Loss of protein phosphatase 6 in oocytes causes failure of meiosis II exit and impaired female fertility

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ABSTRACT

Dynamic protein phosphorylation and dephosphorylation, mediated by a conserved cohort of protein kinases or phosphatases, regulate cell cycle progression. Among the well-known PP2A-like protein phosphatases, PP6 has been analyzed in mammalian mitosis recently identifying Aurora A as its key substrate. However, the functions of PP6 in meiosis are still entirely unknown. To identify the physiological role of PP6 in female gametogenesis, Ppp6c<sup>F/F</sup> mice were first generated and crossed with Zp3-Cre mice to selectively disrupt Ppp6c expression in oocytes. Here we report for the first time that PP6c was dispensable for oocyte meiotic maturation but essential for MII exit after fertilization, since depletion of PP6c caused abnormal MII spindle and disrupted MII cytokinesis, resulting in zygotes with high risk of aneuploidy, defective early embryonic development, thus severe subfertility. We also revealed that PP6 inactivation interfered with MII spindle formation and MII exit due to increased Aurora A activity, and Aurora A inhibition with MLN8237 could rescue the PP6c depletion phenotype. In conclusion, our findings uncover a hitherto unknown role for PP6 as an indispensable regulator of oocyte meiosis and female fertility.
INTRODUCTION

In mammals, it is generally accepted that females are born with a finite number of oocytes contained within primordial follicles. In order to produce mature eggs, dormant primordial follicles are activated and subsequently develop into primary follicles, secondary follicles, and antral follicles (Oktem and Urman, 2010). Throughout this follicular growth process, oocytes are all arrested at prophase of meiosis I (MI) with homologues held together by chiasmata and they only grow in size (commonly referred to as germinal vesicle (GV) stage). Finally, dominant antral follicles reach the preovulatory stage and release the mature egg for fertilization after a gonadotropin surge (Hirshfield, 1991). In the meantime, upon receiving ovulatory signals, these fully-grown, meiotically competent oocytes contained within preovulatory follicles resume meiosis as indicated by germinal vesicle breakdown (GVBD), followed by spindle organization and chromosome alignment for coordinated chromosome segregation (Sun et al., 2009). After the first polar body extrusion (PBE), the oocytes are arrested at metaphase of meiosis II (MII) until being fertilized by sperm. The second meiosis is resumed and the second polar body is extruded upon fertilization (Jones, 2005; Mehlmann, 2005). Thus, a single haploid egg is generated through two consecutive chromosome segregations with only one round of DNA replication from one original diploid germ cell. Aneuploidy might occur in both meioses if chromosomes fail to segregate accurately, which is the leading genetic cause of infertility, pregnancy loss and many developmental disabilities (Hassold and Hunt, 2001).

During meiosis in oocytes, there are dynamic waves of protein phosphorylation and dephosphorylation regulating meiotic cell cycle arrest and progression, chromosome dynamics, and meiotic spindle assembly and disassembly (Schindler, 2011). Many of these phosphorylation and dephosphorylation events are mediated by a conserved cohort of protein kinases or phosphatases. The mouse genome encodes 561 protein kinases compared to only 162 protein phosphatases (Caenepeel et al., 2004). Historically, many studies had focused on protein kinases, resulting in comparatively less information about the roles of protein phosphatases. Serine/threonine phosphoprotein phosphatases (PPPs), a major protein phosphatase family, have been implicated in regulating oocyte meiosis. Within the PPP family, the catalytic subunits
of PP2A, PP4 and PP6 are most closely related and the three proteins form a subfamily called PP2A-like protein phosphatases that also account for the majority of cellular serine/threonine phosphatase activity (Janssens and Goris, 2001; Moorhead et al., 2007). PP2A is involved in regulating chromosome condensation, DNA damage repair, G2/M transition and sister chromatid cohesion, respectively (Ruediger et al., 2011). We have recently shown that PP2A is essential for female meiosis and fertility since oocyte-specific depletion of PP2A facilitates GVBD, causes elongated MII spindles and precocious separation of sister chromatids, resulting in defective early embryonic development, thus subfertility (Hu et al., 2014). Though sharing high homology with PP2A, PP6 does not share the same level of scientific examination and its functions in meiosis still remain unknown.

The PP6 holoenzyme consists of a catalytic subunit, PP6c, one of the three regulatory subunits including SAPS1, 2, 3 (also known as PP6R1, PP6R2 and PP6R3, respectively), and one of the three ankyrin repeat subunits including ARS-A, -B, -C (Stefansson and Brautigan, 2006; Stefansson et al., 2008). PP6 is conserved among all eukaryotic species from yeast to humans, attesting to its fundamental importance. Recently, it has been found that mutations in PP6c existed in 9-12.4% melanomas surveyed and may act as drivers for melanoma development (Hodis et al., 2012; Krauthammer et al., 2012). Sit4, the homologue in yeast, is required for G1/S progression and equal chromosome segregation in yeast (Sutton et al., 1991). The human PP6 has been shown to play a role in DNA damage response, cell cycle, apoptosis and pre-mRNA splicing by acting on DNA-dependent protein kinase (DNA-PK), histone γ-H2AX, Aurora-A, NF-κB and U1 snRNP (Douglas et al., 2014; Douglas et al., 2010; Hammond et al., 2013; Hosing et al., 2012; Kajihara et al., 2014; Kamoun et al., 2013; Stefansson and Brautigan, 2006; Stefansson and Brautigan, 2007; Zeng et al., 2010; Zhong et al., 2011). However, the role of PP6 in reproductive cells remains unclear.

Genetically modified mouse models are powerful tools for studying gene function in vivo (Hu et al., 2012; Sun et al., 2008). Here, we first generated Ppp6c<sup>F/F</sup> mice in which exon II-IV of the Ppp6c gene were flanked with loxp sites, and then used conditional knockout technology by crossing Ppp6c<sup>F/F</sup> mice with Zp3-Cre mice (Wang et al., 2013) to generate mutant mice with specific deletion of Ppp6c in
oocytes from primary follicle stage, in order to investigate the function of PP6 in female meiosis/fertility within ovarian follicles \textit{in vivo}. For the first time, we report that PP6 mutation causes female subfertility by disrupting MII spindle organization and MII exit after fertilization, without affecting follicle growth, ovulation or oocyte meiotic maturation.

