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Journal of Genetics and Genomics 42 (2015) 477–485

JGG

ORIGINAL RESEARCH

ERK1/2 Activities Are Dispensable for Oocyte Growth but Are Required for Meiotic Maturation and Pronuclear Formation in Mouse

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Received 23 June 2015; revised 18 July 2015; accepted 23 July 2015
Available online 1 August 2015

ABSTRACT

Previous studies revealed that extracellular regulated kinase-1 and -2 (ERK1/2) cascade plays pivotal roles in regulating oocyte meiotic cell cycle progression. However, most knowledge about the *in vivo* function of ERK1/2 in mammalian oocytes was indirectly obtained from analyzing the phenotypes of *Mos* knockout mice. In this study, we knocked out *Erk1* and *Erk2* in mouse oocytes as early as the primordial follicle stage using the well-characterized *Gdf9-Cre* mouse model, and for the first time directly investigated the *in vivo* function of ERK1/2 in mouse oocytes. In this novel mouse model, we observed that ERK1/2 activities in oocyte are dispensable for primordial follicle maintenance, activation and follicle growth. Different from the *Mos* null oocytes, the ERK1/2-deleted oocytes had well-assembled spindles at metaphase I (MI), extruded polar body-1 (PB1) with normal sizes, and did not undergo a full parthenogenetic activation characterized for pronuclear formation. However, the ovulated ERK1/2-deleted oocytes had poorly-assembled metaphase II (MII) spindles, spontaneously released polar body-2 (PB2), and were arrested at another metaphase called metaphase III (MIII). In addition, ERK1/2 deletion prevented male pronuclear formation after fertilization, and caused female infertility. In conclusion, these results indicate that ERK1/2 activities are required for not only MII-arrest maintenance, but also efficient pronuclear formation in mouse oocytes.

KEYWORDS: Oocyte; Female reproduction; Meiosis; ERK1/2; Fertilization

INTRODUCTION

Mitogen-activated protein kinase (MAPK) is a family of serine/threonine protein kinases that are widely distributed in eukaryotic cells. Extracellular signal regulated kinase-1 and -2 (ERK1/2), the best studied MAPK family members, are key components of receptor protein tyrosine kinase-induced signaling cascades. Previous studies revealed that ERK1/2 cascade plays pivotal roles in regulating oocyte meiotic cell cycle progression (Fan and Sun, 2004; Liang et al., 2007). In mammals, ERK1/2 activation in cumulus cells is necessary for gonadotropin-induced oocyte meiotic resumption, while ERK1/2 activities in oocyte itself are not required for its spontaneous meiotic resumption *in vitro* (Su et al., 2001, 2002; Fan et al., 2003a, 2009). After germinal vesicle breakdown (GVBD), ERK1/2 are involved in the regulation of microtubule organization and meiotic spindle assembly (Verlhac et al., 1993; Verlhac et al., 1994). Most importantly, ERK1/2 activities are essential for maintenance of metaphase II (MII) arrest (Tong et al., 2003).

MAPK-ERK kinase 1 and 2 (MEK1/2) are dual-specificity protein kinases that activate MAPK through phosphorylation of the threonine-183 and tyrosine-185 within its activation loop (Chen et al., 2001). MEK1/2 are also activated by phosphorylation, and have multiple upstream activators. For example, RAF-A, -B and -C are best studied MEK kinases that are associated with carcinogenesis. Interestingly, the vertebrate oocytes express a specific MEK kinase known as MOS, which is encoded by the proto-oncogene *Mos*. MOS is a 39-kDa germ cell-specific Ser/Thr protein kinase that was first identified in cells transformed by Moloney murine leukemia

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http://dx.doi.org/10.1016/j.jgg.2015.07.004

virus. The *Mos* mRNA is stored as maternal information in the growing oocytes, and its translation into protein activates MAPK cascade during oocyte maturation.

Most knowledge about the *in vivo* function of ERK1/2 cascade in mammalian oocytes was obtained from analyzing the phenotypes of *Mos* knockout mice. *Mos* knockout female mice were less fertile and MII oocytes derived from them were parthenogenetically activated without fertilization (Colledge et al., 1994; Hashimoto et al., 1994). Phenotypically, the first meiotic division of $Mos^{-/-}$ oocytes frequently produced an abnormally large polar body (PB) (Choi et al., 1996; Verlhac et al., 1996). In these oocytes, the spindle shape is altered and the spindle fails to translocate to the cortex, leading to the establishment of an altered cleavage plane. Based on these observations, people concluded that ERK1/2 were not necessary for GVBD and polar body-1 (PB1) emission but were required for normal spindle assembly.

However, not all ERK1/2 functions in oocyte development can be revealed by analyzing MOS-deficient oocytes. Particularly, *Mos* mRNA is only accumulated in fully grown germinal vesical (GV) stage oocytes, and translated into proteins after GVBD. Therefore, it remains unclear if ERK1/2 are activated by other upstream signals, such as oocyte membrane receptors and RAFs, and involved in early events of oocyte development, such as dormancy maintenance and awakening within primordial follicles, and fast oocyte growth in sizes during primordial follicle activation.

This hypothesis is at least partially supported by experimental evidence. For example, KIT ligand secreted from granulosa cells was able to bind with KIT, a receptor protein tyrosine kinase specifically expressed by oocytes, and sequentially activated MEK1/2 and ERK1/2 (Reddy et al., 2005). Another report showed that ERBB2, a member of the EGF receptor family, was expressed in oocytes of primordial follicles, and was required for EGF-induced ERK1/2 activation and growth initiation of primordial follicle (Zheng et al., 2012). These results suggested that other than regulating the final meiotic maturation of fully grown oocytes, ERK1/2 may play a role in enhancing the growth and probably also secretion abilities of partially grown oocytes.

In addition, theoretically MOS may have unknown downstream targets other than ERK1/2 in regulating meiotic divisions. Inactivation of these targets, rather than ERK1/2, may contribute to some phenotypes of $Mos^{-/-}$ oocytes.

In this study, we knocked out *Erk1* and *Erk2* in mouse oocytes as early as the primordial follicle stage using the well-characterized *Gdf9-Cre* mouse model, and for the first time directly investigated the *in vivo* functions of ERK1/2 in mouse oocytes.

RESULTS

ERK1/2 activities in oocyte are dispensable for primordial follicle maintenance, activation and follicle growth

To investigate the *in vivo* function of ERK1/2 in mouse oocytes throughout follicle development, we selectively deleted *Erk2* in oocytes of $Erk1^{-/-}$ background using transgenic mice expressing *Gdf9* promoter-mediated *Cre* recombinase (*Gdf9-Cre*). In *Gdf9-Cre* mice, *Cre* is expressed in oocytes since postnatal day (PD) 3 and in later developmental stages (Lan et al., 2004).

