

CRL4^{DCAF1} is required in activated oocytes for follicle maintenance and ovulation

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ABSTRACT: In mammals, oocytes within the primordial follicles require a number of essential factors to maintain their survival. However, the survival factors for activated oocytes have been poorly characterized. Recently we reported that damaged DNA binding protein-1 (DDB1), the linker subunit of the cullin ring-finger ubiquitin E3 ligase-4 (CRL4) complex, and its substrate adaptor, DDB1-CUL4 associated factor-1 (DCAF1), were essential for primordial follicle maintenance. In this study we specifically deleted these in the oocytes of growing follicles, to investigate if DDB1 and DCAF1 were also survival factors for activated oocytes. In the ovaries of *Ddb1*^{fl/fl};*Zp3-Cre* mice, the primordial follicle pool was intact, but awakened oocytes and growing follicles beyond the primary stage were rapidly depleted. In the ovaries of *Dcaf1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre* and *Ddb1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre* mice, global primordial follicle activation was stimulated by enhanced PI3K signaling, but the awakened oocytes were rapidly lost due to no CRL4^{DCAF1} activity. These mouse models provided original evidence that CRL4^{DCAF1} was essential for maintaining oocyte survival, not only those in dormancy at the primordial follicle stage, but also naturally awakened oocytes and those awakened by hyper-activation of PI3K signaling. Interestingly, the oocyte-specific *Ddb1* or *Dcaf1* knockout mice had ovulation defects even before oocyte exhaustion. CRL4^{DCAF1} within oocytes was required for cumulus expansion and ovulation-related somatic gene expression in a cell non-autonomous manner. Granulosa cells that surrounded these *Ddb1* or *Dcaf1*-deleted oocytes exhibited increased rates of apoptosis and showed poor responses to ovulation signals. These results suggested that CRL4 in oocytes also regulated granulosa cell functions in a cell non-autonomous manner.

Key words: DDB1 / oocyte / ovarian follicle / ovulation / primary ovarian insufficiency

Introduction

In female mammals, the primordial follicle pool at birth provides the germ cell reserve for their entire reproductive lives. A primordial follicle consists of a small oocyte arrested at the diplotene stage of meiosis I and a single layer of flat, non-mitotic somatic cells called granulosa cells. During post-natal ovarian development, some primordial follicles are released from their dormant status; oocytes significantly grow in size and the shape of surrounding pre-granulosa cells changes from flat to cuboid. This is called primordial follicle activation (Adhikari and Liu, 2009). Once activated, follicles undergo an irreversible developmental program and progressively grow into primary, secondary and antral follicle stages.

During the last decade, significant progress has been made in understanding primordial follicle maintenance and activation. Using gene knockout mouse models, it has been found that several transcriptional factors were enriched in the oocytes of primordial follicles, including NOBOX, FIGLA, SOHLH1/2 and LHX8, and were essential for maintaining the primordial follicle pool in post-natal ovaries (Soyal et al., 2000; Rajkovic et al., 2004; Pangas et al., 2006a; Choi et al., 2008). However, another transcription factor, FOXO3, was found to have an indispensable role

in preventing primordial follicle activation (Castrillon et al., 2003). Furthermore, the intra-oocyte activity of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-mechanistic target of rapamycin (mTOR) pathway determines the primordial follicle fates of dormancy, activation or atresia (Reddy et al., 2008, 2009; Adhikari et al., 2009, 2010). In addition, it was previously reported that the PI3K/AKT signaling pathway was important for the survival of both oocytes and granulosa cells (Fan et al., 2008a; Li et al., 2013).

In mice, it takes 6–8 weeks for activated follicles to develop to the antral stage (Zheng et al., 2014). In growing follicles, granulosa cells secrete steroid and peptide hormones to regulate estrous cycles, and oocytes accumulate maternal mRNA and proteins to prepare for meiotic maturation, fertilization and early embryo development. Thus, it is possible that activated oocytes might require a number of essential factors to maintain their survival and normal gene expression. However, these survival factors for activated oocytes have been poorly characterized, as deleting the most essential oocyte genes causes primordial follicle exhaustion and primary ovarian insufficiency, which has prevented the investigation of gene functions in activated oocytes.

Recently, we reported that damaged DNA binding protein-1 (DDB1), the linker subunit of the cullin ring-finger ubiquitin E3 ligase-4 (CRL4) complex, and its substrate adaptor, DDB1-CUL4 associated factor-1 (DCAF1), were essential for primordial follicle maintenance. Specifically deleting *Ddb1* and *Dcaf1* in the oocytes of primordial follicles using *Gdf9-Cre* caused rapid primordial follicle death and resulted in primary ovarian insufficiency in young adult mice (~8 weeks old) (Yu et al., 2013b). In addition to the oocytes in a primordial follicle, DDB1 and DCAF1 proteins were also prominently expressed in the activated oocytes of primary, secondary and antral follicles. To investigate if DDB1 and DCAF1 were also survival factors for activated oocytes, we specifically deleted these in the oocytes of growing follicles using *Zp3-Cre* (Lewandoski et al., 1997). Our results showed that, although the primordial follicle pools remained intact in these conditional gene knockout mice, *Ddb1* or *Dcaf1* deletion in activated oocytes resulted in a rapid loss of growing follicles, which indicated that the CRL4 complex was essential for oocyte survival after their release from dormancy. Furthermore, granulosa cells that surrounded these *Ddb1* or *Dcaf1*-deleted oocytes exhibited increased rates of apoptosis and showed poor responses to ovulation signals. These results suggested that CRL4 in oocytes also regulated granulosa cell functions in a cell non-autonomous manner.

Materials and Methods

Mice

Wild type (WT) C57/B6 mice were from the Zhejiang Academy of Medical Science, China. *Ddb1*^{flox/flox}, *Dcaf1*^{flox/flox}, *Pten*^{flox/flox}, *Gdf9-Cre* and *Zp3-Cre* mice were generated as previously reported (Reddy et al., 2008; Yu et al., 2013b). Mice were maintained under SPF conditions in a controlled environment of 20–22°C, with a 12/12 h light/dark cycle, 50–70% humidity, and food and water *ad libitum*. Animal care and experimental procedures were in accordance with the Animal Research Committee guidelines of Zhejiang University. Mice that lacked *Ddb1* or *Dcaf1* in their oocytes were generated by crossing *Gdf9-Cre* or *Zp3-Cre* mice with previously reported *Ddb1*^{flox/flox} and *Dcaf1*^{flox/flox} mice, respectively (Cang et al., 2006; McCall et al., 2008). All mutant mouse strains were in a C57BL/6 background.

Superovulation

Pubertal mice (21–23 days old) were injected i.p. with 5 IU of pregnant mare serum gonadotrophin (PMSG) (Ningbo Sansheng Pharmaceutical Co., Ltd, P.R. China) and 44 h later with 5 IU hCG (Ningbo Sansheng Pharmaceutical Co., Ltd, P.R. China). After an additional 16 h, oocyte/cumulus masses were surgically removed from oviducts and the numbers of oocytes were counted after digestion with 0.3% hyaluronidase (Sigma-Aldrich).