\textbf{RESULTS}

\textbf{Expression and subcellular localization of PP6 during oocyte maturation}

To study the functions of PP6 in female meiosis, its expression was first analyzed by immunoblot from GV oocytes to 1-cell embryos using an antibody directed against human PPP6c (Fig. 1A). The expression of PP6c throughout oocyte meiotic maturation did not show evident changes with only little up-regulation after the MI stage. Subcellular localization of PP6 was then examined by immunofluorescent staining (Fig. 1B). During oocyte maturation, the localization of PP6c was basically consistent at different stages. At the GV stage, PP6c was concentrated in the GV exhibiting strong punctate staining around the nucleolus. From GVBD to the MII stage, PP6c was always localized to the chromosomes. In particular, PP6c accumulated strongly along the outer chromosome arms when all homologous chromosomes formed bivalents during the MI stage. In 1-cell embryos, PP6c was concentrated in the pronuclei during interphase, but it lost the chromatin localization and dispersed into the cytoplasm when the embryos entered the first mitotic division. This specific localization of PP6c in oocytes suggests its possible role in meiotic progression events, such as spindle organization and chromosome segregation.

\textbf{Generation of mutant mice with oocyte-specific deletion of Ppp6c}

To explore the \textit{in vivo} role of PP6c and its function in oocyte meiotic maturation, we decided to use the conditional knockout approach due to the early lethality of PP6-deficient embryos. The Cre-LoxP site-specific recombination system was used to target \textit{Ppp6c} for oocyte-specific deletion in mice. We first generated \textit{Ppp6c}\textsuperscript{F/F} mice in which exon II-IV of the \textit{Ppp6c} gene were flanked with \textit{Loxp} sites (Fig. S1). To generate the \textit{Ppp6c} targeting vector, a single \textit{LoxP} site was introduced into the upstream of exon 2 of the \textit{Ppp6c} gene, and an \textit{Frt-Neomycin-Frt-LoxP} cassette was inserted into intron 4 (Fig. S1A). This targeting vector was electroporated into mouse
ES cells. The homologous recombinant ES clones were determined by Southern blotting analysis (Fig. S1B), and injected into blastocysts to generate chimeric mice. The chimeric mice exhibited germline transmission of the LoxP-Neo Ppp6c allele (Ppp6cFn/+). Ppp6cFn/+ mouse was bred with the Flpe deleter mouse line (Farley et al., 2000) to excise the Frt-flanked neomycin cassette and generate the Ppp6c floxed heterozygous mouse (Ppp6cF/+; Fig. S1C). After one round of self-cross, Ppp6cF/F mice were obtained.

Then we crossed Ppp6cF/F mice with transgenic mice expressing Zp3 promoter-mediated Cre recombinase to generate oocyte-specific conditional PP6c knockout mice (referred to as Ppp6cF/F;ZCre+ mice, Fig. S1A). In Zp3-Cre mice, Cre is expressed in oocytes of primary follicles from postnatal day 5 on and in later developmental stages (Hu et al., 2012). Immunofluorescent analysis of oocytes from Ppp6cF/F;ZCre+ females revealed loss of PP6c localization on chromosomes, indicating successful disruption of Ppp6c (Fig. 2A). Furthermore, by western blot analysis, we confirmed that expression of the Ppp6c gene in GV oocytes from Ppp6cF/F;ZCre+ females was absent (Fig. 2B).

**PP6c is essential for female fertility**

To investigate the effect of oocyte-specific knockout of PP6c on female fertility, a breeding assay was carried out by mating Ppp6cF/F or Ppp6cF/F;ZCre+ female mice with males of proven fertility for 6 months. As shown in Fig. 2C, female Ppp6cF/F;ZCre+ mice were severely subfertile and gave birth to about 66% fewer pups than Ppp6cF/F mice. The significant decrease of fertility in Ppp6cF/F;ZCre+ mice appeared not to be related to the ovulation rate since the mutant mice could ovulate approximately the same number of eggs (8.5±1.4) compared with control mice (8.7±0.8) in natural ovulation assays (Fig. 2D). Furthermore, we performed histological analysis and compared follicular development in Ppp6cF/F;ZCre+ mice to that in Ppp6cF/F mice. No apparent morphological difference was found in 6-month ovaries of both genotypes, consistent with the follicle counting result (Fig. 2E). These data revealed that Ppp6c deletion from oocytes from the primary follicle stage did not affect follicular development, suggesting that the subfertility of Ppp6cF/F;ZCre+ mice must be caused by defects in oocytes.
Depletion of PP6c did not affect oocyte meiotic maturation progress

To understand the defects of $Ppp6c^{F/F};ZCre^+$ oocytes, we employed oocyte in vitro culture to observe the major events during the meiotic maturation process. The absence of PP6c seemed to have no influence on the oocyte meiotic maturation rate because the $Ppp6c^{F/F};ZCre^+$ oocytes exhibited normal GVBD rates (84.8±3.2%) and PBE rates (94.3±2.8%) compared with the $Ppp6c^{F/F}$ oocytes (86.1±4.8%; 95.5±2.5%) when cultured in vitro (Fig. 3A). Moreover, after 8 hours of in vitro maturation, in $Ppp6c^{F/F};ZCre^+$ oocytes spindles were well-organized with chromosomes all aligned at the equatorial plate, showing no obvious difference compared to $Ppp6c^{F/F}$ oocytes, which was confirmed further by quantification results (90.0±3.0% VS 88.6±7.1%) (Fig. 3B). To test if chromosomes segregated correctly in the first meiosis after PP6c depletion, we employed chromosome spreading of MII oocytes after 13 hours of in vitro culture and counted the number of chromosomes that showed no difference between the mutant group and the control group (92.0±1.8% VS 91.2±5.0%) (Fig. 3C). Besides, when we induced superovulation of the $Ppp6c^{F/F};ZCre^+$ mice with PMSG and hCG treatment, we could collect normal MII oocytes with visible first polar bodies, which suggested that the $Ppp6c^{F/F};ZCre^+$ oocytes could undergo meiotic resumption and polar body emission in vivo, as well as in vitro. Therefore, PP6c might be dispensable for oocyte meiotic resumption and completion of the first meiosis.

