

GYNECOLOGY

Biallelic mutations in *ANAPC13* cause female infertility characterized by oocyte maturation arrest both in humans and mice



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BACKGROUND: Oocyte maturation arrest is an intractable clinical problem, resulting in recurrent failure of assisted reproductive treatments. The anaphase-promoting complex or cyclosome orchestrates a series of proteolytic events to ensure proper cell cycle progression of mitosis in somatic cell proliferation and meiosis during oocyte maturation. Defects in anaphase-promoting complex or cyclosome subunits, such as ANAPC8 and ANAPC12, have been demonstrated linked to oocyte maturation arrest. However, the roles of other anaphase-promoting complex or cyclosome subunits in oocyte maturation remain unclear.

OBJECTIVE: This study aimed to reveal the causal relationship between *ANAPC13* mutations and oocyte maturation arrest, while elucidating the pathogenic mechanism to provide a theoretical basis for clinical diagnosis and treatment.

STUDY DESIGN: Patients diagnosed with oocyte maturation arrest by morphologic assessment during assisted reproductive treatments were recruited and underwent whole-exome sequencing. Mutations in *ANAPC13* were identified in 3 patients and screened out as the candidate. The recurrent mutation c.6C>A was recapitulated in a knock-in mouse model (*Anapc13^{MM}* mice) to clarify its association with oocyte maturation arrest, with wild-type mice (*Anapc13^{+/+}* mice) serving as controls. Further phenotyping experiments with mouse oocytes, proteomic analysis of human oocytes, and molecular experiments with cell lines and plasmids were conducted to determine the role of ANAPC13 in oocyte maturation. *Anapc13* mRNA microinjection was performed as an exploratory rescue treatment.

RESULTS: We identified 2 biallelic *ANAPC13* mutations (NM_001242374.1: c.6C>A; p.D2E and c.71T>G; p.L24R) in 3 infertile females with oocyte maturation arrest at metaphase I. Oocytes from the *Anapc13^{MM}* female mice similarly displayed an extremely low proportion of mature oocytes, whether obtained after superovulation (*Anapc13^{+/+}*: 96.63% $\pm$ 3.40% vs *Anapc13^{MM}*: 1.66% $\pm$ 3.34%, $P < 0.001$) or in vitro maturation (*Anapc13^{+/+}*: 70.30% $\pm$ 1.10% vs *Anapc13^{MM}*: 0.83% $\pm$ 1.66%, $P < 0.001$). An in-depth study of oocytes demonstrated that mutant ANAPC13 disrupts the protein composition of oocytes during metaphase I-to-anaphase I transition by impairing anaphase-promoting complex or cyclosome function, without changing the spindle assembly checkpoint dynamics. Furthermore, a molecular mechanistic study revealed that anaphase-promoting complex or cyclosome dysfunction resulted from abnormal subunit interaction. Moreover, *Anapc13*-mutant oocytes can be partially (49.20% $\pm$ 3.60%) rescued to extrude the first polar body by microinjection of *Anapc13* mRNA.

CONCLUSION: Our study demonstrated the critical role of ANAPC13 in human and mouse oocyte maturation, established a causal relationship between *ANAPC13* mutations and oocyte maturation arrest, and further provided preliminary evidence that microinjection may serve as a potential treatment for these patients with *ANAPC13* mutations.

Key words: *ANAPC13*, anaphase-promoting complex or cyclosome (APC/C), female infertility, metaphase-to-anaphase transition, oocyte maturation arrest

Introduction

Infertility has been a progressively severe global public health problem.¹ With advances in medical technology, assisted reproductive treatments (ARTs) have become the last option to restore the patients' own fertility.² However, oocyte maturation arrest caused by genetic defects is an intractable clinical problem, resulting in repeated failure of ARTs.³ Although significant progress has been made in identifying genes associated with oocyte maturation arrest,^{4–17} the

underlying genetic defects in most patients remain unclear.

The maturation process of oocytes is distinguished from somatic cells and male gametes due to its unique cell cycle process.¹⁸ Before being ready for fertilization, oocytes undergo a prolonged process, including arrest at prophase I diplotene, resumption and completion of meiosis I, and subsequent arrest at metaphase II (MII). Successful completion of meiosis I, which is essential for oocyte nuclear maturation, depends on the orderly regulation of the spindle assembly checkpoint (SAC) and the anaphase-promoting complex or cyclosome (APC/C). Previous studies have extensively elucidated the function of APC/C in the chain events of mitosis progression.^{19,20} In recent years, its

functional category has been extended to meiosis.^{21–23} APC/C orchestrates a series of proteolytic events to ensure proper cell cycle progression in the form of a large multisubunit E3 ubiquitin complex.²⁴ Any subunit is important to the functional integrity of APC/C. So far, only ANAPC8²⁵ and ANAPC12²⁶ have been linked to the cell cycle progression during oocyte maturation. Therefore, the relationship between each APC/C subunit and oocyte maturation remains to be elucidated.

Here, we identified 2 biallelic *ANAPC13* mutations (NM_001242374.1: c.6C>A; p.D2E and c.71T>G; p.L24R) in 3 infertile females with oocyte maturation arrest. A knock-in mouse model (*Anapc13^{MM}* mice) harboring the recurrent mutation (c.6C>A; p.D2E)

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AJOG at a Glance

Why was this study conducted?

This study was conducted to explore a novel pathogenic gene responsible for oocyte maturation arrest and elucidate its pathogenic mechanism, thereby providing a theoretical basis for clinical diagnosis and treatment.

What are the key findings?

ANAPC13 play a critical role in oocyte maturation by composing of anaphase-promoting complex or cyclosome (APC/C) complex to orchestrate protein expression, whose defects caused female infertility characterized by oocyte maturation arrest at metaphase I.

What does this add to what is known?

This study expands the current knowledge on the causal relationship between APC/C subunits and oocyte maturation. The extensive protein network influenced by APC/C directly or indirectly during oocytes development from metaphase I transiting to anaphase I was revealed by utilizing a disease model with APC/C dysfunction.

recapitulates the phenotype of oocyte maturation arrest at metaphase I (MI) observed in our probands. Further functional analysis in vitro and in vivo revealed the important role of ANAPC13 during the transition from MI to anaphase I (AI) in oocyte development. Our study provides new evidence that mutations in previously uncharacterized APC/C subunit can cause female infertility and expands the pathogenic mutational spectrum of MI arrest in oocytes.

Materials and methods**Study participants**

Individuals with primary infertility due to oocyte maturation arrest were recruited from the Reproductive Medicine Center from the First Affiliated Hospital of USTC (University of Science and Technology of China) and Yantai Yuhuangding Hospital Affiliated to Qingdao University. Immature oocytes that would otherwise be discarded in routine clinical practice and peripheral blood samples were collected after obtaining informed consent from each participant.

Genetic studies

Whole-exome sequencing (WES) and bioinformatics analysis were performed as previously described.^{27,28} Briefly, WES was performed on genomic DNA extracted from peripheral blood, and

results were annotated using ANNOVAR against GRCh37. After confirming the absence of mutations in known pathogenic genes associated with oocyte maturation arrest (*CDC20*, *ANAPC8*, *ANAPC12*, etc.), we screened the WES data to identify candidate mutations according to standard filtering criteria. The mutation site and inheritance pattern were validated using polymerase chain reaction (PCR) and Sanger sequencing.

Bioinformatics analyses in silico

Detailed information on ANAPC13 was acquired from the National Center for Biotechnology Information database. Conservation of mutant residues across multiple species was evaluated using MultAlin (<http://multalin.toulouse.inra.fr/multalin/multalin.html>). Allele frequencies and the pathogenicity of mutations in silico were queried from the Var-Cards database (<http://www.genemed.tech/varcards/welcome/index>), which integrates data from population frequency reference databases (e.g., gnomAD and 1000 Genomes) and variant pathogenicity prediction tools (e.g., SIFT, PolyPhen-2, and Mutation Taster). The three-dimensional (3D) structures of wild-type (WT) and mutant ANAPC13 were modeled using Missense3D (<https://missense3d.bc.ic.ac.uk/~missense3d2/>) to visualize

conformational alterations induced by the mutations.

Culture and collection of oocytes

Before culturing the oocytes, pregnant mare serum gonadotropin (PMSG) was used at a dose of 5 IU to induce follicular maturation. Germinal vesicle (GV) oocytes were retrieved from the ovaries of 4-week-old mice at 48 h after intraperitoneal injection. The oocytes were cultured in M16 Medium (Sigma, Saint Louis, USA) in a humidified incubator at 37°C with 5% CO₂. The timing of oocytes collection was determined based on the experimental aims.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA from tissues was extracted using TRIzol, whereas total RNA from oocytes and granulosa cells was extracted using the RNAprep pure Micro Kit (TianGen, Beijing, China). Reverse transcription PCR (RT-PCR) using the Hyper-Script™ III RT SuperMix (NovaBio, Shanghai, China) following the manufacturer's protocol. Subsequently, quantitative PCR (qPCR) was performed as previously described.²⁹

Immunofluorescence (IF)

Ovaries were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned at 3 μm and finally placed on slides. The slides were subsequently subjected to antigen retrieval and permeabilization. Oocytes were collected, fixed, and allowed to penetrate together for 50 min. HeLa cells were cultured while climbing the slides. After 36 h of transfection, the slides were removed and fixed with 4% PFA for 5 min, followed by permeabilization with 0.5% Triton X-100 for 10 min.

