Supplementary information

Supplementary materials and methods

Mice

Pdk1$^{loxP/loxP}$ mice (Hashimoto *et al.*, 2006) and *Pten*$^{loxP/loxP}$ mice (Reddy *et al.*, 2008) with C57BL/6J genomic background were crossed with transgenic mice carrying *Zp3* promoter-mediated Cre recombinase that also had a C57BL/6J background (de Vries *et al.*, 2000). After multiple rounds of crossing, we obtained homozygous mutant female mice lacking *Pdk1* in oocytes (referred to as *Pdk1*$^{loxP/loxP}$*;Zp3-Cre* mice) and mice lacking both *Pdk1* and *Pten* concurrently in oocytes (referred to as *Pdk1*$^{loxP/loxP}$*;Pten*$^{loxP/loxP}$*;Zp3-Cre* mice). C57BL/6J and CD-1 male mice were used to mate with the females. The mice were housed under controlled environmental conditions with free access to water and food. Illumination was on between 0600 and 1800. All experimental protocols were approved by the regional ethics committee of Umeå University, Sweden.

Western blot analysis

For western blot, mouse monoclonal antibody to PDK1 (PKB kinase) and polyclonal antibody to cyclin E were purchased from Santa Cruz. Rabbit polyclonal antibodies to TSC2, Akt, phospho-Akt (S473), S6K1, phospho-S6K1 (T389), Foxo3a, phospho-Foxo3a (S253), and rabbit monoclonal antibody to phospho-TSC2 (T1462) were obtained from Cell Signaling Technologies. Rabbit polyclonal antibody to phospho-S6K1 (T229) was purchased from R&D Systems. Mouse monoclonal antibody to phospho-Akt (T308) was purchased from BD Bioscience. Mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich. Western blots were performed according to the instructions of the suppliers of the different antibodies and visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences).

In vitro kinase assay

Agarose bead-conjugated antibodies to Cdk2, Cdc2, cyclin A, and cyclin B1 (Santa Cruz) were used to precipitate the Cdks and cyclins from lysates of 500 two-cell embryos, and

kinase activity assays were performed as previously described (Rajareddy *et al.*, 2007) using recombinant histone H1 (Roche) as a substrate. Agarose bead-conjugated immobilized Akt antibody (Cell Signaling Technologies) was used to precipitate Akt from lysates of 600 two-cell embryos. The assay was performed as previously described (Bellacosa *et al.*, 1998) using recombinant histone H2B (New England Biolabs) as a substrate. [γ-^{32}P]ATP (Perkin Elmer) was used to indicate the kinase activity.

BrdU and BrUTP incorporation assays for embryos

Embryos were cultured with 10 μM BrdU (Sigma-Aldrich) from late one-cell stage (30 h post hCG) to mid two-cell stage (45 h post hCG), and then fixed with 4% paraformaldehyde at 4°C overnight. The fixed embryos were permeabilized in 0.2% Triton X-100 for 20 min at RT, and blocked with 2% BSA for 1 h at RT. Then the embryos were incubated for 1 h at 37°C with mouse monoclonal anti-BrdU antibody (Dako) and DNase I (Fermentas). The embryos were then incubated with Alexa fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h, and 3 μM DAPI (Sigma-Aldrich) for 5 min at RT. The BrdU incorporated was visualized with a Zeiss Axio Imager Z1 microscope.

Embryos at mid two-cell stage (45 h post hCG) were prepared for BrUTP incorporation assay, as described previously (Aoki *et al.*, 1997). Briefly, the plasma membrane of two-cell embryos was permeabilized for 2 min with 0.05% Triton X-100 in physiological buffer (PB) containing 100 mM potassium acetate, 30 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2HPO_4, 1 mM ATP, 1 mM DTT, 1× complete protease inhibitor cocktail (Roche), and 50 U/ml of RNasin (Promega). Treated embryos were then washed three times in PB and incubated for 10 min at 33°C in PB supplemented with 2 mM $MgCl_2$, 2 mM ATP, and 0.4 mM each of GTP, CTP, and BrUTP (Invitrogen). After three washes in PB, the nuclear membrane was permeabilized in PB containing 0.2% Triton X-100 for 3 min. The embryos were washed again in PB and fixed overnight with 4% paraformaldehyde. The BrUTP incorporated was visualized by immunostaining using the same protocol of BrdU incorporation. The results were quantified with NIH Image J software (National Institutes of Health). Briefly, nuclear signal was outlined to obtain the mean fluorescence

intensity and the cytoplasm background fluorescence was subtracted. The quantified BrUTP level in Em*Pdk1*$^{+/+}$ embryos was set as 100%.

