

Deficiency of *Pdk1* contributes to primordial follicle activation via the upregulation of YAP expression and the pro-inflammatory response

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Abstract. The molecular mechanisms underlying the activation of primordial follicles are poorly understood. The serine/threonine protein kinase phosphoinositide-dependent kinase 1 (PDK1), a pivotal downstream effector of phosphatidylinositol-3 kinase (PI3K) signaling, plays a vital role in cellular signaling. In order to identify the function of PDK1 in ovarian follicle development, this study used conditional *Pdk1* deletion in mouse oocytes by crossing *Pdk1*^{loxP/loxP} mice with transgenic mice carrying Gdf-9 promoter-mediated Cre recombinase and found that *Pdk1*^{flx/flx}*Gdf9Cre* mice were subfertile with increased serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels compared with *Pdk1*^{flx/flx} mice. The deletion of *Pdk1* in oocytes induced massive primordial follicle activation, leading to premature ovarian failure (POF). Further investigation revealed that enhanced Yes-associated protein (YAP) expression and an increased pro-inflammatory response also contributed to massive primordial follicle activation. PDK1 formed the complex with the core kinases of Hippo signaling and regulated the expression levels of YAP. On the whole, the findings of the present study demonstrate that PDK1 serves as an indispensable gatekeeper for maintaining the primordial follicle pool and provide a deeper understanding of POF treatment.

Introduction

Ovaries are female reproductive organs that produce hormones and release ovum. Follicles, the components of the ovary and the pool of primordial follicles, determine the ovarian reserve (1-3). The activity of primordial follicles is highly selective and the majority remain in a quiescent state, determining the female reproductive lifespan. A small number of primordial follicles are activated and develop into growing follicles, while the majority of primordial follicles remain in a quiescent state. When the pool of primordial follicles is exhausted, menopause occurs. Different types of pathological factors can cause the accelerated loss of primordial follicles and the development of premature ovarian failure (POF) (2,4). However, the molecular mechanisms controlling the activation of primordial follicles remain unknown.

In recent years, an increasing number of studies have focused on genetic factors to further investigate premature follicle activation. The ablation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is a negative regulator of phosphatidylinositol 3 kinase (PI3K) in oocytes, has been shown to result in the excessive activation of primordial follicles maintaining them in a dormant state (5). Phosphoinositide-dependent kinase 1 (PDK1) plays a vital role in regulating cell signaling between PI3K and serine/threonine kinase (5). The deletion of *Pdk1* in mouse oocytes has been shown to result in accelerated ovarian aging and premature ovarian failure (POF), due to the compromised lifespan of primordial follicles, which in turn is caused by suppressed PDK1/Akt/S6K1/ ribosomal protein S6 (rpS6) signaling in oocytes (6).

The Hippo signaling pathway controls organ size in animals through the regulation of cell proliferation and apoptosis (7,8). Yes-associated protein (YAP) is one of the key Hippo signaling effectors. When Hippo signaling is disrupted, YAP phosphorylation is decreased, with an increase in YAP nuclear levels (8-10). It has been reported that the Hippo signaling pathway is involved in follicle development (11). The disruption of Hippo signaling may stimulate follicle regeneration and may be used to treat premature ovarian insufficiency through the stimulation of the Akt pathway (12).

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Abbreviations: PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; FSH, follicle-stimulating hormone; LH, luteinizing hormone; YAP, Yes-associated protein; POF, premature ovarian failure; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PGs, prostaglandins; COX, cyclooxygenase

Key words: female fertility, PDK1, ovary, YAP, pro-inflammatory response

Inflammation plays a role in weakening and eventually rupturing the follicle wall, affecting ovulation (13,14). Produced during folliculogenesis, pro-inflammatory cytokines participate in the induction of ovulation. Interleukin (IL)-1 β and tumor necrosis factor α (TNF- α) are vital for follicular growth and oocyte maturation (14). Present in follicular fluid, IL-8 contributes to neutrophil migration (15). It has been reported that nuclear factor- κ B (NF- κ B) also plays a role in causing POF in diabetic rats (16). Chronic inflammation can lead to impaired oocyte quality and an abnormal ovarian physiology.

The aim of the present study was to examine the physiological functions of *Pdk1* in the ovaries of *Pdk1^{flx/flx}Gdf9Cre* mice. PDK1 signaling in oocytes appears to be crucial in maintaining primordial follicle activation, which in turn is vital for determining the duration of fertility, indicating that the deletion of *Pdk1* in oocytes may lead to the onset of ovarian senescence.

Materials and methods

Mice. *Pdk1^{loxP/loxP}* mice with an ICR genomic background were crossed with transgenic mice carrying Gdf-9 promoter-mediated Cre recombinase that also had an ICR background. All the mice were obtained from Jackson Laboratory. There were a total of 100 *Pdk1^{flx/flx}Gdf9Cre* mice. Following multiple rounds of crossing, homozygous mutant female mice we obtained lacking *Pdk1* in oocytes (*Pdk1^{flx/flx}Gdf9Cre*) (weight, 20-30 g; age, 7 days, n=20; 14 days, n=10; 21 days, n=15; 8 weeks, n=10; 12 weeks, n=5; 16 weeks, n=5, 20 weeks, n=5; 24 weeks, n=5). The *Pdk1^{flx/flx}Gdf9Cre* mice exhibited a normal phenotype. Control mice that do not carry the Cre transgene are referred to as *Pdk1^{flx/flx}* mice. The control mice were obtained from Jackson Laboratory and there were 100 *Pdk1^{flx/flx}* mice. The mice were housed under controlled environmental conditions with free access to water and food. All mice were euthanized with carbon dioxide and cervical dislocation was performed to confirm death. The mice were euthanized using 30% volume per minute displacement rates of 100% CO₂. All animal experimental procedures were approved by the Committee on the Use of Live Animals in Teaching and Research of Harbin Medical University. All mice were housed in cages in a constant environment with 55±10% humidity, a temperature of 20±5°C and a 12-h light/dark cycle. All mice exhibited a normal weight and behavior during the feeding period. The mice were sacrificed by cervical dislocation. Ovaries were fixed either with neutral formaldehyde or rapidly frozen in liquid nitrogen.

Antibodies. The antibodies used in the experiments were purchased from the following companies: Rabbit monoclonal anti-YAP (#14074), anti-phosphorylated (p)-YAP (S127, #13008), rabbit monoclonal anti-PDK1 (#5662), rabbit monoclonal TNF- α (#5178), rabbit monoclonal NF- κ B (#8242), rabbit polyclonal histone (#7631), rabbit monoclonal salvador family WW domain containing protein 1 (SAV1; #13301), rabbit monoclonal macrophage stimulating 1 (MST1; #14946), rabbit monoclonal large tumor suppressor kinase 1 (LATS1; #3477), mouse monoclonal anti-GAPDH (#5174) (all from Cell Signaling Technology). Secondary rabbit antibodies (#0114) were purchased from ZhongShan Golden Bridge Biotechnology Co., Ltd.