Samples were then blocked with 2% bovine serum albumin for 1 h at room temperature. Primary antibody incubation was performed at 4°C overnight. Samples were subsequently incubated with fluorescein-conjugated secondary antibodies at 37°C for 2 h. Images were captured using a Zeiss LSM980 confocal microscope (Carl Zeiss, Oberkochen, Germany). Details of the antibodies used are presented Table 1.

TABLE 1

Details of antibody in our study

Name	Manufacturer	Cat.	Dilution
ANAPC13	Sigma	SAB3500481	WB 1:500 IF 1:100
BubR1	Abcam	ab254326	IF 1:200
CyclinB1	Zen-BioScience	340296	WB 1:1000
Securin	Zen-BioScience	R25687	WB 1:500
PRC1	Proteintech	15617-1-AP	WB 1:1000
ASTL	Immunoway	YT6403	WB 1:500
UBE2S	Proteintech	14115-1-AP	WB 1:500
PIN1	Proteintech	10495-1-AP	WB 1:500
Flag	Sigma	F1804	WB 1:5000 IF 1:400
Myc	ABclonal	AE070	WB 1:5000

IF, immunofluorescence; WB, western blotting.

Western blotting (WB)

Samples were collected to lyse total proteins by boiling at 100°C for 10 min in a loading buffer. Proteins were separated by electrophoresis in a suitable concentration of sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The blots were blocked with 5% nonfat milk and incubated with primary antibodies at an applicable dilution ratio at 4°C overnight, followed by incubating the corresponding secondary antibodies for 1 h at room temperature. The signals were evaluated using Super ECL Plus Western Blotting Substrate and a chemiluminescence imaging system. Details of the antibodies used are presented in Table 1.

Chromosome spreading

Oocytes were treated with Tyrode’s solution (Sigma, Saint Louis, USA) to remove the zona pellucida, and then dispersed by hypotonic treatment with a fixation solution containing 1% PFA, 3 mM dithiothreitol, and 0.1% Triton X-100 at pH 9.2 on the slides. IF was performed as described above after drying the slides.

Construction of a knock-in mouse model

CRISPR-Cas9 technology was used to edit the mouse genome by co-microinjecting

Anapc13-targeting gRNA, a donor oligo containing the p.D2E (c.6C>A) mutation, and Cas9 mRNA into fertilized oocytes. F0 founder animals were generated and further genotyped using PCR and Sanger sequencing. Positive F0 founder animals were crossed with WT mice to generate the F1 generation with stable germline transmission. Heterozygous mice were bred until homozygous mice were obtained from the offsprings.

Fertility test

From 8 weeks of age, female mice were mated with WT males in a ratio of 1:1 in at least 3 groups for 3 months. Statistics for litter number and litter size were recorded in details.

Ovarian staining and follicle counts

Ovaries were fixed in 4% PFA, embedded in paraffin, sectioned serially at 5 μm, and finally placed on slides. Each slide was stained with hematoxylin and eosin. Follicles at different stages were counted according to a well-established standard.³⁰

Ovarian superovulation

Superovulation was induced by human chorionic gonadotropin, triggered after intraperitoneal injection of PMSG 48 h, and oocyte retrieval was then scheduled at 16 h after the trigger.

Statistical analysis

Statistical analyses were performed using Graph-Pad PRISM version 9. All experiments were repeated at least three times and all data were expressed as mean ± standard deviation (SD), with sample sizes and P values. Statistical significance of the differences between the mean values for the different genotypes was analyzed using a two-tailed unpaired Student’s t-test. Data were considered statistically significant at P<0.05 (*), P<0.01 (**), or P<0.001 (***).

4D-FastDIA quantitative proteomic analysis

Sample preparation

After controlled ovarian hyperstimulation, 6 immature MI oocytes from 3 independent controls and 6 MI oocytes from individual II-1 in Family 2 (P2) were donated, which would otherwise be discarded in daily medical practice. Oocytes were washed three times in phosphate-buffered saline to remove the culture medium and sonicated three times on ice using a high-intensity ultrasonic processor (Scientz, Ningbo, China) in lysis buffer (8 M urea, 1% protease inhibitor cocktail). Remaining debris was removed by centrifugation at 12,000 g at 4°C for 10 min. Finally, the supernatant was collected, and the protein concentration was determined using a BCA kit according to the manufacturer’s instructions. The protein solution was reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in the dark. The protein sample was then diluted by adding 200 mM TEAB to a urea concentration less than 2 M. Finally, trypsin was added at a 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for the second 4 h digestion. Finally, the peptides were desalted on a Strata X SPE column.

Liquid chromatography-tandem mass spectrometry analysis

The tryptic peptides were dissolved in solvent A, directly loaded onto a home-

made reversed-phase analytical column (25-cm length, 100 μ m i.d.). The mobile phase comprised solvent A (0.1% formic acid and 2% acetonitrile in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were separated using the following gradient: 0 to 5 min, 9% to 24% B; 5 to 6 min, 24% to 35% B; 6 to 7 min, 35% to 80% B; 7 to 8 min, 80% B. All separations were performed at a constant flow rate of 600 nL/min using a NanoElute UHPLC system (Bruker Daltonics). The peptides were introduced into a capillary source followed by timsTOF HT mass spectrometry. Applied electrospray voltage was 1.6 kV. The precursors and fragments were analyzed at the TOF detector. The timsTOF Pro was operated in data-independent parallel accumulation serial fragmentation (dia-PASEF) mode. The full MS scan was set as 300–1500 (MS/MS scan range) and 21PASEF (MS/MS mode)-MS/MS scans were acquired per cycle. The MS/MS scan range was set at 380–1,170, and the isolation window was set at 10 m/z.

Database search

The data-independent acquisition data were processed using DIA-NN search engine (v.1.8). Tandem mass spectra were searched against Homo_sapiens_9606_SP_20231220.fasta (20,429 entries) and concatenated with the reverse-decoy database. Trypsin/P was specified as the cleavage enzyme, allowing for up to one missing cleavage. Excision of N-terminal Met and carbamidomethyl on Cys was specified as a fixed modification. The false discovery rate was adjusted to <1%.

Annotation methods

Gene Ontology (GO) annotation was performed using eggNOG-mapper to extract GO IDs for the identified proteins based on the EggNOG database. Functional classification was then performed according to cellular components, molecular functions, and biological processes. Pathway annotation was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via BLASTP search (E-value $\leq 10^{-4}$). For each sequence, the best hit

was used for annotation. Transcription factors were annotated based on databases of transcription factor-target gene interactions, such as TRRUST and GTRD (limited to humans and mice).

Functional enrichment

The Fisher's exact test was used to analyze the significance of functional enrichment of differentially expressed proteins (DEPs) (using the identified protein as the background). Functional terms with fold enrichment >1.5 and $P < 0.05$ were considered significant.

Enrichment-based clustering

Further hierarchical clustering was performed based on the functional classifications of DEPs, such as GO and KEGG pathways. We first collated all the categories obtained after enrichment along with their P values, and then filtered categories that were enriched in at least one of the clusters with $P < 0.05$. This filtered P value matrix was transformed by the function $x = -\log_{10}(P \text{ value})$. The P values were clustered using one-way hierarchical clustering (Euclidean distance and average linkage clustering) in Genesis. Cluster membership was visualized by a heat map using the "Heatmap" function from the "ComplexHeatmap" R-package.

Protein–protein interaction network

All DEP database accessions or sequences were searched against the STRING database for protein–protein interactions. Only interactions between proteins belonging to the searched dataset were selected, thereby excluding external candidates. STRING defines a metric called "confidence score" to define interaction confidence; we obtained all interactions that had a confidence score >0.7 (high confidence). The interaction network from STRING was visualized in the "vis-Network" R package.

Plasmids construction

Full-length coding sequences of *ANAPC13* and *ANAPC8* were amplified from the total cDNA of HeLa cells and cloned into the 3xFlag-tagged and Myc-

tagged vectors, respectively, using a ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). Site-directed mutagenesis was performed using the Mut Express II Fast Mutagenesis kit V2 (Vazyme, Nanjing, China), according to the manufacturer's instructions. All plasmids were confirmed by Sanger sequencing and transfected into cells using PEI Transfection Reagent (Servicebio, Wuhan, China) for subsequent experiments.

Co-immunoprecipitation (Co-IP)

Cells were harvested after 48 h of transfection with 3xFlag-tagged *ANAPC13* and Myc-tagged *ANAPC8*, and then lysed in radio-immunoprecipitation assay lysis buffer (RIPA lysis buffer) containing the protease and phosphatase inhibitors for 30 min at 4°C. The ultrasonic crusher was used to break the nucleic acid and then the lysates were incubated with anti-Flag beads (Sigma, Saint Louis, USA) on a rotating wheel overnight at 4°C. The co-immunoprecipitated proteins on the beads were eluted with 1x SDS sample loading buffer and analyzed by WB as indicated above.