Loss of PP6c led to defective early embryonic development and subfertility

To find the causes for female subfertility in $Ppp6c^{F/F};ZCre^+$ mice, we extended our observation of the $Ppp6c^{F/F};ZCre^+$ oocytes after fertilization. In vivo zygotes from $Ppp6c^{F/F}$ and $Ppp6c^{F/F};ZCre^+$ females were collected at E0.5 and investigated. In contrast to $Ppp6c^{F/F}$ eggs which had been fertilized with second polar bodies extruded and which had shown two visible pronuclei (yellow arrowheads, Fig 4A), a large proportion of zygotes from $Ppp6c^{F/F};ZCre^+$ mice were abnormal with aberrant second polar body extrusion and no visible pronuclei (Fig. 4A). Further immunofluorescent analysis showed that only $59.4±8.2\%$ mutant zygotes displayed two pronuclei, significantly less than in the control group ($94.4±5.6\%$), and the other $40.6\%$ displayed various kinds of anomalies: Some had more than two small pronuclei and some had accumulated chromatin without formation of pronuclei (Fig. 4A). The $Ppp6c^{F/F};ZCre^+$ oocytes appeared to have defects in completing meiosis II. To
confirm this observation, we performed chromosome spreading of 1-cell embryos that were arrested at metaphase by colchicine. As expected, up to 55.2±4.7% of mutant 1-cell embryos showed aneuploidy, significantly higher than that of control 1-cell embryos which displayed only 12.5±3.6% aneuploidy (Fig. 4B). Therefore, the chances of survival was quite small for the early embryos derived from $Ppp6c^{F/F};ZCre^+$ females mated with WT males. As shown in Fig. 4C, at E4.0, almost all of the early embryos had reached the blastocyst stage with an obvious blastocoel in the control group (93.8±8.8%), but in the mutant group the embryos had barely reached the blastocyst stage (25.2±4.8%) and exhibited obvious malformation (black arrows). The above data indicated that defective early embryonic development derived from mutant zygotes with high aneuploidy rates may account for the main reasons for subfertility in $Ppp6c^{F/F};ZCre^+$ mice. However, questions remained regarding specific defects in the $Ppp6c^{F/F};ZCre^+$ oocytes that caused the aneuploidy concerning MII.

**Depletion of PP6c impaired spindle shape in MII**

To answer the question and unveil the role of PP6c in MII, we collected and observed superovulated MII oocytes using immunofluorescent analysis. As shown in Fig. 5A, $Ppp6c^{F/F}$ MII oocytes showed well-organized bipolar spindles with clearly detectable microtubule fibers and tightly aligned chromosomes at the metaphase plate; surprisingly, $Ppp6c^{F/F}; ZCre^+$ oocyte spindles displayed rather odd formations with several distinct arrays of bundled microtubules, though the chromosomes seemed to be aligned well. We suspected that the microtubules which formed the abnormal spindles in the $Ppp6c^{F/F};ZCre^+$ oocytes might be excessively polymerized. So we cold-treated the MII oocytes from both groups at 4°C to depolymerize the cold-labile microtubules. When cold-treated for 15 minutes, no significant differences were found between $Ppp6c^{F/F}$ oocytes and $Ppp6c^{F/F}; ZCre^+$ oocytes; relatively cold-stable k-fibers still existed in both groups (white arrowheads, Fig. 5B). But after an extended cold treatment of 20 minutes, almost the entire spindle including k-fibers, which are normally resistant to cold over shorter periods, disappeared in the $Ppp6c^{F/F}$ oocytes; however, the spindles of $Ppp6c^{F/F}; ZCre^+$ oocytes still remained relatively intact (26.9±5.6% VS 76.6±6.1%), suggesting that these microtubule bundles were very stable, relatively resistant to cold and could not be depolymerized easily (Fig. 5B).
Therefore, the experiments showed that PP6c is required for maintaining a normal MII spindle organization.

**Oocyte-specific deletion of Ppp6c caused failure of MII exit**

Considering such abnormalities of Ppp6c<sup>F/F</sup>;ZCre<sup>+</sup> oocytes, we asked if they could succeed in MII exit. So we treated oocytes with SrCl<sub>2</sub> activation solution to activate the MII-arrested oocytes and started to observe the completion process of meiosis II by live cell imaging after 30 min of activation. As shown in Fig. 6A, the control MII oocytes were activated and entered anaphase II around time point 1.5h, and completed cytokinesis around time point 2h (Movie 1). In comparison, Ppp6c<sup>F/F</sup>;ZCre<sup>+</sup> oocytes were able to enter anaphase II and had a tendency to extrude the second polar body, but then the extruding polar body retracted and cytokinesis failed eventually with the chromatids still left in the oocytes (Movie 2). In the meantime, the oocytes in both groups were also fixed and stained with α-tubulin and Propidium Iodide (PI) at 2h or 6h of parthenogenetic activation to observe the separation of sister chromatids or pronuclei formation, respectively. Indeed, the immunofluorescent analysis results confirmed the live cell imaging observations in greater detail. At 2h, in both groups sister chromatids had segregated and moved to the spindle poles indicating that anaphase/telophase II had been reached, but spindles in the mutant group appeared twisted or loosened without displaying a visible tight contractile ring. At 6h, unlike control eggs in which large pronuclei had formed with a low abnormality rate (14.8±4.7%), up to 65.7±5.6% mutant eggs displayed two or more spindles in the vicinity of every chromatid accumulation, or much smaller pronuclei were displayed. In addition, we performed in vitro fertilization (IVF) experiments to confirm the above observation and obtained consistent results. At IVF 6h, the abnormal mutant zygotes contained either ≧2 spindles or several small pronuclei (Fig. 6B). Taken together, these data demonstrate that PP6c is indispensable for MII exit.