^{35}S-methionine metabolic labeling of embryos and measurement of *de novo* synthesis of TRC

The synthesis of TRC and spindlin was detected by autoradiography as previously described (Conover *et al.*, 1991). Briefly, one-cell embryos were cultured to late S phase of the two-cell stage (42 h post hCG) with or without the PI3K inhibitor LY294002 (20 μM) (Sigma-Aldrich). Then, 70 two-cell embryos were metabolically labeled with ^{35}S-methionine (Perkin Elmer) in M16 medium (Sigma-Aldrich) for 3 h with or without LY294002. The insoluble proteins were resolved by SDS-PAGE and visualized by Typhoon 9400 scanner (GE Healthcare).

Isolation and culture of oocytes

Growing oocytes (larger than 25 μm) were isolated from ovaries of PD15–18 mice as previously described (Rajareddy *et al.*, 2007). Briefly, to avoid oocytes from primordial follicles where the *Zp3* promoter is not active, isolated oocytes were filtered with a cell dispersing screen with 25.4-μm opening (Bioworld).

To study germinal vesicle breakdown (GVBD) and extrusion of the first polar body (PB1), 23-day-old female mice were treated with 5 IU PMSG and were sacrificed 48 h later. GV-stage oocytes were collected by puncturing the ovaries in M2 medium containing 100 μM dibutyryl cyclic AMP (dbcAMP) (Sigma-Aldrich). The oocytes were then washed twice and cultured in dbcAMP-free M16 medium at 37°C for 6 h in an atmosphere of 5% CO_2 to determine the rate of GVBD. The oocytes that had undergone GVBD were further cultured for 6 h in M16 medium for evaluation of the extrusion of PB1.

Gonadotropin-induced ovulation and collection of ovulated oocytes

To induce synchronized follicular growth and ovulation in order to obtain ovulated oocytes for western blot, 23-day-old female mice were injected 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. Cumulus-oocyte complexes were recovered from oviducts and treated with 0.1% hyaluronidase (Sigma-Aldrich) before oocytes were collected.

Histology

Ovaries were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin-embedded ovaries were sectioned at 8 μm thickness and stained with hematoxylin for morphological observation. Images were taken with a Leica DMLB upright microscope.

Statistical analysis

All experiments were repeated at least 3 times. For comparisons of embryo numbers and litter sizes, differences between the two groups were calculated with Student's t-test, and a difference was considered to be significant at *P*-values of < 0.05.

Supplementary figure legends

Fig S1 Normal follicular development and oocyte maturation in *Pdk1$^{loxP/loxP}$;Zp3-Cre* mice. (**A**–**B**) Western blots showing the absence of PDK1 expression in *Pdk1$^{loxP/loxP}$;Zp3-Cre* growing (larger than 25 μm) and ovulated oocytes. β-actin was the internal control. (**C**) Ovarian histology of 6-week-old control *Pdk1$^{loxP/loxP}$* (panels a and b) and mutant *Pdk1$^{loxP/loxP}$;Zp3-Cre* females (panels c and d). Primary follicles (arrowhead), antral follicles (arrow), and corpora lutea (CL) are shown, indicating that follicular development is not affected by deletion of *Pdk1* in oocytes. (**D**) Examination of maturation of oocytes from *Pdk1$^{loxP/loxP}$;Zp3-Cre* mice. GV-intact oocytes from *Pdk1$^{loxP/loxP}$* and *Pdk$^{loxP/loxP}$;Zp3-Cre* females were cultured *in vitro*, and no significant difference in the rate of GVBD was found. The rate of extrusion of PB1 was also unaltered in *Pdk1$^{loxP/loxP}$;Zp3-Cre* oocytes. Together with our data showing that the numbers of two-cell embryos are similar in *Pdk1$^{loxP/loxP}$* and *Pdk1$^{loxP/loxP}$;Zp3-Cre* females that have mated with wild-type males (Fig 1B), these results suggest that oocyte maturation, ovulation, and fertilization are normal in *Pdk$^{loxP/loxP}$;Zp3-Cre* females.