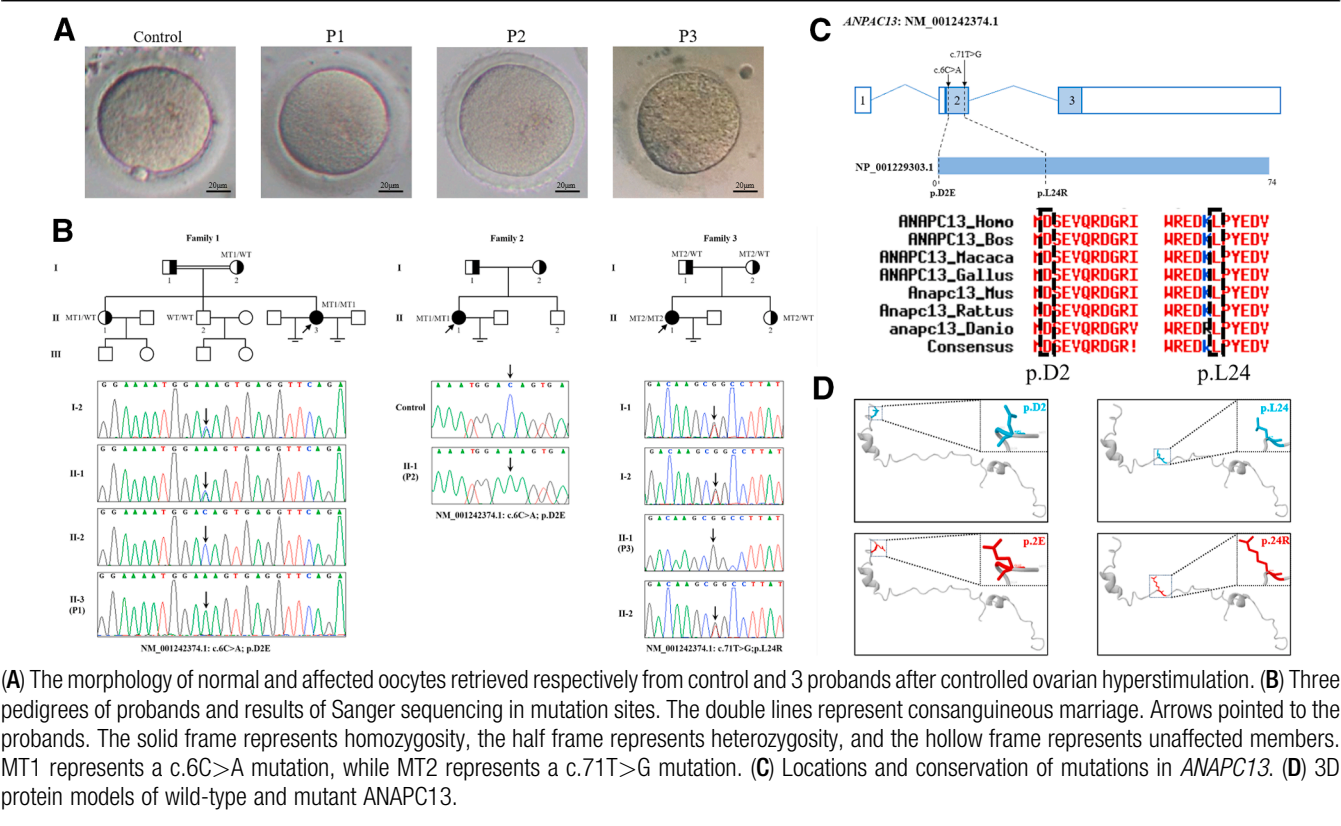
Preparation of mRNA and oocyte microinjection

mRNA was synthesized by in vitro transcription using the WT *Anapc13* plasmid as the template by T7 RNA polymerase, followed by capping and tailing to enhance stability and translation efficiency (Novoprotein, Suzhou, China). After microinjection of WT *Anapc13* mRNA (500 ng/ μ L), the GV oocytes were cultured for 4 h in M16 medium with 2.5 μ M milrinone and then moved to ordinary M16 medium, and oocyte maturation was concurrently observed.

Ethics statement

All studies on human participants were approved by the Medical Research Ethical Committee of the First Affiliated Hospital of USTC (AF/SC-12–2/04.0), while all animal studies were approved by the Laboratory Animal Ethical Committee of the First Affiliated Hospital of USTC (2024-N(A)-29).

FIGURE 1
Identification of *ANAPC13* mutations among patients with oocyte maturation arrest



Results
Identification of *ANAPC13* mutations among patients with oocyte maturation arrest

All the recruited patients were diagnosed with primary infertility without any other systemic diseases. These patients experienced recurrent failures in ARTs caused by the absence of mature oocytes (Figure 1, A), with no apparent link to endocrine abnormalities (Table 2).

The first proband (P1: Family 1, II-3) underwent 3 failed in vitro fertilization (IVF) attempts due to complete maturation arrest of all 23 retrieved oocytes at the MI stage (Figure 1, A). A homozygous missense *ANAPC13* mutation (c.6C>A; p.D2E) was identified from WES as the candidate, given that P1 came from a consanguineous family. Subsequently, PCR and Sanger sequencing verified that the mutation inherited from her heterozygous parents

via an autosomal recessive inheritance model. Her heterozygous sister and WT brother both are fertility (Figure 1, B). WES identified a repeat homozygous mutation (c.6C>A; p.D2E) in a sporadic patient (P2: Family 2, II-1) (Figure 1, B). This proband came from a non-consanguineous family and produced 16 MI-arrested oocytes without the first polar body from 2 IVF cycles (Figure 1, A). Unfortunately, the samples from her parents were unavailable

TABLE 2
Clinical details of patients with *ANAPC13* mutations

No.	Age (y)	FSH (IU/L)	LH (IU/L)	E2 (pg/mL)	AMH (ng/mL)	ARTs cycles (times)	Total number of retrieved oocytes	Ratio of MI oocytes
P1	27	7.75	2.09	43.00	/ ^a	3	23	100%
P2	32	5.31	3.34	18.22	2.89	2	16	100%
P3	33	7.87	5.29	32.86	0.636	5	2	100%

^a The slash represented not available.

for further co-segregation analysis. Based on the autosomal recessive inheritance pattern, we inferred that her parents are likely to harbor a heterozygous mutation at this site.

Moreover, a novel homozygous missense mutation (c.71T>G; p.L24R) was identified by WES in the third proband (P3: Family 3, II-1). Only 2 oocytes were retrieved from 5 IVF cycles in P3 with premature ovarian insufficiency (POI) characterized by low anti-Müllerian hormone level (Table 2). Unfortunately, neither of the 2 oocytes was mature enough to proceed with ARTs (Figure 1, A), which could not be explained by POI. Co-segregation analysis verified by PCR and Sanger sequencing reconfirmed that the biallelic mutations in *ANAPC13* tracked with the phenotype of female infertility, as her fertile parents and sister were heterozygous (Figure 1, B).

Preliminary analysis of the pathogenicity of *ANAPC13* mutations

Two residues (p.D2 and p.L24) are highly conserved across different species (Figure 1, C). Extremely low allele

frequencies of c.6C>A and c.71T>G in human populations were shown by the gnomAD database and 1000 Genomes Project, while high pathogenicity of corresponding p.D2E and p.L24R was predicted by SIFT, Polyphen-2, and Mutation Taster (Table 3).

The 3D models predicted the p.D2E substitution breaks the buried salt bridge formed in the WT protein, which may trigger conformational changes in the protein thereby affecting its biological function (Figure 1, D). However, no structural damage was detected after the substitution of p.L24R (Figure 1, D).

Mice harboring the c.6C>A mutation in *Anapc13* exhibited female infertility

To study the function of *ANAPC13*, we first explored the spatiotemporal expression characteristics of *Anapc13* in WT mice. RT-qPCR and IF revealed that *Anapc13* is highly elevated in oocytes compared to other tissues and granulosa cells (Figure 2, A and B). Further RT-qPCR and WB experiments showed that *Anapc13* is consistently and stably expressed in oocytes across all follicular stages and meiotic phases

(Figure 2, C and D). *Anapc13* is recruited to kinetochores at MI and MII, as demonstrated by oocytes chromosome spreading (Figure 2, E). These results indicated that *Anapc13* plays an important role in oocyte maturation.

Then, we investigated the effect of patient-derived mutations in vivo. Due to the embryonic lethality of *Anapc13* knock-out mice (<http://www.informatics.jax.org>), we produced a mouse model (*Anapc13*^{M/M} mice) harboring the recurrent mutation (c.6C>A) corresponding to two probands (P1 and P2) to further investigate the causal relationship between *ANAPC13* mutations and oocyte maturation arrest (Figure 3, A). Despite the universal expression of *Anapc13* in many tissues (Figure 2, A), *Anapc13*^{M/M} mice survived and developed normally (Figure 3, B and C). However, in contrast to the fertility of male mice (Figure 3, D), *Anapc13*^{M/M} female mice appeared to be completely infertile (Figure 3, E). *Anapc13*^{M/M} female mice have similar ovarian sizes similar to those of WT mice (*Anapc13*^{+/+}) (Figure 3, F). Detection of endogenous *Anapc13* demonstrated that c.6C>A (p.D2E) did not alter its expression level or localization in the oocytes (Figure 3, G and H).