In a superovulation assay, similar numbers of mature oocytes were collected from the oviducts of wide-type (WT), $Erk1^{-/-}$, and $Erk1^{-/-}$;$Erk2^{flox/flox}$;*Gdf9-Cre* (later named as $Erk1/2^{oo-/-}$) mice (Fig. 1A). Primordial follicle activation and oocyte growth in sizes were not affected by ERK1/2 deletion. The ovaries of pubertal $Erk1/2^{oo-/-}$ mice contained normal numbers of primordial follicles and growing follicles (Fig. 1B). The ovarian morphologies of $Erk1/2^{oo-/-}$ mice were indistinguishable from those of control mice, at either puberty (PD 21–23) or late adult (8-month-old) stages (Fig. 1C). Based on these observations, we conclude that ERK1/2 activities in oocyte are physiologically dispensable for primordial follicle maintenance and activation, follicle growth, and ovulation.

While the $Erk1^{-/-}$ and $Erk2^{oo-/-}$ female mice had normal fertility (data not shown), the $Erk1/2^{oo-/-}$ female mice were sterile (Fig. 1D and E). Therefore, we further investigated if the ERK1/2-deleted oocytes had defects in meiotic maturation and fertilization.

ERK1/2 deletion in oocyte does not affect GVBD but delays PB1 extrusion

We isolated fully grown GV-stage oocytes from WT and $Erk1/2^{oo-/-}$ mice, and investigated the *in vitro* oocyte meiotic maturation process. The $Erk1/2^{oo-/-}$ oocytes did not show any delay of GVBD, and formed normal shaped MI spindles, and chromosomes were correctly aligned at the spindle equators (Fig. 2A). Furthermore, ERK1/2-deleted oocytes had normal chromosome configurations and correct localizations of CREST and DNA topoisomerase II (TOP2) on centromeres and chromosome arms (Fig. 2B). Precocious segregations of homologous chromosomes were not observed in these oocytes (Fig. 2B).

However, the $Erk1/2^{oo-/-}$ oocytes showed abnormalities at anaphase I (AI): homologous chromosome migrations to the spindle poles were asynchronous, which might cause delayed PB1 emission (Fig. 2A). At MII stage, spindle formation and chromosome alignment were abnormal in $Erk1/2^{oo-/-}$ oocytes (Fig. 2A). In normal MII oocytes, condensed sister chromatids were hold together by centromeres. In contrast, the chromosome configurations were distorted in $Erk1/2^{oo-/-}$ oocytes (Fig. 2B). Many chromosomes had aberrant localization of centromere components including CREST (Fig. 2C), in ovulated $Erk1/2^{oo-/-}$ oocytes 14 h after human chorionic gonadotropin (hCG) injection. Unpaired, precociously segregated sister chromatids were frequently seen in these oocytes (Fig. 2C, arrows).

After PB1 emission, the WT oocytes entered meiosis II immediately, and their chromosomes maintained the condensed configuration during meiosis I–II transition. However, the chromosomes of ERK1/2-deleted oocytes decondensed after PB1 emission, and only recondensed after

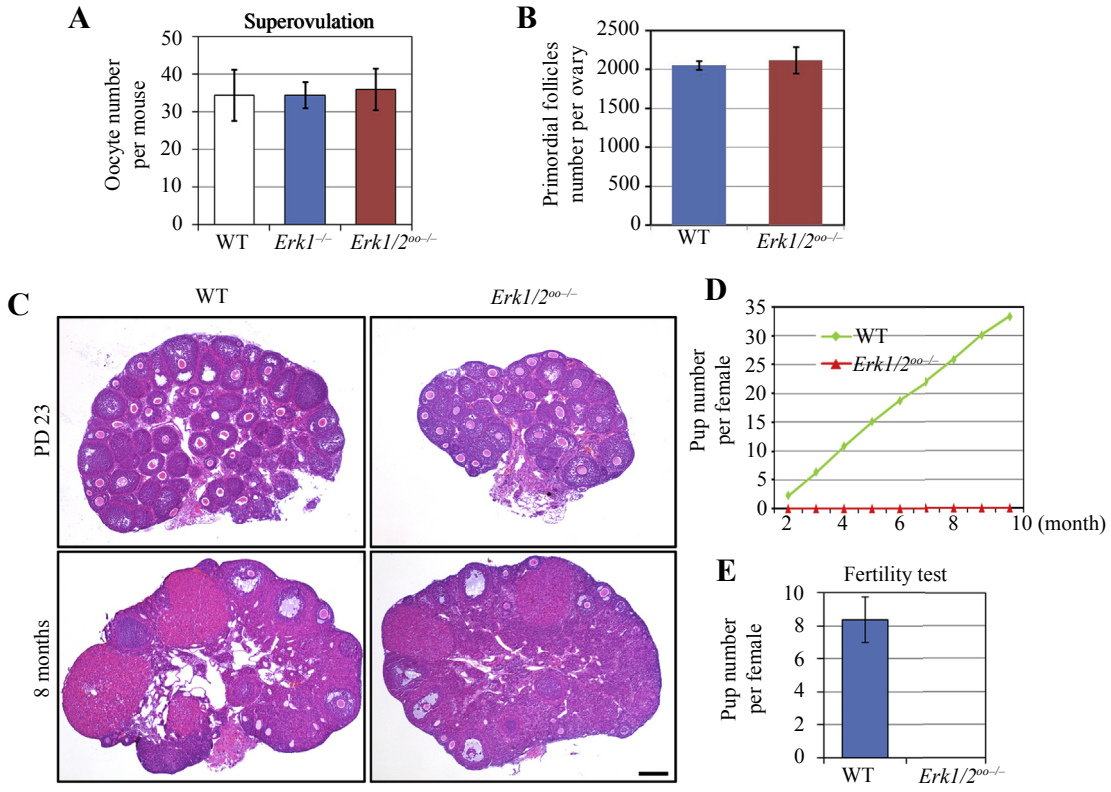

Fig. 1. ERK1/2 activities in oocyte are dispensable for primordial follicle maintenance and follicle growth.
A: The average numbers of oocytes that were ovulated by WT, *Erk1*$^{-/-}$ and *Erk1/2*$^{oo-/-}$ female mice in a superovulation assay, at PD 23. $n = 6$ for each genotype. **B:** Quantification of primordial follicle numbers per ovary in WT and *Erk1/2*$^{oo-/-}$ female mice at PD 23. $n = 3$ for each genotype. **C:** Ovarian histologies of WT and *Erk1/2*$^{oo-/-}$ mice at PD 23 and 8 months after birth. Scale bar = 200 μm. **D** and **E:** The *Erk1/2*$^{oo-/-}$ female mice were infertile. **D** shows the accumulated pup numbers of females at 2−10 months after birth, and **E** shows the average litter sizes of WT and *Erk1/2*$^{oo-/-}$ females.

meiosis II entry (Fig. 2D). These results indicated that ERK1/2 activities were crucial to prevent chromosome decondensation during meiosis I−II transition *in vivo*.

