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RESEARCH PAPER



# Rab24 functions in meiotic apparatus assembly and maturational progression in mouse oocyte

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**ABSTRACT**

Rab GTPases have multiple regulatory functions in intracellular vesicle transport. In recent years, there has been an increasing interest in the roles of Rab proteins in mammalian oocytes. In this paper, we show the specific distribution pattern of Rab24 during mouse oocyte meiosis. Furthermore, we find that Rab24 depletion results in the failure of maturational progression in mouse oocytes. Notably, the frequency of meiotic apparatus abnormality is significantly increased in Rab24-depleted oocytes relative to controls. In addition, lagging chromosomes are readily observed in anaphase/telophase oocytes with Rab24 knockdown. In support of this, the depletion of Rab24 disturbs the kinetochore–microtubule attachments in oocytes, and contributes to the production of aneuploid eggs. Taken together, the results of this study identify Rab24 as a novel factor in the modulation of meiotic apparatus assembly and meiotic progression during mouse oocyte maturation.

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Rab24; meiosis; oocyte; spindle; chromosome

## Introduction

The growth and maturation of oocytes are crucial for the propagation of mammals and other metazoans. Oocytes undergo a delicately regulated maturation process that is needed for fertilization and embryogenesis [1]. The generation of oocytes in the mammalian perinatal ovary coincides approximately with the beginning of meiosis [2]. Oocytes enter into the meiosis I (MI) division and arrest at the diplotene stage of the prophase, containing enlarged nucleus termed germinal vesicles (GV) [3]. Fully grown GV-stage oocytes resume meiosis in response to luteinizing hormone stimulation, marked by nuclear envelope break down (GV breakdown, GVBD) [4]. Microtubules become to nucleate and organize into a bipolar spindle, with chromosomes align in the metaphase spindle equator [5]. At anaphase/telophase I, homologous chromosomes are segregated [6]. After extruding the first polar body (Pb1), oocytes progress uninterruptedly to the metaphase II stage (MII), waiting for the entry of mature sperm cells [7]. Any errors in oocyte meiotic division could give rise to the generation of aneuploid eggs, resulting in a significant increase in infertility, abortion and birth defects. [8,9]. Despite the intensive investigation, the underlying molecular basis of meiotic defects has remained elusive.

Rab (Ras-related proteins in brain) GTPases, belongs to a superfamily of small GTP-binding proteins, are crucial components in vesicle trafficking pathways [10]. Up to now more than 70 Rab and Rab-like proteins have been identified [11,12]. Rab GTPases mostly locate at the intracellular membranes, where they provide connections to motor proteins and to the cytoskeleton. They play vital roles in various steps of the traffic pathways, including the formation and movement of vesicles, endocytosis, recycling, and degradation of proteins [13]. Many Rabs have been proposed to function in mitotic cell division as they participate in cell growth and differentiation, actin cytoskeleton regulation, and spindle pole positioning [14]. Specifically, emerging evidence indicates that some Rabs play roles in meiotic cell division. For example, Rab5a participates in chromosome alignment and spindle organization in mouse oocytes [15]. Rab6a is an essential modulator in oocyte meiosis, especially the metaphase–anaphase transition [16]. Rab24, an atypical Rab protein

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with a low intrinsic GTPase activity and is inefficiently prenylated, has been reported to function in autophagy [17,18]. Recent studies have investigated the relationship between Rab24 and several diseases such as ataxia, tumor and tuberculosis [19]. In a recent study, it was shown that Rab24 takes part in mitotic process, including chromosome segregation and cytokinesis, probably via its interaction with microtubules [20]. However, the role of Rab24 during oocyte meiosis remains unknown.

The purpose of this paper is to obtain insights into the role of Rab24 during meiosis of mouse oocyte. Thus, we employed siRNA knockdown analysis and discovered the involvement of Rab24 in modulating meiotic division and meiotic apparatus, and report our findings here.