Several previous *in vitro* studies have suggested that PDK1-Akt signaling may regulate the maturation and fertilization of oocytes in starfish and mice (Okumura *et al.*, 2002;Hiraoka *et al.*, 2004;Kalous *et al.*, 2006;Han *et al.*, 2006;Hoshino and Sato, 2008). We have, however, shown that ablation of PDK1-Akt signaling in oocytes does not affect oocyte maturation, ovulation, or fertilization. In addition, we have shown in an earlier study that overactivation of Akt in oocytes (by depletion of PTEN) does not affect oocyte maturation, ovulation, and fertilization of female mice (Jagarlamudi et al., 2009). We therefore propose that intra-oocyte PI3K/PTEN-PDK1-Akt signaling is unlikely to play an essential role in regulation of oocyte maturation, ovulation, and fertilization.

Fig S2 Concurrent loss of maternal *Pten* restored the fertility of double-mutant females. (**A**) Numbers of pups per litter from control (*Pdk1$^{loxP/loxP}$*) and double-mutant females (*Pdk1$^{loxP/loxP}$;Pten$^{loxP/loxP}$;Zp3-Cre*). Numbers of females (n) used are indicated. ns, not statistically significant. (**B**) Comparison of average cumulative numbers of pups

per *Pdk1*$^{loxP/loxP}$ female (black line), per *Pdk1*$^{loxP/loxP}$*;Zp3-Cre* female (red line), and per *Pdk1*$^{loxP/loxP}$*;Pten*$^{loxP/loxP}$*;Zp3-Cre* female (blue line). Numbers of females (n) are indicated.

Fig S3 Concurrent loss of maternal *Pten* completely restored TRC synthesis in Em*Pdk1*$^{+/-}$*;Pten*$^{+/-}$ embryos. Representative autoradiograph showing the rescued TRC synthesis in two-cell Em*Pdk1*$^{+/-}$*;Pten*$^{+/-}$ embryos. Note that LY294002 (LY) suppressed the TRC synthesis in both Em*Pdk1*$^{+/-}$*;Pten*$^{+/-}$ and control embryos. Spindlin (SPIN) was used as an internal control.

Supplementary references

Aoki F, Worrad DM, and Schultz RM (1997) Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev Biol,* **181,** 296-307.

Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, and Tsichlis P (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene,* **17,** 313-325.

Conover JC, Temeles GL, Zimmermann JW, Burke B, and Schultz RM (1991) Stage-specific expression of a family of proteins that are major products of zygotic gene activation in the mouse embryo. *Dev Biol,* **144,** 392-404.

de Vries WN, Binns LT, Fancher KS, Dean J, Moore R, Kemler R, and Knowles BB (2000) Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis,* **26,** 110-112.

Han SJ, Vaccari S, Nedachi T, Andersen CB, Kovacina KS, Roth RA, and Conti M (2006) Protein kinase B/Akt phosphorylation of PDE3A and its role in mammalian oocyte maturation. *EMBO J,* **25,** 5716-5725.

Hashimoto N, Kido Y, Uchida T, Asahara S, Shigeyama Y, Matsuda T, Takeda A, Tsuchihashi D, Nishizawa A, Ogawa W, Fujimoto Y, Okamura H, Arden KC, Herrera PL, Noda T, and Kasuga M (2006) Ablation of PDK1 in pancreatic beta cells induces diabetes as a result of loss of beta cell mass. *Nat Genet,* **38,** 589-593.

Hiraoka D, Hori-Oshima S, Fukuhara T, Tachibana K, Okumura E, and Kishimoto T (2004) PDK1 is required for the hormonal signaling pathway leading to meiotic resumption in starfish oocytes. *Dev Biol,* **276,** 330-336.

Hoshino Y and Sato E (2008) Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. *Dev Biol,* **314,** 215-223.

Jagarlamudi K, Liu L, Adhikari D, Reddy P, Idahl A, Ottander U, Lundin E, and Liu K (2009) Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation. *PLoS ONE,* **4,** e6186.