We also analyzed the effect of ERK1/2 depletion on the activation of other pathways that were important for oocyte maturation. Western blot results showed that activation of the phosphorylation of AKT and its downstream target ribosome protein S6 (RPS6) was not affected in ERK1/2-deleted oocytes (Fig. 3A). The phosphorylation of cyclin-dependent kinase-1 (CDK1) at T161 and the accumulation of cyclin B1 were required for CDK1 activation. These were detected during maturation of WT oocytes but were compromised in ERK1/2-deleted oocytes (Fig. 3A). Western blot results also confirmed the deletion of ERK1/2 in oocytes collected from *Erk1/2*$^{oo-/-}$ mice (Fig. 3A). In addition, the MEK1/2 inhibitor U0126 (50 μmol/L) blocked cyclin B1 accumulation, CDK1 phosphorylation, as well as ERK1/2 phosphorylation/activation in matured WT oocytes, indicating these are the direct effect of ERK1/2 inhibition (Fig. 3B).

ERK1/2 are required for spindle assembly and sister chromatid cohesion at MII

Similar to *Mos*$^{-/-}$ oocytes, *Erk1/2*$^{oo-/-}$ oocytes failed to be arrested at metaphase II, and released the polar body-2 (PB2) shortly after ovulation (Fig. 4A and B). After PB2 emission, the *Mos*$^{-/-}$ oocytes underwent a complete parthenogenetic activation and entered interphase. However, only 10% of *Erk1/2*$^{oo-/-}$ oocytes had pronuclei resulted from parthenogenetic activation at 32 h after hCG injection (Fig. 4B, right column panels, and C).

Most ovulated *Erk1/2*$^{oo-/-}$ oocytes (90%, $n = 249$) were arrested at another metaphase after PB2 emission (Fig. 4B, middle column panels). This phenomenon was often observed in cultured rat oocytes, and called "MIII" stage. The typical MIII-arrested oocytes contained condensed chromosomes and a de-shaped spindle. Sister chromatids segregated in these oocytes (Fig. 4D, middle column panels). Only 10% of the ERK1/2-deleted oocytes had decondensed chromatins (Fig. 4D, right column panels). These results indicated that although ERK1/2 activities are required to prevent precocious sister chromatid segregation at MII, ERK1/2 deletion is insufficient for a full interphase entry in mouse oocytes.

In addition, the PB1s emitted by WT oocytes were usually quickly degenerated. But the PB1s released by the ERK1/2-deleted oocytes were maintained, even at 32 h after culture (Fig. 4E), indicating that ERK1/2 activities are required for PB1 degeneration. This result confirmed the previous observation in *Mos*$^{-/-}$ oocytes.

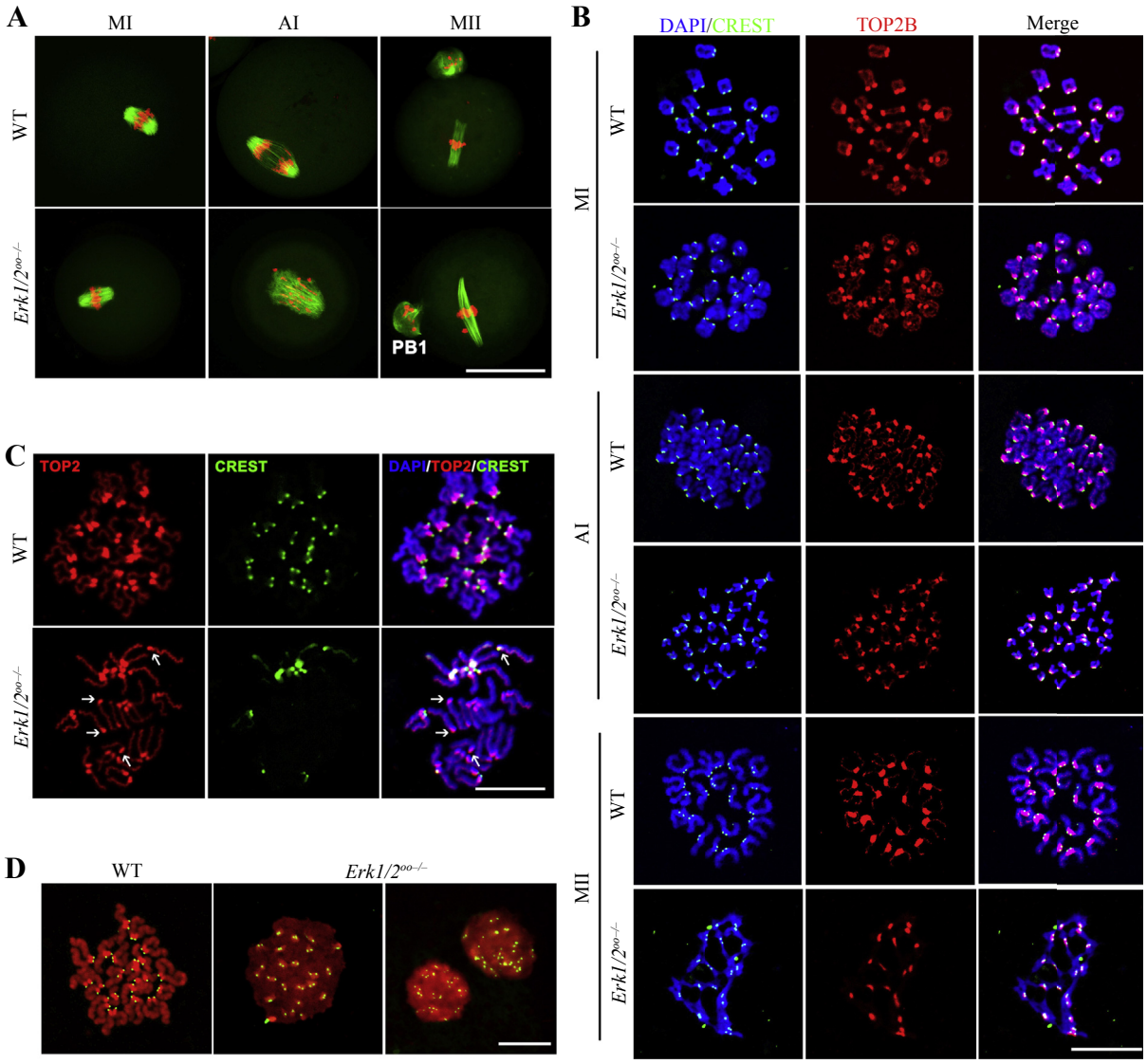

Fig. 2. ERK1/2 deletion in oocyte does not affect germinal vesicle breakdown (GVBD) but causes defects in meiosis I−II transition.