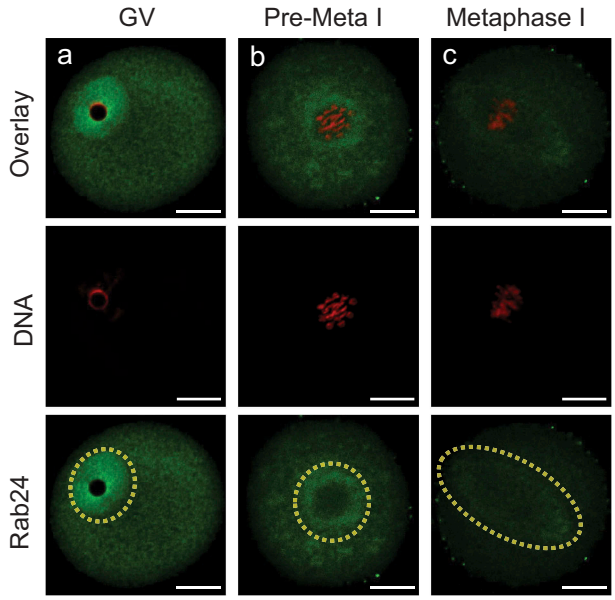


**Figure 1.** Localization of Rab24 during mouse oocyte maturation.

Oocytes at GV, pre-metaphase I, metaphase I were immunolabeled with Rab24 antibody (green) and counterstained with PI to visualize DNA (red). Rab24 signals are denoted by circles. Scale bar, 25 μm.

## Results

### *Subcellular localization of Rab24 during oocyte meiosis*

In an attempt to shed light on the role of Rab24 in meiotic oocytes, we first assessed the distribution of endogenous Rab24 at different stages during maturation. Immunostaining clearly showed the localization of Rab24 in GV, pre-metaphase I, and metaphase I (Figure 1). Samples were co-stained with propidium iodide (PI) to visualize the chromosomes and verify the stages of meiosis. At GV stage, Rab24 displayed a uniformly diffuse pattern in the cytoplasm with an accumulated distribution in nuclear (Figure 1(a)). At pre-metaphase I stage, Rab24 resided in the cytoplasm with a particular concentration around chromosomes (Figure 1(b)). As the oocytes enter into metaphase I, Rab24 located to the spindle region (Figure 1(c)). Similar distribution pattern of Rab24 was also observed in MII oocytes (data not shown). Collectively, these results indicate that Rab24 goes through dynamic distribution changes during meiotic division and probably perform a function in the formation of meiotic apparatus.

### *Rab24 depletion retards meiotic progression in mouse oocytes*

To investigate the role of Rab24 during oocyte maturational progression, exogenous small interfering RNAs (siRNAs) targeting Rab24 were injected into fully grown GV-stage oocytes. After injection, oocytes were incubated in milrinone-supplemented M16 medium for 20 h to inhibit GVBD and allow endogenous Rab24 mRNA degradation. Immunoblotting was performed to examine the effects of Rab24 knockdown (Figure 2(a)). After 3 h *in vitro* culture in milrinone-free medium, we found that the GVBD rate was significantly decreased in Rab24-knockdown (Rab24-KD) oocytes, while more than 90% of control oocytes progressed through GVBD (46.7 ± 2.0% vs. 91.3 ± 1.2% control, $p < 0.05$; Figure 2b), indicating that Rab24 affected meiotic resumption. Moreover, Rab24 depletion notably decreased the Pb1 extrusion percentage of mouse oocytes compared to controls (9.3 ± 2.5% vs. 92.7 ± 2.5% control, $p < 0.05$; Figure 2(c)). In some cases, the symmetrical "2-cell-like" eggs were observed in Rab24-KD group (Figure 2(d), arrows). Altogether, our data provide evidence that Rab24 is crucial for meiotic divisions.