Histological analysis and immunohistochemistry

Ovaries were fixed overnight in 10% phosphate-buffered saline (PBS)-buffered formalin, dehydrated using an ascending series of graded ethanol solutions, and then embedded in paraffin. Ovary samples were serially sectioned at 5 µm thickness and stained with hematoxylin and eosin (H&E). The number of follicles per ovary was counted only when they contained oocytes with clearly visible nuclei. Follicles were classified as previously described (Yu et al., 2013b). For immunohistochemistry, sections were deparaffinised and rehydrated with xylene and an alcohol gradient. Sections were incubated with primary antibodies at room temperature for 1 h,

followed by reaction with biotin-labeled secondary antibodies for 30 min. Staining used Vectastain ABC kits and DAB peroxidase substrate kits (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used and their applications were identical to those in our previous report (Yu et al., 2013b).

TUNEL assays

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays were done with 10% formalin-fixed paraffin-embedded sections using an ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA, USA), according to the manufacturer's instructions.

Immunofluorescent microscopy for ovarian sections

Ovarian tissues were fixed in 4% paraformaldehyde, embedded in O.C.T. compound (Sakura Finetek USA, Inc.), and stored at –80°C before preparing 7 µm sections using a Leica CM1950 cryomicrotome (Leica Microsystems, Wetzlar, Germany). Sections were blocked with PBS that contained 0.3% Triton X-100 and 5% bovine serum albumin (blocking buffer), and were sequentially probed with primary antibodies (indicated in Results) and Alexa Fluor 594- or 488-conjugated secondary antibodies (Molecular Probes). Slides were mounted using VectaShield with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Digital images were acquired using an epifluorescent microscope (Nikon Eclipse 80i) with 4–100× objectives.

Western blot analysis

Ovarian protein extracts were dissolved in sodium dodecyl sulfate (SDS) sample buffer. Protein lysates (30 µg total protein per lane) were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). After probing with primary antibodies, membranes were washed in Tris-buffered saline that contained 0.05% Tween-20 (TBST) and incubated with a horse-radish peroxidase-linked secondary antibody. Finally, bands in the membranes were detected using an Enhanced Chemiluminescence Detection Kit (Amersham).

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions. Real-time PCR analysis used Q Tag SYBR Green Master Mix (Becton Dickinson Medical Devices Co., Ltd, USA) and an Applied Biosystems 7500 Real-Time PCR System. Relative mRNA levels were determined by normalizing to endogenous *Gapdh* mRNA levels (used as an internal control) using Microsoft EXCEL[®]. For each of the indicated genes, the relative transcript level of a control sample was set at 1. The relative transcript levels of other samples were compared with the control and the fold-changes are shown in the graphs. Quantitative RT-PCR reactions were done in triplicate for each experiment. Sequences of PCR primers are shown in Table I.

Statistical analysis

Quantitative RT-PCR results are given as mean ± SD; each experiment included at least three independent samples and was repeated at least three times. Group comparisons were made by two-tailed unpaired Student's *t*-tests using Microsoft EXCEL[®].

Table 1 Sequences of primers (5'–3') used in quantitative RT–PCR in this study.

<i>Areg</i>	F: TTTATCTTCACACATCTCTTTATGTACAG R: GAGGCTTCGACAAGAAAACG
<i>Ereg</i>	F: GTCCCTGAGGTCCTCTCTC R: AACTGGTCTGCGATGTGAG
<i>Btc</i>	F: CTCCTCCTGCATCTGTGAG R: CTCTGAATATCTTCACTTATGGGAG
<i>Ptgs2</i>	F: TGTACAAGCAGTGGCAAAGG R: CCCCAAAGATAGCATCTGGA
<i>Has2</i>	F: GTTGGAGGTGTTGGAGGAGA R: ATTCCAGAGGACCGCTTAT
<i>Tnfrif6</i>	F: TTCCATGTCTGTGCTGCTGGATGG R: AGCCTGGATCATGTTCAAGGTCAA
<i>Fshr</i>	F: ACCATGGCTTAGAAAATCTGAAG R: GATCCCCAGGCTGAGTCATA
<i>Npr2</i>	F: AGGGCAGCAACCTCAGTGT R: AGACATAGTCCCCATTGGTCAG
<i>Egfr</i>	F: GGAATGACGGGTAGAAATTTT R: TGGAATCATGGTTGTTCTTTGA
<i>Gapdh</i>	F: ACACCTGAGGACCAGTTGTCTC R: TACTCCTTGAGGCCATGTAG

F: forward; R: reverse.

Results

Dcaf1 deletion in activated mouse oocytes results in a loss of growing follicles

We selectively deleted *Dcaf1* in activated follicles by crossing *Dcaf1*^{fl/fl} mice with *Zp3-Cre* mice. Immunohistochemistry results showed that in the ovaries of *Dcaf1*^{fl/fl};*Zp3-Cre* mice, which were harvested at post-natal day (PD) 21, DCAF1 protein expression was specifically deleted in activated oocytes but not in dormant oocytes within primordial follicles (Fig. 1A). At PD21, *Dcaf1*^{fl/fl};*Zp3-Cre* mice had comparable numbers of activated follicles compared with their control litter mates (Fig. 1A and B). However, a rapid loss of growing follicles was found in *Dcaf1*^{fl/fl};*Zp3-Cre* mice at 4 weeks of age. No activated follicles beyond the primary stages were found in the ovaries of mice that were older than 6 weeks (Fig. 1B). Immunohistochemical staining for the germ cell marker MVH (mouse VASA homolog) indicated normal numbers of primordial follicles in the ovaries of adult *Dcaf1*^{fl/fl};*Zp3-Cre* mice (12–20 weeks old; Fig. 1C), suggesting that the primordial follicle pool was not affected by *Zp3-Cre* mediated *Dcaf1* deletion.

At PD28, increased granulosa cell apoptosis (Fig. 2A, upper panels) and follicle atresia (Fig. 2A, lower panels) were detected by immunohistochemical staining of cleaved caspase 3 (CC3) and TUNEL assay, in the ovaries of *Dcaf1*^{fl/fl};*Zp3-Cre* mice compared with ovaries in control mice. Quantifying atretic follicles versus total follicles on ovarian sections indicated that the ovaries of *Dcaf1*^{fl/fl};*Zp3-Cre* mice had more atretic follicles at PD28 (Fig. 2B; *n* = 6 for each genotype).

Thus, we investigated the levels of phosphorylated ribosome protein S6 (pRPS6), a well-established downstream marker of PI3K/AKT pathway activity, in the ovaries of WT and *Dcaf1*^{fl/fl};*Zp3-Cre* mice by immunohistochemistry. WT and *Dcaf1*-deleted oocytes had similar

levels of pRPS6, whereas the granulosa cells of growing follicles of *Dcaf1*^{fl/fl};*Zp3-Cre* mice had significantly reduced pRPS6 levels compared with those in control mice (Fig. 2C). These results suggested that *Dcaf1* deletion in oocytes had impaired PI3K signaling in the surrounding granulosa cells and facilitated follicle atresia.