Anapc13 c.6C>A mutation leads to MI arrest in oocytes

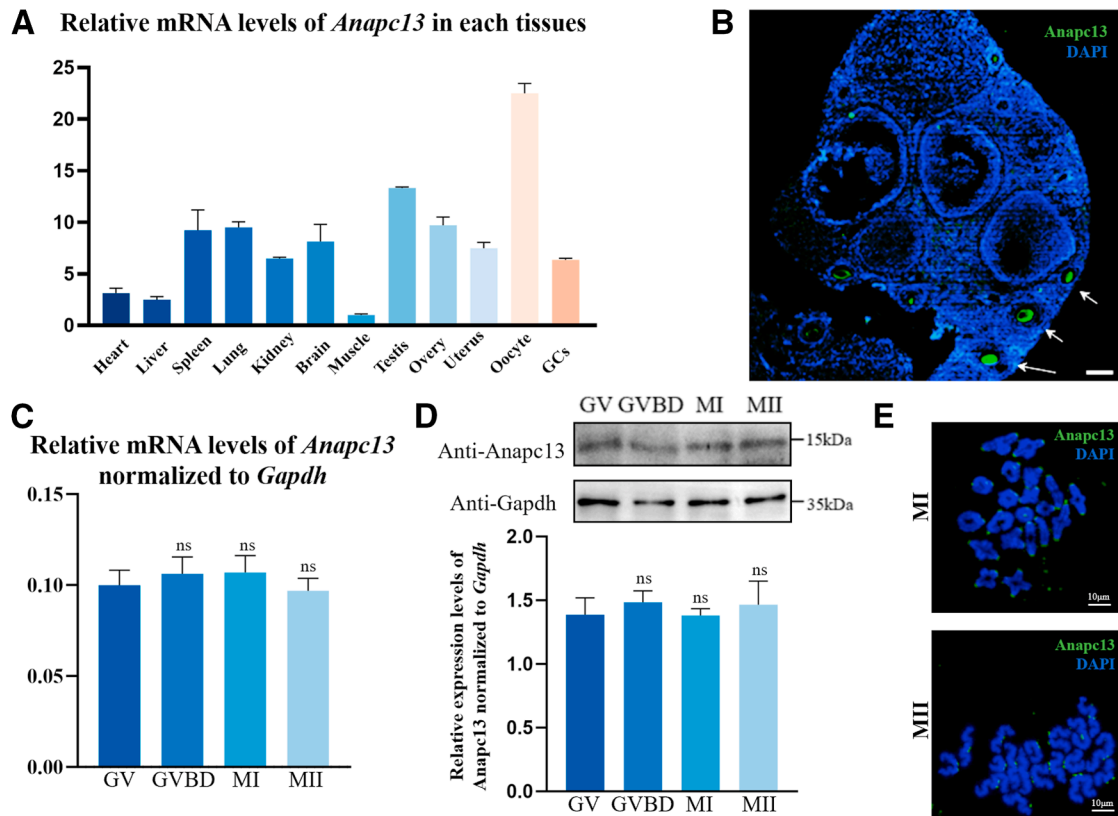
To explore the reason for infertility, we first examined the *Anapc13*^{M/M} mouse ovaries. No obvious abnormalities in proliferation or apoptosis were observed in the *Anapc13*^{M/M} mouse ovaries (Figure 3, I and J). *Anapc13*^{+/+} and *Anapc13*^{M/M} mice exhibited similar trends in follicle count (Figure 3, K).

Oocytes were collected to further analyze the cause of female infertility. After superovulation, *Anapc13*^{M/M} mice retrieved a similar number of oocytes on average compared with the *Anapc13*^{+/+} mice, but almost all oocytes from the *Anapc13*^{M/M} mice did not exhibit the first polar body extrusion (PBE) (*Anapc13*^{+/+}: 96.63% ± 3.40% [n=93] vs *Anapc13*^{M/M}: 1.66% ± 3.34% [n=89], *P*<0.001), recapitulating the phenotype in the affected patients (Figure 4, A). Cell cycle analysis of these obtained

TABLE 3
Overview of the *ANAPC13* mutations identified in 3 probands

Subject	P1	P2	P3
Chromosome coordinates	chr3:134,201,741	chr3:134,201,741	chr3:134,201,676
cDNA alteration	c.6C>A	c.6C>A	c.71T>G
Genotype	Homozygous	Homozygous	Homozygous
Amino acid alteration	p.D2E	p.D2E	p.L24R
Variation type	missense	missense	missense
Allele frequency			
GnomAD_exome_All	0.0000006843	0.0000006843	^a
GnomAD_exome_East Asian	0.00002519	0.00002519	.
1000genomes_All	.	.	.
1000genomes_East Asian	.	.	.
Pathogenic prediction			
SIFT	0.011, D	0.011, D	0, D
Polyphen2	0.643, P	0.643, P	0.999, D
Mutation Taster	0.999998, D	0.999998, D	1, D

^a The dot represented not available.

FIGURE 2
Physiological function of Anapc13 in WT mice

(A) Relative mRNA levels of *Anapc13* in each tissue, oocytes, and granulosa cells in WT mice. (B) A representative IF image of an ovary stained with anti-ANAPC13 antibody (Green) and DAPI (Blue). Scale bar=200 μ m. (C) Relative mRNA levels of *Anapc13* in oocytes at GV, GVBD, MI, and MII stages. (D) Relative protein expression levels of *Anapc13* in oocytes at GV, GVBD, MI, and MII stages. (E) Anapc13 localization in oocytes at MI and MII.

GVBD, germinal vesicle breakdown.

oocytes revealed that *Anapc13* mutation caused oocyte meiotic arrest at MI (*Anapc13*^{+/+}: 97.73% $\pm$ 2.27% [n=93] vs *Anapc13*^{M/M}: 1.66% $\pm$ 3.34% [n=89], $P < 0.001$) (Figure 4, B). To further validate the phenotype of oocyte maturation arrest caused by *Anapc13* mutation, we collected GV oocytes for in vitro maturation (IVM). Consistent with the results in vivo, *Anapc13* mutation did not affect oocyte germinal vesicle breakdown (*Anapc13*^{+/+}: 97.63% $\pm$ 7.13% [n=108] vs *Anapc13*^{M/M}: 98.25% $\pm$ 5.25% [n=120], $P > 0.05$) (Figure 4, C), but induced maturation arrest without PBE (*Anapc13*^{+/+}: 70.30% $\pm$ 1.10% [n=108] vs *Anapc13*^{M/M}: 0.83% $\pm$ 1.66% [n=120], $P < 0.001$) (Figure 4, D). Subsequent IF analysis suggested that the

mutant oocytes were arrested at MI, with failure in the MI-to-AI transition and PBE (Figure 4, E).

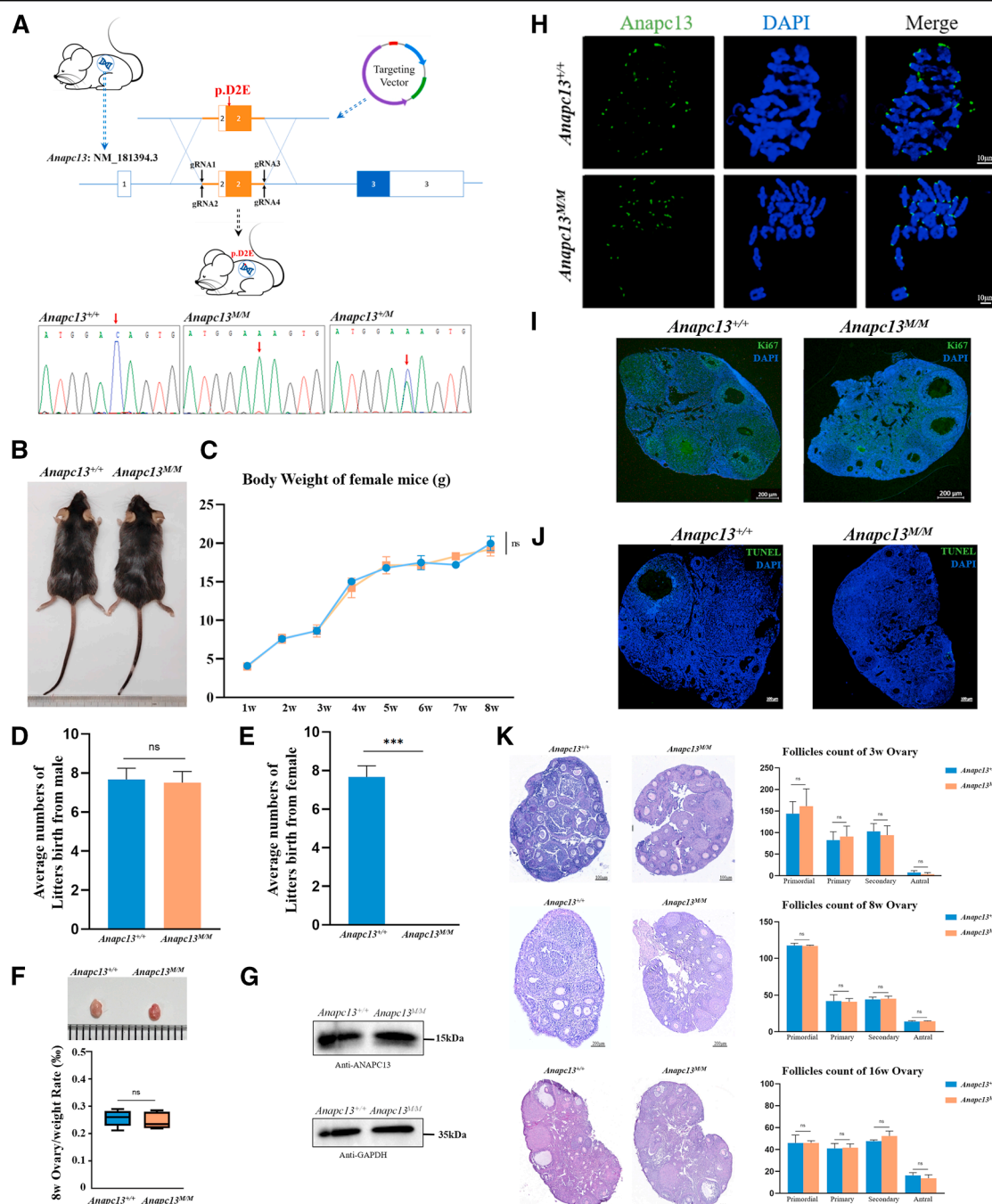
MI arrest in oocytes from *Anapc13*^{M/M} female mice caused by APC/C dysfunction