### *Rab24 is required for chromosome alignment and spindle assembly*

The particular localization of Rab24 to the spindle/chromosome region led to the hypothesis

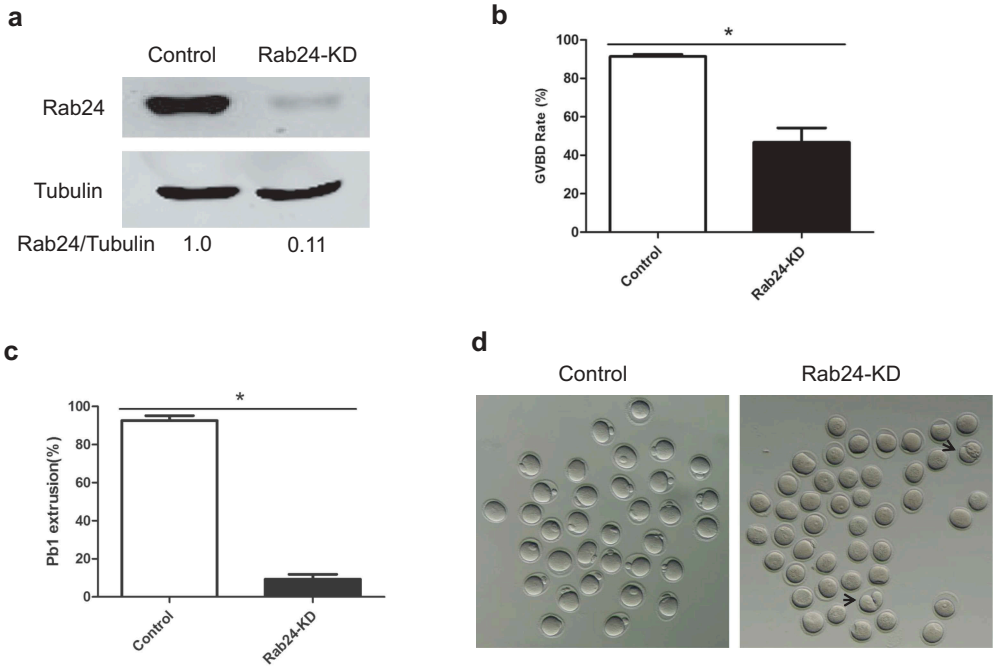


**Figure 2.** Effects of Rab24 knockdown on oocyte maturation.

Fully grown oocytes were microinjected with Rab24-siRNA, which were then arrested at GV stage with milrinone to facilitate mRNA degradation. (a) Knockdown of endogenous Rab24 protein (Rab24-KD) after siRNA injection was confirmed by western blot. Band intensity was calculated using Image J software, and the ratio of Rab24/Tubulin expression was normalized. (b, c) Quantitative analysis of the GVBD rate and Pb1 extrusion rate in control and Rab24-KD oocytes. (d) Phase-contrast images of control and Rab24-KD oocytes. Arrows indicate the oocytes with symmetrical division. Data are expressed as mean percentage ± SD from three independent experiments in which at least 100 oocytes were analyzed. *$p < 0.05$ vs. control

that Rab24 may serve as a regulator in the meiotic apparatus assembly. To test this, oocytes were collected and immunolabel was performed to detect the spindle and chromosomes. Using high-resolution confocal fluorescence microscopy, we noted that in the majority of control cells at metaphase, the spindle was barrel-shaped and chromosomes were congressed to the metaphase plate (Figure 3(a)). Strikingly, a high percentage of Rab24-KD oocytes exhibited abnormal spindles and misaligned chromosomes (41.7 ± 1.5% vs. 10.6 ± 2.0% control, $p < 0.05$; Figure 3(a-b)). Moreover, spindle lengths of Rab24-KD oocytes were significantly longer than controls (38.33 ± 2.52% vs. 22.33 ± 1.53% control, $p < 0.05$; Figure 3(c)). To further analyze the role of Rab24 during chromosome separation, control and Rab24-KD cells at anaphase/telophase were immunolabeled and observed under a confocal microscope. The percentage of lagging chromosomes was dramatically increased in Rab24-KD group compared to control (20.7 ± 3.1% vs. 7.3 ± 1.5% control, $p < 0.05$; Figure 3(d-e), arrowheads). These observations indicate that Rab24 is required for proper organization of meiotic apparatus and chromosome segregation.