Histological analysis and quantification of ovarian follicles. The ovaries used for histological analysis were fixed in 4% paraformaldehyde (pH 7.5) overnight at 4°C, dehydrated and embedded in paraffin. Paraffin-embedded ovaries were serially sectioned at 8 μ m and every 5th section was mounted on slides. Hematoxylin and eosin staining was performed to identify the morphology of ovaries. Ovarian follicles at different developmental stages, including primordial, primary, early secondary, late secondary and antral follicles were counted in the collected sections of an ovary, based on the well-accepted standards established by Peterson and Peters under a microscope (DP25, Olympus) (17). In each section, only those follicles in which the nucleus of the oocyte was clearly visible were scored and the cumulative follicle counts were multiplied by a correction factor of 5 to represent the estimated number of total follicles in an ovary.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the oocytes or ovaries using TRIzol reagent (Invitrogen; Thermo Fisher Scientific). RNA was reverse transcribed into complementary DNA (cDNA) using oligo(dT) and the ImProm-II Reverse Transcription System (Promega). qPCR was conducted using the SYBR-Green PCR reagent (Life Technology; Thermo Fisher Scientific) and StepOnePlus System (Applied Biosystems). The mRNA expression of target genes was normalized to GAPDH. The primer sequences are listed as Table I. The PCR conditions for reverse transcription were as follows: 65°C, 5 min; 4°C, 5 min; 37°C, 50 min; 70°C, 15 min; 4°C, 30 min. The fragment size for each primer was as follows: Cellular communication network factor 1 (CCN1, also known as and referred to herein as CYR61), 109 bp; cellular communication network factor 2 (CCN2, also known as and referred to herein as CTGF), 151 bp; ankyrin repeat domain 1 (ANKRD1), 108 bp; IL-1 β and IL-6, 81 bp; PDK1, 110 bp; TNF α , 684 bp; and GAPDH, 123 bp. The cycling conditions for qPCR were as follows: 95°C, 10 min, 1 cycle; 95°C, 15 sec, 60°C, 1 min, 40 cycles. Data analyses were performed using the comparative Cq ($\Delta\Delta Cq$) method for calculating relative gene expression (18).

Western blot analysis. Oocytes or ovary lysate were prepared from minced ovaries following the removal of suspended granulosa cells by centrifugation (300 x g at 4°C for 5 min) for western blot analysis. The total protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). A total of 30 μ g ovary protein or 200 oocytes were mixed with SDS sample buffer and boiled for 5 min at 100°C for SDS-PAGE. Equal quantities of protein (40 μ g/lane) were resolved by 10% SDS-PAGE, followed by transferred onto polyvinylidene difluoride (PVDF) membranes with a transfer apparatus (Bio-Rad Laboratories, Inc.). The PVDF membranes were incubated in 5% non-fat milk containing Tris-buffered saline containing Tween-20 (TBST) for 3 h at room temperature. The PVDF membranes were sequentially incubated with primary antibodies against PDK1 (cat. no. 5662), phosphorylated (p)-YAP (S127, #13008), YAP (cat. no. 14074), TNF- α (cat. no. 5178), NF- κ B p50 (cat. no. 8242), histone (cat. no. 7631), SAV1 (cat. no. 13301), MST1 (cat. no. 14946), LATS1 (cat. no. 3477) (all from Cell

Table I. List of oligonucleotides used for RT-qPCR.

Prime	Sequence (5'-3')
CYR61	Forward: CTGCGCTAAACAACCTCAACGA Reverse: GCAGATCCCTTTTCAGAGCGG
CTGF	Forward: GGGCCTCTTCTGCGATTTC Reverse: ATCCAGGCAAGTGCATTGGTA
ANKRD1	Forward: GGATGTGCCGAGGTTTCTGAA Reverse: GTCCGTTTATACTCATCGCAGAC
IL-1 β	Forward: CTTTCATGGTCCGTGGTACCGCCCTGGAGTCT Reverse: TGCCCCAGGGCATGGCACCAATCACCCCTCTGAC
IL-6	Forward: GGTACATCCTCGACGGCATCT Reverse: GTGCCTCTTTGCTGCTTTCAC
IL-8	Forward: GTAGCGAAGGACCTGTCAAACATTGGAAGATCA Reverse: CAACTCAGTGACCTTGCCCTCCTCATGGTAATAC
PDK1	Forward: CTATGC TGTGTTACTTCTTGAGACACAG Reverse: TGCCGAATATCATGGTGGAAAATGGCCG
TNF- α	Forward: GCCTCTTCTCATTCTGCTTG Reverse: CTGATGAGAGGGAGGCCATT
GAPDH	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTC

CYR61, cellular communication network factor 1 (CCN1; also known as and referred to herein as CYR61); CTGF, cellular communication network factor 2 (CCN2, also known as and referred to herein as CTGF); and ANKRD1, ankyrin repeat domain 1; IL, interleukin; PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; YAP, Yes-associated protein; TNF, tumor necrosis factor.

Signaling Technology) and GAPDH (cat. no. AF0006; Shanghai Boyun Biotech Co., Ltd.) at a dilution of 1:1,000 overnight at 4°C. Following incubation with the primary antibodies, the membranes were washed in a TBST 4 times for 10 min and incubated with mouse anti-rabbit IgG (H&L)-horseradish peroxidase conjugate (1:1,500 dilution; cat. no. sc2357; Santa Cruz Biotechnology, Inc.) for 3 h at room temperature. The blots were visualized using Enhanced Chemiluminescence Western Blotting Luminol Reagent (GE Healthcare) and were semi-quantified using Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc.).

In vivo injections with YAP inhibitor. The PD7 *Pdk1^{flx/flx}Gdf9^{Cre}* mice were administered DMSO (vehicle) or 100 mg/kg verteporfin (VP), which is a potent YAP inhibitor, intraperitoneally at 3-day intervals for 14 days. A total of 10 mice were used with 5 mice in each group. Ovaries were collected and embedded in paraffin, and serial sections of 8 μ m thickness were prepared and stained with hematoxylin and eosin. The morphology of the ovaries was observed under a microscope (DP25, Olympus), and number of primordial follicles and activated follicles were counted.

Nuclear extraction. Nuclear protein extraction was performed according to the following protocol: Oocytes were lysed using buffer I containing 10 mmol/l HEPES, 10 mmol/l KCl, 1 μ mol/l EDTA, 1 μ mol/l EGTA, 15% Nonidet, protease and phosphatase inhibitors. Following centrifugation (12,000 \times g at 4°C for 15 min) the supernatant (cytosolic fraction) was

transferred to a new tube and stored or immediately used for western blot analysis. The remaining pellet containing the nuclear fraction was dissolved in 40 μ l buffer II consisting of 20 mmol/l HEPES, 400 mmol/l NaCl, 0.01 mol/l EDTA, 0.01 mol/l EGTA, 15% Nonidet, and protease and phosphatase inhibitors. Subsequently, this solution was sonicated 2 times for 5 sec at 50 Watts at 4°C. Following centrifugation (12,000 \times g at 4°C for 15 min) the supernatant containing the nuclear fraction was transferred to a new tube and stored at -80°C or was immediately used.

