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PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles

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The molecular mechanisms that control reproductive aging and menopausal age in females are poorly understood. Here, we provide genetic evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) signaling in oocytes preserves reproductive lifespan by maintaining the survival of ovarian primordial follicles. In mice lacking the PDK1-encoding gene *Pdk1* in oocytes, the majority of primordial follicles are depleted around the onset of sexual maturity, causing premature ovarian failure (POF) during early adulthood. We further showed that suppressed PDK1–Akt–p70 S6 kinase 1 (S6K1)–ribosomal protein S6 (rpS6) signaling in oocytes appears to be responsible for the loss of primordial follicles, and mice lacking the *Rps6* gene in oocytes show POF similar to that in *Pdk1*-deficient mice. In combination with our earlier finding that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in oocytes suppresses follicular activation, we have now pinpointed the molecular network involving phosphatidylinositol 3 kinase (PI3K)/PTEN–PDK1 signaling in oocytes that controls the survival, loss and activation of primordial follicles, which together determine reproductive aging and the length of reproductive life in females. Underactivation or overactivation of this signaling pathway in oocytes is shown to cause pathological conditions in the ovary, including POF and infertility.

INTRODUCTION

In humans, the pool of ovarian primordial follicles is formed during fetal development. From a peak of 6–7 million at 20 weeks of gestation, the oocyte count falls dramatically so that by the time of onset of puberty, the number of oocytes that are enclosed in primordial follicles has decreased to around 400 000 in the ovaries, serving as the source of fertilizable ova (1–3). In order to ensure the proper length of reproductive life, certain primordial follicles must survive in the ovary for decades, in a resting state (Fig. 1A-1) (1,2,4). At the same time, progressive loss of primordial follicles, which is initiated by death of the oocyte (5), occurs in the ovary (Fig. 1A-2), dictating the process of reproductive aging (1,2,4). Only limited numbers of primordial follicles are continuously recruited into the growing follicle pool (Fig. 1A-3) (3,6). In humans, menopause occurs at about 50 years of age when the pool of primordial follicles has been virtually exhausted. The length of reproductive life and the timing of menopause in a woman

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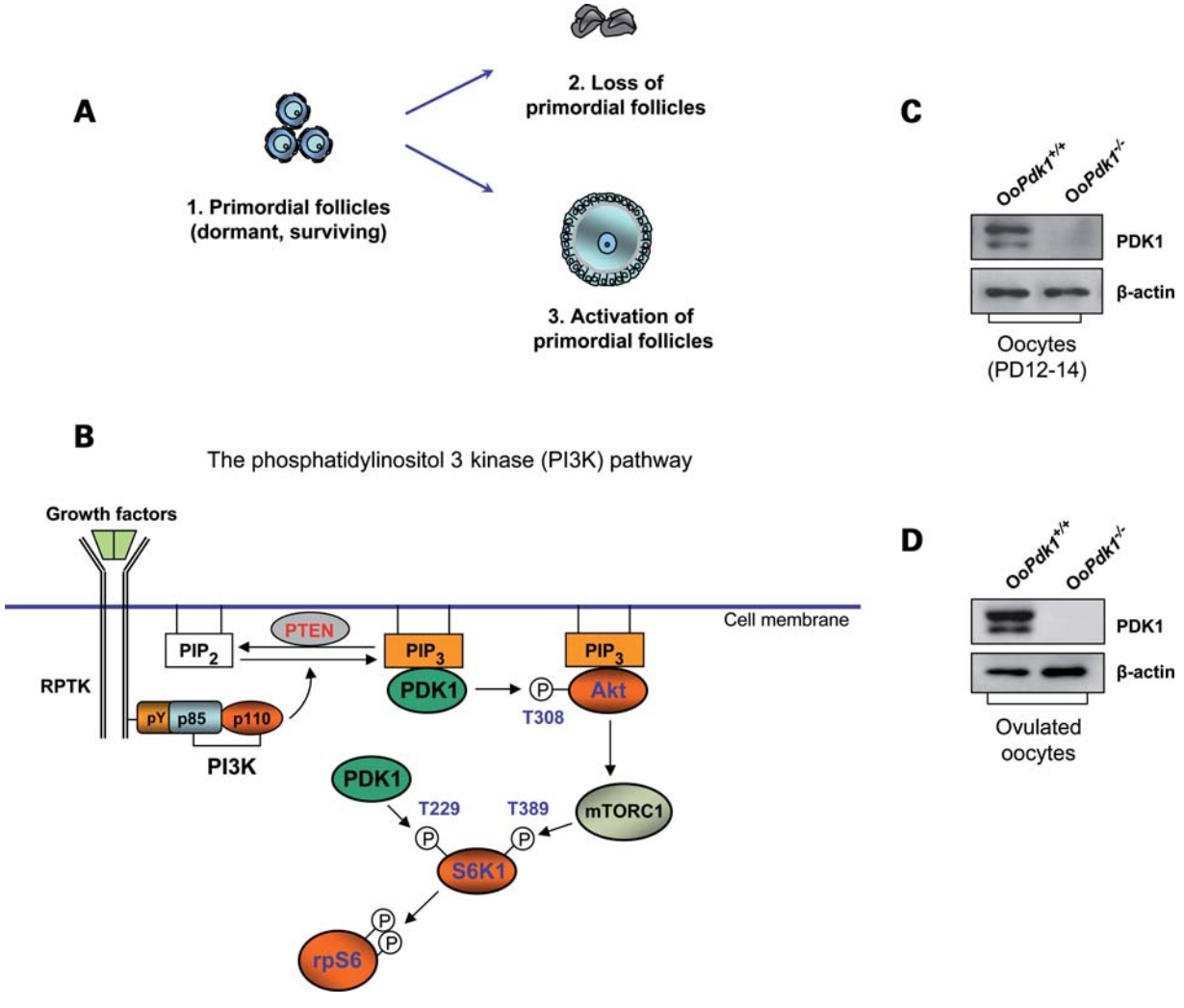

Figure 1. Infertility and POF in Oo*Pdk1*$^{-/-}$ mice. (**A**) Schematic illustrations of the courses of development of primordial follicles. In the mammalian ovary, the pool of dormant primordial follicles serves as the source of fertilizable ova for the entire duration of reproductive life. Three different courses of development for primordial follicles are illustrated: (1) they remain dormant (not growing but surviving); (2) they progressively die out directly from their dormant state, causing ovarian aging and (3) limited numbers of primordial follicles are continuously activated into the growing follicle pool. The reproductive lifespan and timing of menopause in a woman are decided by the duration of survival and the rate of loss of primordial follicles. (**B**) Illustration of the PI3K signaling pathway, with class IA PI3K as an example. Upon ligand binding (e.g. of growth factors), the receptor protein tyrosine kinases (RPTK) are in most cases dimerized and autophosphorylated and present one or several phosphorylated tyrosine residues (pY) that are capable of binding to the SH2 domain of the p85 regulatory subunit of PI3K. This recruits PI3K from the cytoplasm to the inner membrane area of the cell and enables the p110 catalytic subunit of PI3K to phosphorylate the 3′-OH group of the inositol ring of inositol phospholipids, leading to the production of PIP_3 from PIP_2. The phosphatase PTEN converts PIP_3 to PIP_2, thereby negatively regulating PI3K activity. Kinases containing pleckstrin homology domain (PH domain), such as PDK1 and Akt, are recruited through binding of their PH domains to PIP_3. A considerable proportion of the signaling mediated by PI3Ks converges at PDK1. PDK1 phosphorylates Akt at T308 and activates it; PDK1 also functions as a master kinase to activate other protein kinases of the AGC family, such as S6K1, via phosphorylation of T229 in S6K1. Akt is a serine/threonine kinase with many substrates (not shown). Akt can phosphorylate and inactivate tuberous sclerosis complex 2 (Tsc2, or tuberin) (not shown), which leads to the activation of mTOR complex 1 (mTORC1). mTORC1 can phosphorylate S6K1 at T389, which is also important for the activation of S6K1. S6K1 subsequently phosphorylates and activates rpS6, which enhances protein translation that is needed for cell growth. Note that this illustration is a simplified version of PI3K signaling. For detailed descriptions, see recent reviews (8–11). (**C** and **D**) Oocyte-specific deletion of *Pdk1* in mice. Western blots showing the absence of PDK1 protein expression in Oo*Pdk1*$^{-/-}$ mouse oocytes. Oocytes were isolated from ovaries of 12–14-day-old Oo*Pdk1*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice (C) or recovered from oviducts of Oo*Pdk1*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice after PMSG-hCG primed ovulation (D), as described in Materials and Methods. For each experiment in (C), material from three to five mice was used per lane. For each lane, around 20 μg of protein was loaded. For experiments in (D), 100 ovulated oocytes were used in each lane. Levels of β-actin were used as internal controls. The experiments were repeated three times, and representative images are shown. (**E**) Comparison of average cumulative numbers of pups per Oo*Pdk1*$^{-/-}$ (red line) and Oo*Pdk1*$^{+/+}$ female (black line). (**F**–**K**) POF in Oo*Pdk1*$^{-/-}$ females during early adulthood. Ovaries from 8- and 15-week-old Oo*Pdk1*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice were embedded in paraffin, and sections of 8 μm thickness were prepared and stained with hematoxylin. Virtually, all follicular structures were depleted in Oo*Pdk1*$^{-/-}$ ovaries (G and J). Arrows in the upper inset of (I) indicate primordial follicles. (**L**) Average weights of ovaries obtained from young adult (8–12 weeks old) Oo*Pdk1*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice. (**M** and **N**) Levels of FSH and LH in sera of young adult (8–12 weeks old) Oo*Pdk1*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice. For (E) and (L–N), the numbers of mice used (*n*) and *P*-values are shown in the figures.