### *Rab24 depletion disrupts K-MT attachments*

Chromosome alignment and segregation in cell division require a proper interaction between spindle microtubule and kinetochore, which is a proteinaceous complex that gathers at the centromeric regions of chromosomes [21,22]. The unusual spindle/chromosome defects in Rab24-KD oocytes may possibly result from disrupted kinetochore–microtubule (K-MT) interaction. To assess the attachment of microtubules to kinetochores, metaphase oocytes were immunolabeled using CREST serum (kinetochore, pink) and anti-tubulin antibody (microtubule, green). Hoechst 33,342 was used to detect DNA (blue). Among control oocytes, amphitelic attachment is the predominant form of K-MT interaction (kinetochores attached to the opposite pole; Figure 4aa). However, we found that in Rab24-

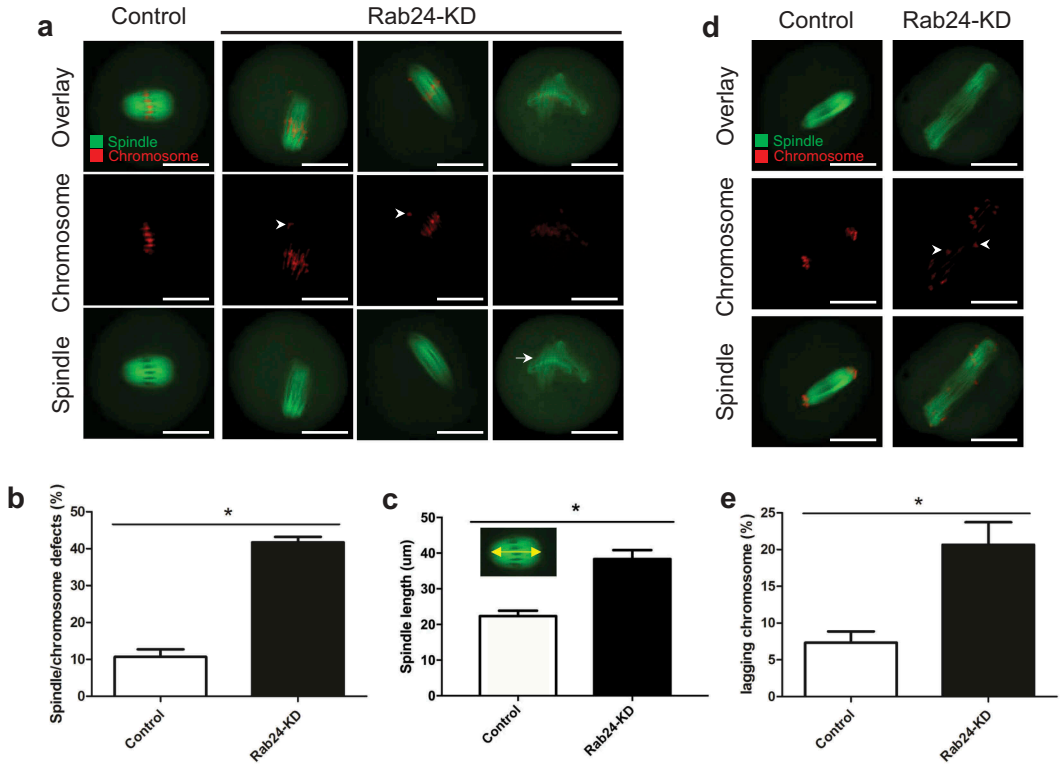


**Figure 3.** Effects of Rab24 knockdown on meiotic apparatus in mouse oocytes.

Oocytes at metaphase and anaphase were stained with α-tubulin antibody to visualize spindle (green) and counterstained with PI to visualize chromosomes (red). (a) The control metaphase oocyte show a typical barrel-shape spindle and well-aligned chromosomes. Spindle defects (arrow) and chromosome misalignment (arrowheads) were frequently observed in Rab24-KD oocytes. (b) Quantification of control and Rab24-KD oocytes with spindle/chromosome abnormalities. (c) Quantification of spindle lengths in control and Rab24-KD oocytes. (d) Chromosome separation in control and Rab24-KD oocytes during anaphase/telophase stage. Arrowheads indicate the lagging chromosomes. (e) Quantification of control and Rab24-KD oocytes with lagging chromosomes. Data are expressed as mean percentage ± SD from three independent experiments in which at least 60 oocytes were analyzed. *$p < 0.05$ vs. control. Scale bar, 25 μm.