To gain insight into the potential molecular mechanisms of MI arrest in oocytes, we analyzed the crucial metaphase-specific events. As with WT oocytes, most *Anapc13* mutation oocytes exhibited a typical barrel spindle with neatly arranged chromosomes (*Anapc13*^{+/+}: 19.35% $\pm$ 7.25% [n=67] vs *Anapc13*^{M/M}: 22.9% $\pm$ 10.4% [n=72], $P > 0.05$), suggesting that MI arrest in *Anapc13* mutation-induced oocytes was not caused by abnormal spindle assembly (Figure 4, F). After refrigerating

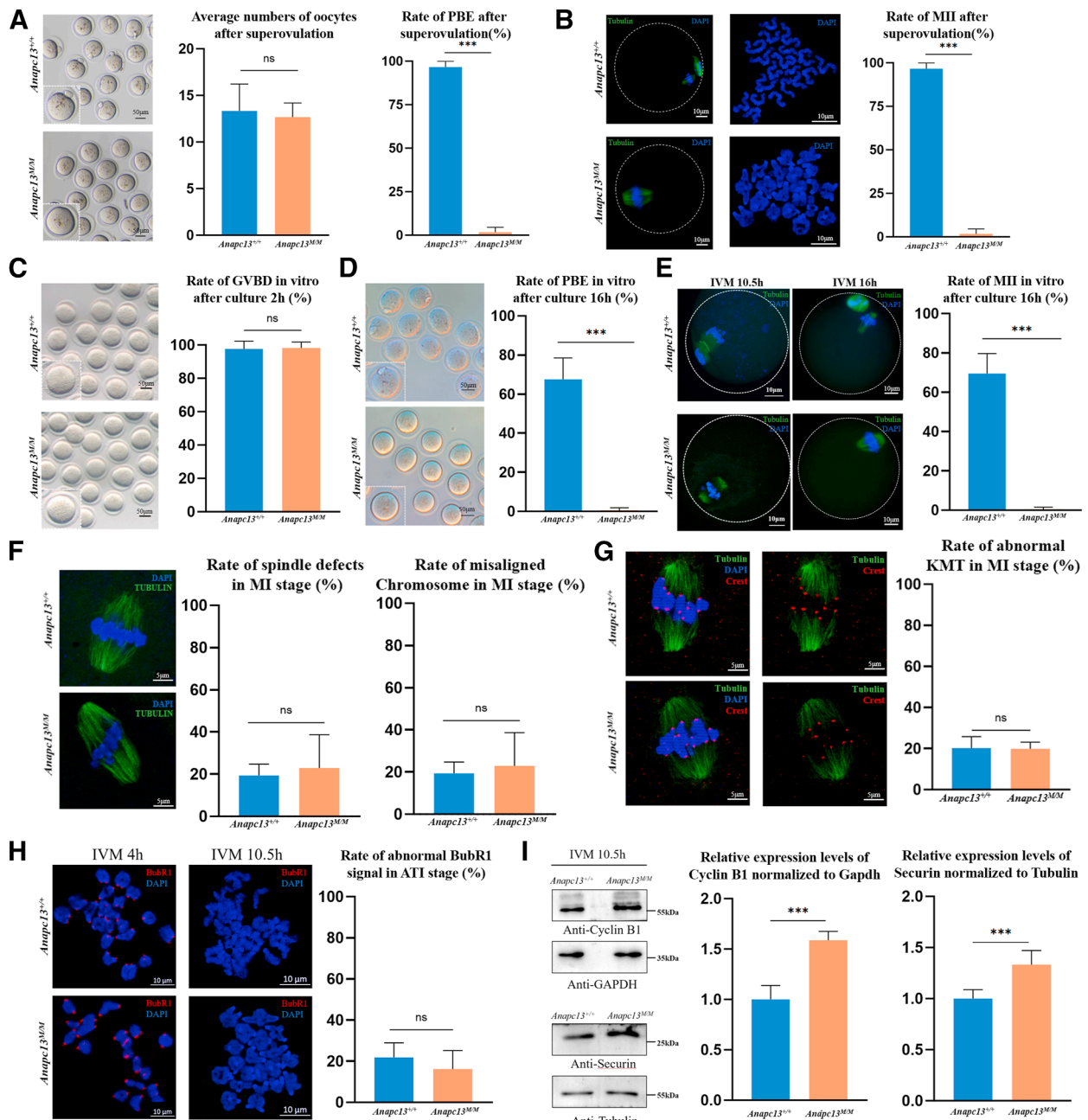
treatment, the stable existence of kinetochore-microtubules further confirmed the correct connection between kinetochore and microtubule (*Anapc13*^{+/+}: 20.28% $\pm$ 6.00% [n=64] vs *Anapc13*^{M/M}: 19.91% $\pm$ 3.25% [n=68], $P > 0.05$) (Figure 4, G). Subsequently, the normal inactivation of SAC remained unaffected by *Anapc13* mutation did not affect, as BubR1 signal, the key component of SAC,³¹ was detected at 4 h (pre-metaphase of WT and *Anapc13* mutated oocytes) and undetected at 10.5 h (anaphase of WT and arrested metaphase of *Anapc13* mutated oocytes) (*Anapc13*^{+/+}: 21.90% $\pm$ 8.10% [n=67] vs *Anapc13*^{M/M}: 16.25% $\pm$ 9.11% [n=66], $P > 0.05$) (Figure 4, H).

Given that APC/C activation occurs downstream of SAC inactivation, the

FIGURE 3

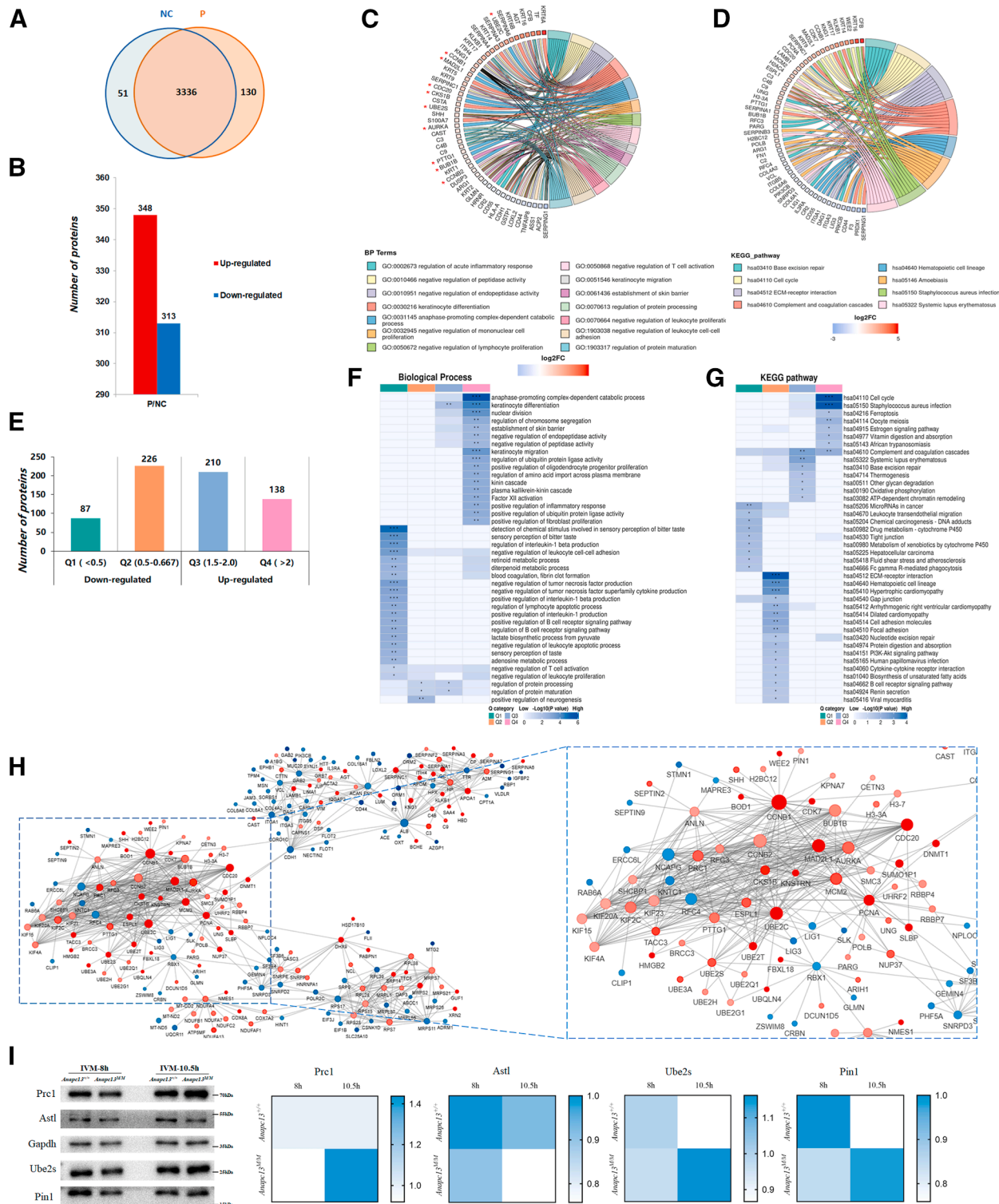
Construction and phenotype identification of the mouse model harboring the p.D2E mutation in *Anapc13*