Co-immunoprecipitation. The protein concentration prepared from oocytes was measured using the BCA protein assay kit. Subsequently, IP was conducted using a Pierce Classic IP kit (cat. no. 26146; Pierce; Thermo Fisher Scientific, Inc.). For 1 mg lysate, 80 μ l Control Agarose Resin slurry was added to pre-clear the lysate. Subsequently, PDK1 (1:50, cat. no. 5662), SAV1 (1:50, cat. no. 13301), MST1 (1:50, cat. no. 14946), LAST1 (1:50, cat. no. 3477) (all from Cell Signaling Technology) and GAPDH (1:50, cat. no. AF0006; Shanghai Boyun Biotech Co., Ltd.) was added to the pre-cleared cell lysate in a microcentrifuge tube; the antibody/lysate solution was diluted to 300-600 μ l with PBS. Rabbit IgG was used as a negative control (1:50, cat. no. 2729; Cell Signaling Technology). The solution was incubated overnight at 4°C to form the immune complex in the spin column. To capture the immune complex, the antibody/lysate sample was added to Protein A/G Plus Agarose in the spin column (supplied in the IP kit), and the column was incubated with gentle end-over-end shaking for 1 h at 4°C. Subsequently, the sample

was centrifuged 100 x g for 1 min 4°C and the flow-through was saved. The spin column containing the resin was transferred into a new collection tube and 2X reducing sample buffer was added and incubated at 100°C for 5-10 min. The samples were further centrifuged 100 x g for 1 min 4°C to collect the eluate. Finally, the eluate and flow-through were separated by 10% SDS-PAGE and examined by western blot analysis.

Serum analysis. A mixture of 0.75 ml ketamine (150 mg/kg) and 0.25 ml xylazine (10 mg/kg) was prepared and diluted to 5 ml with sterile 1X PBS (19). Mice (20 g) were administered a 0.125 ml mixture for anesthesia via i.p. injection. Once the rodents no longer responded to an interdigital pinch, they were transferred to a temperature-controlled surgical plate. Blood (700 μ l) was collected via cardiac puncture. A total of 10 mice were used with 5 in each group. Mouse blood was centrifuged at 453 x g at 4°C for 10 min. The supernatants were collected and these serum samples were used for ELISA. ELISAs were performed according to the manufacturer's instructions for the mouse FSH ELISA kits (Westang Bio-Tech Co., Ltd.). The human Luteinizing Hormone ELISA kit was purchased from Abcam (ab108651, Abcam). Briefly, 100 μ l serum, along with mouse E2 standards (at concentrations of 8,000, 4,000, 2,000, 1,000, 500, 250 and 125 pg/ml) and mouse FSH standards (at concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 ng/ml), was added to pre-coated 96-well detection plates and incubated for 60 min at 37°C. The solution was discarded and the plates were washed 3 times with wash solution. Horseradish peroxidase-conjugated detection antibodies (dilution 1:500; ab205718, Abcam) were added and incubated for 60 min at 37°C. Subsequently, stop solution was added and the optical density of each well in the 96-well plate was measured at a wavelength of 450 nm.

Statistical analysis. The experiments were performed in 5 mice every time and repeated for at least 3 times. The reproducibility rate of this study is 90%. Statistical analysis of the experimental results was performed using GraphPad Prism 5 (GraphPad Software, Inc.), ImageJ 1.8.0 (National Institutes of Health), Image-Pro Plus 6, and SPSS 22.0 (IBM Corp.) statistical software. All data are expressed as the means \pm SEM. Differences among groups were analyzed by one-way analysis of variance followed by Tukey's post hoc test using SPSS 22.0 software. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Depletion of *Pdk1* in oocytes causes massive primordial follicle activation. To investigate the function of PDK1, a conditional knockout mice were generated by crossing *Pdk1^{flx/flx}* mice with transgenic mice expressing Gdf9 promoter-mediated Cre recombinase. *Gdf9Cre* is highly expressed in oocytes and causes the inactivation of *Pdk1* in primordial follicles and in later developmental stages. *Pdk1^{flx/flx}Gdf9Cre* mice were used to examine the effects of the conditional deficiency of *Pdk1* on primordial follicle activation. The successful depletion of *Pdk1* in the mouse oocytes was confirmed by RT-qPCR and western blot analysis (Fig. 1A and B). A breeding assay was then performed to examine the effects of *Pdk1* deletion on

female fertility. As shown in Fig. 1C, female *Pdk1^{flx/flx}Gdf9Cre* mice were significantly infertile compared with the wild type (*Pdk1^{flx/flx}* mice). In order to investigate the potential causes of the decreased fertility in the mutant mice, the levels of gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) level were measured in mouse serum, which may indicate ovary function. The levels of FSH and LH were significantly increased in the *Pdk1^{flx/flx}Gdf9Cre* mice, indicating that follicle growth may be accelerated (Fig. 1D and E). The morphology of the ovaries from the *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mice was also observed. It was found that at postnatal day (PD)7 and PD14, massive primordial follicles were activated, with an increased number of primary or secondary follicles (Fig. 2A-E). At PD7, the *Pdk1^{flx/flx}Gdf9Cre* mice had 201 \pm 43 secondary follicles compared to 89 \pm 22 in their counterparts. The number of primordial follicles was 1,817 \pm 58 in the control littermates and was reduced to 1,243 \pm 89 in the *Pdk1^{flx/flx}Gdf9Cre* mice (Fig. 2C).

At PD14, the *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries contained 257 \pm 75 primary follicles and 323 \pm 49 secondary follicles, compared to 103 \pm 10 and 156 \pm 25 the controls, respectively (Fig. 2D-F). The number of primordial follicles was significantly decreased from 1,453 \pm 164 in the counterparts to 900 \pm 48 in the *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries (Fig. 2F). At PD21, the secondary follicle number was increased along with a diminished number of antral follicles and the total number of follicles in the *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries (Fig. 2G-I). Almost all primordial follicles were activated by 2 months of age and only a small number of primordial follicles and growing follicles existed in the *Pdk1^{flx/flx}Gdf9Cre* mice, whereas most of the follicles in the control mouse ovaries were still at the primordial stage (Fig. 2J-L). The results of this study thus indicate that the deletion of *Pdk1* leads to massive primordial activation and causes POF.

YAP expression is upregulated in *Pdk1^{flx/flx}Gdf9Cre* mice. The activation of YAP is determined by the hypophosphorylation of YAP at the Ser127 residue, an increase in YAP and their target gene expression, and the nuclear localization of YAP. As a key mediator of chemoresistance in various types of cancer, it has been reported that the disruption of YAP expression also plays a role in the development of POF (12). To investigate whether YAP contributes to massive primordial follicle loss in *Pdk1^{flx/flx}Gdf9Cre* mice, YAP expression was examined by western blot analysis. The results demonstrated that there was a significant increase in YAP expression and a significant decrease in YAP phosphorylation in the ovaries of *Pdk1^{flx/flx}Gdf9Cre* mice (Fig. 3A). RT-qPCR analysis also revealed that the expression levels of YAP target genes, including *CYR61*, *CTGF* and *ANKRD1*, were significantly lower in the ovaries of *Pdk1^{flx/flx}Gdf9Cre* mice compared with the controls (Fig. 3B). These data thus indicated that YAP activity was enhanced in the *Pdk1^{flx/flx}Gdf9Cre* mice.