Kalous J, Solc P, Baran V, Kubelka M, Schultz RM, and Motlik J (2006) PKB/AKT is involved in resumption of meiosis in mouse oocytes. *Biol Cell,* **98,** 111-123.

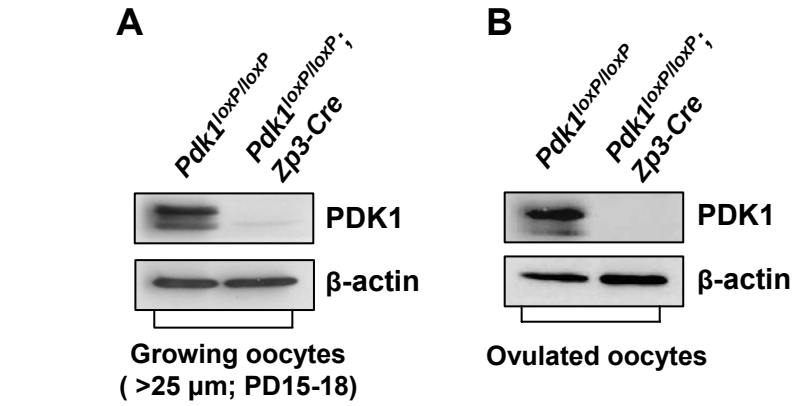
Okumura E, Fukuhara T, Yoshida H, Hanada SS, Kozutsumi R, Mori M, Tachibana K, and Kishimoto T (2002) Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. *Nat Cell Biol,* **4,** 111-116.

182 Rajareddy S, Reddy P, Du C, Liu L, Jagarlamudi K, Tang W, Shen Y, Berthet C, Peng
183 SL, Kaldis P, and Liu K (2007) p27kip1 (cyclin-dependent kinase inhibitor 1B)
184 controls ovarian development by suppressing follicle endowment and activation and
185 promoting follicle atresia in mice. *Mol Endocrinol,* **21,** 2189-2202.

186 Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W,
187 Hamalainen T, Peng SL, Lan ZJ, Cooney AJ, Huhtaniemi I, and Liu K (2008) Oocyte-
188 specific deletion of Pten causes premature activation of the primordial follicle pool.
189 *Science,* **319,** 611-613.