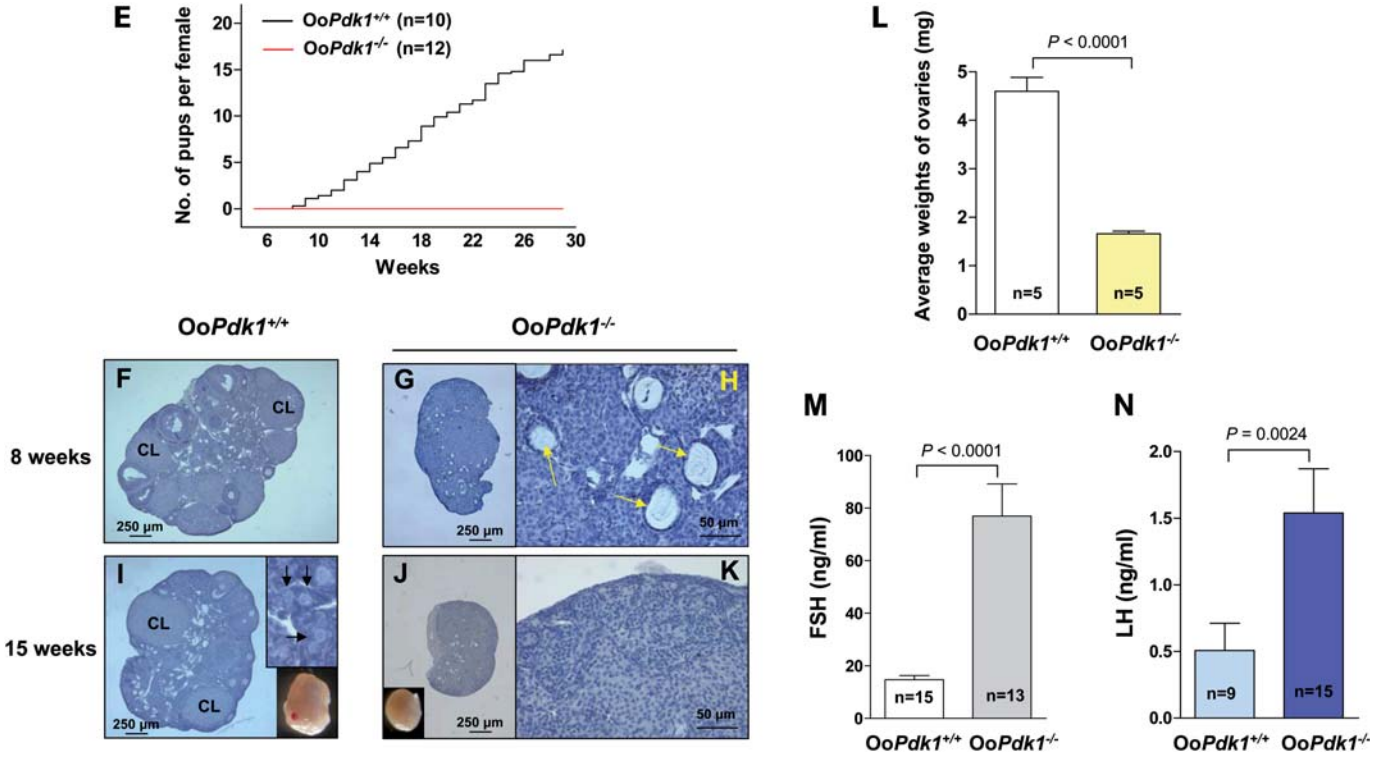

Figure 1. Continued.

are thus determined by the size and persistence of her primordial follicle pool (1–4).

The molecular mechanisms controlling the balance between the survival and loss of primordial follicles are, however, poorly defined. In recent years, genetic factors have received increasing attention as determinants of ovarian aging and menopausal age (1). In a recent study, we showed that ablation of *phosphatase and tensin homolog deleted on chromosome 10* (*Pten*) in oocytes results in excessive activation of primordial follicles (7). PTEN is a negative regulator of phosphatidylinositol 3 kinase (PI3K) (Fig. 1B) (8–10), and it appears to suppress the activation of primordial follicles and maintain them in a dormant state (7). However, whether or not the PI3K signaling in oocytes has direct effects on the survival and maintenance of the primordial follicle pool is not yet clear.

A considerable proportion of the signaling mediated by PI3Ks converges at 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Fig. 1B). PDK1 activates Akt through co-binding to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) generated by PI3Ks (11,12). Apart from this, PDK1 also functions as a master kinase to activate other protein kinases of the AGC family (denoting protein kinases A, G and C), such as p70 S6 kinase 1 (S6K1) (Fig. 1B), p90 ribosomal S6 kinase (RSK) and protein kinase C (PKC) (11). The activation of substrates other than Akt does not, however, involve binding of PDK1 to the PI3K generated PIP_3 (11). Previous reports have shown that conventional deletion of the PDK1-encoding gene *Pdk1* (also known as *Pdpk1* or *Pkb kinase*—Mouse Genome Informatics) causes embryonic lethality (13), and conditional deletion of *Pdk1* from heart, pancreas, liver and T cells results in heart failure (14), diabetes (15), defective postprandial glucose disposal and liver failure (16,17) and impaired T cell differentiation (18), respectively.

In the present study, we found that deletion of *Pdk1* in mouse oocytes resulted in accelerated ovarian aging and premature ovarian failure (POF), due to the severely compromised lifespan of primordial follicles, which was caused by suppressed PDK1–Akt–S6K1–ribosomal protein S6 (rpS6) signaling in oocytes. This finding was further corroborated by our results that mice lacking the *Rps6* gene in oocytes exhibited POF similar to that seen in *Pdk1*-deficient mice. Thus, PDK1 signaling in oocytes appears to be indispensable for maintaining the survival of primordial follicles, which is in turn crucial for determining the duration of female fertility. As the demise of non-growing follicles is initiated by oocyte death (5), our results suggest that the suppression of PDK1 signaling in oocytes contributes to follicular loss, which eventually leads to the onset of ovarian senescence.

RESULTS

Infertility, accelerated ovarian aging and POF caused by deletion of *Pdk1* in oocytes

To study how PDK1 signaling in oocytes controls the different courses of development of primordial follicles, we generated mutant mice in which the *Pdk1* gene was deleted in oocytes of primordial and further developed follicles (referred to as Oo*Pdk1*$^{-/-}$ mice). This was achieved by crossing *Pdk1*$^{loxP/loxP}$

mice (15,19) with transgenic mice expressing *growth differentiation factor 9* (*Gdf-9*) promoter-mediated Cre recombinase (20). The *Gdf-9* promoter becomes active in oocytes of primordial follicles (20), and primordial follicles in mice start to form on postnatal day (PD) 1–2 and finish on PD7–8 (21). By western blot analysis, we found that expression of PDK1 protein was almost completely absent in oocytes that were isolated from ovaries of 12–14-day-old Oo*Pdk1*$^{-/-}$ mice (Fig. 1C), and it was completely absent in ovulated Oo*Pdk1*$^{-/-}$ oocytes (Fig. 1D).

During a testing period from 5 to 30 weeks of age, the Oo*Pdk1*$^{-/-}$ females were found to be infertile (Fig. 1E). To determine how the deletion of *Pdk1* from oocytes impedes fertility, we studied the ovarian morphology of young adult mutant mice. As shown in Figure 1G, in ovaries of 8-week-old Oo*Pdk1*$^{-/-}$ mice, basically all follicular structures were diminished, with some degenerating oocytes in growing follicles being seen (Fig. 1H, arrows). Ovaries from older Oo*Pdk1*$^{-/-}$ mice showed a similar morphology; they were devoid of oocytes and follicles (Fig. 1J and K). In comparison, ovaries from adult Oo*Pdk1*$^{+/+}$ ovaries (Fig. 1F and I) contained follicles and corpora lutea (CL), and most importantly, primordial follicles that would retain their fertility for the rest of the reproductive life (Fig. 1I, upper inset, arrows).