YAP activation promotes pro-inflammatory responses in *Pdk1^{flx/flx}Gdf9Cre* mouse oocytes. To investigate whether YAP mediates the pro-inflammatory responses in *Pdk1^{flx/flx}Gdf9Cre* mouse oocytes, the expression levels of inflammatory cytokine genes were examined by RT-qPCR and western blot analysis. It was observed that the expression of TNF- α was elevated

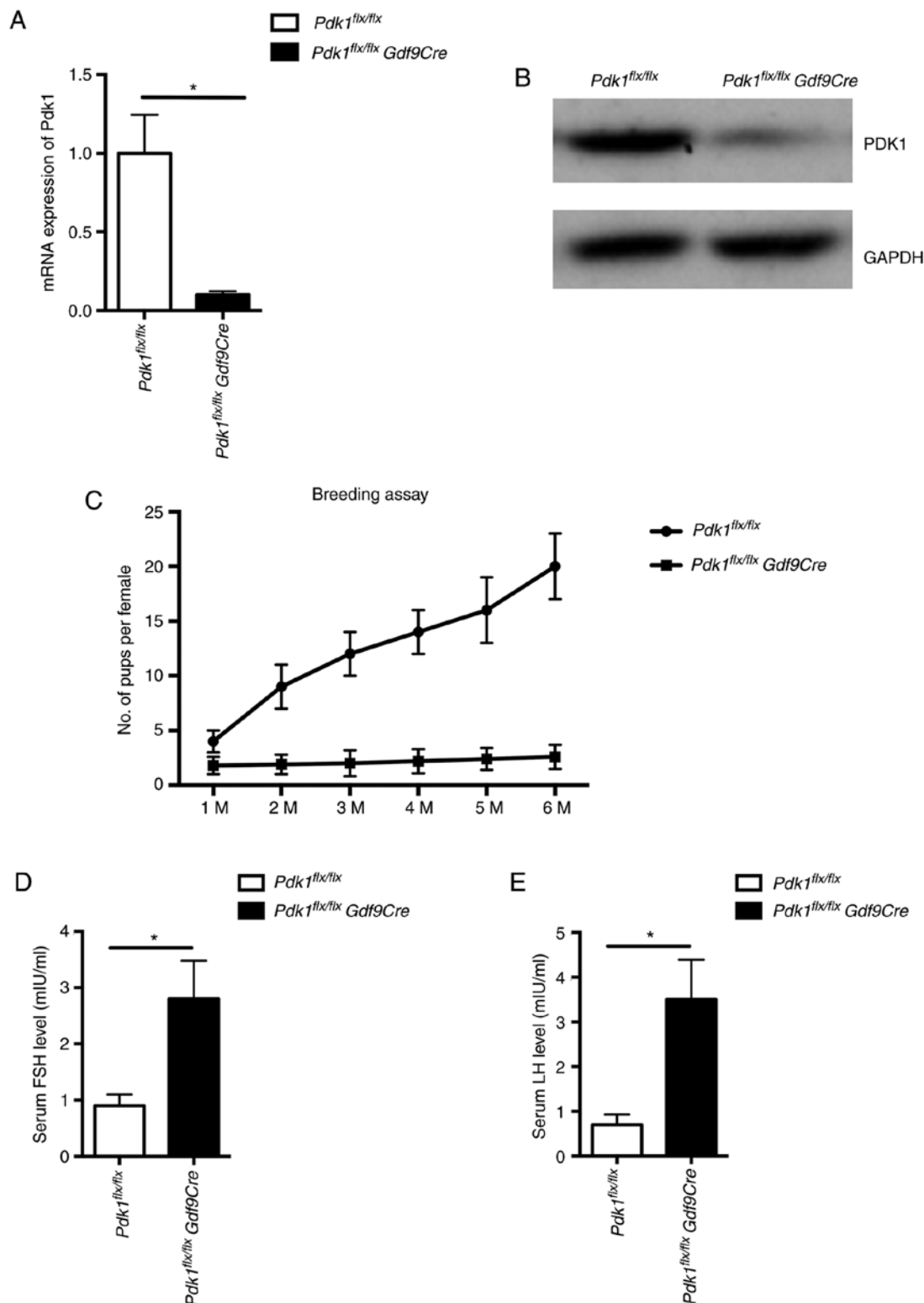


Figure 1. PDK1 is essential for female fertility. Oocytes were isolated from ovaries from 21-day-old *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mice. (A) *Pdk1* expression was examined by RT-qPCR and normalized to GAPDH. (B) Western blot analysis was performed to detect PDK1 and GAPDH protein levels. (C) Breeding assay was performed and the cumulative number of progeny/female in *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mice was compared. (D) Sera from 2-month-old *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mice were collected for the measurement of FSH. (E) Sera from 2-month-old *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mice were collected for the measurement of LH. n=5, *P<0.05 compared with control. PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

in the *Pdk1^{flx/flx}Gdf9Cre* mouse oocytes at both the mRNA and protein level (Fig. 4A and B). The mRNA expression of cytokines was also measured, and it was found that there was a significant elevation in the levels of IL-1 β , IL-6 and IL-8 in the

Pdk1^{flx/flx}Gdf9Cre mouse oocytes (Fig. 4C). Furthermore, to examine the association between *Pdk1* deletion and the activation of NF- κ B, the expression of NF- κ B p50 was examined at the protein level (Fig. 4D). The results revealed that the