Dcaf1 deletion results in oocyte death independent of PI3K pathway activity

Because recent studies showed that PI3K signaling was essential for oocyte survival and awakening from dormancy within primordial follicles, it was possible that DCAF1 might maintain oocyte survival by interacting with the PI3K pathway. To test this, we enhanced PI3K signaling activity in *Dcaf1*-deleted oocytes by deleting *Pten*, a key repressor of the PI3K signaling pathway. As shown by H&E staining (Fig. 3) and MVH immunohistochemistry (Fig. 4), *Pten* deletion in the oocytes of primordial follicles (*Pten*^{fl/fl};*Gdf9-Cre*) stimulated global follicle activation. This was in agreement with the results in previous reports (Reddy et al., 2008).

In the ovaries of these mice, activated follicles continued to grow and survived up to 10–12 weeks after birth. However, *Pten* deletion within a *Dcaf1* deletion background (*Dcaf1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre*) failed to rescue the rapid loss of activated follicles (Figs 3 and 4), and resulted in a typical primary ovarian insufficiency phenotype (complete depletion of oocytes and follicles in the ovary of young adults) at ~6 weeks after birth (Fig. 3). Residues of dead oocytes within activated follicles were frequently observed in the ovaries of *Dcaf1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre* mice, but not in those of WT or *Pten*^{fl/fl};*Gdf9-Cre* mice between PD21 and PD28 (Fig. 4, arrows). This indicated that *Pten/Dcaf1* double-deleted oocytes underwent death *en masse* shortly after their awakening from dormancy. *Pten* deletion also did not rescue the primary ovarian insufficiency phenotype (8–10 weeks after birth) caused by *Ddb1* deletion in oocytes (Fig. 3). The ovaries of *Ddb1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre* mice had a similar histology as those of the *Pten*^{fl/fl};*Gdf9-Cre* mice at 3 and 4 weeks after birth (data not shown). We started to see differences between these two strains as early as 6 weeks after birth. We also observe dead oocytes in activated follicles in ovaries from *Ddb1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre* mice. The phenotype was similar to that of the *Dcaf1*^{fl/fl};*Pten*^{fl/fl};*Gdf9-Cre* mice (data not shown). These results indicated that CRL4^{DCAF1} activity was indispensable for oocyte survival, but was independent of PI3K signaling in oocytes.

DDB1 and DCAF1 in activated mouse oocytes regulates granulosa cell function during ovulation by a cell non-autonomous mechanism

In the ovaries of *Ddb1*^{fl/fl};*Gdf9-Cre* and *Dcaf1*^{fl/fl};*Gdf9-Cre* mice, activated oocytes were maintained for 6 weeks after birth. However, exogenous PMSG/hCG treatment failed to induce efficient ovulation in mice of both strains (Fig. 5A). Because the two mouse strains were phenocopies of each other, we only used ovaries of *Ddb1*^{fl/fl};*Gdf9-Cre* mice to analyze the molecular changes underlying the phenotype of defective ovulation. At 8 h after hCG treatment, cumulus expansion was significantly impaired in the pre-ovulatory follicles of *Ddb1*^{fl/fl};*Gdf9-Cre* mice when compared with that in WT mice (Fig. 5B). Subsequently, pre-ovulatory follicles did not rupture at 16 h after hCG treatment (Fig. 5B) and non-ovulated oocytes were trapped within the corpus luteum at 48 h post-hCG treatment (Fig. 5B).

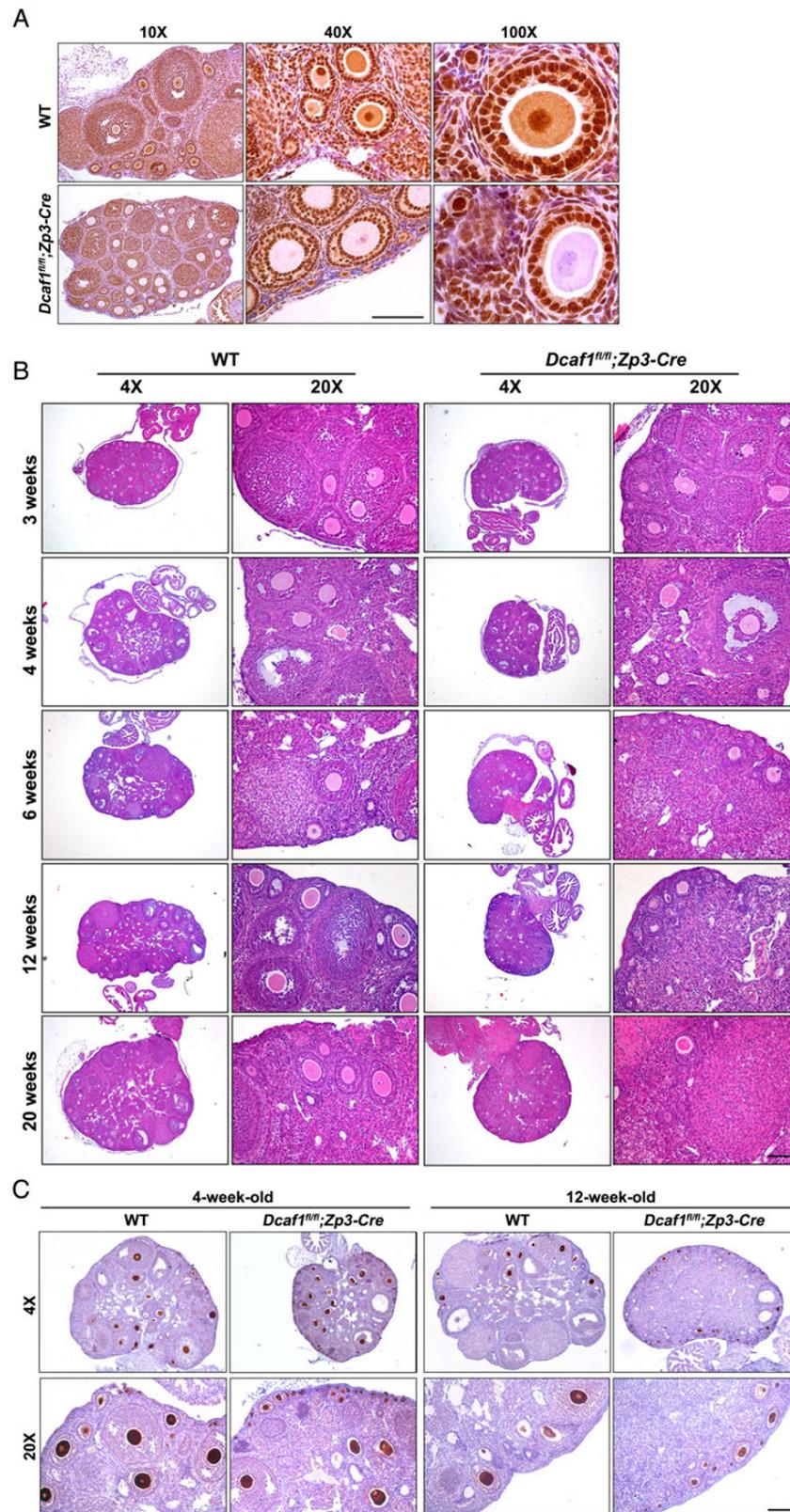


Figure 1 DDB1-CUL4 associated factor I (*Dcaf1*) deletion in activated mouse oocytes results in a loss of growing follicles. **(A)** DCAF1 immunohistochemistry showing specific DCAF1 protein depletion in the activated oocytes of *Dcaf1^{fl/fl};Zp3-Cre* mice. Scale bar, 100 μ m. **(B)** Hematoxylin and eosin (H&E) staining showing representative ovarian histology of wild type (WT) and *Dcaf1^{fl/fl};Zp3-Cre* mice at 3, 4, 6, 12 and 20 weeks after birth. Scale bar, 100 μ m. **(C)** Mouse VASA homolog (MVH) immunohistochemistry showing oocytes in the ovaries of WT and *Dcaf1^{fl/fl};Zp3-Cre* mice. Scale bar, 100 μ m.