In young adult Oo*Pdk1*$^{-/-}$ mice, the ovaries were lighter (Fig. 1L), which was accompanied by significantly elevated serum levels of follicle-stimulating hormone (FSH) (Fig. 1M) and luteinizing hormone (LH) (Fig. 1N). These results show that deletion of *Pdk1* from oocytes causes POF in Oo*Pdk1*$^{-/-}$ mice.

PDK1 in oocytes is required to maintain the survival of primordial follicles

The follicular resource available in the ovary is the direct determinant of reproductive lifespan (1,2). To investigate how the absence of *Pdk1* in oocytes leads to POF, we studied postnatal follicular development in Oo*Pdk1*$^{-/-}$ mice. We found that at PD8 (Fig. 2A, B and G) and PD23 (Fig. 2H), the numbers of primordial follicles and of all follicles were similar in Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ ovaries, indicating that comparable numbers of follicles are formed in the wild-type and mutant ovaries and that deletion of *Pdk1* in oocytes does not have an immediate effect on follicular survival up to PD23. This may be caused by some temporary compensatory mechanisms that take over the functions of PDK1 signaling in oocytes. Alternatively, as the deletion of *Pdk1* in oocytes only occurs after the primordial follicles are formed, it may take some time for PDK1-mediated signals to become downregulated or inactivated in oocytes.

However, at PD35, the time of onset of sexual maturity, the Oo*Pdk1*$^{-/-}$ ovaries were smaller (Fig. 2D), due to markedly reduced numbers of follicles (Fig. 2I). Specifically, compared with Oo*Pdk1*$^{+/+}$ ovaries where clusters of primordial follicles were seen (Fig. 2E, arrows), the numbers of healthy-looking primordial follicles in Oo*Pdk1*$^{-/-}$ ovaries were substantially reduced (Fig. 2F, arrow), corresponding to 27.7% of those in Oo*Pdk1*$^{+/+}$ ovaries (Fig. 2I). In Oo*Pdk1*$^{-/-}$ ovaries, the total numbers of follicles corresponded to 33.7% of those in Oo*Pdk1*$^{+/+}$ ovaries at PD35 (Fig. 2I), with also reduced numbers of activated follicles (including type 3b, type 4, type 5 and type 6 follicles) (61.6%) (Fig. 2I). Thus, primordial follicles were more susceptible to the loss of *Pdk1* in oocytes and were the main population of follicles to disappear from Oo*Pdk1*$^{-/-}$ ovaries.

At 8 weeks of age, primordial follicles that normally survive as the source of ova for the entire reproductive lifetime had been completely abolished in Oo*Pdk1*$^{-/-}$ ovaries (Fig. 1G). Consequently, healthy-looking growing follicles were not observed in the mutant ovaries at this age (Fig. 1G). These results show that the accelerated depletion of primordial follicles is the direct cause of POF in Oo*Pdk1*$^{-/-}$ mice.

In this mutant mouse model, the *Gdf-9* promoter used to mediate Cre recombinase expression becomes active in oocytes of primordial follicles (20). As a synchronous wave of follicular activation takes place once the primordial follicles are formed (3,22), it is most likely that the first wave of postnatal follicular activation has occurred in Oo*Pdk1*$^{-/-}$ ovaries before the complete clearance of *Pdk1* mRNA and PDK1 protein in oocytes.

At PD23, female Oo*Pdk1*$^{-/-}$ mice can respond normally to gonadotropin treatment and ovulate. At the age of 5–6 weeks, some type 5–6 follicles from the first wave of postnatal follicular activation were still present in Oo*Pdk1*$^{-/-}$ ovaries (Fig. 2D, arrows), indicating that PDK1 in oocytes mainly maintains the survival of primordial follicles in a stage-specific manner, but may not greatly affect the follicles that have entered the growing phase. The Oo*Pdk1*$^{-/-}$ females had a normal vaginal opening at the age of 5–6 weeks (which is the appropriate age) and they showed normal estrous cycles. These mice were found to ovulate normally when mated with wild-type male mice, based on the fact that similar numbers of oocytes/embryos were recovered from their oviducts (Supplementary Material, Fig. S1A). This indicates that before the follicles become depleted in Oo*Pdk1*$^{-/-}$ ovaries, ovulation *per se* is not affected even if *Pdk1* is deleted from oocytes. However, zygotes from the Oo*Pdk1*$^{-/-}$ females mated with wild-type males showed arrest of development at the two-cell stage, making the Oo*Pdk1*$^{-/-}$ females completely infertile (Supplementary Material, Fig. S1B).

Deletion of *Pdk1* in oocytes reverses the excessive follicular activation and survival caused by loss of *Pten*

The rapid depletion of primordial follicles in Oo*Pdk1*$^{-/-}$ ovaries makes it difficult to study the function of oocyte PDK1 in mediating follicular activation. To circumvent this problem, we crossed Oo*Pdk1*$^{-/-}$ mice with mice lacking *Pten* in oocytes, which exhibit premature activation of the primordial follicle pool (referred to as Oo*Pten*$^{-/-}$ mice in this study) (7), and studied follicular development in progeny mice with concurrent loss of *Pdk1* and *Pten* in oocytes (referred to as Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ mice).

In Oo*Pten*$^{-/-}$ ovaries, all primordial follicles were activated by PD23, with noticeably enlarged oocytes (Fig. 3A, arrows), and the percentage of primordial follicles was 0% (Fig. 3D) (7). In Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ ovaries at PD23, however, clusters of primordial follicles were observed (Fig. 3B, arrows), as with Oo*Pten*$^{+/+}$ ovaries (Fig. 3C,