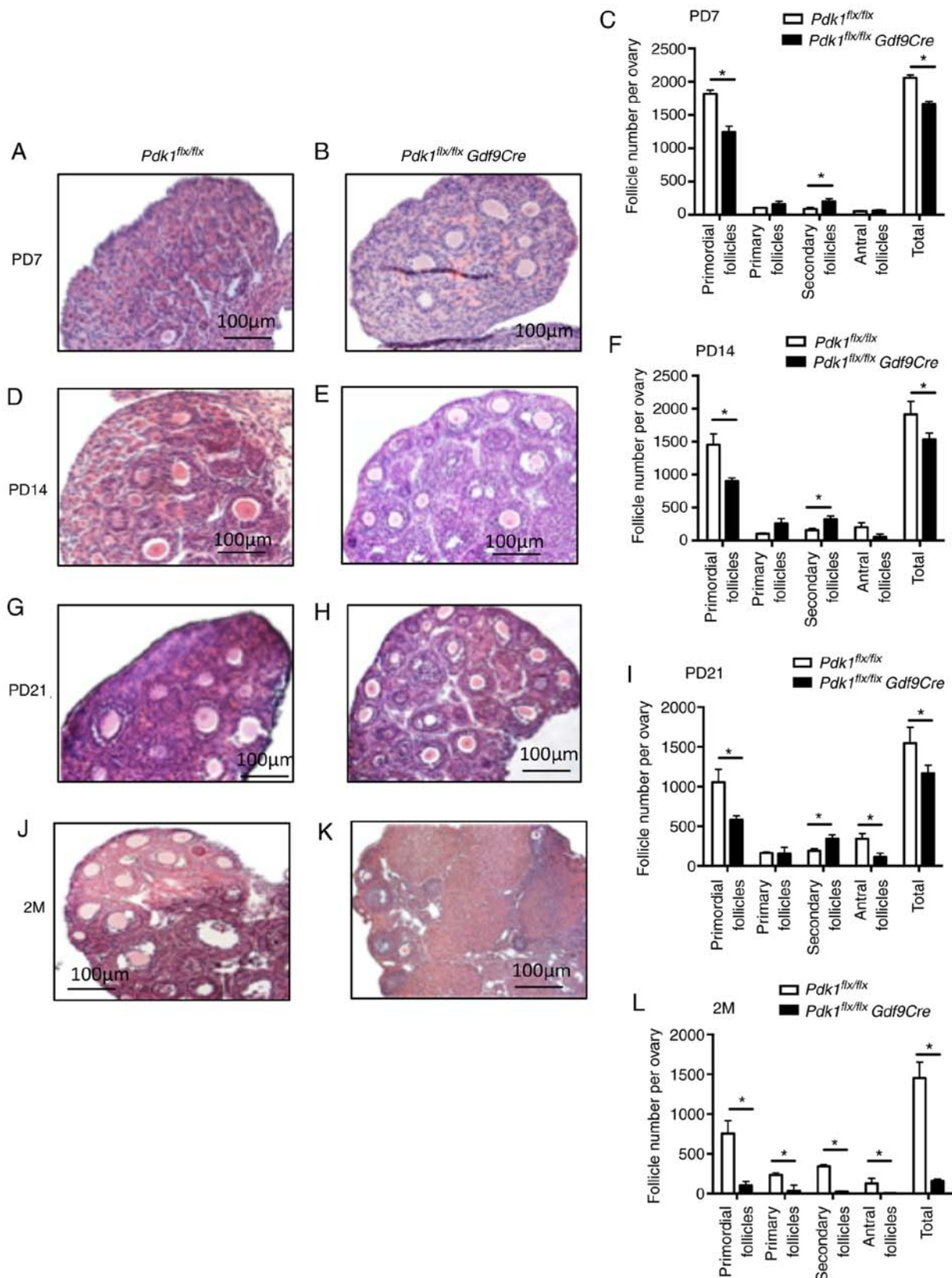


Figure 2. Depletion of *Pdk1* in oocytes causes massive primordial follicle activation. Ovaries from *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mice were embedded in paraffin, and serial sections of 8 μ m thickness were prepared and stained with hematoxylin and eosin. (A) Morphological analysis of ovaries from *Pdk1^{flx/flx}* littermates at PD7. (B) Morphological analysis of ovaries from *Pdk1^{flx/flx}Gdf9Cre* littermates at PD7. (C) Quantification of follicle numbers in ovaries of PD7 old *Pdk1^{flx/flx}* mice and their *Pdk1^{flx/flx}Gdf9Cre* littermates. (D) Morphological analysis of ovaries from *Pdk1^{flx/flx}* littermates at PD14. (E) Morphological analysis of ovaries from *Pdk1^{flx/flx}Gdf9Cre* littermates at PD14. (F) Quantification of follicle numbers in ovaries of PD14 old *Pdk1^{flx/flx}* mice and their *Pdk1^{flx/flx}Gdf9Cre* littermates. (G) Morphological analysis of ovaries from *Pdk1^{flx/flx}* littermates at PD21. (H) Morphological analysis of ovaries from *Pdk1^{flx/flx}Gdf9Cre* littermates at PD21. (I) Quantification of follicle numbers in ovaries of PD21 old *Pdk1^{flx/flx}* mice and their *Pdk1^{flx/flx}Gdf9Cre* littermates. (J) Morphological analysis of ovaries from *Pdk1^{flx/flx}* littermates at 2-month old. (K) Morphological analysis of ovaries from *Pdk1^{flx/flx}Gdf9Cre* littermates at 2-month old. (L) Quantification of follicle numbers in ovaries of 2-month old *Pdk1^{flx/flx}* mice and their *Pdk1^{flx/flx}Gdf9Cre* littermates. $n=5$, * $P<0.05$ compared with the control. PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; PD, postnatal day; M, months.

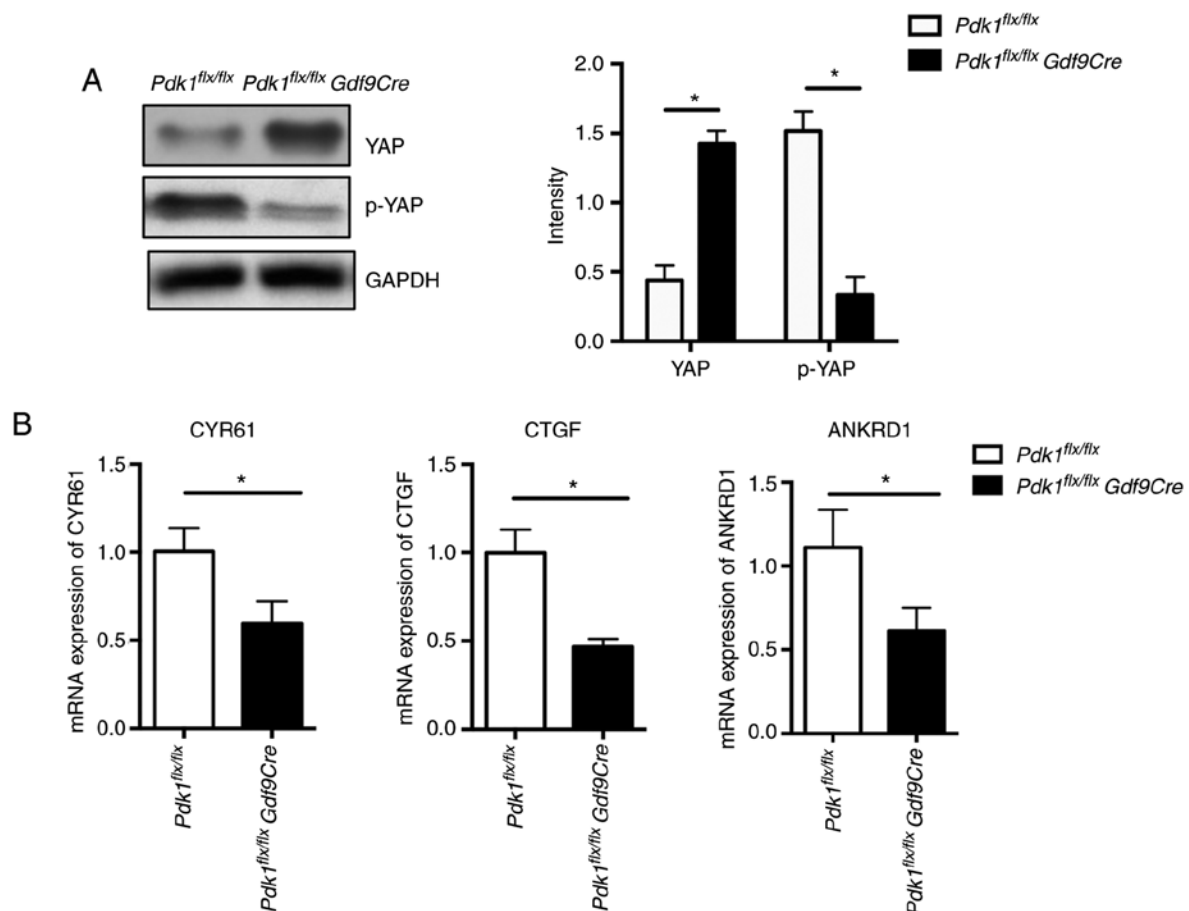


Figure 3. Depletion of *Pdk1* in oocytes enhances YAP expression. (A) Secondary and antral follicles were isolated from PD7 *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries, protein was extracted, and western blot analysis was performed using antibodies against YAP and phosphorylated YAP. GAPDH was used as a control. (B) Secondary and antral follicles were isolated from PD7 *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries and RNA was extracted for cDNA conversion and RT-qPCR. The mRNA expression of CYR61, CTGF and ANKRD1 was measured. Data were normalized to GAPDH expression and are presented as the mean relative quantity (compared with control). n=5, *P<0.05 compared with the control. PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; YAP, Yes-associated protein; CYR61, cellular communication network factor 1 (CCN1; also known as and referred to herein as CYR61); CTGF, cellular communication network factor 2 (CCN2, also known as and referred to herein as CTGF); and ANKRD1, ankyrin repeat domain 1.

expression of NF- κ B was upregulated in the *Pdk1^{flx/flx}Gdf9Cre* mouse oocytes compared with the wild-type control. Taken together, these results indicate that *Pdk1* deletion may induce inflammation in oocytes, which in turn contributes to POF.