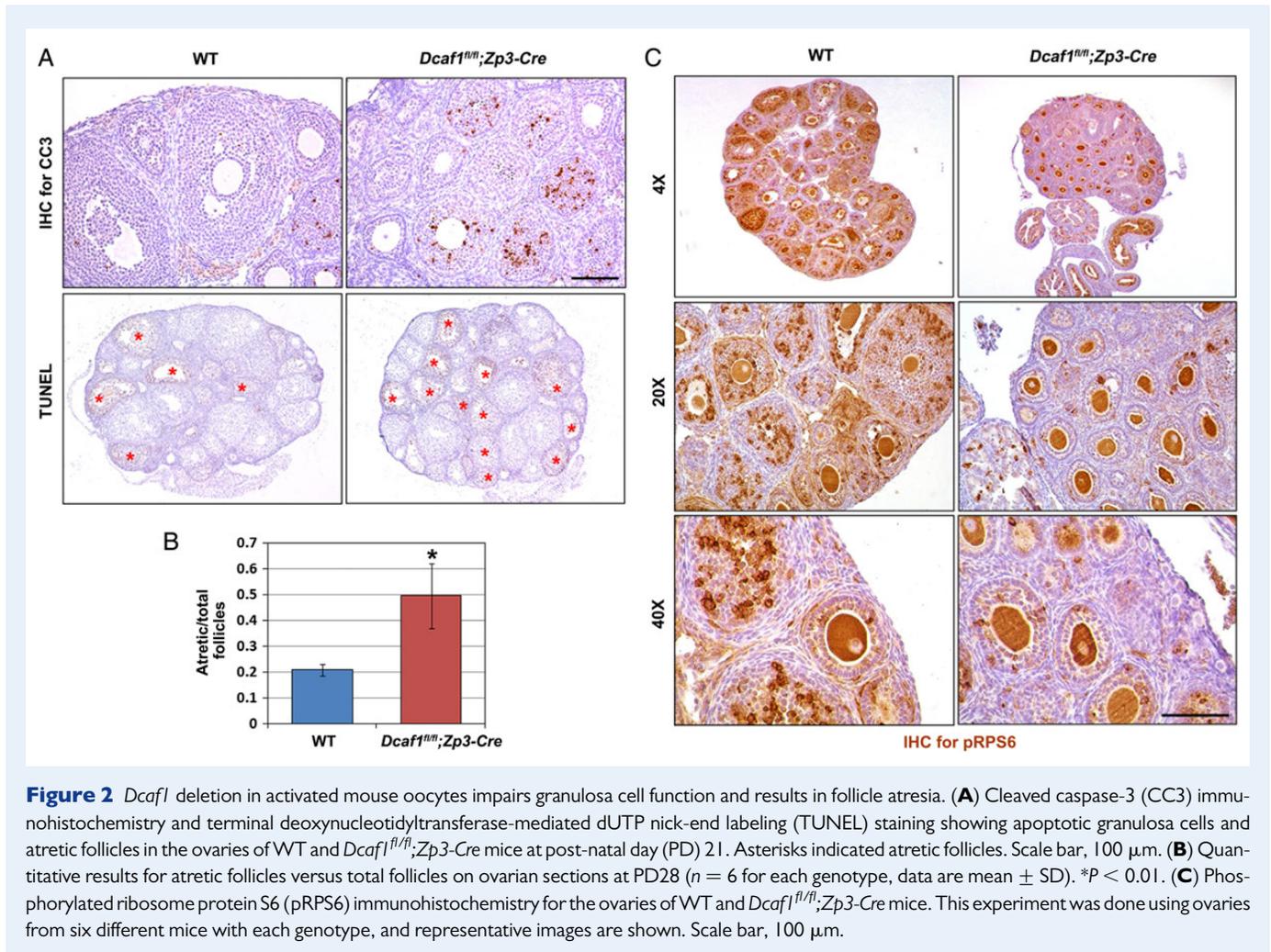


Figure 2 *Dcaf1* deletion in activated mouse oocytes impairs granulosa cell function and results in follicle atresia. **(A)** Cleaved caspase-3 (CC3) immunohistochemistry and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining showing apoptotic granulosa cells and atretic follicles in the ovaries of WT and *Dcaf1^{fl/fl};Zp3-Cre* mice at post-natal day (PD) 21. Asterisks indicated atretic follicles. Scale bar, 100 μ m. **(B)** Quantitative results for atretic follicles versus total follicles on ovarian sections at PD28 ($n = 6$ for each genotype, data are mean \pm SD). * $P < 0.01$. **(C)** Phosphorylated ribosome protein S6 (pRPS6) immunohistochemistry for the ovaries of WT and *Dcaf1^{fl/fl};Zp3-Cre* mice. This experiment was done using ovaries from six different mice with each genotype, and representative images are shown. Scale bar, 100 μ m.

IHC staining indicated that in the pre-ovulatory follicles of *Ddb1^{fl/fl};Gdf9-Cre* mice, DDB1 protein was only deleted in oocytes but not in mural granulosa or cumulus cells (Fig. 5B), which suggested that DDB1 in oocytes was required for ovulation in a cell non-autonomous manner. Furthermore, the mRNA expression of numerous genes in granulosa/cumulus cells known to be essential for ovulation, including those encoding for EGF-like paracrine factors (*Areg*, *Ereg*, *Btc*) and signaling molecule receptors (*Fshr*, *Npr2*, *Egfr*), as well as those essential for cumulus expansion (*Ptgs2*, *Has2*, *Tnfrsf11b*) were compromised, after DDB1 deletion in oocytes (Fig. 5C).