depleted oocytes the proportion of K-MT mis-attachment, such as monotelic attachment (one kinetochore attached while another not; Figure 4ab), merotelic/lateral attachment (one kinetochore attached to both poles; Figure 4ac) and loss attachment (kinetochore attached to neither of poles; Figure 4ad), was significantly increased (Figure 4(b)). K-MT attachment errors give rise to the failure of chromosome biorientation, which could contribute to the spindle/chromosome abnormalities in Rab24-KD oocytes. These findings indicate that Rab24 depletion disturbs K-MT attachments, which could induce the failure of chromosome alignment observed in our experiments.

### *Loss of Rab24 increases incidence of aneuploid egg*

Aneuploidy is a chromosome abnormality in which the chromosome number is not a multiple of the haploid number [23]. Errors in meiotic chromosome segregation occur frequently in mammalian oocytes, could result in the production of aneuploid eggs [9]. It can therefore be assumed that the spindle defects and chromosome misalignments in Rab24-KD cells would result in the generation of aneuploid eggs. Chromosome spreading was performed to analyze the karyotype of MII oocytes. Representative images of euploidy and aneuploidy are shown in Figure 5(a). Aneuploidy was observed in about 50% of Rab24-depletion oocytes, which was greatly higher than that of control oocytes (49.3 ± 2.1% vs. 10.3 ± 1.5% control, $p < 0.05$; Figure 5b). These results suggest that depletion of Rab24 in mouse oocytes could lead to the production of aneuploid eggs.

## Discussion

Rab GTPases are common regulators of intracellular membrane transport systems. Here we report