A: Representative immunostaining images for spindle (green) and DNA (red) showing normal progression to MI, defective homologous chromosome separation in AI, and MII spindle assembly failure in ERK1/2-deleted oocytes. PB1, polar body 1. Scale bar = 50 μm. **B:** Representative immunostaining images for DNA topoisomerase II (TOP2, red), CREST (green) and DNA (blue) in chromosome spreads of MI (8 h after culture), AI (10 h after culture), and MII (14 h after culture) oocytes. Scale bar = 50 μm. **C:** Representative immunostaining images for TOP2 (red), CREST (green) and DNA (blue) in chromosome spreads of ovulated oocytes at 14 h after hCG injection. Arrows indicate precociously separated sister chromatids. Scale bar = 50 μm. **D:** Representative immunostaining images for CREST (green) and DNA (red) in chromosome spreads of telophase I (11−12 h after culture) oocytes, showing decondensed chromosomes in oocytes of *Erk1/2*$^{oo-/-}$ mice. Scale bar = 50 μm.

Embryos derived from *Erk1/2*$^{oo-/-}$ oocytes fail to develop

We then determined if the *Erk1/2*$^{oo-/-}$ oocytes can be activated by fertilization. Superovulated *Erk1/2*$^{oo-/-}$ females were mated with fertile WT males, and successful coitus was confirmed by the presence of virginal plugs. At 24 h after hCG injection, only 22% zygotes (n = 32) from *Erk1/2*$^{oo-/-}$ females contained two well-developed pronuclei, 26% (n = 37) had single pronuclei, and 43% (n = 62) did not have pronucleus (Fig. 5A). In the single pronucleus zygotes, sperm nuclei were present in the ooplasm and remained condensed (Fig. 5B).

In male and female pronuclei, DNA and histones are differentially marked by epigenetic modifications. These maternal- and paternal-specific epigenetic modifications are essential for embryonic development. In male pronuclei of control zygotes, the 5′-methylated cytosine (5mC) is changed into 5′-hydroxymethylated cytosine (5hmC) (Gu et al., 2011; Yu et al., 2013). Although abnormally small male pronuclei were present in zygotes derived from *Erk1/2*$^{oo-/-}$ oocytes, the female pronuclei were normal in size (Fig. 5C). Interestingly, the small male pronuclei still had elevated 5hmC levels (Fig. 5C). Moreover, in zygotes derived from both WT and *Erk1/2*$^{oo-/-}$ oocytes, levels of tri-methylated histone H3 at Lys-4 (H3K4me3) and Lys-9 (H3K9me3) in female pronuclei

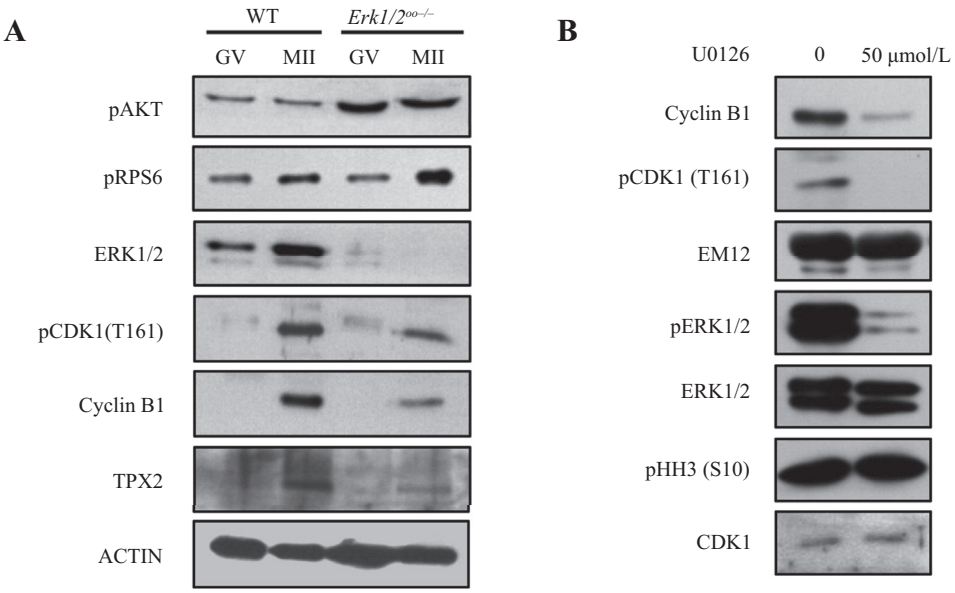

Fig. 3. CDK1 activation during oocyte maturation is compromised in ERK1/2-deleted oocytes.
A: Western blot results showing levels of indicated proteins in WT and ERK1/2-deleted oocytes at GV and MII stages. Total proteins from 200 oocytes were loaded in each lane. **B:** Western blot results showing levels of indicated proteins in WT oocytes at MII stages, with or without U0126 treatment (50 μmol/L). Total proteins from 200 oocytes were loaded in each lane.

were significantly higher than those in male pronuclei (Fig. 5D and E). These results indicate that although pronuclear development is negatively affected by maternal ERK1/2 deletion, ERK1/2 activities are not essential in zygotes for establishing maternal- and paternal-specific epigenetic modifications.

DISCUSSION

Oocyte meiotic maturation is one of the important physiological requirements for species survival. A number of studies have demonstrated that ERK1/2 play a pivotal role in the regulation of meiotic cell cycle progression in oocytes, but controversial findings have been reported in both lower vertebrates and mammals (Roy et al., 1996). In this study, we directly investigated the *in vivo* function of ERK1/2 in oocyte, from primordial follicle stage to zygotic activation.

Our results rule out the possibility that ERK1/2 might have functions in dormant or growing oocytes. The previously reported *Mos*$^{-/-}$ mouse is not perfectly suitable to evaluate ERK1/2 functions *in vivo*: first, MOS protein only exists in fully grown oocytes that resume meiotic maturation; in addition, MOS activity could be bypassed, as shown in *Xenopus* oocytes (Frank-Vaillant et al., 1999). RAF is an alternative kinase existing in *Xenopus* oocytes to activate ERK1/2 through a MOS-independent pathway (Schulze et al., 2004). Injection of *Xenopus* oocytes with constitutively active H-RAS-V12 or its downstream kinase RAF can trigger ERK1/2 activation independent of MOS, while dominant-negative forms of RAF can impair MAPK activation induced by RAS and progesterone in *Xenopus* oocytes (Barrett et al., 1990). Although the current study in mouse showed that ERK1/2 activities were not required for primordial follicle survival and oocyte growth, RAS may still contribute to primordial follicle activation by interacting with phosphatidylinositol-3 kinase (PI3K) because PI3K pathway is crucial for primordial follicle maintenance and oocyte awakening.