(A) Construction strategy of the knock-in mouse model and the results of Sanger sequencing to confirm the genotypes. (B) Representative appearance picture of 8-week-old *Anapc13*^{+/+} and *Anapc13*^{M/M} female mice. (C) Weight curve recording of the developmental stages of *Anapc13*^{+/+} and *Anapc13*^{M/M} female mice. (D) Fertility tests were performed on *Anapc13*^{+/+} and *Anapc13*^{M/M} female mice. The average number of litters was counted. (E) Fertility tests were performed on *Anapc13*^{+/+} and *Anapc13*^{M/M} male mice. (F) Appearance pictures and sizes quantified by the weights of ovaries from *Anapc13*^{+/+} and *Anapc13*^{M/M} adult female mice. (G) Anapc13 expression levels of Anapc13 in *Anapc13*^{+/+} and *Anapc13*^{M/M} ovaries. (H) Anapc13 localization in oocytes at MI from *Anapc13*^{+/+} and *Anapc13*^{M/M} female mice. (I) Proliferation of ovaries from *Anapc13*^{+/+} and *Anapc13*^{M/M} adult female mice by staining Ki67 (green) and DAPI (blue). (J) Apoptosis of ovaries from *Anapc13*^{+/+} and *Anapc13*^{M/M} adult female mice by staining TUNEL (green) and DAPI (blue). (K) H&E staining and follicle counts of ovaries from *Anapc13*^{+/+} and *Anapc13*^{M/M} female mice at different ages.

FIGURE 4
Anapc13 c.6C>A mutation leads to MI arrest in mouse oocytes

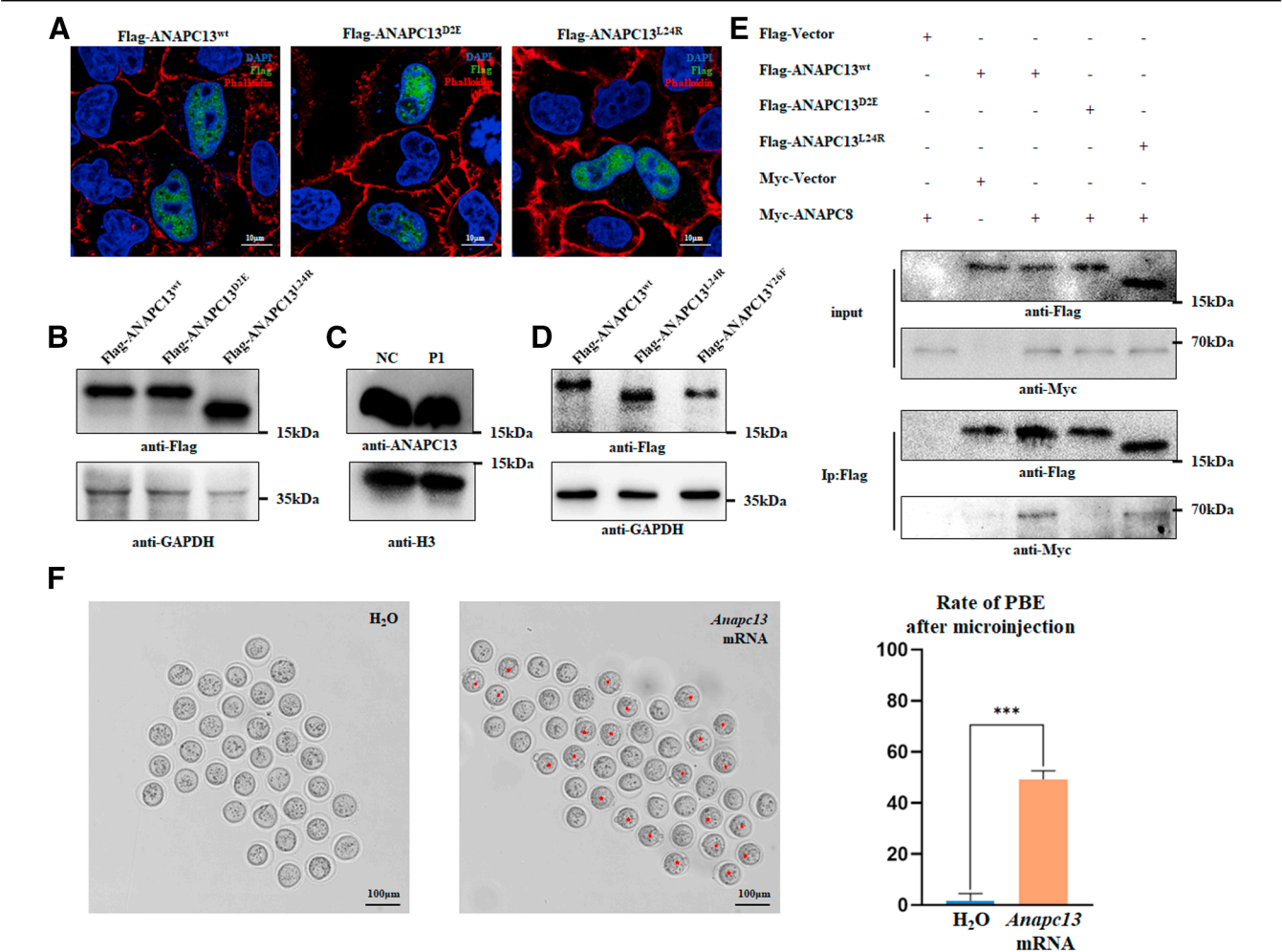
(A) Oocytes from 8-week-old *Anapc13*^{+/+} and *Anapc13*^{MM} mice after superovulation. The average numbers of retrieved oocytes and PBE rate is shown. Statistical analysis was performed with an unpaired *t*-test. Data are presented as mean $\pm$ SD. ****P* < 0.001; ns, not significant. (B) Cell cycle analysis of oocytes from 8-week-old *Anapc13*^{+/+} and *Anapc13*^{MM} mice after superovulation by IF and chromosome spreading. The rate of oocytes at MII is shown. (C) Oocytes from 4-week-old *Anapc13*^{+/+} and *Anapc13*^{MM} mice were cultured in vitro for 2 h (normal theoretical time for GVBD). The rate of GVBD oocytes is shown. (D) Oocytes from 4-week-old *Anapc13*^{+/+} and *Anapc13*^{MM} mice were cultured in vitro for 16 h (normal theoretical time to complete PBE). The rate of PBE oocytes is shown. (E) Cell cycle analysis of oocytes from 4-week-old *Anapc13*^{+/+} and *Anapc13*^{MM} mice cultured in vitro for 10.5 h (normal theoretical time point of anaphase) and 16 h by IF. The rate of oocytes at MII stage is shown. (F) Spindle morphology and chromosome alignment of oocytes from *Anapc13*^{+/+} and *Anapc13*^{MM} cultured in vitro for 8 h (normal theoretical time point of metaphase). (G) Stability of K-MT in oocytes from *Anapc13*^{+/+} and *Anapc13*^{MM} cultured in vitro for 8 h and placed in 4°C for 5 min. (H) Chromosome spreading of oocytes from 4-week-old *Anapc13*^{+/+} and *Anapc13*^{MM} mice cultured in vitro for 10.5 h (anaphase of WT and arrested metaphase of *Anapc13* mutated oocytes), compared with positive control cultured for 4 h (pre-metaphase of WT and *Anapc13* mutated oocytes). The rate of the abnormal activated BubR1 signal is shown. (I) Western blot images of oocytes from *Anapc13*^{+/+} and *Anapc13*^{MM} mice cultured in vitro for 10.5 h. Quantifications of the protein levels of Cyclin B1 and Securin were shown. GVBD, germinal vesicle breakdown; IF, immunofluorescence; PBE, polar body extrusion.