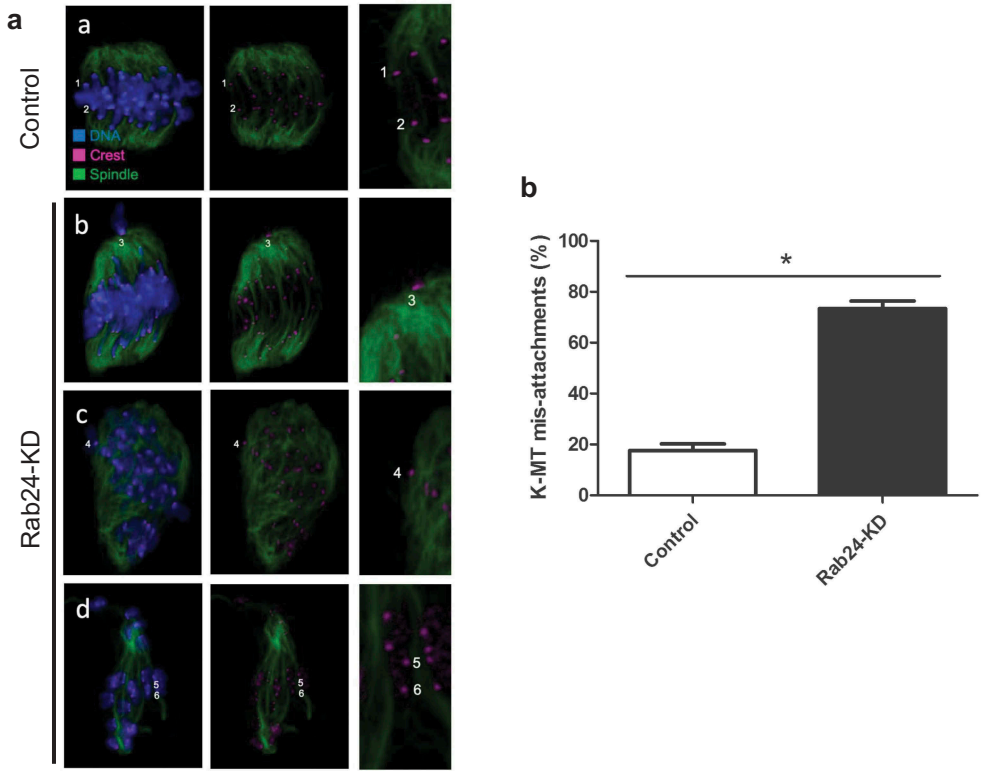


**Figure 4.** Effects of Rab24 knockdown on kinetochore-microtubule attachments during oocyte meiosis.
(a) Control and Rab24-KD metaphase oocytes were labeled with Hoechst 33,342 for chromosome (blue), CREST for kinetochores (pink) and anti-tubulin antibody for microtubules (green). Chromosomes 1 and 2 represent amphitelic attachment, chromosome 3 represents monotelic attachment, chromosome 4 represents merotelic/lateral attachment, chromosomes 5 and 6 represent lost attachment. (b) Quantification of control (n = 45) and Rab24-depleted (n = 40) oocytes with K-MT mis-attachments. The graph shows the mean ± SD of results obtained in three independent experiments. *p < 0.05 vs. control.

a novel role for Rab24 in mouse oocytes: the involvement of chromosome/spindle assembly and meiotic maturation.

Rab24 was first characterized as a perinuclear protein, which interacted with Golgi markers and late endosome structures [24]. Rab24 also combined with autophagic protein LC3 in CHO cells in face of starvation, which suggests a role for Rab24 in the autophagic pathway [25]. Furthermore, Rab24 plays a part in the degradation of misfolded cellular proteins or trafficking of proteins to the nuclear envelope [26]. It has been found that Rab24 locates at the mitotic spindle in metaphase, at the midbody during telophase and in the furrow during cytokinesis [20]. However, the distribution of Rab24 in meiotic oocytes has not been reported previously. In this paper, we show that Rab24 undergoes dynamic localization changes during oocyte meiotic progression (Figure 1). Our further analysis revealed the failure of meiotic resumption and Pb1 extrusion of oocytes when Rab24 protein was knockdown. In particular, symmetrical "2-cell-like" eggs were frequently observed in Rab24-KD groups (Figure 2), indicating Rab24 is essential for meiotic division. Stable biorientation of chromosomes should be established to achieve proper segregation during cell division. Chromosome alignment and segregation depends on the interaction between spindle microtubules and kinetochores [27,28]. Disturbance in this specific chromosome movement in meiotic division leads to the generation of aneuploid eggs, thus cause miscarriage in humans [8]. In line with this conception, we found that Rab24 knockdown causes an alteration of K-MT interactions (Figure 4), thus results in the failure of meiotic apparatus assembly and chromosome segregation (Figure 3). In addition, a high frequency of aneuploidy production was detected when Rab24 was knocked down (Figure 5).