**Aurora A activity was up-regulated in PP6c-deficient oocytes**

Based on the above results, the main cause for the PP6c depletion phenotype appeared to be defects in the MII spindle. As a critical regulator of spindle organization, Aurora A is activated by the spindle assembly factor TPX2 and they form a complex together (Bayliss et al., 2003). PP6 was reported to be a T-loop phosphatase for the Aurora
A-TPX2 complex at T288 of Aurora A in mitosis (Zeng et al., 2010), and the effects of PP6 inactivation on the Aurora A-TPX2 complex were therefore investigated. Western blot analysis demonstrated that Aurora A activity was significantly amplified in \( Ppp6c^{F/F}; \) \( ZCre^+ \) MII oocytes using the phosphorylated T288 antibody, though the expression of Aurora A and TPX2 seemed unchanged (Fig. 7A). We also performed immunofluorescent experiments to test the effect of PP6c deficiency on Aurora A localization and activity. In \( Ppp6c^{F/F} \) MII oocytes, the staining of Aurora A was spread at the spindle poles, while in \( Ppp6c^{F/F};ZCre^+ \) oocytes the staining was concentrated into a big dot at each pole but not stronger (Fig. 7B). As for staining of P-Aurora A T288 at the spindle poles, it was clearly elevated and spread to the spindle microtubules in PP6c-deficient oocytes in contrast to the control oocytes (Fig. 7C, yellow arrowheads). Results from both methods confirmed that Aurora A activity was up-regulated in MII oocytes when PP6 function was abolished.

**Aurora A inhibition rescued the PP6c depletion phenotype**

MLN8237 has been proved to be a highly specific small molecule inhibitor of Aurora A (Manfredi et al., 2011), and we used this drug as a rescue strategy for the PP6c depletion phenotype. A low dose of MLN8237 (20nM) was added to M2 culture medium and the oocytes were matured *in vitro* and collected for western blot and immunofluorescent experiments. Addition of 20nM MLN8237 reversed the increase of pT288 form of Aurora A in MII oocytes after PP6c depletion (Fig. 8A). Importantly, this partial Aurora A inhibition also rescued the impaired spindle shape in \( Ppp6c^{F/F};ZCre^+ \) MII oocytes that now showed normal barrel-shaped spindles with normal levels of p-Aurora A staining just like the \( Ppp6c^{F/F} \) oocytes (Fig. 8B). To further test the rescue effect of Aurora A inhibition, we carried out IVF experiments with super-ovulated MII oocytes after a short treatment with 20 nM MLN8237 for 15 minutes. As seen in Fig. 8C, after MLN8237 treatment, many more zygotes from mutant groups had formed normal pronuclei (white arrows) compared to those without treatment. Statistically, the Aurora A inhibition treatment had significantly improved the normal pronuclei formation rate from 42.7% to 72.3%. The reversal of the PP6c depletion phenotype by reduction of Aurora A activity using MLN8237 indicated that Aurora A is a major substrate of PP6 during MII in mouse oocytes.
Collectively, these findings are in support of PP6 acting as a critical regulator for MII spindle organization by limiting the activity of Aurora A and that it is essential for efficient MII exit and embryo euploidy, providing an evident explanation for female subfertility in the absence of functional PP6c.

**DISCUSSION**

In female reproduction, production of quality eggs requires both successful ovulation and precise oocyte meiotic completion. By crossing $P_{pp6c}^{+/+}$ mice with $Zp3^{-Cre}$ mice to generate mutant mice with specific deletion of $P_{pp6c}$ in oocytes, we were able to investigate the roles of PP6c in both ovulation and meiosis. We found that mutant mice with $P_{pp6c}$ deletion in oocytes from primary follicle stages could ovulate normally, but still suffered severe female subfertility. After the step-by-step investigation of the meiosis process in $P_{pp6c}^{+/+};Z^{Cre+}$ oocytes, disorganized MII spindle, failed MII exit and high-frequency aneuploidy in zygotes turned out be the underlying causes for this subfertility.

Meiosis is the cellular process by which haploid gametes are generated from diploid cells. Strikingly, female germ cell meiosis is characterized by two rounds of cell cycle arrests and asymmetric cell divisions in both meioses. The first meiotic division with the separation of homologous chromosomes is termed reductional division. The second division, which takes place immediately after meiosis I without an intervening S-phase, is equational, with the separation of sister chromatids, similar to mitosis (Schindler, 2011; Wassmann, 2013). Although unique, oocyte meiosis, especially meiosis II still appears to adopt many of the same proteins and mechanisms described for mitosis, with necessary modifications to accommodate their special needs (Liu, 2012). Among numerous kinases in somatic cells, Aurora kinases are a family of S/T protein kinases required for successful execution of cell division by ensuring the formation of a bipolar mitotic spindle, accurate segregation of chromosomes and the completion of cytokinesis (Crane et al., 2004). Aurora A is the most abundantly expressed Aurora kinase in mouse oocytes (Swain et al., 2008). As an important regulator involved in the G2/M transition, centrosome maturation and separation, and spindle formation in somatic cells, not surprisingly, Aurora A also functions in meiosis. Similar to mitotic cells, Aurora A localizes to MTOCs and spindle poles during MI and MII and its activated form (phosphorylated on T288) associates with
poles prior to and after GVBD (Saskova et al., 2008). Overexpression of Aurora A leads to increased numbers of MTOCs, formation of an abnormal MI spindle and failed metaphase I-metaphase II transition (Saskova et al., 2008; Yao et al., 2004). Importantly, we showed that PP6 appeared to act as an Aurora A suppressor and PP6 dysfunction would up-regulate Aurora A activity and damage spindle formation during MII, similar to effects of Aurora A overexpression. So the question arises as to why only MII but not MI was affected in PP6c-depleted oocytes? Now we have to consider TPX2, a known activator of Aurora A in mitotic cells. PP6 specifically recognizes and acts upon the Aurora A-TPX2 complex as the T-loop phosphatase regulating Aurora A activity (Zeng et al., 2010). Along with the specific expression pattern of TPX2 which accumulates from MI and expresses most at MII (Brunet et al., 2008), we could assume that the Aurora A-TPX2 complex forms and functions mainly in MII, which would make the spindle formation in MII more sensitive to the absence of PP6 than in MI. Another possibility is that Aurora A activity can be regulated by PP1 or PP2A in MI and mainly by PP6 in meiosis II since PP1 and PP2A can act as free Aurora A phosphatases (Bayliss et al., 2003; Eyers et al., 2003; Tsai et al., 2003). This could explain why the PP6c-deficient phenotype was only exhibited during meiosis II and thereafter. Furthermore, our evidence also confirms that the mechanisms for spindle formation in MI and MII may be different, which exhibits the complexity and unique charm of meiosis in oocytes.