Previously, most knowledge regarding to the *in vivo* function of ERK1/2 was obtained from the phenotypical investigations of *Mos*$^{-/-}$ mice. As a maturation-inducing factor, the function of MOS is mainly based on the activation of MEK and subsequent ERK1/2. Fully grown *Mos*$^{-/-}$ oocytes fail to activate ERK1/2 after meiotic resumption, while CDK1 activity is normal until late in MII when it decreases prematurely. Moreover, the first polar body persists instead of degrading and sometimes undergoes an additional cleavage. Similar phenotypes were observed in *Erk1/2*-deleted oocytes.

It has been reported that in *Mos*$^{-/-}$ oocytes extraordinarily long spindles were formed in meiosis I, which caused symmetric meiotic divisions and large PB1 formation (Choi et al., 1996; Verlhac et al., 1996; Tong et al., 2003). People concluded that this phenotype was caused by defective ERK1/2 activation. Surprisingly, the ERK1/2-deleted oocytes and *Mos*$^{-/-}$ oocytes had different phenotypes. Oocytes of the *Erk1/2*$^{oo-/-}$ mice did not form extraordinarily long spindles at MI, and did not release giant PB1s either *in vitro* or *in vivo*.

When *in vivo* ovulated oocytes were collected 21−32 h after hCG injection, almost all WT oocytes remained arrested at MII, but about 63% of the *Mos*$^{-/-}$ oocytes were parthenogenetically activated as evidenced by their pronuclear formation. In the ovary of *Mos*$^{-/-}$ mice, many oocytes were not only activated with nuclear formation, but also underwent several divisions. As a result, the *Mos*$^{-/-}$ female mice developed ovarian teratomas at a high frequency. However, the *Erk1/2*-deleted oocytes have low frequency of parthenogenetic activation characterized by pronuclear formation (less than

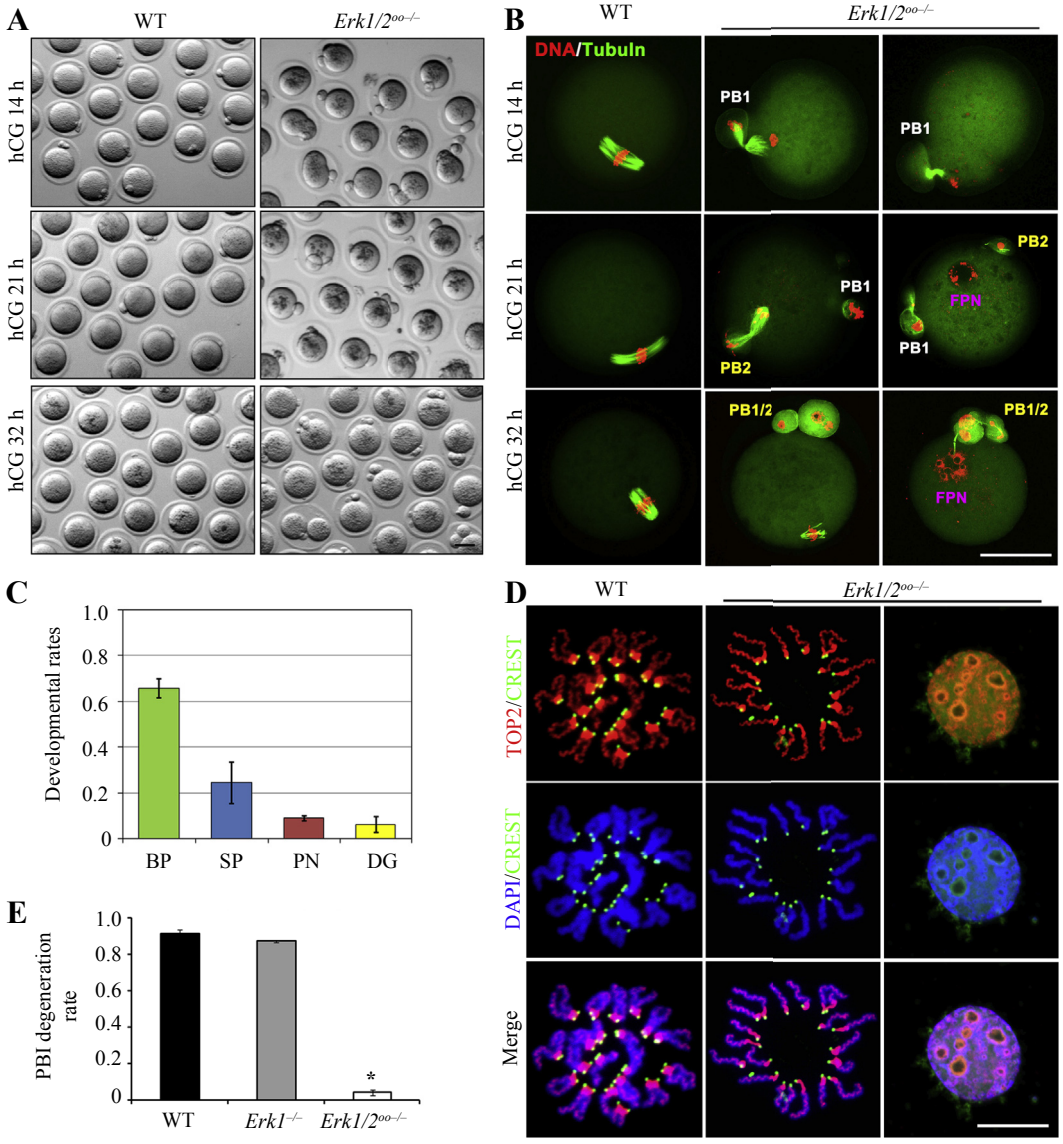

Fig. 4. ERK1/2 are required for spindle assembly and sister chromatid cohesion at MII.

A: Representative images showing the morphology of WT and *Erk1/2*$^{oo-/-}$ oocytes harvested at 14 h, 21 h and 32 h after hCG injection. Scale bar = 50 μm. **B:** Representative images of immunostaining for spindle (green) and DNA (red) in ovulated oocytes of WT and *Erk1/2*$^{oo-/-}$ mice at 14 h, 21 h and 32 h after hCG injection, showing spontaneous PB2 emission, followed by arresting at the MIII stage or entering interphase characterized by the female pronucleus (FPN) formation in ERK1/2-deleted oocytes. PB1 and PB2, polar body 1 and 2. Scale bar = 50 μm. **C:** Quantifications of ovulated *Erk1/2*$^{oo-/-}$ oocytes with indicated spindle/chromosome configurations at 32 h after hCG injection. BP, bipolar spindle; SP, single polar spindle; PN, pronuclei; DG, degeneration. **D:** Immunofluorescent staining results for TOP2 (red), CREST (green), and DNA (blue) in chromosome spreads made from ovulated oocytes at 32 h after hCG injection. Scale bar = 50 μm. **E:** Quantifications of PB1 degeneration rates in WT and *Erk1/2*$^{oo-/-}$ oocytes at 32 h after hCG injection.*, $P < 0.01$.