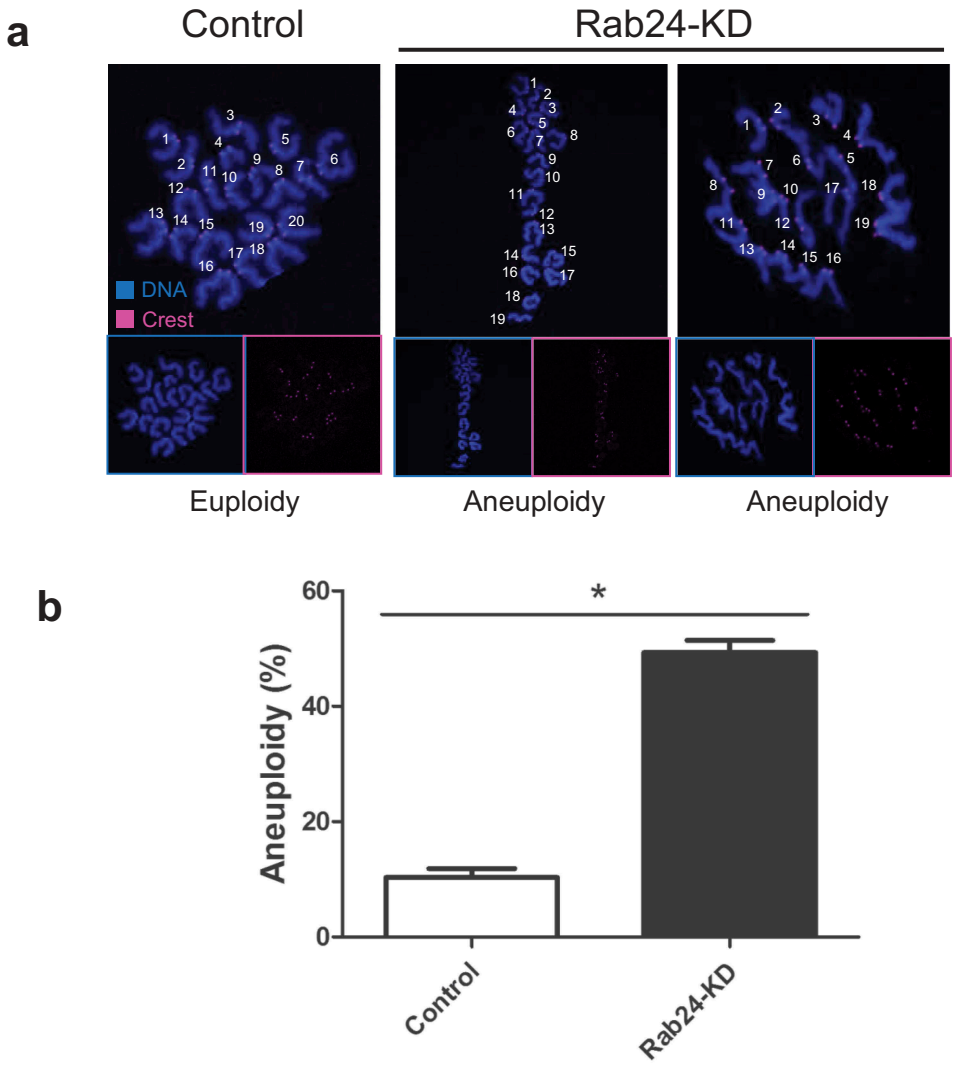


**Figure 5.** Rab24 knockdown increases the incidence of aneuploidy in mouse oocytes.

(a) Chromosome spread of control and Rab24-KD metaphase II oocytes. Chromosomes were stained with Hoechst 33,342 (blue) and kinetochores were labeled with CREST (pink). Representative confocal images showing the euploid control oocyte, and aneuploid Rab24-KD oocytes. (b) Histogram shows the incidence of aneuploidy in control (n = 45) and Rab24-depleted (n = 40) oocytes. Data are expressed as mean percentage ± SD from three independent experiments. *p < 0.05 vs. control.

However, further study is needed to elucidate how Rab24 modulates meiotic apparatus organization at the molecular level during oocyte meiosis.

Depletion of Rab24 results in the high frequency of lagging chromosomes during telophase and failures in cytokinesis, suggesting that Rab24 is necessary for normal cell division, perhaps through its interaction with microtubules [20]. Survivin, a member of the chromosomal passenger complex, is crucial for proper chromosome segregation and cell cycle control through its interaction with several elements of mitotic apparatus, such as centrosomes, microtubules, and midbodies [29–31]. It has recently been revealed a co-localization of Rab24 and survivin at prophase, indicating that survivin may be the potential effector of Rab24 mediating K-MT attachments [14,20]. Rab5 is crucial for proper chromosome congression in mitosis through its interaction with CENP-F and Lamin [32,33]. Lambright *et al.* have noted the association between Rabenosyn-5 (a Rab5 effector) and Rab24 [34]. It is plausible to speculate that Rab24 might participate in the degradation of nuclear lamin or the regulation of CENP-F, thus affecting meiotic resumption and polar body emission.

In summary, our work uncovers an unexpected role for a Rab GTPase during meiosis: depletion of Rab24 protein in oocytes compromises the

interaction between kinetochore and microtubule, which in turn causes spindle/chromosome defects, inducing the production of aneuploid eggs.

## Materials and methods

Chemicals were obtained from Sigma unless otherwise noted. All experiments were authorized by the Animal Care and Use Committee of Guangdong Second Provincial General Hospital and were performed in accordance with institutional guidelines.