In previous studies we showed that the pre-ovulatory LH surge induced transient activation of extracellular signal regulated kinase-1 and -2 (ERK1/2) in granulosa and cumulus cells, and that ERK1/2 activity was indispensable for LH-target gene expression and ovulation (Fan et al., 2008b, 2009). Thus, we determined protein levels of phosphorylated/activated ERK1/2 and its downstream target prostaglandin synthase-2 (PTGS2) in the granulosa cells of *Ddb1^{fl/fl};Gdf9-Cre* mice by western blotting (Fig. 5D) and immunofluorescent staining (Fig. 5E). These results showed that ERK1/2 phosphorylation levels were remarkably decreased in the granulosa/cumulus cells of *Ddb1^{fl/fl};Gdf9-Cre* mice at 4 h after hCG injection. Previous studies also showed that oocyte-secreted growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) induced phosphorylation/activation of their downstream

signaling molecules, including SMAD2/3 and SMAD1/5, in adjacent cumulus cells, and that this transforming growth factor (TGF)- β signaling was a prerequisite for successful cumulus expansion and ovulation (Pangas et al., 2006b; Yu et al., 2013a). Moreover, expression of *Gdf9*, *Bmp15* and *Fgf8*, were decreased in *Ddb1* or *Dcaf1*-deleted oocytes, suggesting that TGF- β signaling between a CRL4^{DCAF1} deficient oocyte and its surrounding granulosa cells was impaired (Yu et al., 2013b). As shown in Fig. 5E, pSMAD1/5 and pSMAD2 levels were significantly lower in cumulus cells that surrounded *Ddb1*-deleted oocytes when compared with those that surrounded WT oocytes. Similarly, the protein expression of oocyte factor (GDF9, BMP15, FGF8)-regulated, ovulation-related genes in cumulus cells, including PTGS2 and pantraxin-3 (PTX3), were decreased in the pre-ovulatory follicles of *Ddb1^{fl/fl};Gdf9-Cre* mice (Fig. 5E).

Discussion

DDB1/DCAF1 involvement in maintaining oocyte survival in activated follicles

The molecular mechanisms that control reproductive aging and menopausal age in females are poorly understood. PI3K signaling in oocytes has received increasing attention in the last decade as a determinant of

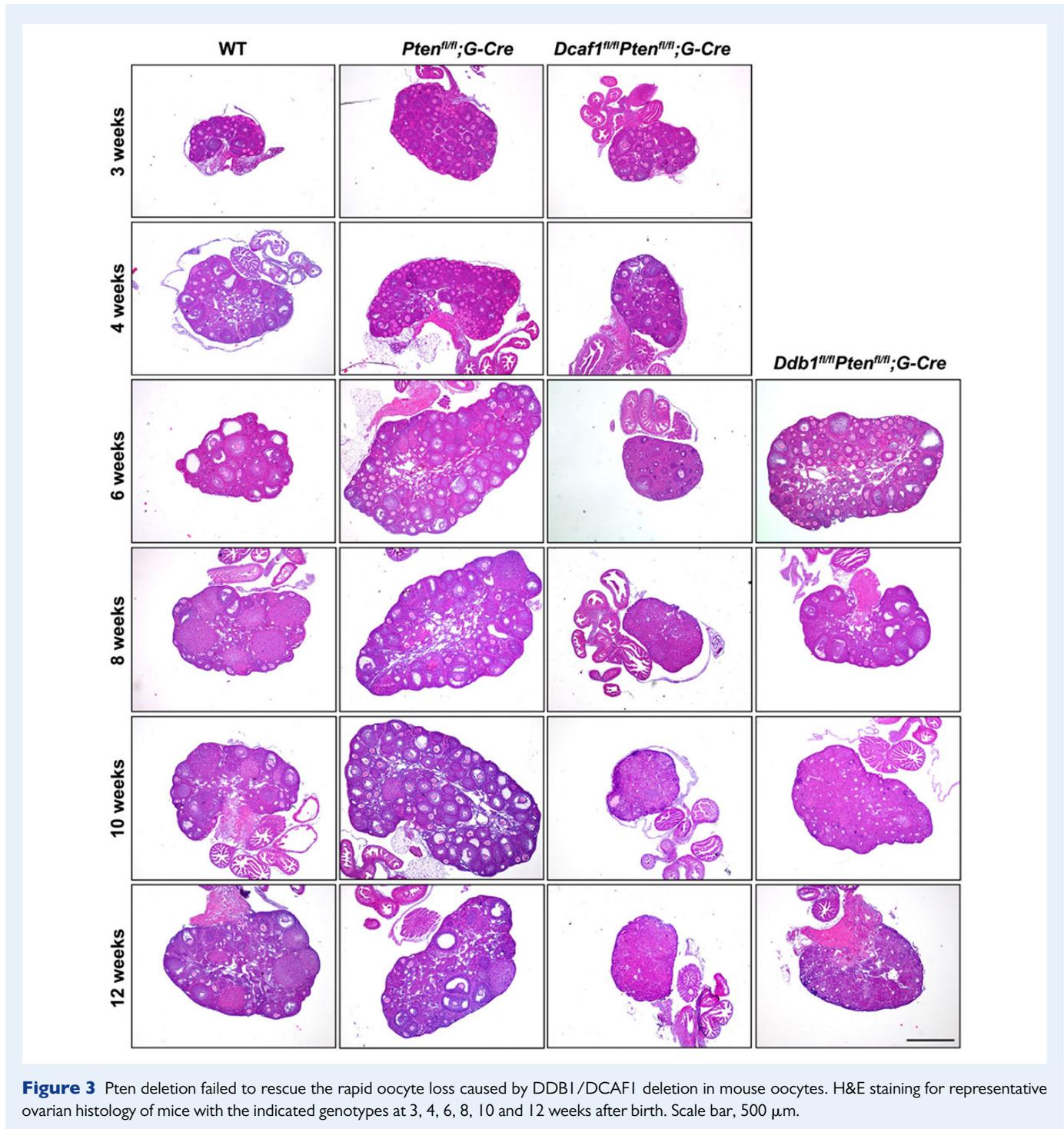


Figure 3 Pten deletion failed to rescue the rapid oocyte loss caused by DDB1/DCAF1 deletion in mouse oocytes. H&E staining for representative ovarian histology of mice with the indicated genotypes at 3, 4, 6, 8, 10 and 12 weeks after birth. Scale bar, 500 μm .

ovarian aging and menopausal age (Castrillon et al., 2003; Reddy et al., 2008, 2009). However, whether other pathways in oocytes have any direct effects on the survival and maintenance of the primordial follicle pool remains uncertain. In a recent study we identified CRL4^{DCAF1} E3 ligase as a crucial factor for oocyte survival and that deficiencies in DDB1 or DCAF1 caused primary ovarian insufficiency and infertility (Yu et al., 2013b). Those results indicated that a protein ubiquitination system regulated survival or loss, other than the activation, of primordial follicles to determine reproductive aging and menopause in females.