PDK1 directly interacts with the Hippo complex. To investigate whether PDK1 is involved in forming the complex in Hippo signaling pathway to regulate YAP activity, co-immunoprecipitation with endogenous PDK1 in oocytes was performed. The results revealed that SAV1, MST1 and LATS1, co-immunoprecipitated with endogenous PDK1. The interaction of PDK1 with all of these components was normal in the *Pdk1^{flx/flx}* mouse oocytes with or without PDK1 inhibitor, while there was no detection of interaction in the *Pdk1^{flx/flx}Gdf9Cre* mouse oocytes (Fig. 5). This may be due to the reason that *Pdk1* deletion caused the disruption of interaction with the complex in Hippo signaling. The results demonstrated that SAV1, MST1 and LATS1, coimmunoprecipitated with endogenous PDK1 in the wild-type oocytes, suggesting that PDK1 is a component of the Hippo pathway complex.

Suppressed expression of YAP reverses the overactivation of primordial follicles in *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries.

In order to provide *in vivo* evidence that increased YAP activity accelerates the overactivation of primordial follicles in *Pdk1^{flx/flx}Gdf9Cre* mice, this study intraperitoneally injected PD7 *Pdk1^{flx/flx}Gdf9Cre* mice with VP, a potent YAP inhibitor, at 3-day intervals for 14 days. The mice were sacrificed, and ovarian morphology was examined.

It was indicated that in the PD21 *Pdk1^{flx/flx}Gdf9Cre* mice injected with VP, the expression of YAP was suppressed with an increased level of phosphorylated YAP (Fig. 6A). Typical primordial follicles were found in the *Pdk1^{flx/flx}Gdf9Cre* mice treated with VP, while in the ovaries of PD21 *Pdk1^{flx/flx}Gdf9Cre* mice that were treated with the vehicle (control), the majority of primordial follicles was activated (Fig. 6B). The quantification of the aforementioned results of follicle numbers revealed that the proportion of primordial follicles in the VP-treated *Pdk1^{flx/flx}Gdf9Cre* ovaries was upregulated by 60%, which was similar to its counterparts (61%) (Fig. 6C). These results demonstrated that increased YAP activity in oocytes was the major factor that activated the primordial follicle pool in *Pdk1^{flx/flx}Gdf9Cre* mice, suggesting that a low expression of YAP is indispensable for the preservation of primordial follicles in a dormant state.

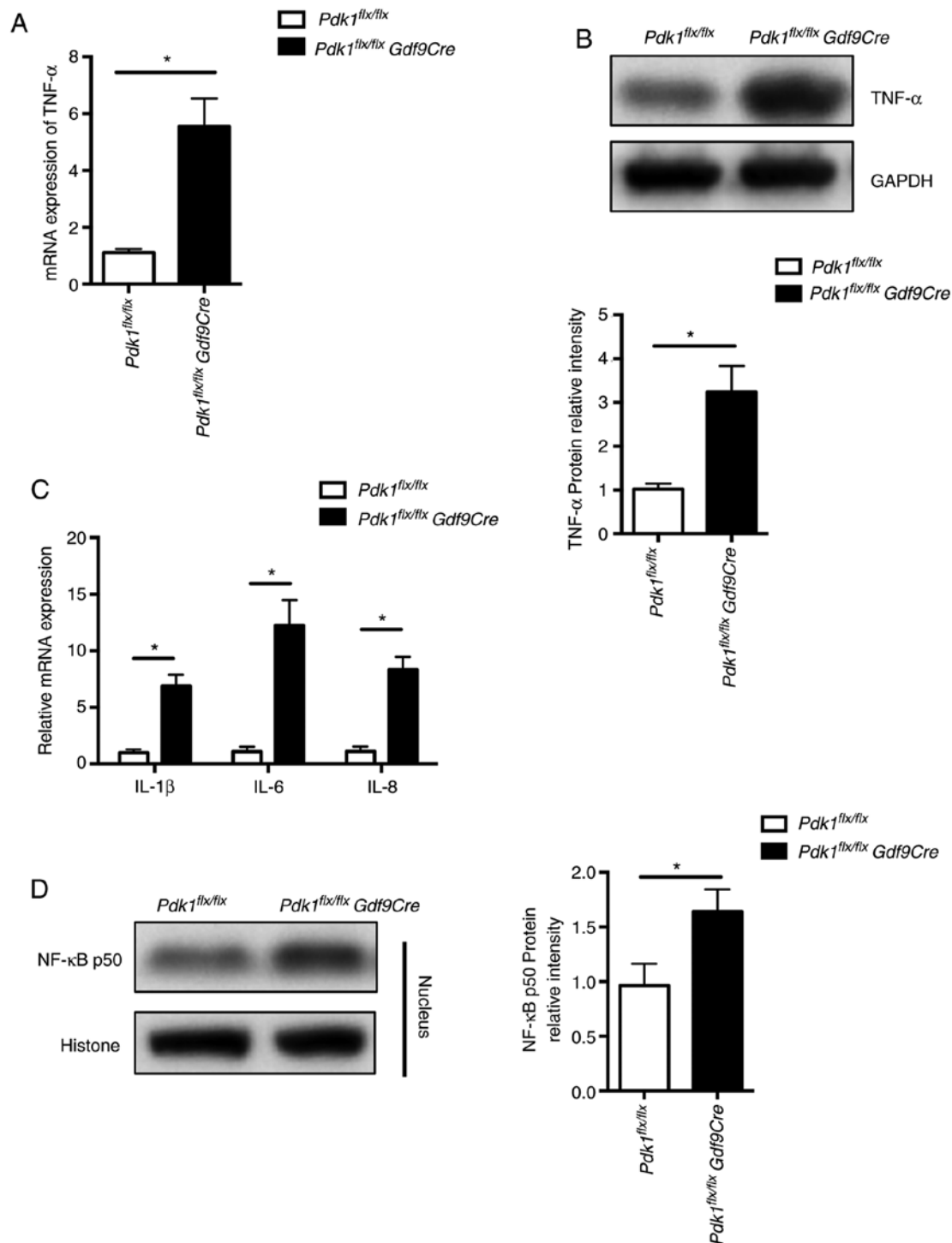


Figure 4. YAP activation promotes pro-inflammatory responses in *Pdk1^{flx/flx}Gdf9Cre* mouse oocytes. Oocytes were isolated from PD7 *Pdk1^{flx/flx}* and *Pdk1^{flx/flx}Gdf9Cre* ovaries, and protein was extracted. (A and C) Total RNA was analyzed by RT-qPCR to determine the mRNA expression profiles of TNF- α , IL-1 β , IL-6 and IL-8. The expression was normalized to GAPDH. (B) Protein from oocytes were subjected to western blot analysis for TNF- α analysis. The protein profiles were normalized to GAPDH. (D) The nuclear proteins of NF- κ B p50 were extracted and were subjected to western blot analysis. The protein profiles were normalized to histone. n=5, *P<0.05 vs. the control. PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; YAP, Yes-associated protein; TNF- α , tumor necrosis factor- α ; IL, interleukin.