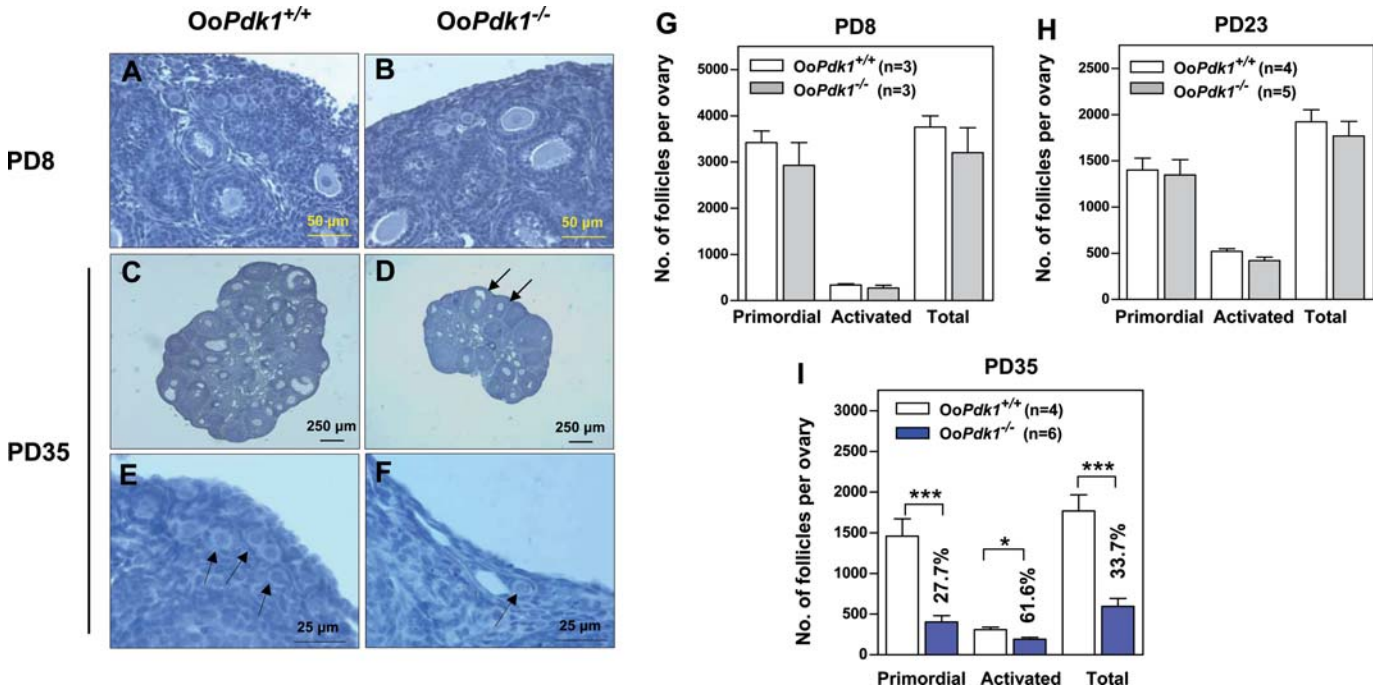

Figure 2. Deletion of *Pdk1* from oocytes causes accelerated depletion of primordial follicles in Oo*Pdk1*$^{-/-}$ mice. (**A**–**F**) Morphological analysis of ovaries from Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ littermates at PD8 and PD35. Ovaries from 8- and 35-day-old Oo*Pdk1*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice were embedded in paraffin, and serial sections of 8 μm thickness were prepared and stained with hematoxylin. At PD8, similar ovarian morphologies were seen in Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ littermates (A and B). At PD35, however, the Oo*Pdk1*$^{-/-}$ ovaries were smaller (D), and a dramatic loss of primordial follicles took place in Oo*Pdk1*$^{-/-}$ ovaries (F, arrow) compared with Oo*Pdk1*$^{+/+}$ ovaries (E, arrows). The experiments were repeated more than three times, and for each time and each age, ovaries from one mouse of each genotype were used. (**G**–**I**) Quantification of follicle numbers in ovaries of PD8, PD23 and PD35 Oo*Pdk1*$^{-/-}$ mice and their Oo*Pdk1*$^{+/+}$ littermates. Ovaries were embedded in paraffin, and sections of 8 μm thickness were prepared and stained with hematoxylin. The numbers of follicles per ovary (mean ± SEM) were quantified as described in Materials and Methods. Note that although similar numbers of follicles were seen in both genotypes at PD8 (G) and PD23 (H), the numbers of primordial follicles, activated follicles (including type 3b, type 4, type 5 and type 6 follicles) and all follicles per Oo*Pdk1*$^{-/-}$ ovary were significantly reduced at PD35, representing 27.7%, 61.6% and 33.7% of those per Oo*Pdk1*$^{+/+}$ ovary, respectively (I). It is notable that the reduced follicle numbers in Oo*Pdk1*$^{-/-}$ ovaries were mostly due to reduction in the number of primordial follicles (I). The numbers of mice used (*n*) and results of statistical analyses are given. $^{*}P < 0.05$, $^{***}P < 0.001$.

arrows). The proportion of primordial follicles in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ ovaries was elevated to 71.3%, which was similar to the proportions in Oo*Pten*$^{+/+}$ ovaries (69.2%) and Oo*Pdk1*$^{-/-}$ ovaries (76.2%) (Fig. 3D). In addition, the temporarily enhanced postnatal follicular survival seen in Oo*Pten*$^{-/-}$ ovaries, as judged by the elevated total number of follicles at PD23 (Fig. 3E) (7), was found to be efficiently reversed in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ ovaries, to levels similar to those seen in Oo*Pten*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ ovaries (Fig. 3E).

The above results indicate that the excessive follicular activation and elevated postnatal follicular survival observed in Oo*Pten*$^{-/-}$ ovaries (7) were mostly, if not all, mediated by PDK1, suggesting that the activation and survival of primordial follicles are dependent on PDK1 signaling in oocytes.

Deletion of *Pdk1* impairs PDK1–Akt–S6K1–rpS6 signaling in Oo*Pdk1*$^{-/-}$ oocytes

PDK1 plays an essential role in phosphorylating and activating Akt and S6K1 (11). To study the signaling pathways underlying the accelerated loss of follicles in Oo*Pdk1*$^{-/-}$ ovaries, we first studied Akt signaling in mutant and control oocytes. We stimulated starved oocytes with Kit ligand (KL), which has been shown to activate PI3K–Akt signaling in oocytes through its receptor Kit (23). We found that KL was able to trigger phosphorylation of Akt (p-Akt, T308) in Oo*Pdk1*$^{+/+}$ oocytes, but not in Oo*Pdk1*$^{-/-}$ oocytes (Fig. 4A). This shows that the phosphorylation of Akt at threonine 308 by PDK1, which is essential for activation of Akt (11,24), is disabled in Oo*Pdk1*$^{-/-}$ oocytes. As an indicator of Akt activity, elevated phosphorylation of Foxo3a at serine 253 (p-Foxo3a, S253) upon stimulation by KL was seen in Oo*Pdk1*$^{+/+}$ oocytes, but not in Oo*Pdk1*$^{-/-}$ oocytes (Fig. 4A), implying that Foxo3a can not be efficiently phosphorylated and suppressed by Akt (25) in the mutant oocytes, which can be a reason for the accelerated follicular loss, as previously proposed (26). Elevated phosphorylation of another Akt substrate, tuberin/Tsc2, was triggered by treatment with KL in Oo*Pdk1*$^{+/+}$ oocytes, but again not in Oo*Pdk1*$^{-/-}$ oocytes (Fig. 4A, p-Tsc2, T1462), indicating that Tsc2 may not be efficiently suppressed by Akt in Oo*Pdk1*$^{-/-}$ oocytes. Thus, the loss of *Pdk1* in oocytes leads to suppressed Akt signaling.

Activation of S6K1 requires phosphorylation of its threonine 229 by PDK1 (11). S6K1 can then phosphorylate and activate one of the 40S ribosomal proteins, rpS6, which is needed for protein translation and ribosome biogenesis during cell growth (27,28). In an earlier study (7), we reported that overactivation of PI3K signaling in Oo*Pten*$^{-/-}$ oocytes leads to