In the same report, mechanistic studies suggested that CRL4^{DCAF1} maintained oocyte genome DNA methylation levels and regulated the expression of essential oocyte genes, including *Nobox*, *Figla*, *Sohlh1/2* and *Lhx8*, in both dormant oocytes of primordial follicles and activated oocytes of growing follicles (Yu et al., 2013b). In mice null for the genes *Nobox*, *Figla*, *Sohlh1/2* and *Lhx8*, oocytes within primordial follicles were rapidly depleted after birth (Rajkovic et al., 2004; Pangas et al., 2006a; Choi et al., 2008). The young adult knockout (KO) mice had similar primary ovarian insufficiency phenotypes as those of *Ddb1*^{fl/fl};

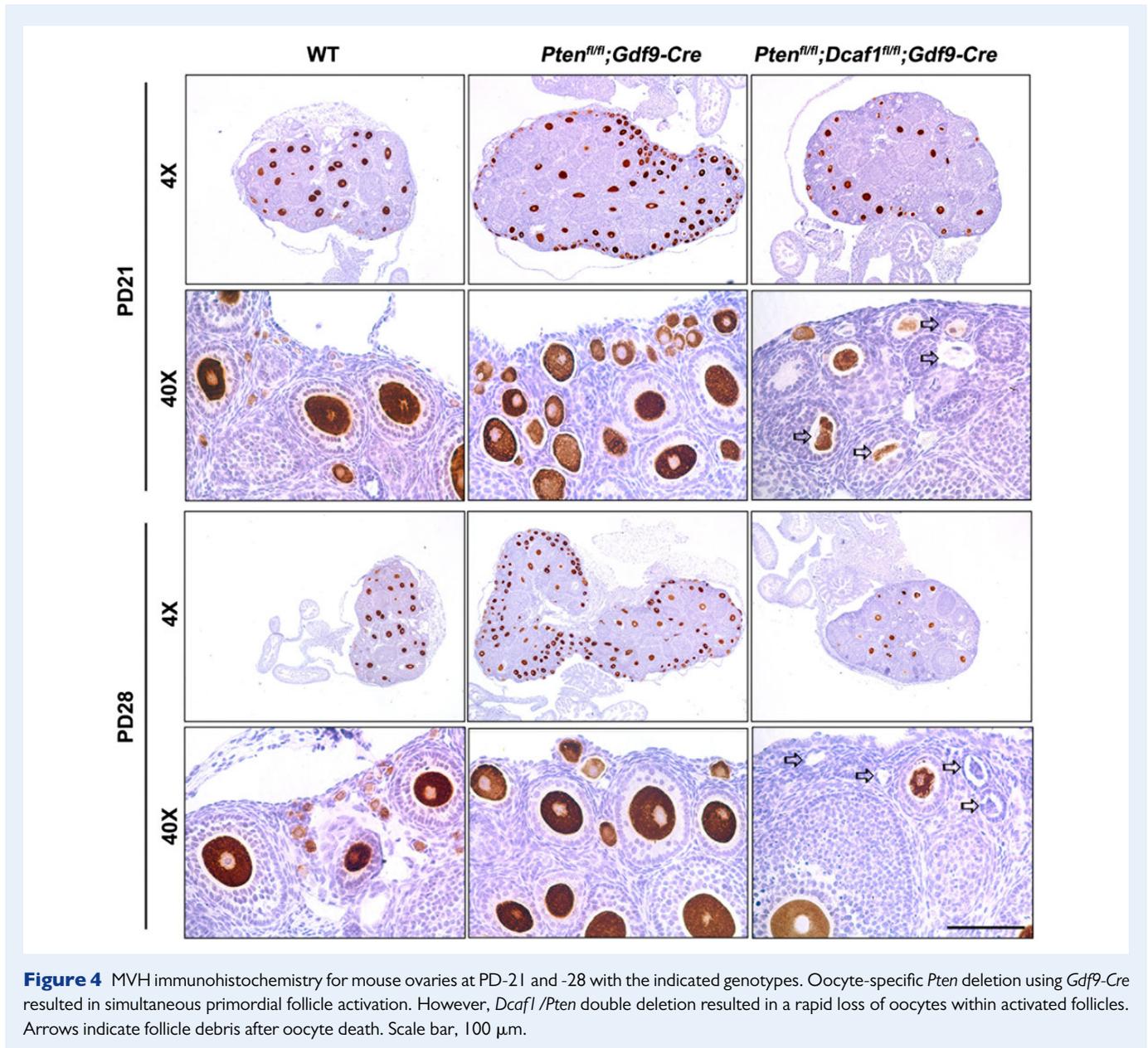


Figure 4 MVH immunohistochemistry for mouse ovaries at PD-21 and -28 with the indicated genotypes. Oocyte-specific *Pten* deletion using *Gdf9-Cre* resulted in simultaneous primordial follicle activation. However, *Dcaf1*/*Pten* double deletion resulted in a rapid loss of oocytes within activated follicles. Arrows indicate follicle debris after oocyte death. Scale bar, 100 μm .

Gdf9-Cre or *Dcaf1*^{fl/fl};*Gdf9-Cre* mice. However, it remained unclear if CRL4 and its targets were also essential for oocyte survival after they were awakened from their dormant status.

Our current study of activated oocyte-specific DCAF1-deficient mice provides new evidence that CRL4^{DCAF1} is a survival factor for fully grown oocytes and that its depletion in these oocytes causes primary ovarian insufficiency without affecting the primordial follicle reserves within adult ovaries. In addition, we explored possible gene interactions between CRL4^{DCAF1} and PI3K signaling in oocytes, which is a well-established master regulator of oocyte maintenance and awakening. Our results showed that they functioned independently in oocytes.

Pten deletion caused global primordial follicle activation, as was previously described, even though DDB1 or DCAF1 was absent in oocytes. DDB1- or DCAF1-deletion caused rapid oocyte losses that could not be rescued by *Pten* deletion or hyper-activation of PI3K signaling. This

is important because it indicated that during natural reproductive processes or in assisted reproduction therapies, both the activities of the PI3K pathway and CRL4 E3 ubiquitin ligase must be simultaneously maintained for successful follicle growth and oocyte maturation.

DDB1/DCAF1 involvement in follicle growth and ovulation

Interestingly, our oocyte-specific *Ddb1* or *Dcaf1* KO mice had ovulation defects even before oocyte exhaustion. Using these animal models, we found that CRL4^{DCAF1} within oocytes was required for cumulus expansion and ovulation-related somatic gene expression in a cell non-autonomous manner. Our previous results suggested that the underlying mechanism was that *Ddb1*/*Dcaf1* deletion impaired the expression of genes encoding oocyte-derived paracrine factors (*Gdf9*, *Bmp15* and