Recently the role of PP6 in mitosis has been studied in great detail (Hammond et al., 2013; Zeng et al., 2010), and the T-loop of Aurora A has been identified as the key target of PP6 in mitosis. The knowledge gained from these studies has benefited our analysis of Aurora A as a potential substrate in our study, and in fact our study also confirmed that Aurora A is a critical target for PP6, not only in mitosis but also in meiosis, which is a very interesting and remarkable finding. Aside from sharing the same target, PP6 also has similar behaviors in mitosis and meiosis; for example, PP6 regulates spindle formation in both kinds of cell division, because efficient PP6c depletion caused spindle formation and chromosome segregation defects and resulted in high risk of micronucleation or binucleation in mitosis/egg aneuploidy in meiosis. However, there are several differences of PP6 behavior in mitosis and meiosis as well.

1) Subcellular localization of PP6 is not the same. In mitotic cells PP6 is localized to the cytoplasm; however, it has specific chromatin localization in oocytes. In our study,
the loss of the chromatin localization in mitotic 1-cell embryos (Fig. 1B) also confirmed that PP6 has a unique location in meiosis. 2) The PP6c depletion phenotypes are not quite the same. PP6C-depleted somatic cells showed delayed spindle formation and defective spindle integrity with highly fragmented and disordered spindle poles and failed to efficiently align chromosomes at the metaphase plate. But these defects were not found in PP6c-depleted oocytes. In fact, without PP6c in oocytes, the MI spindles were totally normal and MII spindles though abnormal still remained in intact and bipolar organization with well-aligned chromosomes. Moreover, the hyperstable spindles with excessively polymerized microtubules in PP6c-deficient oocytes had not been discovered in mitotic cells. The above different spindle defects might be due to functional variation of Aurora A in mitosis and meiosis II. Taken together, our study unveils the role of PP6 in meiosis that was hitherto unknown and provides a significant advance of our knowledge on PP6 function.

As members of PP2A-like subfamily, PP6 does share common features with PP2A or PP4. They not only have similar structure, similar sensitivities to small-molecule inhibitors such as okadaic acid, but they also have similar functions. Like PP2A, these similarities might be the reason for unexplained PP6 functions for many years. Our study, by using genetically modified mouse models, provides convincing evidence showing important roles for PP6 in oocyte meiosis. From previous reports and our two conditional knockout studies of PP2A and PP6 in oocytes, we have demonstrated that PP6 is not merely “PP2A-like”. In oocyte meiosis, PP2A mainly functions in meiosis I as a GVBD inhibitor and centromeric cohesion protector, and plays a role in MII arrest and egg activation as well (Chang et al., 2011); however, PP6 appears to mainly function in meiosis II as an Aurora A kinase inhibitor that ensures proper spindle formation.

Female meiosis is error-prone in humans. It is estimated that 20% of human oocytes are aneuploid, and mistakes in meiotic chromosome segregation account for 1/3 of all pregnancy losses (Hassold and Hunt, 2001; Qiao et al., 2014; Wang et al., 2011). Both MI and MII exits are key events in maintaining euploidy of oocytes. Interestingly, all PP2A-like protein phosphatases have essential roles in preventing oocyte aneuploidy. Our previous study has reported that oocyte-specific deletion of ppp2r1a resulted in
precocious separation of sister chromatids and oocyte aneuploidy, which provided direct \textit{in vivo} evidence that PP2A ensures accurate chromosome segregation and euploidy during the MI exit (Hu et al., 2014). PP4 has been reported to be involved in the establishment or maintenance of chiasmata during meiotic prophase I and in regulating embryo microtubule severing in C. elegans (Han et al., 2009; Sumiyoshi et al., 2002). Here we have shown that PP6c depletion in oocytes leads to defective MII spindle function and unfaithful chromatid segregation in meiosis II, which proves that PP6 also acts as an important antagonist to oocyte aneuploidy during the MII exit. Our study contributes to elucidating the underlying mechanisms for proper spindle formation and accurate chromosome segregation.

In conclusion, for the first time our study provides strong \textit{in vivo} evidence from the whole animal level to the molecular level that PP6 in oocytes plays a unique role as an Aurora A suppressor in MII and is critical for MII exit, euploid egg production and female fertility.
MATERIALS AND METHODS

Mice

Mice lacking Ppp6c in oocytes (referred to as Ppp6c<sup>F/F</sup>;ZCre+) were generated by crossing Ppp6c<sup>F/F</sup> mice with Zp3-Cre mice. Generation of Ppp6c floxed heterozygous mouse: A 12 kb genomic fragment, comprising a 2.2 kb 5’ arm of homology, an 8 kb core region containing exons 2-4, and a 2 kb 3’ arm homology, was cloned into the Pfrt1 vector containing an Frt-floxed neomycin resistance cassette and a TK cassette. The neomycin cassette was inserted downstream of exon 4, and the other LoxP was inserted upstream of exon 2. NotI-linearized targeting vector was electroporated into mouse embryonic stem (ES) cells. Correct targeted ES cells were confirmed by Southern blot analysis. The chimeric mice were generated by microinjection of targeted ES cells into C57BL/6J blastocysts. The mice were housed under controlled environmental conditions with free access to water and food. Light was provided between 08:00 and 20:00. Animal care and handling were conducted according to the guidelines of the Animal Research Committee of the Institute of Zoology, Chinese Academy of Sciences.