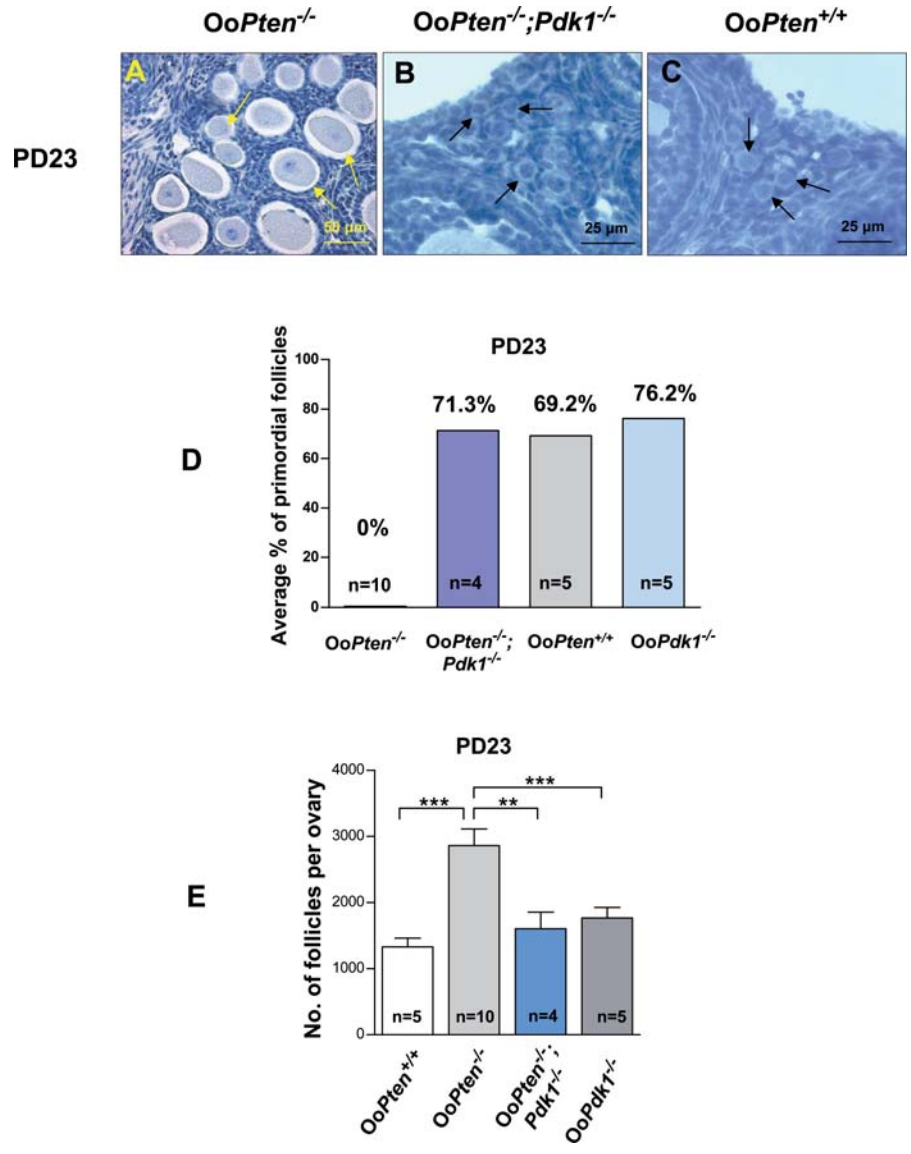

Figure 3. Concurrent loss of *Pdk1* and *Pten* in oocytes reverses the excessive follicular activation and survival caused by loss of *Pten*. (**A**–**C**) Morphological analysis of ovaries from Oo*Pten*$^{-/-}$, Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ and Oo*Pten*$^{+/+}$ mice at PD23. In Oo*Pten*$^{-/-}$ ovaries, no primordial follicles were seen, and all follicles were activated with enlarged oocytes (A, arrows). However, when *Pten* and *Pdk1* were simultaneously deleted from oocytes, clusters of primordial follicles were observed (B, arrows), which were similar to those in the control Oo*Pten*$^{+/+}$ mice (C, arrows). (**D**) Proportions of primordial follicles (compared with the total number of follicles) in Oo*Pten*$^{-/-}$, Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$, Oo*Pten*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ ovaries at PD23. Ovaries from Oo*Pten*$^{-/-}$, Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ doubly deficient mice, Oo*Pten*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ mice (all at PD23) were embedded in paraffin, and sections of 8 μm thickness were prepared and stained with hematoxylin. Numbers of primordial follicles and other types of follicles were quantified as described in Materials and Methods. The average percentages of primordial follicles in Oo*Pten*$^{-/-}$, Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$, Oo*Pten*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ ovaries are shown. The proportion of primordial follicles in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ ovaries was elevated to 71.3% when compared with that in Oo*Pten*$^{-/-}$ ovaries (0%), which was similar to the proportions in Oo*Pten*$^{+/+}$ ovaries (69.2%) and Oo*Pdk1*$^{-/-}$ ovaries (76.2%). The numbers of mice used (*n*) are shown in the figure. (**E**) Concurrent loss of *Pdk1* and *Pten* in oocytes prevented the enhanced postnatal follicular survival seen in Oo*Pten*$^{-/-}$ ovaries. Total numbers of follicles per ovary (mean ± SEM) at PD23 are shown. The postnatal follicular survival in Oo*Pten*$^{-/-}$ ovaries was elevated compared with that in Oo*Pten*$^{+/+}$ ovaries (7). However, follicle numbers in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ doubly deficient ovaries at PD23 were similar to those in Oo*Pten*$^{+/+}$ and Oo*Pdk1*$^{-/-}$ ovaries. The numbers of mice used (*n*) and results of statistical analyses are shown in the figure. $^{**}P < 0.01$, $^{***}P < 0.001$.

elevated activation of rpS6, indicating that rpS6 may act downstream of PI3K–Akt–S6K1 signaling to mediate the protein translation needed for oocyte growth during follicular activation. In this study, we investigated whether the S6K1–rpS6 signaling is deregulated in Oo*Pdk1*$^{-/-}$ oocytes. As shown in Figure 4B, compared with Oo*Pdk1*$^{+/+}$ oocytes where phosphorylation of S6K1 (p-S6K1, T229) was triggered by KL treatment, the phosphorylation of S6K1 at T229 was completely abolished in Oo*Pdk1*$^{-/-}$ oocytes, showing that S6K1 cannot be activated in the mutant oocytes. Moreover, in Oo*Pdk1*$^{-/-}$ oocytes, phosphorylation of rpS6 was substantially downregulated (Fig. 4B, p-rpS6, S240/4), indicating that there were low levels of rpS6 activation and of rpS6-mediated protein translation and ribosome biogenesis in the mutant oocytes.

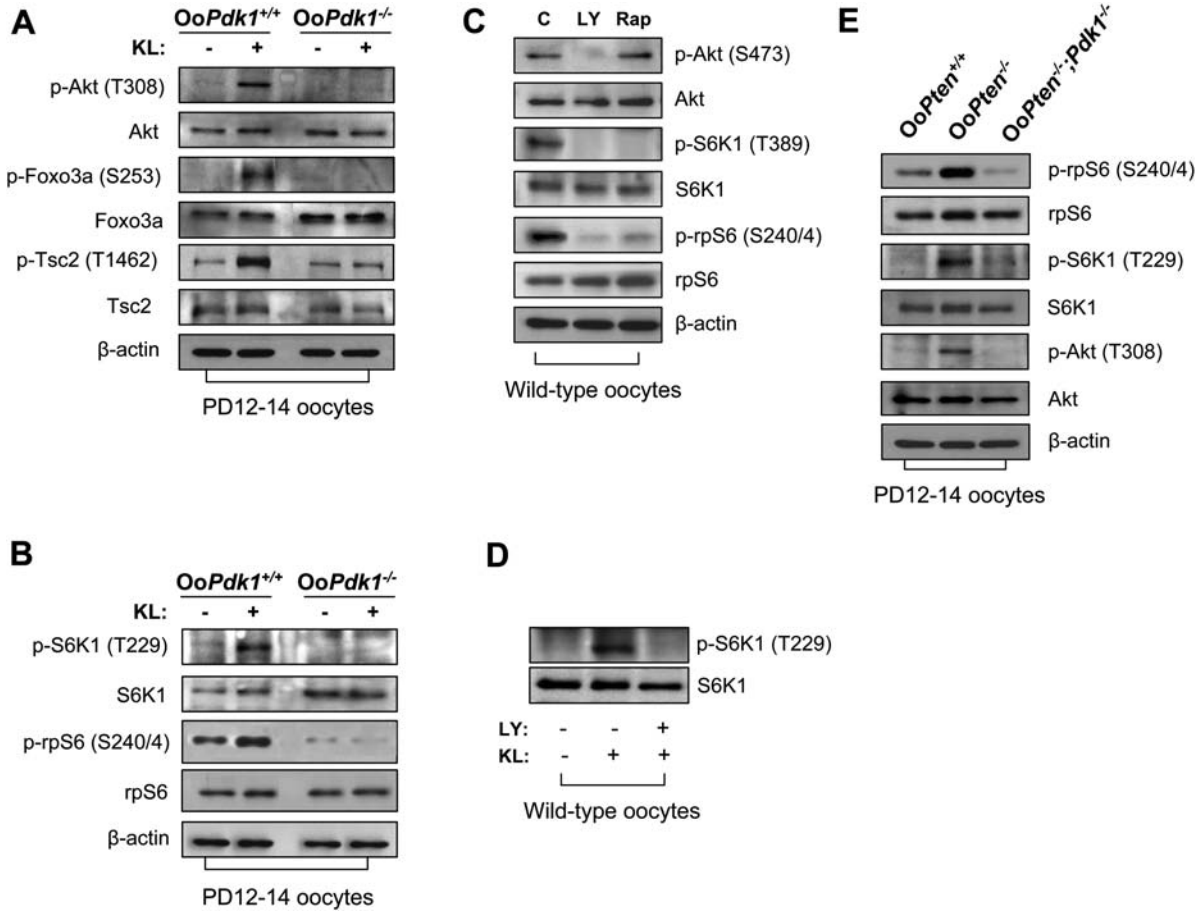

Figure 4. Studies of PDK1–Akt–S6K1–rpS6 signaling in Oo*Pdk1*$^{-/-}$, Oo*Pdk1*$^{+/+}$, Oo*Pten*$^{-/-}$, Oo*Pten*$^{+/+}$, Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ and C57BL/6J oocytes. Oocytes were isolated from ovaries of mice at PD12–14, and western blots were performed as described in Materials and Methods. (**A**) Studies of Akt signaling in Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ oocytes. KL treatment (100 ng/ml, 2 min) was found to lead to rapid phosphorylation of Akt at T308 in Oo*Pdk1*$^{+/+}$ oocytes, but not in Oo*Pdk1*$^{-/-}$ oocytes, indicating that Akt cannot be activated in Oo*Pdk1*$^{-/-}$ oocytes. Consequently, KL can trigger elevated phosphorylation of Akt substrates Foxo3a (S253) and Tsc2 (T1462) in Oo*Pdk1*$^{+/+}$ oocytes, but not in Oo*Pdk1*$^{-/-}$ oocytes. Levels of total Akt, Foxo3a, Tsc2, and β-actin were used as internal controls. (**B**) Studies of S6K1–rpS6 signaling in Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ oocytes. Phosphorylation of S6K1 (p-S6K1, T229) was triggered by treatment with KL (100 ng/ml, 2 min) in Oo*Pdk1*$^{+/+}$ oocytes, but not in Oo*Pdk1*$^{-/-}$ oocytes, indicating that S6K1 cannot be activated in the mutant oocytes. As a result, the phosphorylation of rpS6 (p-rpS6, T240/4) is drastically suppressed in Oo*Pdk1*$^{-/-}$ oocytes. Levels of total S6K1, rpS6 and β-actin were used as internal controls. All experiments were repeated at least three times. For each experiment, material from three to five mice was used per lane. In each lane, 20–30 μg of protein sample was loaded. Representative images are shown. (**C** and **D**) Activation of S6K1–rpS6 in oocytes is dependent on PI3K–Akt signaling. Oocytes isolated from ovaries of C57BL/6J mice at PD12–14 were cultured with various inhibitors or stimulated with KL (100 ng/ml, 2 min) as described in Materials and Methods. (C) Treatment of oocytes with the PI3K-specific inhibitor LY294002 (LY, 50 μM) for 1 h largely suppressed the levels of p-Akt (S473), p-S6K1 (T389) and p-rpS6 (240/4). However, treatment with the mTORC1-specific inhibitor rapamycin (Rap, 50 nM) for 1 h only suppressed the levels of p-S6K1 (T389) and p-rpS6 (S240/4) in oocytes. (D) KL-triggering of p-S6K1 (T229) in oocytes was completely blocked by a 1 h pretreatment with LY294002 (LY, 50 μM). Levels of total Akt, S6K1, rpS6 and β-actin were used as internal controls for loading of equal amounts of protein. (**E**) The elevated levels of p-rpS6 (S240/4), p-S6K1 (T229) and p-Akt (T308) in Oo*Pten*$^{-/-}$ oocytes were prevented by the concurrent loss of *Pten* and *Pdk1* in Oo*Pten*$^{-/-}$; *Pdk1*$^{-/-}$ oocytes. Levels of total S6K1, Akt and β-actin were used as internal controls. All experiments were repeated three times. For each experiment, material from three to five mice was used per lane. In each lane, 20–30 μg of protein sample was loaded. Representative images are shown.