## Antibodies

The following antibodies were used in this study: mouse monoclonal anti-Rab24 antibody (Cat#: MA5-26,188, Thermo Fisher Scientific); goat anti-mouse IgG secondary antibody conjugated Invitrogen Alexa Fluor Plus 488 (Cat#: A32723, Thermo Fisher Scientific); mouse monoclonal FITC-conjugated anti-α-tubulin antibody (Cat#: F2168, Sigma); propidium iodide (PI) (Cat#: P4170, Sigma); Hoechst 33,342 (Cat#: B2261, Sigma); human anti-centromere CREST antibody (Cat#: 15–234, Antibodies Incorporated); Cy5-conjugated donkey anti-human IgG (Cat#: 709–605-149, Jackson ImmunoResearch Laboratory).

## Oocyte collection and culture

To retrieve fully grown GV oocytes, 6- to 8-week-old ICR female mice were given 5 IU pregnant mare serum gonadotropin (PMSG) intraperitoneally; 46–48 h after PMSG treatment, cumulus-enclosed oocytes were harvested from ovaries by puncturing antral follicles with a sterile needle in M16 medium. Denuded oocytes were isolated by repeatedly pipetting through a narrow-bore glass pipette. Oocytes were transferred into new M16 medium, covered with mineral oil, and matured in a stable environment with saturated humidity, 5% CO2 and 95% air at 37°C.

## Rab24 knockdown experiment

Microinjections were performed using a Narishige microinjector; 2.5 pl Rab24 siRNA (1 mM) was microinjected into the cytoplasm of GV-stage oocytes. Negative siRNA was injected as control. Milrinone was added to M16 medium to prevent spontaneous meiotic resumption. After injections, oocytes were arrested at GV stage for 20 h to facilitate the degradation of Rab24 mRNA, and then transferred to milrinone-free medium for maturation.

Rab24-siRNA sequence:
5′- –3′: CCAGUGGAAUUAGAUGAAUTT
5′- –3′: AUUCAUCUAAUUCCACUGGTT
Control siRNA sequence:
5′- –3′: UUCUCCGAACGUGUCACGUTT
5′- –3′: ACGUGACACGUUCGGAGAATT

## Immunofluorescence

All operations were performed at room temperature unless otherwise stated. Oocytes were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100 followed by blocking in 1% BSA. All samples were incubated overnight at 4°C with antibody against Rab24. Tubulins were labeled with FITC-conjugated anti-tubulin antibody. To evaluate kinetochores, oocytes were co-stained with CREST (1:500) for two nights. Chromosomes were visualized by staining with propidium iodide (1:350) or Hoechst 33,342 (1:300). Samples were mounted on anti-fade medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). Fluorescence was detected by LSM710 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

## Western blotting

Samples were subjected to 10% SDS–polyacrylamide gels prior to being transferred to a PVDF membrane. Membranes were pretreated with 5% non-fat dry milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for blocking and then probed with primary antibodies overnight at 4°C (Rab24 antibody, 1:1,000; Tubulin antibody, 1:2,000). After being incubated with HRP-conjugated secondary antibodies for 10 min, the protein bands were scanned using an ECL Plus Western Blotting Detection System (GE Healthcare, Little Chalfont, UK).

## Chromosome spread

MII oocytes were treated with Tyrode's buffer (pH 2.5) for 1 min at 37°C to remove zona pellucida.

Samples were then fixed in 1% paraformaldehyde with 0.15% Triton X-100 followed by air drying for 1 h. Kinetochores were labeled with CREST and chromosomes were detected using Hoechst 33,342. Laser scanning confocal microscope was used to assess chromosome numbers in oocytes.

## Statistical analysis

Data are presented as means ± SD. Statistical comparisons were made with Student's t-test using Prism 5 software (GraphPad, San Diego, CA, USA). Results were considered to be significant when $p < 0.05$.

## Author contributions

DQ and XO designed research; DQ, SL, GL, and RY performed research; DQ and XO analyzed data; DQ and XO wrote the paper.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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