Reagents and antibodies

Commercial antibodies were used to detect α-tubulin (mouse, DM1A; Sigma-Aldrich), PPP6C (rabbit, A300-844A; Bethyl Laboratories, Inc.), Aurora-A (rabbit, BS1840; Bioworld Technology, Inc.), phospho-Aurora-A/B/C pT288/232/198 (rabbit, 2914; Cell Signaling Technology), TPX2 (rabbit, 11741-1-AP; Proteintech Group, Inc.) and β-actin (mouse, sc-47778, Santa Cruz). Secondary antibodies were purchased from ZhongShan Golden Bridge Biotechnology Co., LTD (Beijing). Aurora A kinase inhibitor was obtained from Selleck Chemicals (MLN8237; 10mM of 500000× stock).

Natural ovulation and superovulation

For the natural ovulation assay, 2-4 month-old female mice were mated with fertile males overnight. Successful mating was confirmed by the presence of vaginal plugs. Fertilized eggs were harvested from oviducts, counted and analyzed after treatment of
the cumulus mass with 1mg/ml hyaluronidase (Sigma-Aldrich) in M2 medium (Sigma-Aldrich). Blastocysts were harvested from the uterus at E 3.5 and counted.

To induce ovulation and collect MII oocytes, each female mouse was injected with 10 IU of PMSG followed by 10 IU of hCG 48h to promote ovulation. Mice were killed at 12-14h of hCG treatment and cumulus-oocyte complexes were recovered from each oviduct. After a 5-min treatment with hyaluronidase (1mg/ml) in M2 medium, oocytes were collected.

**Histological analysis and quantification of ovarian follicles**

Ovaries used for histological analysis were collected from adult female mice. Then, they were fixed in 4% paraformaldehyde (pH 7.5) overnight at 4°C, dehydrated, and embedded in paraffin. Paraffin-embedded ovaries were sectioned at a thickness of 8-μm for hematoxylin and eosin (H&E) staining. One or both ovaries from more than three mice of each genotype were used for the analysis. Quantification of ovarian follicles was performed as previously described (Hu et al., 2014).

**Parthenogenetic activation and in vitro fertilization (IVF)**

For Sr\(^{2+}\) activation, super-ovulated MII oocytes were first washed twice in M2 medium and once in activation medium and then cultured in activation medium (Ca\(^{2+}\)-free CZB medium supplemented with 10 mM SrCl\(_2\)) for 6 h.

*In vitro* fertilization (IVF) was performed by recovering fresh sperm from C57BL6 male mice from the epididymis into HTF medium for at least 0.5h. Then the dispersed sperm cells were added into the HTF drops containing the mature COCs. After co-incubation for 2h, presumptive zygotes were washed three times to remove cumulus cells and excess sperm and placed into 20 μl drops of HTF medium under mineral oil. Embryos were cultured at 37°C in a humidified atmosphere of 5% O\(_2\) and 6% CO\(_2\) in air for another 4h and collected for assessment.
Live imaging of oocyte activation

For live cell imaging, super-ovulated MII oocytes were activated and cultured in activation medium supplemented with Hoechst 33342 (5 ng/ml) for 0.5 h and then transferred to the Perkin Elmer precisely Ultra VIEW VOX Confocal Imaging System (PerkinElmer, Waltham, MA, USA) equipped with 37°C incubator and 5% CO₂ supply (Jiang et al., 2014). The observation lasted for at least 7 hours. The DNA in oocytes was labeled in blue using Hoechst 33342, and changed into red in the figures and movies.

Oocyte collection and culture

GV stage oocytes were isolated from ovaries of 6-9 week-old female mice and cultured in M2 medium under paraffin oil at 37°C, 5% CO₂ in air. For Aurora A inhibition, 20nM MLN8237 or control DMSO was added to M2 medium for oocyte culture. Oocytes were collected at different times of culture for immunofluorescent staining, western blot and chromosome spreads.

Immunofluorescent analysis and chromosome spread

Oocytes for immunofluorescent staining were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Then the fixed oocytes were transferred to membrane permeabilization solution (0.5% Triton X-100) for 20 min and blocking buffer (1% BSA-supplemented PBS) for 1 h. At last, oocytes were incubated overnight at 4°C with antibodies described above in appropriate dilutions. Then the oocytes were mounted on glass slides and examined with a laser scanning confocal microscope (Zeiss LSM 780 META, Germany).

For chromosome spreads, the oocytes were first freed of the zona pellucida by acid Tyrode’s solution (Sigma-Aldrich). After a brief recovery in M2 medium, the oocytes were transferred onto glass slides and fixed in a solution of 1% paraformaldehyde in distilled H₂O (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol. DNA on the slides was stained with DAPI and slides were mounted for observation with immunofluorescence microscopy.
Western Blot analysis

A total of 200 mouse oocytes or zygotes per sample were mixed with 2× SDS sample buffer and boiled for 5 min at 100°C for SDS-PAGE. Western blot was performed as described previously (Qi et al., 2013), using the antibody dilution anti-PPP6c/Aurora A/TPX2: 1:500; anti-p-Aurora A/B/C: 1:1000; anti-β-actin: 1:2000.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed using SPSS. Data were expressed as mean ± SEM and p < 0.05 was considered statistically significant.