To determine the upstream and downstream relationships between Akt and S6K1 signaling in oocytes, we treated cultured wild-type oocytes with the PI3K-specific inhibitor LY294002. As shown in Figure 4C, LY294002 (LY) largely suppressed the phosphorylation of Akt (p-Akt, S473), S6K1 (p-S6K1, T389) and rpS6 (p-rpS6, S240/4) in oocytes. As a control, treatment of cultured oocytes with the mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin (Rap) only suppressed the phosphorylation of S6K1 and rpS6, but not that of Akt (Fig. 4C). In addition, the phosphorylation of S6K1 at T229 (p-S6K1, T229) that was triggered by KL treatment was completely blocked by pretreatment of the oocytes with LY294002 (Fig. 4D). These results indicate that activation of S6K1–rpS6 in primary mouse oocytes is downstream of PI3K–Akt signaling.

Therefore, we conclude that the absence of *Pdk1* in oocytes impairs the PDK1–Akt–S6K1–rpS6 signaling, which appears to be responsible for the premature loss of primordial follicles in Oo*Pdk1*$^{-/-}$ ovaries.

To determine whether the premature loss of primordial follicles in Oo*Pdk1*$^{-/-}$ ovaries occurred through accelerated

apoptosis in the mutant oocytes, we measured the levels of some apoptotic molecules in Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ oocytes that were isolated from PD23–25 mice, which is a time point that is prior to the disappearance of primordial follicles. However, the levels of cleaved poly (ADP-ribose) polymerase [PARP] and cleaved caspase-7 were found to be unaltered in Oo*Pdk1*$^{-/-}$ oocytes as compared to Oo*Pdk1*$^{+/+}$ oocytes (Supplementary Material, Fig. S2).

rpS6 is the key downstream effector of PTEN/PI3K–PDK1–Akt–S6K1 signaling in oocytes

rpS6 is an important substrate of S6K1. When PI3K signaling was overactivated in Oo*Pten*$^{-/-}$ oocytes, elevated phosphorylation (indicating activation) of both rpS6 (Fig. 4E, p-rpS6, S240/4) and S6K1 (Fig. 4E, p-S6K1, T229) could be seen. In the present study, we also found that the elevated phosphorylation of rpS6 in Oo*Pten*$^{-/-}$ oocytes was prevented by the concurrent loss of *Pdk1* in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ oocytes (Fig. 4E, p-rpS6), which was most likely caused by downregulated activation of S6K1 (Fig. 4E, p-S6K1, T229) and of Akt (Fig. 4E, p-Akt, T308) in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ oocytes, when compared with that in Oo*Pten*$^{-/-}$ oocytes. Thus, downregulation of phosphorylation of rpS6 in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ oocytes may be the reason behind the prevention of excessive follicular activation and survival seen in Oo*Pten*$^{-/-}$ ovaries. We propose that the phosphorylation and activation of rpS6, as downstream events of PTEN/PI3K–PDK1–Akt–S6K1 signaling in oocytes, are important for maintaining the survival and activation of primordial follicles.

Oocyte-specific deletion of *Rps6* causes POF in Oo*Rps6*$^{-/-}$ mice

Of the 33 40S ribosomal proteins, rpS6 is the only ribosomal protein that is known to be a substrate of S6K1, and it is activated through phosphorylation by S6K1 (28). To determine whether rpS6 in oocytes is indispensable for maintenance of the normal reproductive lifespan, we conditionally deleted the *Rps6* gene from mouse oocytes by crossing *Rps6*$^{loxP/loxP}$ mice (28) with the *Gdf-9-Cre* mice (20). The mutant animals are referred to as Oo*Rps6*$^{-/-}$ mice.

Our results showed that rpS6 protein expression was almost absent in Oo*Rps6*$^{-/-}$ oocytes (Fig. 5A). At PD8, the morphology of Oo*Rps6*$^{-/-}$ ovaries was similar to that of Oo*Rps6*$^{+/+}$ ovaries (Fig. 5B and C). At PD23, however, the mutant ovaries were much smaller (Fig. 5E) and lighter (Fig. 5L), apparently due to dramatically reduced numbers of follicles (Fig. 5E). By PD35, even fewer healthy primordial follicles or other types of follicles were found in Oo*Rps6*$^{-/-}$ ovaries (Fig. 5G and H). At 8 weeks of age, the follicular structure completely disappeared in ovaries of Oo*Rps6*$^{-/-}$ mice (Fig. 5J and K). In accordance with this, the Oo*Rps6*$^{-/-}$ mice were found to be infertile due to lack of preovulatory follicles (Fig. 5E and G) and ovulation. It is thus clear that the loss of *Rps6* in oocytes causes POF in Oo*Rps6*$^{-/-}$ mice, which was corroborated by the elevated levels of FSH and LH in sera (Fig. 5M and N).

Collectively, these results strongly suggest that rpS6 in oocytes plays an important role in maintaining the survival of primordial and developing follicles. The PDK1–Akt–S6K1–rpS6 network in oocytes is therefore crucial for preservation of the normal length of reproductive life in females.

DISCUSSION

In this study, by using a mouse model with oocyte-specific deletion of *Pdk1*, we found that PDK1 signaling in oocytes plays an essential role in preserving the normal reproductive lifespan in females by maintaining the survival of primordial follicles during their long dormancy. Suppressed PDK1–Akt–S6K1–rpS6 signaling in oocytes was shown to be responsible for the clearance of non-growing follicles, which causes reproductive aging and eventually ovarian senescence. We therefore propose that maintenance of the primordial follicle pool, which is a prerequisite for preservation of the normal length of reproductive life in females, is dependent on PDK1 signaling in oocytes (Fig. 6A).

Recently, PTEN, the upstream negative regulator of PI3K–PDK1 signaling, was identified as a suppressor of follicular activation (7). PTEN in oocytes prevents primordial follicles from being activated prematurely, and it also promotes their death (7). It is likely that PTEN activity adjusts the 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 (Fig. 6A). On the other hand, loss of primordial follicles can be caused by suppressed PDK1 signaling in oocytes (Fig. 6C); and activation of primordial follicles can be a result of elevated PDK1 signaling in oocytes (Fig. 6B). Based on the evidence accumulated, we believe that the PTEN/PI3K–PDK1 signaling network in oocytes controls the survival during dormancy, the activation, and the loss of primordial follicles, which together govern the length of reproductive life in females.

Signal transduction studies with oocytes lacking *Pdk1*, *Pten* or both *Pdk1* and *Pten* revealed that the phosphorylation and activation of rpS6 in oocytes via PDK1–Akt–S6K1 signaling are key downstream events in determining the fates of primordial follicles. Underactivation of rpS6 in oocytes by deletion of *Pdk1*, or deletion of *Rps6*, leads to accelerated loss of primordial follicles. In contrast, overactivation of rpS6 in oocytes as a result of loss of *Pten* is accompanied by enhanced follicular activation and survival (7). Furthermore, simultaneous ablation of *Pdk1* and *Pten* in oocytes reverses the excessive follicular activation and survival seen in Oo*Pten*$^{-/-}$ ovaries, which is likely achieved by downregulation of rpS6 phosphorylation in Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ oocytes. We therefore propose that the up- and downregulation of rpS6-mediated protein translation and ribosome biogenesis in oocytes may be critical for controlling the courses of development of primordial follicles regarding their survival during dormancy, their activation or their loss.