10%). Ovarian teratomas were not observed in aged *Erk1/2*$^{oo-/-}$ female mice. Instead, the *Erk1/2*-deleted oocytes spontaneously released PB2 and then were arrested at MIII stage. The result indicated that ERK1/2 activities are required for MII arrest, but its loss is not sufficient for a full parthenogenetic activation. It has been previously reported that a large portion of *in vitro* cultured rat oocytes were not arrested at MII and developed to MIII (Fan et al., 2003b). Therefore, it will be interesting to check if ERK1/2 are inactivated in these rat oocytes.

There are two possibilities that the *Mos*$^{-/-}$ and *Erk1/2*$^{oo-/-}$ oocytes have different phenotypes. First, MOS might activate targets other than ERK1/2, particularly other MAPK family members, such as stimulus activated protein kinases (SAPKs) and p38MAPKs. In fact, the specificity of MOS in activating different branches of MAPK cascade is not sufficiently addressed among the existed references. Secondly, small amount of ERK1/2 might still be activated by other MAPK kinase kinases (MAPKKKs) in *Mos*$^{-/-}$ oocytes, at least transiently. In contrast, the *Erk1/2*$^{oo-/-}$ mouse is a more appropriate model to directly analyze ERK1/2 functions in mammalian oocytes. The *in vivo* functions of ERK1/2 revealed by the novel oocyte-specific *Erk1/2* knockout mouse model are summarized in Fig. 6.

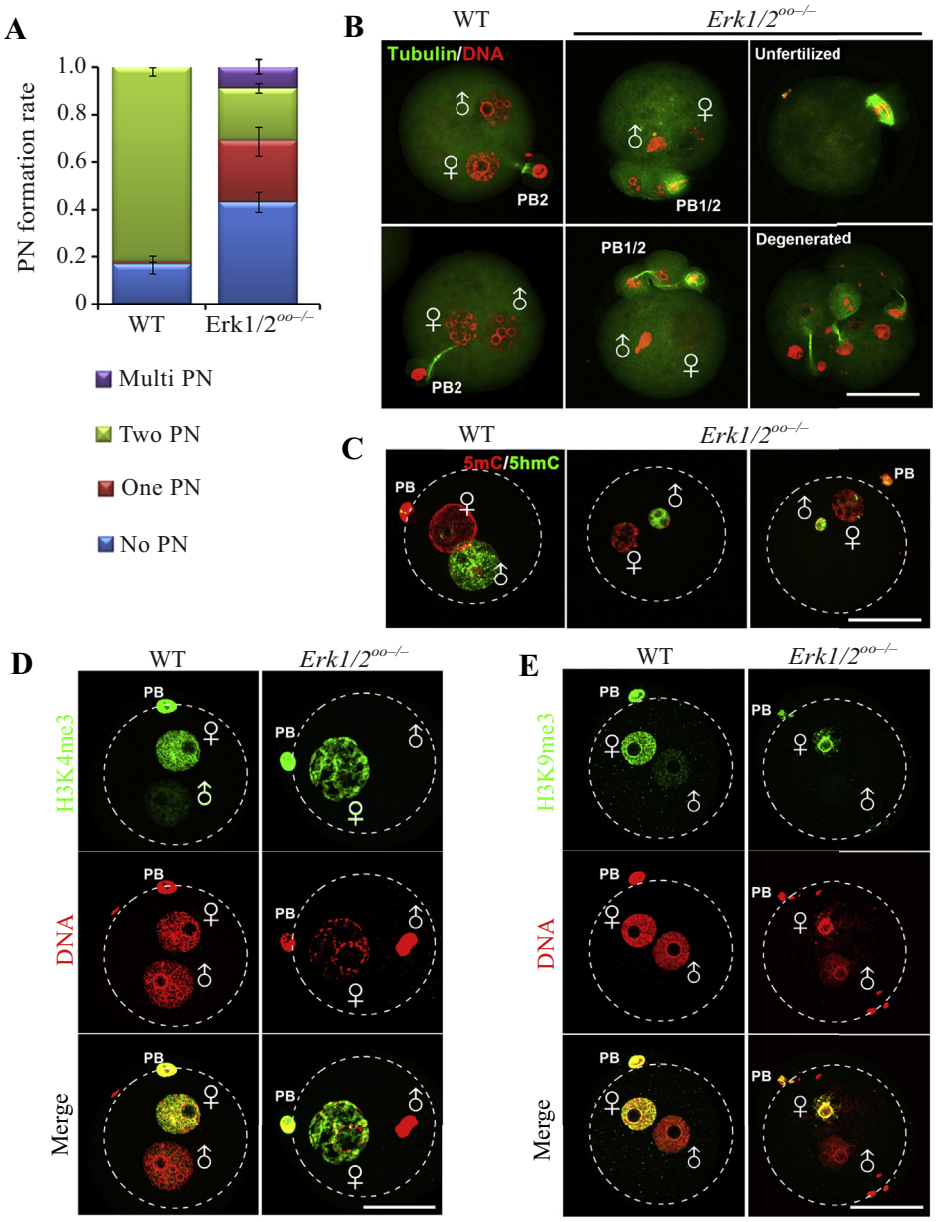

Fig. 5. Embryos derived from ERK1/2-deleted oocytes fail to develop.

A: Percentages of WT and ERK1/2-deleted oocytes/zygotes that have no pronucleus (PN), one PN, two PNs, or multiple PNs, at 32 h after hCG injection. **B:** Immunofluorescent staining results for tubulin (green) and DNA (red) in WT and *Erk1/2*$^{oo-/-}$ zygotes, at 24 h after hCG injection. Scale bar = 50 μm. **C:** Representative images of 5hmC (green)/5mC (red) double-immunostaining of WT and *Erk1/2*$^{oo-/-}$ zygotes at 24 h after hCG injection. PB, polar body. Female and male symbols indicate female and male pronuclei, respectively. Scale bar = 50 μm. **D** and **E:** Immunofluorescent staining results fortri-methylated histone H3 at Lys-4 (H3K4me3, green) (**D**) and Lys-9 (H3K9me3, green) (**E**) in WT and *Erk1/2*$^{oo-/-}$ zygotes at 24 h after hCG injection. Scale bar = 50 μm.