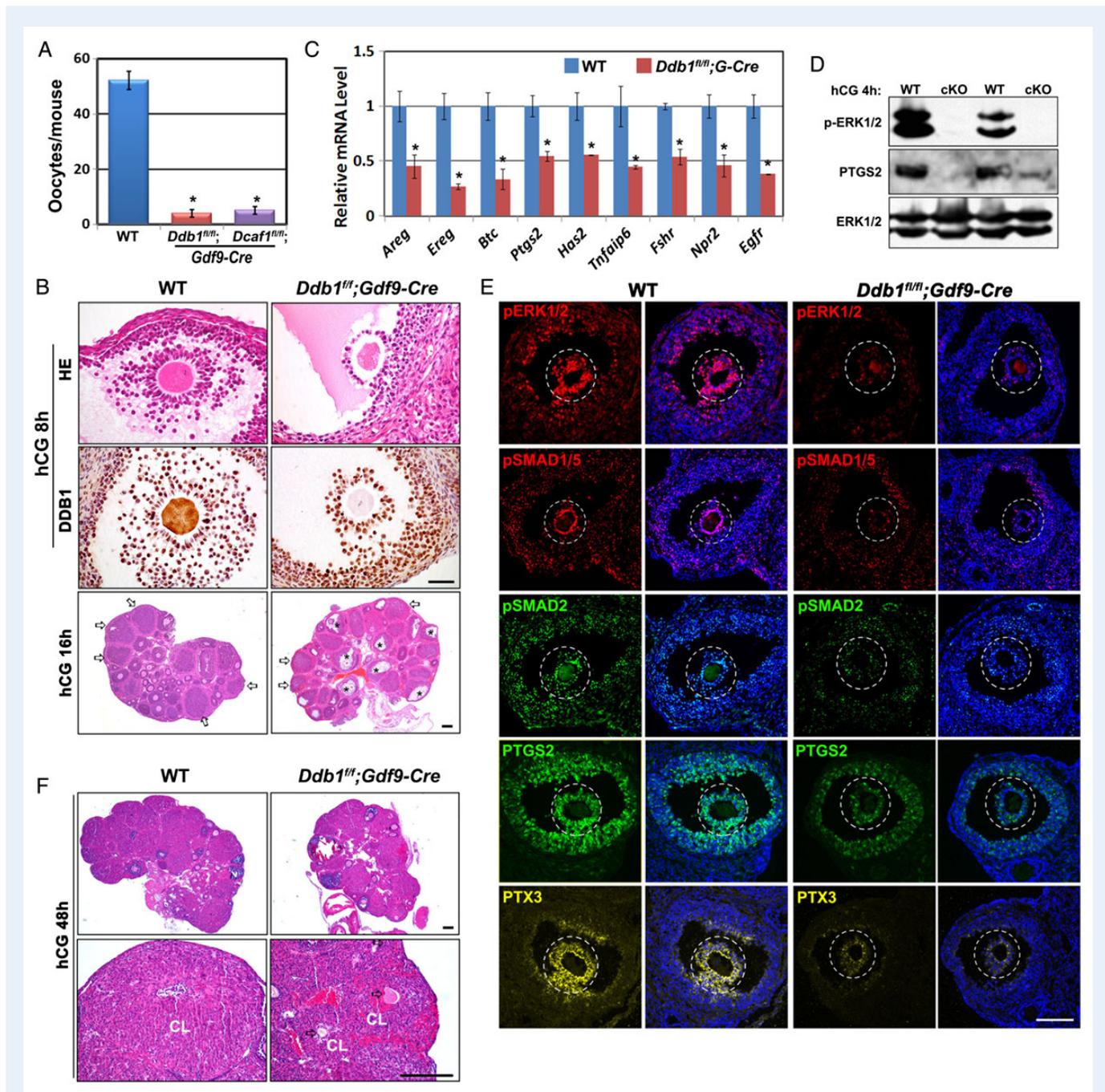


Figure 5 *Ddb1* and *Dcaf1* deletion in mouse oocytes results in ovulation defects. **(A)** Super-ovulation assay for the numbers of ovulated oocytes from 5-week old mice with the indicated genotypes. **(B)** H&E (upper and lower panels) and DDB1 immunohistochemistry staining (middle panels) showing the cell non-autonomous effect of DDB1 deletion on cumulus expansion and follicle rupture at 8 and 16 h post-hCG injection, respectively. Arrows and asterisks indicated ovulated and non-ovulated follicles, respectively. Scale bar, 50 μ m. * $P < 0.001$ when compared with WT control mice. **(C)** Real-time RT-PCR results for the mRNA expression levels of the indicated genes in granulosa cells isolated from WT and *Ddb1^{fl/fl}; Gdf9-Cre* mice at 4 h post-hCG injection. * $P < 0.001$ when compared with WT control mice. **(D)** Western blot results showing impaired extracellular signal regulated kinase 1/2 (ERK1/2) phosphorylation and decreased expression of ovulation-related protein prostaglandin synthase 2 (PTGS2) in the ovaries of *Ddb1^{fl/fl}; Gdf9-Cre* mice at 4 h after hCG treatment. The proteins were loaded on the gel in replicates. Total ERK1/2 was blotted as loading control. **(E)** Immunofluorescent staining results showing reduced ERK1/2 phosphorylation and expressions of ovulation-related genes' products in the pre-ovulatory follicles of *Ddb1^{fl/fl}; Gdf9-Cre* mice. In control ovaries, phospho-ERK1/2, PTGS2 and pentraxin 3 (PTX3) were detected in granulosa cells, particularly in cumulus cells of pre-ovulatory follicles at 4 h post-hCG injection, but had weaker signals in *Ddb1^{fl/fl}; Gdf9-Cre* ovaries. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in blue. COCs were circled by white dashed lines. Scale bar, 100 μ m. **(F)** H&E staining results showing that in the ovaries of *Ddb1^{fl/fl}; Gdf9-Cre* mice, non-ovulated oocytes were trapped within the corpus luteum at 48 h post-hCG. Arrows indicate non-ovulated oocytes trapped within the corpus luteum (CL).