One remaining question is that the upstream regulation of PTEN/PI3K–PDK1–Akt–S6K1–rpS6 signaling in oocytes, such as the extra-oocyte signals from the surrounding somatic cells that activate or suppress the signaling cascades within the oocyte, is not completely understood.

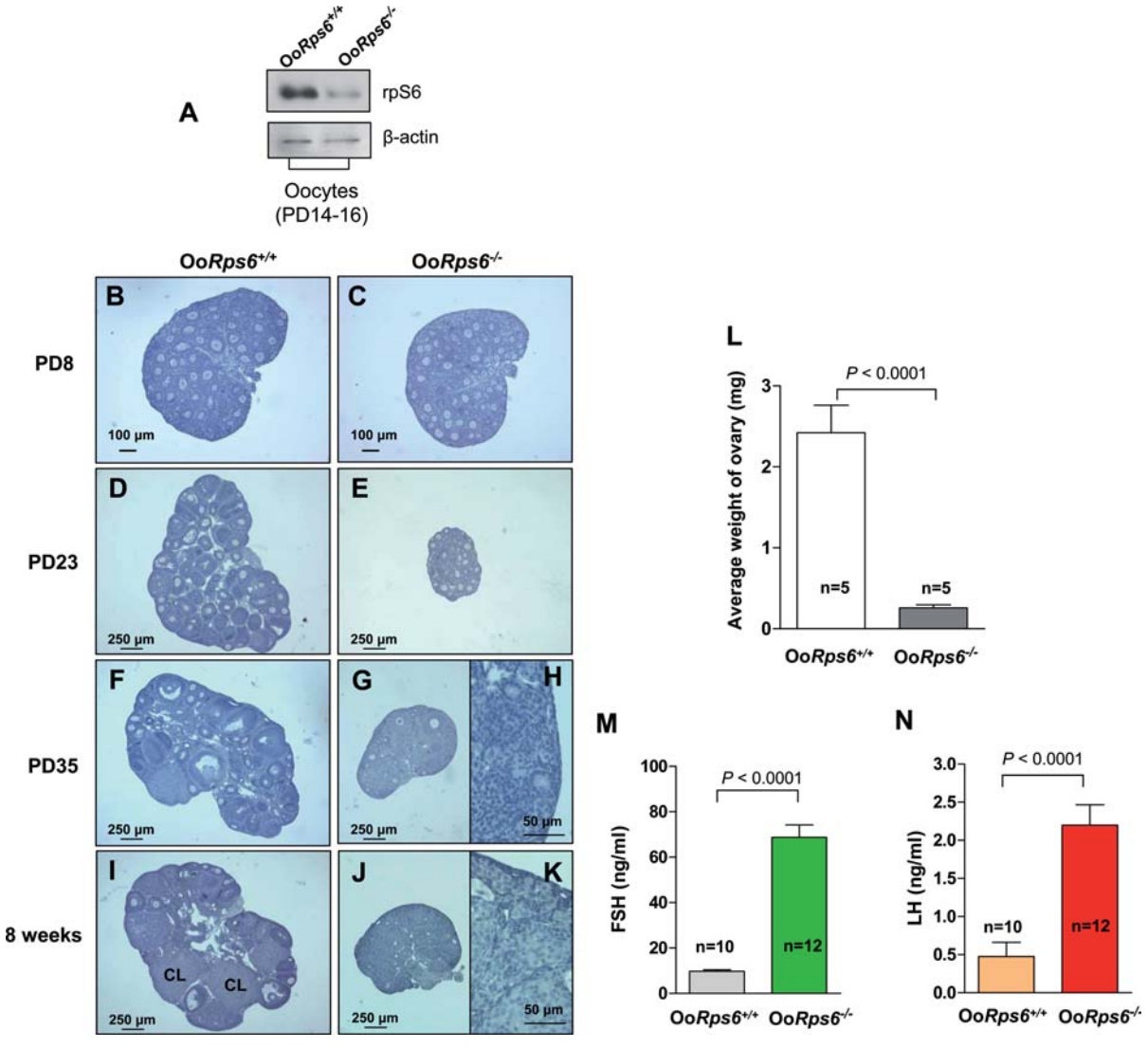

Figure 5. Deletion of *Rps6* in mouse oocytes leads to POF. (**A**) Effective removal of rpS6 protein expression from mouse oocytes. Oocytes were isolated from ovaries of 14–16-day-old Oo*Rps6*$^{+/+}$ and Oo*Rps6*$^{-/-}$ mice as described in Materials and Methods, and the extracted proteins were used for western blot. The experiments were repeated three times. For each experiment, material from five to ten mice was used per lane. In each lane, about 20 μg of protein sample was loaded. Levels of β-actin were used as internal controls. Representative images are shown. (**B**–**K**) Morphological analysis of ovaries from Oo*Rps6*$^{+/+}$ and Oo*Rps6*$^{-/-}$ littermates. Ovaries were embedded in paraffin, and sections of 8 μm thickness were prepared and stained with hematoxylin. At PD8, similar ovarian morphologies were seen in Oo*Rps6*$^{+/+}$ and Oo*Rps6*$^{-/-}$ mice (B and C). At PD23, however, the Oo*Rps6*$^{-/-}$ ovaries appeared to be much smaller (E), due to dramatic loss of primordial and activated follicles. By PD35, the follicular structures in Oo*Rps6*$^{-/-}$ ovaries were almost depleted, and few primordial or other types of follicles could be identified (G and H). Ovaries from 8-week-old Oo*Rps6*$^{-/-}$ mice showed a complete loss of follicular structures (J and K). The experiments were repeated more than three times, and for each time and each age, ovaries from one mouse of each genotype were used. (**L**) Average weights of ovaries that were obtained from 23- to 25-day-old Oo*Rps6*$^{+/+}$ and Oo*Rps6*$^{-/-}$ mice. The numbers of mice used (*n*) and *P*-values are given. (**M** and **N**) Elevated levels of FSH and LH in 8–12-week-old Oo*Rps6*$^{-/-}$ mice. This confirms the POF in Oo*Rps6*$^{-/-}$ mice, as shown in (J) and (K). Measurements of FSH and LH levels in mouse serum were performed as described in Materials and Methods. The numbers of mice used (*n*) and *P*-values are shown.

Also, it is not yet clear whether deletion of *Pdk1* in oocytes triggers the loss of primordial follicles via apoptosis of the oocytes. From a practical point of view, it has been difficult to detect apoptosis in mouse primordial follicles using normal apoptotic markers, such as by TUNEL assay or by detection of cleaved PARP (our own data; Dr Jodi Anne Flaws, personal communications, University of Illinois, USA). This is likely due to the rapid and transient nature of the apoptotic process. On the other hand, although apoptosis has been suggested as the mechanism underlying oocyte death during primordial and primary follicle atresia in the postnatal ovary (29,30), other studies, however, show no apoptosis in primordial follicles in postnatal life (31). Some previous studies have suggested that there are more differences than similarities between physiological oocyte cell death and apoptosis, suggesting that oocyte death should be assigned to a different class of cell death other than apoptosis (32,33). It has been shown recently that the clearance of primordial follicles in postnatal mouse ovaries does not occur via apoptosis (34). Whether suppressed PDK1 signaling in oocytes triggers fol-