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Figure 1. Characterization of PP6c during mouse oocyte meiotic maturation. (A) Western blots showing expression pattern of PP6c at different stages of
oocytes/zygotes. A total of 200 oocytes were collected after being cultured for 0, 4, 8 and 13 hours, corresponding to GV, GVBD, MI and MII stages, respectively. A total of 200 1-cell embryos were collected at 26-28h after hCG treatment with successful mating. Samples were immunoblotted using anti-PP6c and anti-β-actin antibodies. Molecular mass is given in kilodaltons. (B) Representative images of subcellular localization of PP6c during oocyte meiotic maturation and after fertilization. Oocytes were double stained for PP6c (green) and DNA (red) at GV, GVBD, Pro-MI, MI, AI-TI and MII stages; 1-cell embryos were double stained for PP6c (green) and DNA (red) at interphase and metaphase stages of the first mitotic division. Magnification of the boxed region is shown on the top right corner. GV, Germinal vesicle; N, Nucleolus; PN, pronuclei; PB, polar body. Bar = 20μm.
Figure 2. Disruption of Ppp6c in oocytes led to female subfertility without impacting ovulation. (A) Localization of PP6c in Ppp6c<sup>F/F</sup> and Ppp6c<sup>F/F;ZCre+</sup> oocytes, respectively. GV oocytes were cultured for about 4 hours and those that had undergone GVBD were fixed, followed by immunofluorescent staining for PP6c (green) and DNA (red). Bar = 20μm. All of the experiments were repeated at least three times, and representative results are shown. (B) Western blots showing the
absence of PP6c protein expression in $Ppp6c^{F/F};ZC{\text{re}}+$ oocytes. Lysate from 200 GV oocytes was loaded in each lane. Levels of β-actin was used as internal controls. Molecular mass is given in kilodaltons. All of the experiments were repeated at least three times, and representative results are shown. (C) Subfertility of the female $Ppp6c^{F/F};ZC{\text{re}}+$ mice. Continuous breeding assessment showed the cumulative number of progeny per female mouse for 6 months. (D) Normal ovulation rate in $Ppp6c^{F/F};ZC{\text{re}}+$ mice. Fertilized eggs were collected and counted from female mice of each genotype with vaginal plugs after mating overnight. At least 6 mice of each genotype were used. (E) Representative hematoxylin and eosin (H&E) staining and follicle counting results of ovaries from 6-month-old mice of each genotype. Bar = 500 μm; CL, corpus luteum. At least 3 mice of each genotype were used.
Figure 3. PP6c depletion did not impair oocyte meiotic progression during first meiosis. (A) Comparable GVBD rates and PBE rates of Ppp6c^{F/F} oocytes and Ppp6c^{F/F};ZCre+ oocytes. GV oocytes were isolated and matured in vitro, oocytes that resumed meiosis I (GVBD) and extruded first polar body (PBE) were counted at 4h and 13h, respectively. Representative DIC images are shown. Data are presented as mean ± SEM. In vitro maturation (IVM) experiments were repeated at least 3 times.
≥150 oocytes of each genotype were analyzed for each time point. (B) Representative images of staining for DNA (red) and immunostaining for α-tubulin (green) showing normal spindle assembly in Ppp6c<sup>F/F</sup>; ZCre<sup>+</sup> oocytes at the MI stage. Bar=10 μm. GV oocytes were isolated, cultured for 8h to the MI stage and then fixed. Percentages of oocytes with normal spindle at the MI stage of each genotype are presented as mean ± SEM. The numbers of analyzed oocytes are indicated (n). (C) Chromosome spread of MII oocytes from Ppp6c<sup>F/F</sup> and Ppp6c<sup>F/F</sup>; ZCre<sup>+</sup> mice, showing chromosomes stained with DAPI (blue). Representative images are shown. Bar = 10μm. GV oocytes were isolated and cultured for 13h and MII oocytes with PB1 were fixed. Number of chromosomes of each oocyte was counted and percentages of euploidy (20 pairs of chromatids) in MII oocytes of each genotype are presented as mean ± SEM. The total numbers of analyzed oocytes are indicated (n).
Figure 4. Aneuploidy in zygotes led to defective early embryonic development and subfertility in Ppp6c\(^{+/+}\);ZCre+ mice. (A) Representative images of zygotes at E0.5 from Ppp6c\(^{+/+}\) and Ppp6c\(^{+/+}\);ZCre+ females. Yellow arrowheads showed visible pronuclei. Representative images of immunostaining for DNA (red) and α-tubulin.
(green) showing pronuclei formation in zygotes from $Ppp6c^{F/F}$ and $Ppp6c^{F/F};ZCre+$ females were presented in the lower panel. Yellow arrows showed normal pronuclei. PN, pronuclei. Bar = 20μm. Percentages of zygotes with normal pronuclei formation at E0.5 in $Ppp6c^{F/F}$ and $Ppp6c^{F/F};ZCre+$ mice are presented as mean ± SEM. At least 5 mice of each genotype were used and the total numbers of analyzed zygotes are indicated (n). *, P<0.05. (B) Chromosome spread of 1-cell embryos blocked at metaphase with colchicine from $Ppp6c^{F/F}$ and $Ppp6c^{F/F};ZCre+$ female mice, showing chromosomes stained with DAPI (blue). Representative images are shown. Bar = 10μm. Number of chromosomes of each embryo was counted and percentage of 1-cell embryos with aneuploidy in the control group and mutant group. The total numbers of analyzed embryos are indicated (n). Data are presented as mean ± SEM. *, P<0.05. (C) Representative images of embryos at E4.0 from $Ppp6c^{F/F}$ and $Ppp6c^{F/F};ZCre+$ females. Black arrows showed apoptotic embryos. Percentages of blastocyst formation at E4.0 in $Ppp6c^{F/F}$ and $Ppp6c^{F/F};ZCre+$ mice are presented as mean ± SEM. At least 4 mice of each genotype were used and the total numbers of analyzed embryos are indicated (n). ***, P<0.001.
Figure 5. Deficiency of PP6c resulted in abnormal spindle microtubules in MII.