MATERIALS AND METHODS

Mice

WT C57/B6 mice were from the Shanghai SLAC laboratory, China. *Erk1*$^{-/-}$, *Erk2*$^{flox/flox}$ and *Gdf9-Cre* mice in C57BL/6 background were reported ago (Lan et al., 2004; Fan et al., 2009). Mice were maintained in a controlled environment of 20°C−22°C, with a 12 h/12 h light/dark cycle, 50%−70% humidity, and food and water provided *ad libitum*. Animal care and experimental procedures were in accordance with the Animal Research Committee guidelines of Zhejiang University.

Oocyte and embryo harvesting and culture

Three- to four-week-old female mice were intraperitoneally injected with 5 IU of pregnant mare serum gonadotropin (PMSG) (Ningbo Sansheng Pharmaceutical, China) and humanely sacrificed 44 h later. Oocytes at GV stage were harvested in M2 medium (Sigma, USA) and cultured in mini-

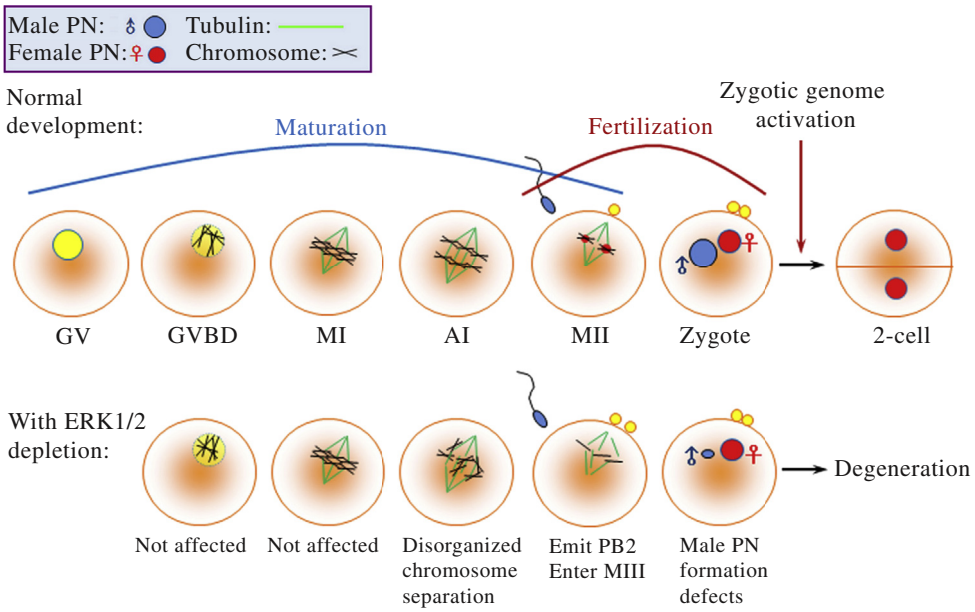

Fig. 6. A summary of ERK1/2 functions in mouse oocytes and zygotes.
In GV oocytes, endogenous ERK1/2 are not required for meiotic resumption (GVBD) and spindle assembly at MI. However, *Erk1/2*$^{oo-/-}$ oocytes showed abnormalities at AI: homologous chromosome migrations to the spindle poles were asynchronous, followed by delayed PB1 emission. Although *Erk1/2*$^{oo-/-}$ oocytes were able to develop to MII, the spindle formation and chromosome alignment were abnormal. These oocytes failed to be arrested at MII. They released the PB2s shortly after ovulation, and were arrested at another metaphase called "MIII". Furthermore, zygotes derived from ERK1/2-deleted oocytes showed defects in male pronucleus formation, indicating that ERK1/2 are required for efficient oocyte−embryo transition.

drops of M16 medium (Sigma) covered with mineral oil (Sigma) at 37°C in a 5% CO_2 atmosphere.

To harvest MII oocytes, mice were injected with PMSG and then hCG (Ningbo Sansheng Pharmaceutical) 44 h later. Oocytes were obtained by tearing the ampulla of oviducts at 14 h, 21 h and 32 h after hCG injection. Cumulus cells were removed by a brief exposure to 300 IU/mL of hyaluronidase (Sigma).

To collect fertilized eggs and early embryos, the superovulated females were mated with sexually mature WT males. Successful mating was confirmed the following morning by the presence of virginal plugs. At 18−20 h after hCG injection, zygotes were collected from the oviducts. The blastocysts were flushed from the uterus at 96 h after hCG treatment.

Immunofluorescent microscopy

Oocytes or zygotes were fixed in PBS-buffered 4% paraformaldehyde for 30 min at room temperature. After blocking with 1% BSA (Sangon Biotech, China) in PBS, oocytes were incubated overnight at 4°C with primary antibodies diluted in blocking solution. After washes in PBS, oocytes were labeled with secondary antibodies for 45 min, and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) or propidium iodide (PI) (Life Technologies, USA) for 10 min. Oocytes were mounted on glass slides using SlowFade® Gold Antifade Reagent (Life Technologies) and examined with a confocal laser scanning microscope (Carl Zeiss, Germany).

The primary antibodies used and dilution factors are listed as the followings: anti-5mC (#61226, Active Motif, USA, 1:500), anti-5hmC (#39769, Active Motif, 1:500), anti-H3K4me3 (#8580, Abcam, UK, 1:100), anti-H3K9me3 (#8898, Abcam, 1:100) and FITC-labeled anti-α-tubulin antibodies (Sigma, USA). The secondary antibody was Alexa Fluor 594-conjugated goat anti-rabbit IgG (Life Technology) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, USA).

Histological analysis

Ovaries were fixed overnight in 10% PBS-buffered formalin, dehydrated, embedded in paraffin, and serially sectioned at 5 μm thickness and stained with hematoxylin and eosin. The number of follicles per ovary was counted only when they contained oocytes with clearly visible nuclei.

Chromosome spreading and immunofluorescence

ZP-free oocytes were fixed in a solution containing 1% paraformaldehyde, 0.15% Triton X-100, and 3 mmol/L DTT (Sigma) on glass slides for 30 min and air dried. Immunofluorescent staining was performed as in oocytes described above. The primary antibodies used and dilution factors are anti-TOP2 (#3747-1, Epitomics, USA, 1:200) and anti-CREST antibodies (Fitzgerald Industries International, USA, 1:50).

Western blot analysis

Oocytes were lysed with SDS sample buffer (200 oocytes per sample) and heated for 5 min at 95°C. Total oocyte proteins were separated by SDS-PAGE and electrophoretically transferred to

PVDF membranes (Millipore, USA), followed by blocking in TBST containing 5% defatted milk (BD, USA) for 30 min. After probing with primary antibodies, the membranes were washed in TBST, incubated with a HRP-linked secondary antibody for 1 h, followed by washing with TBST three times. Bound antibodies were detected using SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher, USA).