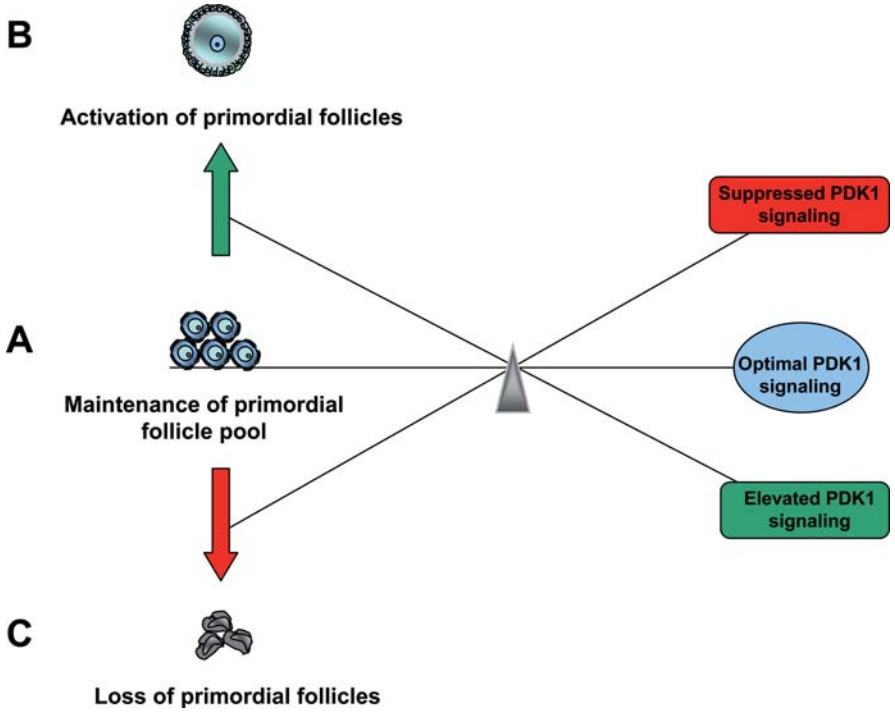

Figure 6. Suggested roles of PDK1 signaling in oocytes in controlling the survival during dormancy, the activation and the loss of primordial follicles. (**A**) Optimal PDK1 signaling in oocytes is proposed to be essential for maintaining the survival of primordial follicles during their long dormancy and is therefore indispensable for preservation of the length of reproductive life. (**B**) If PDK1 signaling is elevated in oocytes—for example, in Oo*Pten*$^{-/-}$ oocytes where the PI3K negative regulator PTEN is deleted (7)—oocyte growth is initiated and follicular activation takes place. (**C**) In contrast, if PDK1 signaling is suppressed in oocytes, such as in the case of Oo*Pdk1*$^{-/-}$ mice, the survival of primordial follicles is compromised, leading to loss of primordial follicles that causes reproductive aging. We propose that finely balanced PDK1 signaling in oocytes is essential for the maintenance of the primordial follicle pool and for preservation of the reproductive lifespan in females.

licle loss through apoptosis of oocytes will be revealed by *in vivo* studies; for example, by crossing mice lacking the pro-apoptotic molecule *Bax* to Oo*Pdk1*$^{-/-}$ mice, to see whether the accelerated loss of primordial follicles in Oo*Pdk1*$^{-/-}$ mice can be rescued by the simultaneous loss of *Bax*.

Ablation of either *Pten* or *Pdk1* in mouse oocytes results in POF, but from opposite directions. *Pten* deficiency in oocytes leads to POF due to excessive follicular activation that is followed by follicular atresia (7), whereas deletion of *Pdk1* causes POF as a result of accelerated clearance of primordial follicles directly from their dormant state. The two types of POF, resulting from loss of *Pten* or *Pdk1*, may represent distinct etiologies of POF in humans. In women, the normal length of reproductive life and menopausal age are determined by the reserve of primordial follicles in the ovaries. It is possible that PTEN/PI3K–PDK1 signaling in oocytes also controls the courses of development of primordial follicles in humans, thereby maintaining the duration of fertility and contributing to the timing of menopause in women. Underactivation or overactivation of the PDK1–Akt–S6K1–rpS6 signaling in oocytes may also cause defects in primordial follicle survival and development in humans, resulting in pathological conditions in the ovary, including POF and infertility. In this sense, our work may have broad physiological and clinical implications. We believe that comprehension of the signaling networks in oocytes will open up new avenues for a better understanding of ovarian physiology and pathology.

MATERIALS AND METHODS

Mice

Pdk1$^{loxP/loxP}$ mice (15,19), *Pten*$^{loxP/loxP}$ mice (7) and *Rps6*$^{loxP/loxP}$ mice (28) with C57BL/6J genomic background were crossed with transgenic mice carrying *Gdf-9* promoter-mediated Cre recombinase that also had a C57BL/6J background (7,20). After multiple rounds of crossing, we obtained homozygous mutant female mice lacking *Pdk1* in oocytes (Oo*Pdk1*$^{-/-}$ mice), mice lacking *Pten* in oocytes (Oo*Pten*$^{-/-}$ mice), mice lacking both *Pdk1* and *Pten* concurrently in oocytes (Oo*Pten*$^{-/-}$;*Pdk1*$^{-/-}$ mice) and mice lacking *Rps6* in oocytes (Oo*Rps6*$^{-/-}$ mice). Control mice that do not carry the Cre transgene are referred to as Oo*Pdk1*$^{+/+}$, Oo*Pten*$^{+/+}$ or Oo*Rps6*$^{+/+}$ mice. The mice were housed under controlled environmental conditions with free access to water and food. Illumination was on between 06:00 and 18:00 h. Experimental protocols were approved by the regional ethical committee of Umeå University, Sweden.

Reagents, antibodies and immunological detection methods

Mouse monoclonal antibodies to PDK1 (PKB kinase) and rpS6 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to Akt, phospho-Akt (S473), S6K1, phospho-S6K1 (T389), phospho-rpS6 (S240/244), tuberin/Tsc2, cleaved PARP,

cleaved caspase-7, and also rabbit monoclonal antibody to phospho-tuberin/Tsc2 (T1462), were obtained from Cell Signaling Technologies (Beverly, MA, USA). Mouse monoclonal antibody to phospho-Akt (T308) was purchased from BD Bioscience (Franklin Lakes, NJ, USA). The polyclonal antibody to phospho-S6K1 (T229) was purchased from R&D Systems (Minneapolis, MN, USA). Pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG) and mouse monoclonal antibody to β-actin were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). The PI3K-specific inhibitor LY294002, the mTORC1-specific inhibitor rapamycin and recombinant mouse KL were obtained from EMD Biosciences (San Diego, CA, USA). Western blots were carried out according to the instructions of the suppliers of the different antibodies and visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Uppsala, Sweden).

Quantification of ovarian follicles and histological analysis

Quantification of ovarian follicles was performed as previously described (7). Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. To count the numbers of follicles, paraffin-embedded ovaries were serially sectioned at 8 μm thickness and stained with hematoxylin for morphological observation. Ovarian follicles at different stages of development, including primordial follicles and activated follicles (including transient follicles containing enlarged oocytes surrounded by flattened pregranulosa cells, type 3b, type 4, type 5 and type 6 follicles), were counted in all sections of an ovary, based on the well-accepted standards established by Pedersen and Peters (35). Follicles that contained oocytes with clearly visible nuclei were scored in each section, as previously reported (36). Judged from careful morphological analysis, the incidence of counting the same follicle twice or of missing a follicle was low.

Isolation of oocytes from postnatal mouse ovaries and stimulation of starved oocytes with KL

Isolation and lysis of oocytes were performed as previously described (7). For stimulation with KL, equal amounts of oocytes were aliquoted into wells of a 24-well plate. Typically, each well contained oocytes obtained from three to five mice that were 12–14-days old. The oocytes were first starved by culturing them in serum-free DMEM/F12 medium for 4 h, followed by treatment with 100 ng/ml KL for 2 min. After stimulation with KL, the 24-well plate was chilled on ice and oocytes were lysed for western blot analysis.

Measurement of serum hormone levels

Adult female Oo*Pdk1*$^{-/-}$ or Oo*Rps6*$^{-/-}$ mice from weeks 8–12 were killed randomly due to lack of regular estrus cycles; control Oo*Pdk1*$^{+/+}$ or Oo*Rps6*$^{+/+}$ female mice of similar ages were killed at the estrus stage based on vaginal smears, in order to measure gonadotropin levels during the follicular growth phase but not the ovulation phase. Serum hormone levels were determined by immunoassay as described previously for FSH (37) and LH (38).

Gonadotropin-induced ovulation

To induce synchronized follicular growth and ovulation in order to obtain ovulated oocytes for western blot, immature 23-day-old female mice were injected intraperitoneally with 5 IU of PMSG to stimulate follicular development and with 5 IU hCG 48 h later to induce ovulation. Ovulation normally takes place 10–12 h after hCG treatment (39). Cumulus-oocyte complexes were recovered from oviducts and treated with hyaluronidase (0.1%) before oocytes were collected.

Natural ovulation and embryo culture

Oo*Pdk1*$^{-/-}$ and Oo*Pdk1*$^{+/+}$ female mice of 5–6 weeks old were housed with wild-type males, and vaginal plugs were checked every morning. Embryonic day 0.5 (E0.5) refers to the day that a vaginal plug was found. The mated female mice were sacrificed at E1.5 and zygotes/oocytes were recovered from their oviducts; these were cultured further for 72 h in KSOM medium supplemented with amino acids (Chemicon) in an incubator at 37°C with an atmosphere of 5% CO_2. The numbers and stages of development of the zygotes/oocytes were recorded.

Statistical analysis

All experiments were repeated at least three times. For comparisons of follicle numbers, ovarian weights and hormone levels, differences between the two groups were calculated with Student's *t*-test, and a difference was considered to be significant if $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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