FIGURE 5

Proteome changes caused by *ANAPC13* c.6C>A mutations during oocyte development

(A) Chart showing the total number of proteins identified in *ANAPC13*-mutant oocytes from P2 compared with the normal. (B) Chart showing numbers of significantly up-regulated (red) and significantly down-regulated (blue) proteins. (C) The relationships between DEPs and biological process revealed through GO analysis. (D) The relationships between significantly enriched KEGG pathways and DEPs. (E) Four quarters parted in the DEPs according to fold change. (F) The macro perspective in biological process analyzed by GO based on the 4 quarters. (G) The macro perspective of enriched KEGG

FIGURE 6
Molecular mechanisms of APC/C dysfunction induced by *ANAPC13* mutations and rescue in mutant oocytes



(A) The location of WT and mutant ANAPC13 proteins expressed in corresponding plasmid-transfected HeLa cells revealed by IF. (B) The expression level of WT and mutant ANAPC13 proteins in corresponding plasmid-transfected HEK293T cells revealed by WB. (C) The expression level of endogenous ANAPC13 proteins in peripheral blood from normal control (NC) and P1 revealed by WB. (D) The apparent molecular weight of the mutant ANAPC13 harboring the patient's mutant p.L24R and phospho-deficient mutant p.Y26F revealed by WB. (E) Co-IP analysis of ANAPC13 and ANAPC8 in HEK293T cells. FLAG-antibody was incubated with the cell lysate, and Myc-antibody was used to quantify the level of precipitated ANAPC8 protein. (F) Representative mouse oocytes microinjected with *Anapc13* mRNA followed by IVM for 16 h H₂O was microinjected as a control. Red asterisks indicate the *Anapc13*-mutant oocytes with PBE after microinjection.

APC/C, anaphase-promoting complex or cyclosome; IF, immunofluorescence; IVM, in vitro maturation; WB, western blotting; WT, wild type.

substrates, Cyclin B1 and Securin, were monitored as indicators of APC/C activity. Cyclin B1 and Securin accumulated significantly in oocytes from

Anapc13^{M/M} mice after IVM for 10.5 h, compared with *Anapc13*^{+/+} mice, which revealed that *Anapc13* mutation prevented the normal degradation of

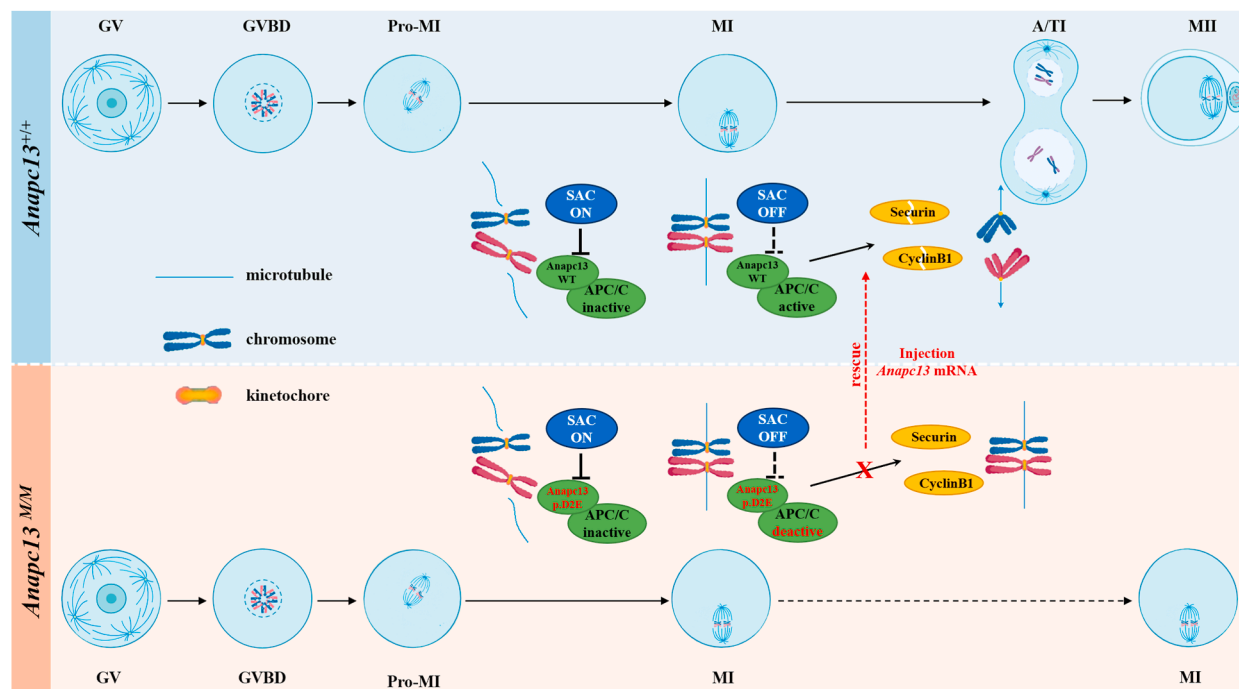
Cyclin B1 and Securin during MI-to-AI transition (Figure 4, I). These results strongly suggested that the MI arrest phenotype in oocytes from *Anapc13*^{M/M}

pathways based on the 4 quarters. (H) Protein–protein interaction network of DEPs. (I) The picked DEPs were verified in oocytes at MI (8 h) and AI (10.5 h) from *Anapc13*^{+/+} and *Anapc13*^{M/M} mice. The heat map displayed the changes in protein expression levels normalized with Gapdh.

DEPs, differentially expressed proteins; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

FIGURE 7

Speculated mechanism of the p.D2E mutation in ANAPC13 leading to MI arrest in oocytes



mice was resulted from the dysfunction of APC/C, rather than the abnormal activation of SAC.

Anapc13 c.6C>A mutation disturbed the proteome during oocyte development

To identify the altered proteins associated with ANAPC13 dysfunction and to increase the proteome coverage and depth, we collected 6 MI-arrested oocytes from the proband II-1 and 6 immature MI oocytes from 3 independent control individuals that would otherwise be discarded in the ICSI procedure, and further utilized 4D-FastDIA quantitative proteomic analysis. We identified a total of 24,786 peptides from 3517 proteins. Among them, 51 were unique to the control group, 151 were unique to the patient group, and 3336 were deemed quantitatively comparable (Figure 5, A).

To screen potential proteins in response to ANAPC13 dysfunction, we applied the criteria of fold change (FC) > 1.5 or <1/1.5 and identified 661 DEPs, of

which 348 were upregulated and 313 were downregulated in oocytes with mutant ANAPC13 (Figure 5, B and Supplemental Table 1). A drastic difference in protein composition was indicated between the proteomes, despite both oocyte samples being at the same MI stage.

GO and KEGG analyses of these DEPs showed that enrichment of biological processes highlighted APC/C-dependent catabolic process (Figure 5, C and D). Associated proteins showed significant accumulation, such as APC/C substrates Cyclin B1 (CCNB1), Cyclin B2 (CCNB2), and Securin (PTTG1), coordinated E2 ubiquitin-conjugating enzymes UBE2C and UBE2S, and the APC/C co-activator CDC20.

To evaluate the relationship between FC and function enrichment, the DEPs were partitioned into 4 quarters according to FC (Q1: FC<0.5, Q2: FC 0.5–0.667, Q3: FC 1.5–2.0 and Q4: FC>2.0), and GO and KEGG analyses were performed in each quarter (Figure 5, E–G). Q1 and Q2 represent downregulated protein, and Q3 and Q4

represent upregulated proteins. Proteins related to APC/C-dependent catabolic process, nuclear division, regulation of chromosome segregation, and keratinocyte differentiation, as well as pathways related to cell cycle, oocyte meiosis, estrogen signaling pathway, and ferroptosis, were frequently detected in Q4, suggesting that those dramatic abnormally upregulated proteins (FC>2.0) and their linked molecular pathways may be the most predominant part of the proteomic imbalance responsible for the impairment of MI-to-AI transition in oocytes with mutant ANAPC13.

Some well-characterized proteins known to be related to oocyte maturation, oocyte meiosis, fertilization or early embryonic development in humans (ASTL, TUBA1C, TACC3, WEE2, CDC20, KPNA7, etc.) and mice (PIN1, UBE2S, ZAR1L, PRC1, DPPA3, NLRP4, etc.), and other oocyte-specific proteins (OOSP4A, IZUMO1R, ACTL8, etc.) were also significantly altered in oocytes with mutant ANAPC13 (Figure 5, H). To verify the reliability and repeatability

of the proteomics data, we detected Prc1,³² Astl,¹⁰ Ube2s³³ and Pin1³⁴ explicitly linked to oocyte maturation in *Anapc13*-mutant oocytes. WB showed a consistent trend of the proteome between patient and mice (Figure 5, I). The above data suggest that the *ANAPC13* c.6C>A mutation disturbed the proteome during oocyte development from MI to AI both in humans and mice.