The primary antibodies used and dilution factors are anti-ERK1/2 (Santa Cruz, USA, 1:1000) and anti-ACTIN antibodies (Cell Signaling, USA, 1:1000). The secondary antibody was HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories).

Statistical analysis

Results were given as means ± SDs. Each experiment included at least three independent samples and was repeated at least three times. Group comparisons were made by unpaired Student's two-tailed *t*-test. *P*-values of <0.05 were considered significant.

ACKNOWLEDGMENTS

This study was supported by the National Basic Research Program of China (Nos. 2011CB944504 and 2012CB944403) and the National Natural Science Foundation of China (Nos. 81172473 and 31371449).

REFERENCES

Barrett, C.B., Schroetke, R.M., Van der Hoorn, F.A., Nordeen, S.K., Maller, J.L., 1990. Ha-rasVal-12, Thr-59 activates S6 kinase and p34cdc2 kinase in *Xenopus* oocytes: evidence for c-mosxe-dependent and -independent pathways. Mol. Cell. Biol. 10, 310−315.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., Cobb, M.H., 2001. MAP kinases. Chem. Rev. 101, 2449−2476.

Choi, T., Fukasawa, K., Zhou, R., Tessarollo, L., Borror, K., Resau, J., Vande Woude, G.F., 1996. The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proc. Natl. Acad. Sci. USA 93, 7032−7035.

Colledge, W.H., Carlton, M.B., Udy, G.B., Evans, M.J., 1994. Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. Nature 370, 65−68.

Fan, H.Y., Liu, Z., Shimada, M., Sterneck, E., Johnson, P.F., Hedrick, S.M., Richards, J.S., 2009. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science 324, 938−941.

Fan, H.Y., Sun, Q.Y., 2004. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biol. Reprod. 70, 535−547.

Fan, H.Y., Tong, C., Lian, L., Li, S.W., Gao, W.X., Cheng, Y., Chen, D.Y., Schatten, H., Sun, Q.Y., 2003a. Characterization of ribosomal S6 protein kinase p90rsk during meiotic maturation and fertilization in pig oocytes: mitogen-activated protein kinase-associated activation and localization. Biol. Reprod. 68, 968−977.

Fan, H.Y., Tong, C., Teng, C.B., Lian, L., Li, S.W., Yang, Z.M., Chen, D.Y., Schatten, H., Sun, Q.Y., 2003b. Characterization of Polo-like kinase-1 in rat oocytes and early embryos implies its functional roles in the regulation of meiotic maturation, fertilization, and cleavage. Mol. Reprod. Dev. 65, 318−329.

Frank-Vaillant, M., Jessus, C., Ozon, R., Maller, J.L., Haccard, O., 1999. Two distinct mechanisms control the accumulation of cyclin B1 and mos in *Xenopus* oocytes in response to progesterone. Mol. Biol. Cell 10, 3279−3288.

Gu, T.P., Guo, F., Yang, H., Wu, H.P., Xu, G.F., Liu, W., Xie, Z.G., Shi, L., He, X., Jin, S.G., Iqbal, K., Shi, Y.G., Deng, Z., Szabo, P.E., Pfeifer, G.P., Li, J., Xu, G.L., 2011. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 477, 606−610.

Hashimoto, N., Watanabe, N., Furuta, Y., Tamemoto, H., Sagata, N., Yokoyama, M., Okazaki, K., Nagayoshi, M., Takedat, N., Ikawatll, Y., Aizawai, S., 1994. Parthenogenetic activation of oocytes in c-mos-deficient mice. Nature 370, 68−71.

Lan, Z.J., Xu, X., Cooney, A.J., 2004. Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. Biol. Reprod. 71, 1469−1474.

Liang, C.G., Su, Y.Q., Fan, H.Y., Schatten, H., Sun, Q.Y., 2007. Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. Mol. Endocrinol. 21, 2037−2055.

Reddy, P., Shen, L., Ren, C., Boman, K., Lundin, E., Ottander, U., Lindgren, P., Liu, Y.X., Sun, Q.Y., Liu, K., 2005. Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. Dev. Biol. 281, 160−170.

Roy, L.M., Haccard, O., Izumi, T., Lattes, B.G., Lewellyn, A.L., Maller, J.L., 1996. Mos proto-oncogene function during oocyte maturation in *Xenopus*. Oncogene 12, 2203−2211.

Schulze, A., Nicke, B., Warne, P.H., Tomlinson, S., Downward, J., 2004. The transcriptional response to raf activation is almost completely dependent on mitogen-activated protein kinase kinase activity and shows a major autocrine component. Mol. Biol. Cell 15, 3450−3463.

Su, Y.Q., Rubinstein, S., Luria, A., Lax, Y., Breitbart, H., 2001. Involvement of MEK-mitogen-activated protein kinase pathway in follicle-stimulating hormone-induced but not spontaneous meiotic resumption of mouse oocytes. Biol. Reprod. 65, 358−365.

Su, Y.Q., Wigglesworth, K., Pendola, F.L., O'Brien, M.J., Eppig, J.J., 2002. Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology 143, 2221−2232.

Tong, C., Fan, H.Y., Chen, D.Y., Song, X.F., Schatten, H., Sun, Q.Y., 2003. Effects of MEK inhibitor U0126 on meiotic progression in mouse oocytes: microtuble organization, asymmetric division and metaphase II arrest. Cell Res. 13, 375−383.

Verlhac, M.H., de Pennart, H., Maro, B., Cobb, M.H., Clarke, H.J., 1993. MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. Dev. Biol. 158, 330−340.

Verlhac, M.H., Kubiak, J.Z., Clarke, H.J., Maro, B., 1994. Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development 120, 1017−1025.

Verlhac, M.H., Kubiak, J.Z., Weber, M., Geraud, G., Colledge, W.H., Evans, M.J., Maro, B., 1996. Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. Development 122, 815−822.

Yu, C., Zhang, Y.L., Pan, W.W., Li, X.M., Wang, Z.W., Ge, Z.J., Zhou, J.J., Cang, Y., Tong, C., Sun, Q.Y., Fan, H.Y., 2013. CRL4 complex regulates mammalian oocyte survival and reprogramming by activation of TET proteins. Science 342, 1518−1521.

Zheng, L.P., Huang, J., Zhang, D.L., Xu, L.Q., Li, F., Wu, L., Liu, Z.Y., Zheng, Y.H., 2012. c-erbB2 and c-myb induce mouse oocyte maturation involving activation of maturation promoting factor. DNA Cell Biol. 31, 164−170.