Discussion

In this study, it was found that the PDK1-YAP signaling pathway plays an essential role in maintaining the survival of primordial follicles and the activation of growing follicles. The deletion of *Pdk1* contributed to an enhanced YAP expression and its cytoplasm-nucleus translocation, causing a

pro-inflammatory response, which was responsible for primordial follicle activation. Therefore, this study suggests that the maintenance of the primordial follicle pool is dependent on PDK1 in mouse ovaries (Fig. 7).

It has been reported that the deletion of *Pdk1* in oocytes can cause the accelerated loss of primordial follicles through rpS6 inactivation, which is vital for mediating ribosome biogenesis

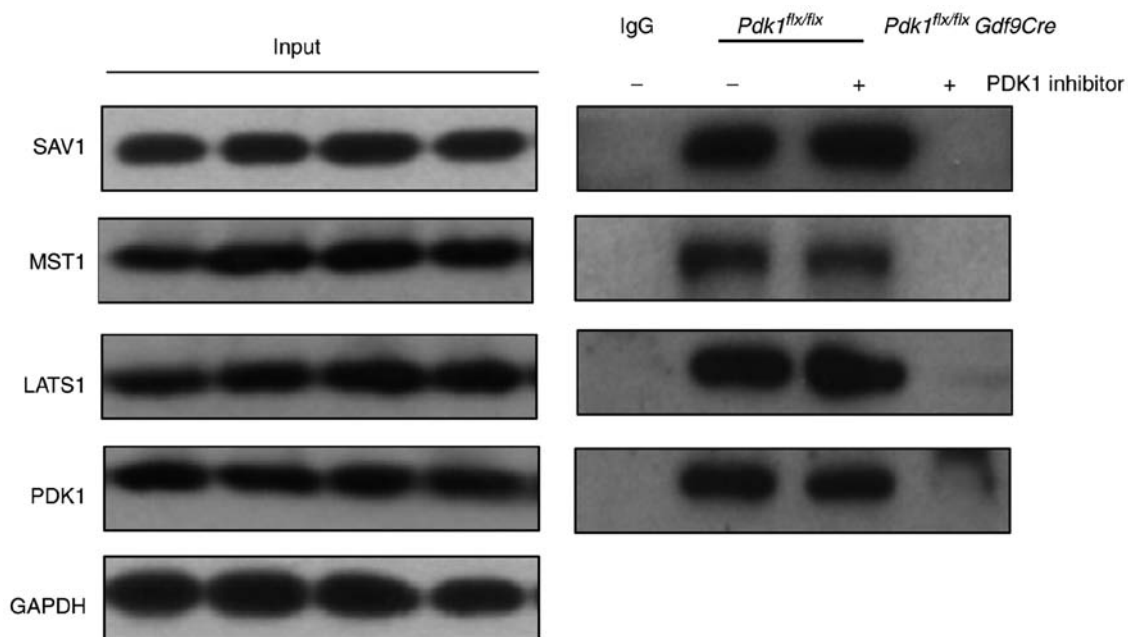


Figure 5. PDK1 directly interacts with the Hippo complex. Oocytes were isolated from PD7 *Pdk1*^{flx/flx} and *Pdk1*^{flx/flx}*Gdf9*^{Cre} mouse ovaries, and protein was extracted. Co-immunoprecipitation was performed in endogenous PDK1 binding to the Hippo complex proteins. The precipitated PDK1 immunocomplex input samples were subjected to western blot analysis with the indicated antibody. n=5. PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; SAV1, salvador family WW domain containing protein 1; MTS1, macrophage stimulating 1; LATS1, large tumor suppressor kinase 1.

and protein translation in oocytes (18). PTEN activity adjusts PI3K/PDK1 signaling in oocytes to an optimal level, so that the pool of primordial follicles can be maintained in a dormant and surviving condition. In this study, in our mouse model, it was found that the deletion of *Pdk1* in oocytes activated primordial follicles to transition into growing follicles. It was further indicated that the expression of YAP, which controls cell proliferation, was enhanced in *Pdk1* oocyte-specific knockout mice. The suppression of YAP in *Pdk1* knockout mice may maintain the number of primordial follicles compared with the vehicle control, indicating that YAP plays an essential role in regulating POF. The expression of the core components of Hippo pathway was altered with follicle development, and the trend of this alteration was consistent with that in follicles cultured *in vitro* in a recent study (19). Primordial follicle activation was accompanied by the attenuation of the Hippo pathway, therefore suggesting a close association of the Hippo pathway and primordial-to-primary transition.

Prostaglandins (PGs) may also serve as non-negligible factors that may contribute to premature follicle activation. PGs have been reported to be involved in female reproductive functions, including ovulation, fertilization and implantation (20). The cyclooxygenase (COX) isozymes, COX-1 and COX-2, are considered to be the primary producers of PGs (21). Oocytes are arrested at the germinal vesicle stage before the surge of pituitary luteinizing hormone, inducing epidermal growth factor-like growth factors, which can promote meiotic resumption and cumulus expansion by depositing via COX-2 expression (22). It has been reported that PGs can mediate inflammation by regulating YAP expression (23). PGs can also activate PKD1 expression to regulate cell proliferation (24).

The Hippo signaling pathway controls organ size in mammals by regulating apoptosis and cell proliferation (25). The nuclear accumulation and activation of YAP, the main transcriptional effector of the Hippo signaling pathway, is associated with

organ growth, the loss of contact inhibition, cellular proliferation and inhibition of apoptotic signals. Emerging studies have revealed the critical role of YAP in ovarian follicle development. In mammals, there are 4 core components of the Hippo pathway, which includes serine/threonine kinases MST1/2 (homologs of Hippo/Hpo), LATS1/2 (homologs of Warts/Wts), their adaptor proteins SAV1 (homolog of Salvador/Sav) and MOB (homologs of MATS) (26). It is indispensable to form the MST1, SAV1 and LATS1 complex for the Hippo signaling cascade. The disruption of the Hippo signaling pathway and the stimulation of Akt can preserve the ovarian follicles and may serve as a potential treatment option for infertile women (12). However, the underlying mechanisms through which Hippo signaling regulates follicle activation remain unclear. In this study, it was found that PDK1 coimmunoprecipitated with Hippo signaling kinases, including SAV1, MST1 and LATS1 in wild-type mouse oocytes. Whereas, in *Pdk1*^{flx/flx}*Gdf9*^{Cre} mouse oocytes, THE deletion of *Pdk1* caused the dissociation of kinase complex and the dephosphorylation and the nuclear translocation of YAP.

Inflammation serves a number of physiological roles in folliculogenesis, and mammalian ovulation has been reported to share similarities properties with the inflammatory response (27). IL-8, a leukocyte chemotactic and neutrophil-activating cytokine, is present in preovulatory follicles and plays a role in ovulation (27). IL-1 α , IL-6, IL-8 and TNF- α expression is increased in granulosa cells of antral follicles compared with dormant follicles (28). In this study, in our mouse model, there was a significant increase in the levels of IL-1 α , IL-6, IL-8 and TNF- α in *Pdk1*^{flx/flx}*Gdf9*^{Cre} mouse ovaries, indicating that an enhanced pro-inflammatory response contributed to premature follicle activation.

