out with informed consent.

Consent for publication

The participant has consented to the submission of the study to the journal.

References

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Supplemental Figure Legends

Figure S1. TUBA4A is conserved among mammals.

(A) Genome locus of human *TUBA4A* and conserved sequences among different species.

(B) The amino acid sequence around TUBA4A E284 among different mammals.

(C and D) Translation efficiency of TUBA4A in human (C) and mouse (D) oocytes.

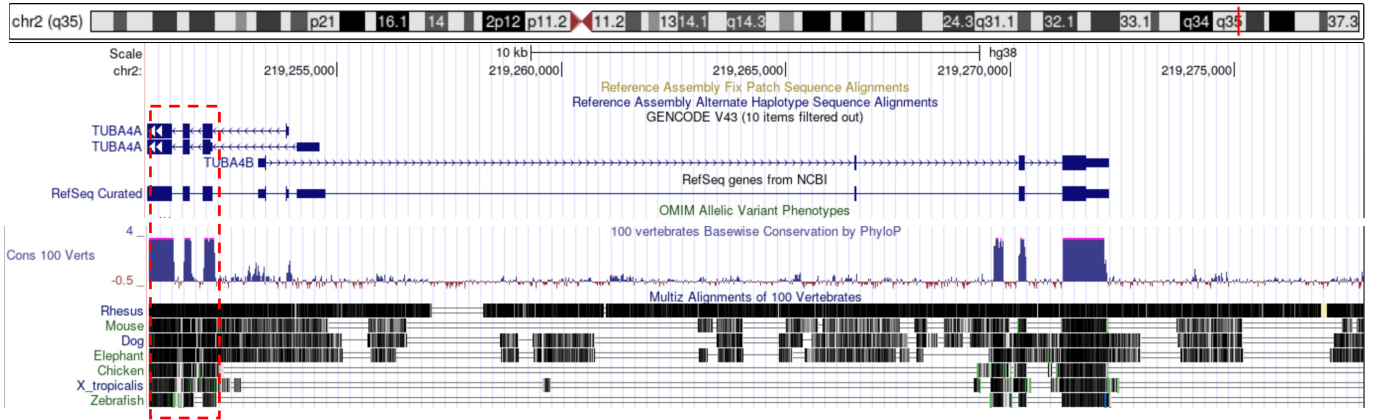
(E and F) Immunostaining of TUBA4A in mouse (E) and human (F) sperms. Cyan, DAPI; yellow, α -tubulin; magenta, TUBA4A. Scale bar, 10 μ m.

Figure S2. Overexpression of TUBA4A E284K and E284G mutants leads to reduced 1st PB protrusion in mouse oocytes.

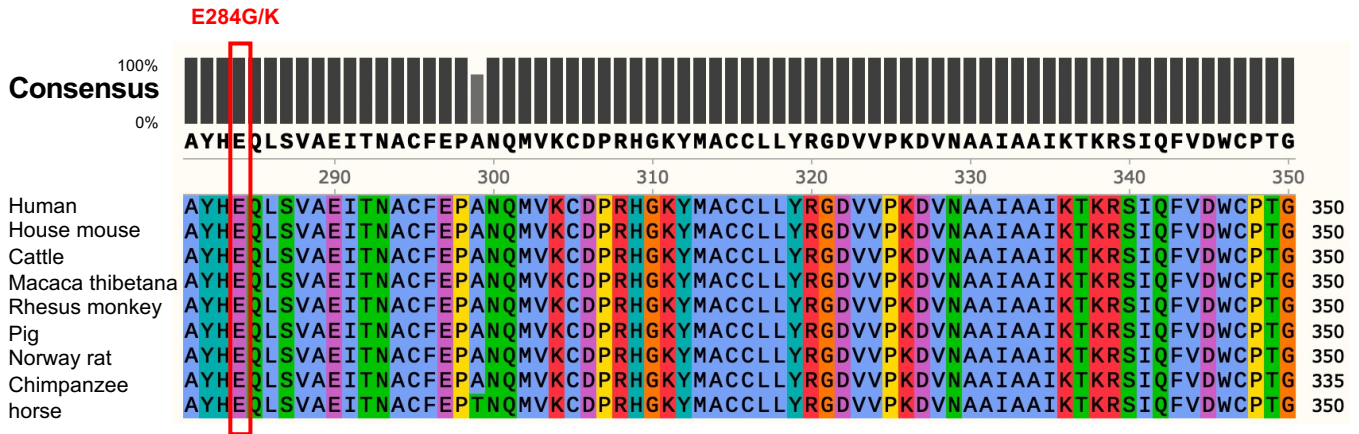
(A and B) Bright-field and GFP images of oocytes injected with TUBA4A WT, E284K, and E284G mutants (as indicated). Images were taken 4 or 20 h after injection. Oocytes with the 1st polar body were marked with a red *.

A

TUBA4A gene locus: 2q35

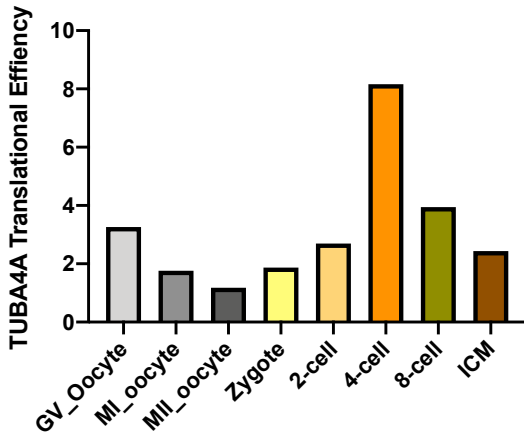


B



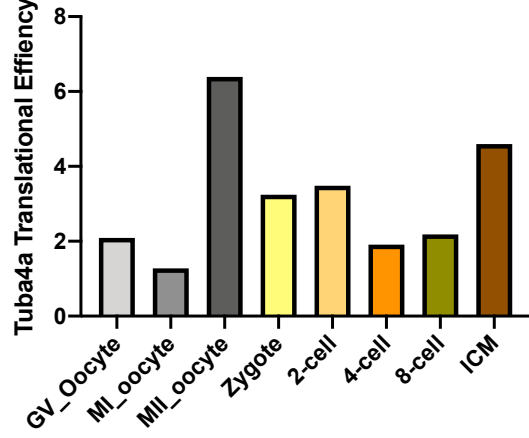
C

Human

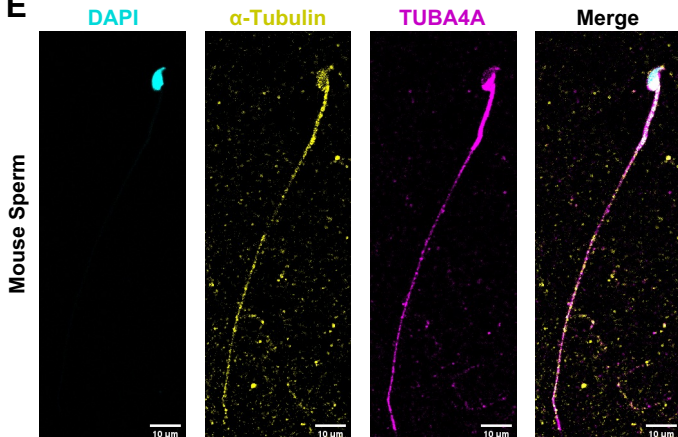


D

Mouse



E



F