(A) Representative images of staining for DNA (red) and immunostaining for α-tubulin (green) showing spindle organization in super-ovulated MII oocytes from *Ppp6c<sup>F/F</sup>* and *Ppp6c<sup>F/F</sup>;ZCre+* mice after hCG. Bar = 20μm. Magnifications of the boxed regions are shown. All of the experiments were repeated at least three times, and ≥50 oocytes of each genotype were analyzed. (B) Super-ovulated MII oocytes from *Ppp6c<sup>F/F</sup>* and *Ppp6c<sup>F/F</sup>;ZCre+* mice were cold-treated at 4°C for 20 minutes and
then fixed and double-stained for DNA (red) and α-tubulin (green) to show spindle microtubule depolymerization. All of the experiments were repeated at least three times, and representative results are shown. Bar = 10μm. Representative images of cold treatment for 15 minutes were used as negative control to demonstrate that $P_{pp6c}^{F/F}$ oocytes do indeed have k-fibers. White arrowheads show k-fibers which are relatively cold-stable microtubules associated with kinetochores. Percentages of oocytes with relatively intact spindle in the control group and mutant group after 20 minutes of cold treatment are presented as mean ± SEM. The numbers of analyzed oocytes are indicated (n).*, $P< 0.05$. 
Figure 6. *Ppp6c* deletion in oocytes caused failure of MII exit. (A) Dynamics of MII exit in *Ppp6c^{F/F}* oocytes and *Ppp6c^{F/F};ZCre+* oocytes. Super-ovulated MII oocytes were parthenogenetically activated and cultured in SrCl\textsubscript{2} activation medium supplemented with Hoechst 33342 (5 ng/ml) for 0.5 h and then were live imaged. Representative still images from Movies 1 and 2 are shown. Timestamps indicate hours after activation. Yellow arrows pointing at second polar body extrusion. Red, DNA. Representative images of staining for DNA (red) and immunostaining for α-tubulin (green) showing spindle organization and chromatids separation at telophase II (2h) and pronucleus formation (6h) in activated oocytes of each genotype are also presented aside. All of the experiments were repeated at least three times, and ≥100 oocytes of each genotype were analyzed. PN, pronuclei. Bar = 20μm. (B) Representative images of immunostaining for DNA (red) and α-tubulin (green) showing pronuclei formation in zygotes from *Ppp6c^{F/F}* and *Ppp6c^{F/F};ZCre+* mice.
Super-ovulated MII oocytes of each genotype were collected and fertilized *in vitro* with active spermatozoa. Zygotes were cultured for 6h after *In Vitro* Fertilization (IVF) and then fixed. All of the experiments were repeated at least three times, and ≥110 oocytes of each genotype were analyzed. PN, pronuclei. Bar = 20μm.
Figure 7. Amplified Aurora A activity in PP6c-deficient MII oocytes. (A) Western blots showing up-regulated p-Aurora A T288 in Ppp6c<sup>F/F</sup>;ZCre<sup>+</sup> oocytes. Each sample (200 MII oocytes) was collected after being cultured for 13 hours in vitro, and immunoblotted for p-Aurora A T288, Aurora A, TPX2 and β-actin. Level of β-actin was used as internal control. Molecular mass is given in kilodaltons. (B) Representative images of immunostaining for Aurora A (red), α-tubulin (green) and staining of DNA (blue) showing comparable signals of total Aurora A in MII oocytes from Ppp6c<sup>F/F</sup> and Ppp6c<sup>F/F</sup>;ZCre<sup>+</sup> mice. Bar = 10μm. Experiments were repeated at least 3 times and ≥30 oocytes of each genotype were analyzed. (C) Representative immunofluorescent staining showing amplified p-Aurora A T288 signal at spindle poles in PP6c-depleted MII oocytes. Red, p-Aurora A/B/C; Green: α-tubulin; Blue, DNA. Yellow arrowheads showed specific signals of p-Aurora A T288. Bar = 10μm. Quantification of the p-Aurora A T288 signal at spindle poles were presented as mean ± SEM. Experiments were repeated at least 3 times and the numbers of analyzed oocytes are indicated (n). **, P<0.001.
Figure 8. Aurora A inhibition by MLN8237 rescued PP6c depletion phenotype.

(A) Western blots showing that enhanced Aurora A activity in Ppp6c<sup>F/F</sup>;ZCre<sup>+</sup> oocytes was down-regulated to normal level after MLN8237 treatment. Each sample (200 MII oocytes) was collected after being cultured for 13 hours with or without treatment (20 nM DMSO for Ppp6c<sup>F/F</sup> oocytes and 20 nM MLN8237 for Ppp6c<sup>F/F</sup>;ZCre<sup>+</sup> oocytes), and immunoblotted for p-Aurora A T288, Aurora A, TPX2 and β-actin. Level of β-actin was used as internal control. Molecular mass is given in kilodaltons. (B) Representative immunofluorescent staining showing rescued spindle shape and reduced p-Aurora A T288 signal at spindle poles in PP6c-depleted MII oocytes after MLN8237 treatment. Red, p-Aurora A/B/C; Green: α-tubulin; Blue, DNA. Yellow arrowheads pointing at signals of p-Aurora A T288. Experiments were repeated at least 3 times and ≥30 oocytes of each genotype were analyzed. Bar = 10μm. (C) Representative immunofluorescent staining showing improved pronuclei formation in mutant zygotes at IVF 6h after MLN8237 treatment. Super-ovulated MII oocytes of each genotype were collected and cultured in M2 medium with or without treatment for 15 minutes (20 nM DMSO for Ppp6c<sup>F/F</sup> oocytes and 20 nM MLN8237
for Ppp6c<sup>F/F</sup>;ZCre+ oocytes). Then the oocytes were washed and fertilized <em>in vitro</em> with active spermatozoa. Zygotes were cultured for 6h before fixation. Green: α-tubulin; Red, DNA. White arrows showing zygotes with normal pronuclei formation. Bar = 100μm. Percentages of zygotes with normal pronuclei formation in each group are presented as mean ± SEM. All of the experiments were repeated at least three times and the total numbers of analyzed zygotes are indicated (n). *, P<0.05; **, P<0.001.