However, this study also presented with a number of limitations. PDK1 is one of the vital gates for the Akt/mTOR

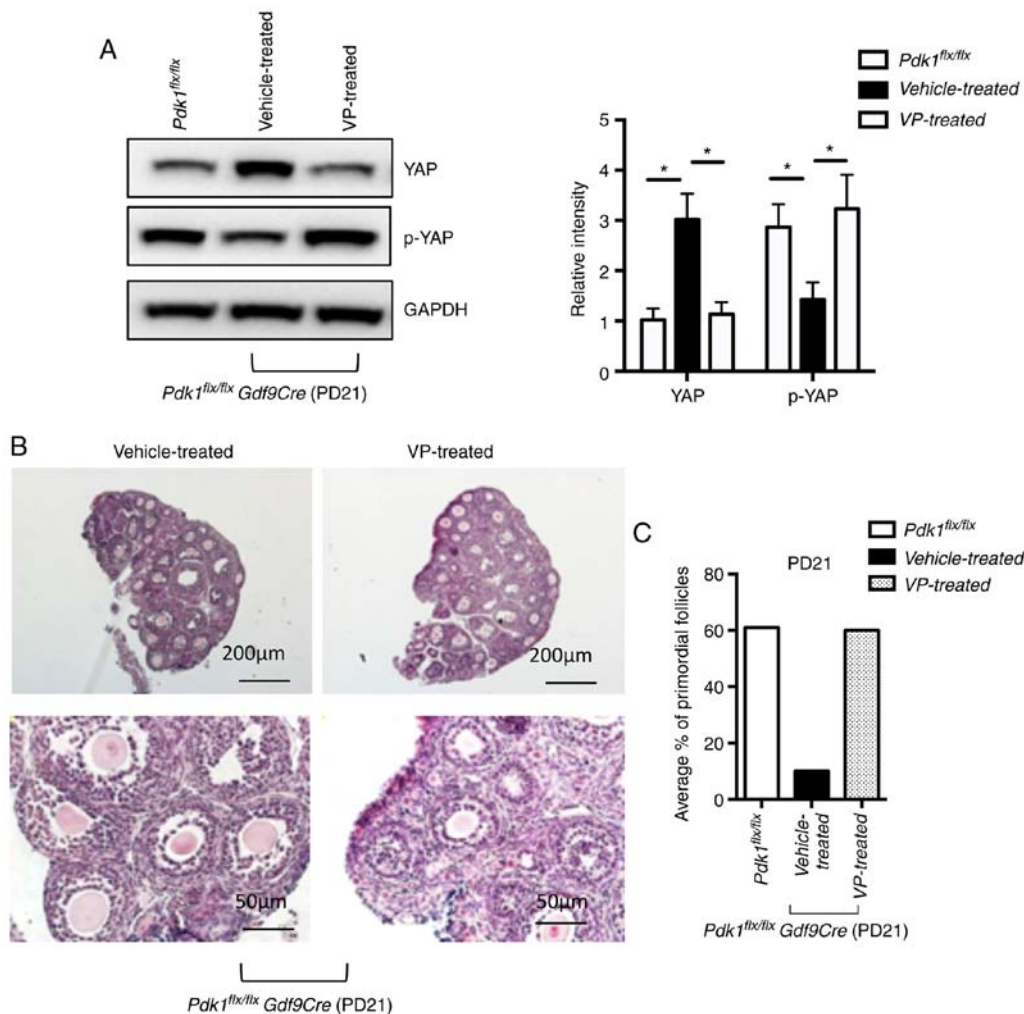


Figure 6. The suppressed expression of YAP reverses the overactivation of primordial follicles in *Pdk1^{flx/flx}Gdf9Cre* mouse ovaries. PD21 *Pdk1^{flx/flx}Gdf9Cre* mice were administered DMSO (vehicle) or 100 mg/kg verteporfin, which is a potent YAP inhibitor, intraperitoneally at 3-day intervals for 14 days. (A) Ovaries were collected, and protein was extracted, and western blot analysis was performed using antibodies against YAP and phosphorylated YAP. GAPDH was used as a control. Expression intensity was quantified using ImageJ software. (B and C) Ovaries were collected and embedded in paraffin, and serial sections of 8 μm thickness were prepared and stained with hematoxylin and eosin. Morphology of ovaries were observed, and number of primordial follicles were counted. n=5, *P<0.05. PDK1, serine/threonine protein kinase phosphoinositide-dependent kinase 1; VP, verteporfin.

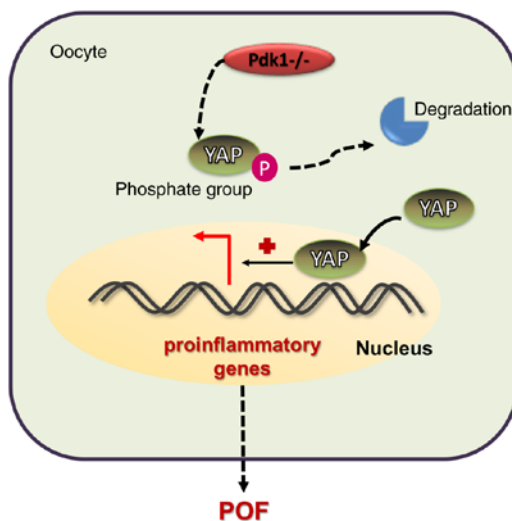


Figure 7. Working model. Oocyte-specific deletion of *Pdk1* caused enhanced expression of YAP, leading to increased proinflammatory response, contributing to premature ovarian failure. *Pdk1*, serine/threonine protein kinase phosphoinositide-dependent kinase 1; YAP, Yes-associated protein; POF, premature ovarian failure.

signaling pathway; therefore, the deletion of *Pdk1* may cause other molecular alterations, apart from YAP, which may also contribute to premature follicle activation. Furthermore, the deletion of the *Pdk1* gene cannot be performed in humans; therefore the importance of *Pdk1* in human physiology cannot be fully explained. Future studies are required to focus on the collection of human oocyte samples from premature ovarian failure and examine *Pdk1* expression in these samples. In addition, the treatment of mice with PDK1 inhibitor or activator may be performed to observe the effects on the ovaries. The identification of the role of the PDK1/YAP signaling in regulating follicle development may provide novel prospects for understanding the specific mechanisms of the activation of primordial follicles in mammalian ovaries. This study may serve as a valuable clinical indicator for designing treatments for POF.

In conclusion, this study found that PDK1 was pivotal for maintaining female fertility, and the deletion of *Pdk1* in mouse oocytes induced the overactivation of the primordial follicles, resulting in follicular exhaustion and POF in adulthood. Furthermore, these results suggested that the deletion of *Pdk1* enhanced proinflammatory reaction and increased YAP

nuclear location, which resulted in massive follicular activation in knockout mice and eventually led to premature ovarian failure. Thus, PDK1 is a critical gatekeeper for regulating primordial follicle dormancy and/or activation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JG and PL were involved in the conception of the study. JG was involved in the study methodology. TS was involved in data analysis. DC was involved in data validation. CL was involved in formal analysis. JJ was involved in the investigative aspect of the study. JP and YY were responsible for the experimental design. JG was involved in the writing of and preparation of the original draft. PL was involved in the writing, reviewing and editing of the manuscript. PL supervised the study, was responsible for project administration and funding acquisition. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental procedures were approved by the Committee on the Use of Live Animals in Teaching and Research of Harbin Medical University (Harbin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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