Supplementary figure

Figure S1



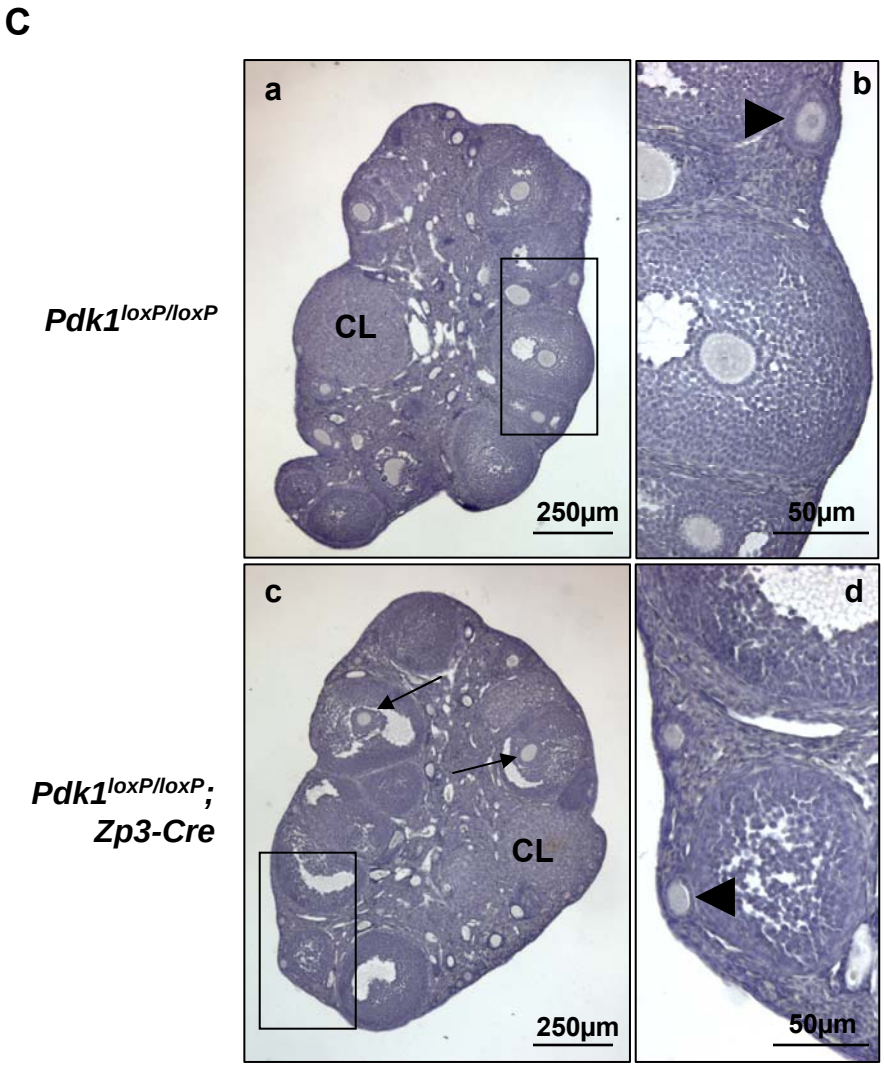


Figure S1 continued

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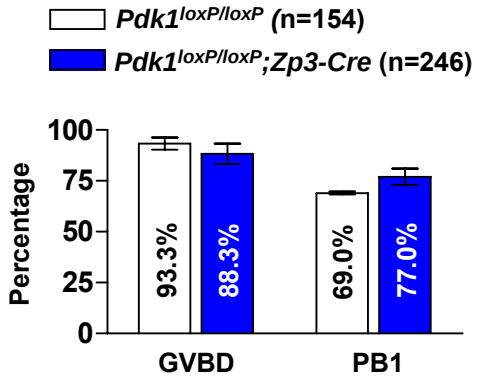

Figure S2

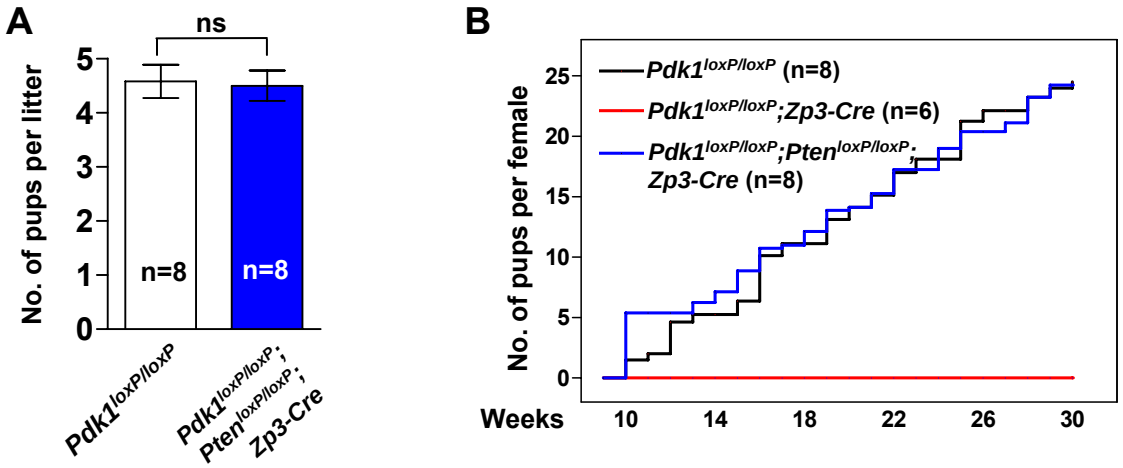
A
ns
No. of pups per litter
5
4
3
2
1
0
n=8
n=8
Pdk1loxP/loxP
Pdk1loxP/loxP; PtenloxP/loxP; Zp3-Cre
B
No. of pups per female
25
20
15
10
5
0
Pdk1loxP/loxP (n=8)
Pdk1loxP/loxP;Zp3-Cre (n=6)
Pdk1loxP/loxP;PtenloxP/loxP; Zp3-Cre (n=8)
Weeks
10
14
18
22
26
30

Figure S3