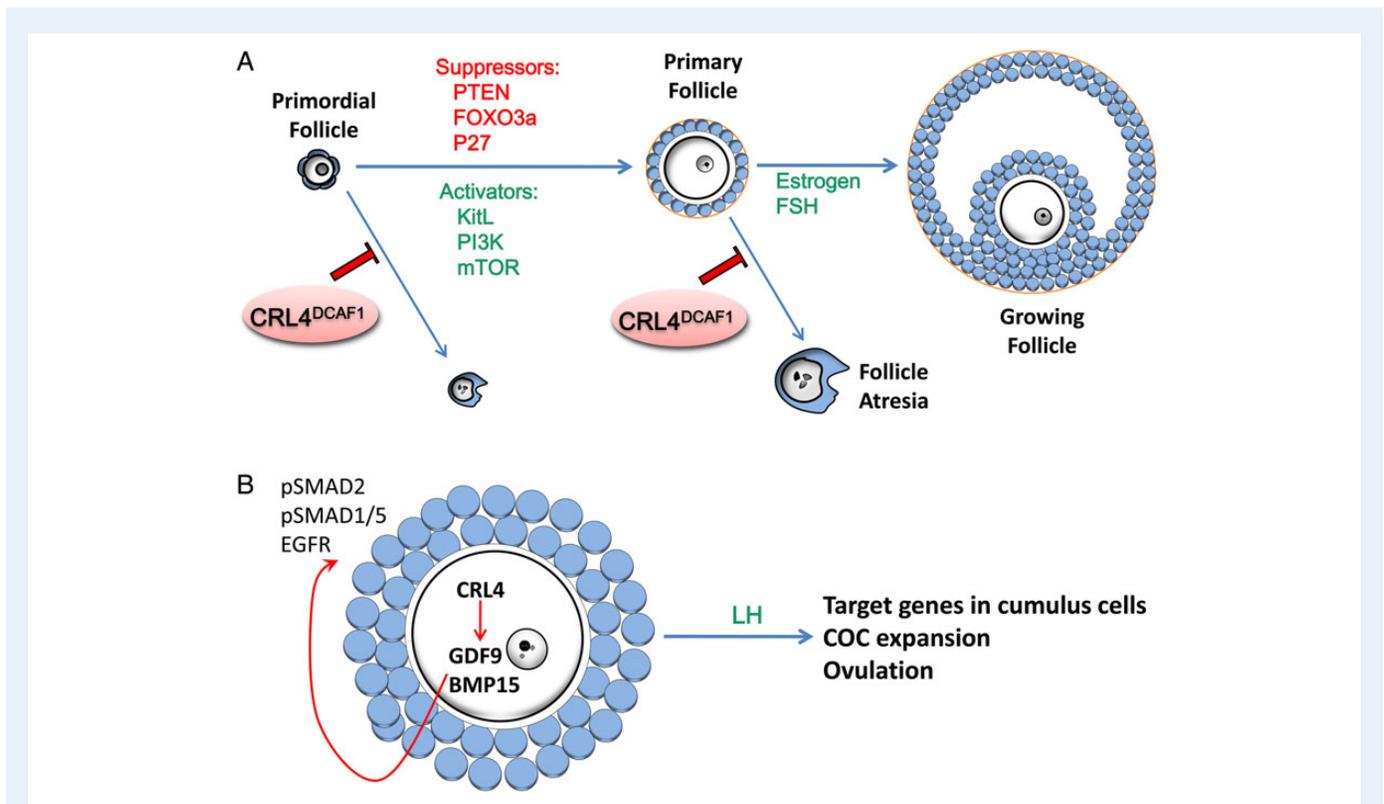


Figure 6 Schematic of CRL4^{DCAF1} functions in awakened mouse oocytes of growing and pre-ovulatory follicles. **(A)** Independent of known suppressors (PTEN, FOXO3a and p27) and activators (Kit ligand, PI3K/AKT pathway and mTOR pathway) of primordial follicle activation in mouse, CRL4^{DCAF1} activity is required for mouse oocyte survival within both primordial follicles and activated growing follicles. **(B)** In fully grown mouse oocytes of pre-ovulatory follicles, CRL4^{DCAF1} activity is required for oocyte expression of the cumulus enabling factors GDF9 and BMP15, which activate TGF- β signaling in the surrounding cumulus cells and maintain COC responsiveness to the ovulation signal triggered by LH.

Fgf8) due to hyper-methylation of their promoter regions (Yu *et al.*, 2013b). Indeed, in oocytes that were isolated from *Ddb1^{fl/fl};Gdf9-Cre* mice, *Gdf9*, *Bmp15* and *Fgf8* mRNA levels were all decreased.

Previous studies showed that oocyte-secreted paracrine factors, including GDF9, BMP15 and FGF8, were essential for cumulus expansion and ovulation (Eppig *et al.*, 2005; Diaz *et al.*, 2007). GDF9 and BMP15 are TGF- β family ligands that promote SMAD2/3 and SMAD1/5/8 phosphorylation in granulosa cells. Furthermore, oocyte-secreted GDF9 and BMP15 are required for maintaining EGF receptor expression and ERK1/2 phosphorylation in cumulus cells (Su *et al.*, 2010). Both the TGF- β and EGFR-ERK1/2 signaling pathways are required for the induced expression of ovulation-related genes, such as *Ptgs2* and *Ptx3* (Diaz *et al.*, 2007; Fan *et al.*, 2009). We also recently showed that SMAD4, a key component of TGF- β signaling pathways, was required in granulosa cells for healthy follicle growth and responsiveness to ovulation signals (Yu *et al.*, 2013a).

In the current study, we found *in vivo* evidence that CRL4^{DCAF1} activity was required for fully grown oocytes to govern normal follicle growth and ovulation. In granulosa/cumulus cells that surround DDB1-deleted oocytes, the phosphorylation levels of SMAD1/5 and SMAD2 were decreased and LH/hCG-stimulated ERK1/2 activation was compromised. Taken together, these results suggest that CRL4^{DCAF1} activity maintains the expression and secretion of paracrine factors from fully grown oocytes to support follicle growth and provide permissive

signals for ovulation in a cell non-autonomous manner. The phenotypes we observed in these mouse models clearly demonstrated the physiological importance of this regulation mechanism, which is summarized in Fig. 6: Independent of known suppressors (PTEN, FOXO3a and p27) and activators (Kit ligand, PI3K/AKT pathway, and mTOR pathway) of primordial follicle activation, CRL4^{DCAF1} activity is required for oocyte survival within both primordial follicles and activated growing follicles (Fig. 6A). Furthermore, in fully grown oocytes of pre-ovulatory follicles, CRL4^{DCAF1} activity is also required for oocyte expression of the cumulus enabling factors GDF9 and BMP15, which activate TGF- β signaling in the surrounding cumulus cells and maintain the responsiveness of cumulus-oocyte complexes to the ovulation signal triggered by LH (Fig. 6B).

Implications for human primary ovarian insufficiency

Mouse models have been extensively used to study human primary ovarian insufficiency. However, the definitions of primary ovarian insufficiency used for gene mutated mice and humans are conceptually different. The primary ovarian insufficiency phenotype of gene mutated mice is usually defined as the complete depletion of oocytes, particularly those in dormant primordial follicles, at early ages in the ovaries of these mice, as judged by ovarian histological analyses. However, in humans, primary

ovarian insufficiency is usually diagnosed by elevated gonadotrophin levels and low estrogen levels in the blood circulation and by decreased ovary sizes, as determined by bio-imaging. Therefore, in some human patients, primary ovarian insufficiency could be caused by the absence of growing follicles beyond the primary stage, rather than by primordial follicle loss and, to date, no mouse models that mimic this category of primary ovarian insufficiency patients have been developed.

In this study, we reported on several primary ovarian insufficiency mouse models. In the ovaries of *Ddb1^{fl/fl};Zp3-Cre* mice, the primordial follicle pool was intact, but awakened oocytes and growing follicles beyond the primary stage were rapidly depleted. In the ovaries of *Dcaf1^{fl/fl};Pten^{fl/fl};Gdf9-Cre* and *Ddb1^{fl/fl};Pten^{fl/fl};Gdf9-Cre* mice, global primordial follicle activation was stimulated by enhanced PI3K signaling, but the awakened oocytes were rapidly lost due to lack of CRL4^{DCAF1} activity. These mouse models provided original evidence that CRL4^{DCAF1} ubiquitin E3 ligase was essential for maintaining oocyte survival, not only those in dormancy at the primordial follicle stage, but also in naturally awakened oocytes and those awakened by hyper-activation of PI3K signaling.

Recently, small molecules that modulate the PI3K pathway have been used for treating the ovarian tissues of human primary ovarian insufficiency patients for assisted reproduction purposes (Li et al., 2010). In comparison, inhibitors of the CRL family ubiquitin E3 ligases have been used for cancer therapy (Pan et al., 2013). Our observations in these novel mouse models suggest that the key role of CRL4^{DCAF1} in oocytes of growing follicles should be considered when using these new drugs in medical practice.

Authors' roles

C.Y., Y.-W.X. and Q.-Q.S. made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. H.-Y.F. drafted the article and gave final approval of the version to be published.

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Conflict of interest

None declared.

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