Molecular mechanism underlying *ANAPC13* mutations-induced APC/C dysfunction

For further mechanistic studies, we constructed the WT and mutant plasmids containing the corresponding sequences of *ANAPC13* to verify their effects in vitro. IF analysis showed that the mutations did not affect the subcellular localization of *ANAPC13* in the cell nucleus (Figure 6, A). WB showed that p.D2E had no effect on protein expression compared with WT *ANAPC13* (Figure 6, B), which was consistent with the detection of the protein extracted from the peripheral blood of the normal control and P1 (Figure 6, C). However, p.L24R caused an obvious change in the apparent molecular weight of *ANAPC13* (Figure 6, B). Since p.Y26 is a phosphorylation site retrieved from the PhosphoSitePlus database and is adjacent to the mutation site (p.L24), this could potentially explain the altered protein mobility observed in our WB analysis. To verify this hypothesis, we engineered expression plasmids encoding phospho-deficient mutants (p.Y26F) to abrogate phosphorylation, as a reference for the change caused by p.L24R. Consistent with our hypothesis, the mutant *ANAPC13* proteins harboring the p.L24R and p.Y26F displayed the same apparent molecular weight (Figure 6, D), suggesting the presence of a similar modification defect.

ANAPC13 contacts the successive tetratricopeptide repeat (TPR) subunits of the TPR lobe in APC/C, while its N-terminus inserts into the TPR groove formed by the N-terminal TPR helix of *ANAPC8*.³⁵ To further investigate how *ANAPC13* mutations induce APC/C

dysfunction, we performed Co-IP of *ANAPC13* and *ANAPC8*, as both mutations are located in the N-terminal region of *ANAPC13*. The results showed that the amount of precipitated *ANAPC8* dramatically decreased in the mutant groups compared with that in the WT group (Figure 6, E), suggesting that p.D2E and p.L24R considerably reduced the interaction between *ANAPC13* and *ANAPC8*, resulting in APC/C dysfunction.

Anapc13 mRNA injection rescues oocyte maturation arrest phenotype

Building upon the finding that *ANAPC13* mutation disrupted the meiotic process by impairing APC/C function, we investigated whether supplementation with WT *ANAPC13* is sufficient to rescue APC/C activation and subsequently complete meiosis I. WT *Anapc13* mRNA was injected into GV oocytes from *Anapc13*^{M/M} mice, whereas H₂O was injected as a control. As expected, a significant portion of oocytes extruded the first polar body after being microinjected with WT *Anapc13* mRNA, while the control group failed (*Anapc13* mRNA: 49.2% ± 3.60% [n=87] vs H₂O: 7.76% ± 5.54% [n=75], *P*<0.001) (Figure 6, F). This result reaffirmed that the defect in *ANAPC13* was responsible for oocyte maturation arrest and suggested that mRNA injection could be a future potential therapeutic treatment for patients with MI arrest in oocytes caused by *ANAPC13* mutations.

Comment Principal findings

In this study, we identified 2 biallelic *ANAPC13* mutations (NM_001242374.1: c.6C>A; p.D2E and c.71T>G; p.L24R) in 3 infertile females with oocyte maturation arrest. *ANAPC13*-mutant oocytes were characterized by arrest at MI both in humans and mice. *ANAPC13* was demonstrated to play a critical role in MI-to-AI transition during oocyte meiosis by functioning collectively within APC/C to regulate the downstream protein network, independent of SAC dynamics (Figure 7).

Results in the context of what is known

The ubiquitination regulation mediated by APC/C, as a crucial event in cell cycle control, has expanded its research scope from mitosis to meiosis.^{19–22} Despite extensive research on APC/C subunits (*ANAPC8*,²⁵ *ANAPC12*),²⁶ co-activator (CDC20),⁸ and collaborative partner (UBE2C,UBE2S)^{33,36} in oocytes, the contributions of many other APC/C pathway components to female infertility remain elusive, owing to the pathway's complexity.

ANAPC13 is an accessory subunit of the TPR lobe subcomplex.³⁷ Consistent with the most APC/C pathway-related proteins, *ANAPC13*-mutant oocytes exhibited a similar MI arrest phenotype. As canonical substrate of APC/C, Cyclin B1 and Securin also obviously accumulate and thereby prevent the cell cycle from proceeding.

Interestingly, both *ANAPC12* and *ANAPC13* are accessory subunits of the TPR arm, but they exhibit distinct phenotypic patterns. *ANAPC12* mutations cause embryonic lethality in homozygotes and oocyte maturation arrest in heterozygotes,²⁶ whereas *ANAPC13* mutations result in oocyte arrest only in homozygotes with no apparent phenotype in heterozygotes. Moreover, unlike *ANAPC8* defects associated with abnormal SAC activation,²⁵ *ANAPC13* mutations exclusively disturb APC/C function independent of SAC dynamics, which is more akin to UBE2S.³³ It suggested that different proteins in the APC/C pathway play precise roles. Thus, it is crucial to map the complete functional architecture APC/C by characterizing each subunit individually. Based on data from the current study, we speculate that compared with *ANAPC8* and *ANAPC12*, which are core subunits essential for overall APC/C function, *ANAPC13* is more likely to be a modulator of APC/C activity.

Clinical implications

Oocyte maturation arrest is an intractable clinical problem due to recurrent failure of ARTs. Our study revealed a causal relationship between *ANAPC13*

mutations and oocyte maturation, and further elucidated its pathogenic mechanism. These findings provide a theoretical basis for clinical diagnosis and treatment.

Meanwhile, the extensive protein network directly or indirectly regulated by APC/C, uncovered by the disease model, provided a new insight into oocyte maturation. Beyond the well-characterized proteins known to be related to oocyte maturation, oocyte meiosis, fertilization or early embryonic development, we believe that many uncharacterized proteins intimately linked to oocyte development are harbored in this extensive protein network. This offers fertile ground for future investigations into the genetic causes of oocyte maturation arrest.

Moreover, the success of *Anapc13* mRNA injection in rescuing the phenotype shown in our results reaffirmed that a defect in ANAPC13 was responsible for oocyte maturation arrest. This suggests that mRNA injection may be a future potential therapeutic treatment for patients with MI arrest in oocytes caused by ANAPC13 mutations.

Research implications

These preliminary findings highlight the value of screening for ANAPC13 mutations in a larger cohort of patients with oocyte maturation arrest to further reveal the pathogenic mechanism of ANAPC13 mutations.

Strengths and limitations

Strengths

Our study made the following key contributions. First, we established a causal relationship between ANAPC13 mutations and human oocyte maturation using a knock-in mouse model. Second, we first revealed the extensive protein network directly or indirectly regulated by APC/C during MI-to-AI transiting in oocytes meiosis, utilizing the disease model of APC/C dysfunction caused by ANAPC13 mutation. Notably, obtaining oocytes at MI-to-AI transition was challenging due to the extremely efficiency and transient nature of APC/C-regulated processes, which are tightly coordinated by co-activators following

SAC inactivation.^{38,39} Finally, we verified that *Anapc13* mRNA injection could be a potential therapeutic treatment for patients with MI arrest in oocytes caused by ANAPC13 mutations.

Limitations

Our study has several limitations that need to be addressed. First, although APC/C plays an important role in mitosis and meiosis, ANAPC13 mutations only exhibited an effect in oocytes without any abnormalities in somatic and spermatogenic cells in humans and mice, whereas *Anapc13* knockout mice were embryonic lethal. According to the spatial expression of WT mice, Anapc13 is highly elevated in oocytes compared with that in other tissues and granulosa cells (Figure 2, A). This might indicate that the phenotypic difference between oocytes and other cells may be caused by different thresholds of Anapc13 amount required. Female infertility caused by Anapc13 defects may be dosage-dependent, whereas mitosis and male gamete meiosis appear to be more tolerant of these defects than meiosis in oocytes, a phenomenon similar to that observed in infertile females with ANAPC8 and CDC20 mutations.^{8,25}

However, our study did not address the threshold amounts of ANAPC13 required for mitosis versus meiosis. Additionally, whether distinct regions of ANAPC13 perform different biological functions, thereby resulting in variable manifestations between somatic cells and gametes, remains to be elucidated.

Second, only a reduced interaction between ANAPC13 and ANAPC8 was confirmed, and further investigation is needed to provide a clearer explanation for the relationship between ANAPC13 mutations and oocyte maturation arrest. All the mechanisms by which ANAPC13 mutations affected APC/C function, specifically whether by impairing interactions with other APC/C subunits or by disturbing the proteolysis of substrates, needs to be studied further. Whether the downstream proteins are regulated directly by the ANAPC13 mutation or indirectly via secondary signaling cascades remain elusive.

Moreover, the DEPs in our proteomic analysis were not only related to oocyte meiosis as expected, but also involved in fertilization, early embryonic development, and other ambiguous biological processes. An in-depth analysis of protein–protein interaction networks and a comprehensive validation of the underlying downstream proteins are needed to provide valuable mechanistic insights. Although *Anapc13* mRNA injection could rescue the phenotype of oocyte maturation arrest, additional work in rescued *Anapc13*-mutant oocytes needs to be conducted in subsequent fertilization and early embryonic development, considering its extensive effects.

Conclusions

Our study established a causal relationship between ANAPC13 mutations and oocyte maturation arrest and elucidated the critical role of ANAPC13 in oocyte maturation. This provides new insights into the genetic causes of oocyte maturation arrest and a compelling theoretical basis to promote genetic counseling and clinical diagnosis and treatment strategies.

Data sharing statement

All data collected for this study is presented within this manuscript, and available to others on request from the corresponding authors. ■

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