Mastl is required for timely activation of APC/C in meiosis I and Cdk1 reactivation in meiosis II

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In mitosis, the Greatwall kinase (called microtubule-associated serine/threonine kinase like [Mastl] in mammals) is essential for prometaphase entry or progression by suppressing protein phosphatase 2A (PP2A) activity. PP2A suppression in turn leads to high levels of Cdk1 substrate phosphorylation. We have used a mouse model with an oocyte-specific deletion of Mastl to show that Mastl-null oocytes resume meiosis I and reach metaphase I normally but that the onset and completion of anaphase I are delayed. Moreover, after the completion of meiosis I, Mastl-null oocytes failed to enter meiosis II (MII) because they reassembled a nuclear structure containing decondensed chromatin. Our results show that Mastl is required for the timely activation of anaphase-promoting complex/cyclosome to allow meiosis I exit and for the rapid rise of Cdk1 activity that is needed for the entry into MII in mouse oocytes.

Introduction

Meiotic maturation of mammalian oocytes consists of two consecutive M phases, meiosis I and meiosis II (MII), without an intervening S phase. As in mitosis, entry into both of the meiotic M phases is driven by the activity of Cdk1 in association with cyclin B (Jones, 2004). The resumption of oocyte meiosis I, as characterized by germinal vesicle (GV) breakdown (GVBD), is considered to be equivalent to the mitotic G2/M transition (Eppig et al., 2004; Jones, 2004).

After GVBD in mouse oocytes, Cdk1 activity increases gradually during the lengthy prometaphase of meiosis I (Polanski et al., 1998; Kitajima et al., 2011; Davydenko et al., 2013). Once metaphase I is reached, a transient decrease in Cdk1 activity leads to the extrusion of the first polar body (PB1) as a result of the degradation of cyclin B1. This degradation is mediated by the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) that is loaded with its targeting subunit Cdc20. In mitosis, APC/C activity is regulated by the modulation of Cdc20 through phosphorylation, degradation, or the direct binding to inhibitory proteins (Pesin and Orr-Weaver, 2008). The regulation of APC/C activity in mammalian meiosis remains poorly understood, but recent findings show that mitogen-activated protein kinase and Cdk1 are involved in the regulation of APC/C during meiosis I progression in mouse oocytes (Nabti et al., 2014). Once anaphase I is completed, Cdk1 activity is up-regulated rapidly, and this is essential for entry into MII. The elevated Cdk1 activity peaks at metaphase II (MetII) and remains high until fertilization (Kubiak et al., 1992).

Recent studies have indicated that an increase in Cdk1 activity alone is not sufficient for mitotic progression and that a simultaneous suppression of antagonizing protein phosphatase activity is also required (Virshup and Kaldis, 2010). Greatwall (Gwl) kinase or its mammalian orthologue microtubule-associated serine/threonine kinase like (Mastl) is the key regulator that suppresses protein phosphatase activity and thus plays an important role in mitotic progression in all model systems tested so far, including Drosophila melanogaster, Xenopus laevis egg.

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Supplemental Material can be found at: /content/suppl/2014/09/22/jcb.201406033.DC1.html
Results and discussion

Infertility of OoMastl−/− mice and delayed onset of anaphase I in oocytes

We first investigated the expression pattern of the Mastl protein during oocyte maturation and transition into embryos. As shown in Fig. S1 A, Mastl was expressed in mouse oocytes throughout the maturation process and in early embryos. Notably, Mastl protein levels were elevated in the oocytes at MetII, and Mastl exhibited slower migration (Fig. S1 A) as a result of its phosphorylation status in prometaphase I and MetII oocytes (Fig. S1 B). Immunofluorescence microscopy indicated that Mastl was localized in the GV before meiotic resumption but was found throughout the ooplasm after GVBD and in MetII oocytes (Fig. S1 C).

To determine the functions of Mastl during oocyte maturation, we generated a mouse model in which Mastl was deleted specifically in oocytes. Our results demonstrate that although Mastl is not required for the resumption of meiosis and progression to metaphase I, it plays an important role in the timely activation of APC/C at the end of meiosis I. Moreover, Mastl is indispensable for the rapid Cdk1 reactivation that is essential for MII entry.

Infertility of OoMastl−/− females was found to be infertile (Fig. S2 A), but the ovarian development and ovulation of OoMastl−/− females were normal (not depicted). The requirement of Mastl/Gwl during the entry into meiosis I in Xenopus oocytes (Dupré et al., 2013), the entry and progression of meiosis I in Drosophila (Kim et al., 2012; Von Stetina et al., 2008; Kim et al., 2012) and porcine oocytes (Li et al., 2013), and the progression of meiosis in starfish oocytes (Okumura et al., 2014) led us to assume that the Mastl-deficient oocytes would fail to progress through meiosis I. However, we found that in vitro–cultured OoMastl−/− oocytes underwent GVBD with kinetics and efficiencies that were indistinguishable from those of OoMastl+/+ oocytes (Fig. 1 A). OoMastl−/− oocytes progressed to metaphase I with chromosome condensation and spindle formation comparable with those of OoMastl+/+ oocytes (Fig. 1 B, Fig. S2 B, and Videos 1 and 2). The spindles migrated toward the cortex normally in Mastl-deficient oocytes (Fig. 2 D, right; and Video 2). These results suggest that Mastl has no essential role during meiotic resumption or prometaphase I progression in mouse oocytes.

In OoMastl+/+ oocytes, PB1 extrusion (PBE), an event marking the completion of meiosis I, had occurred by 12 h after GVBD (n = 60) in vitro. In contrast, only ~60% of OoMastl−/− oocytes extruded PB1 at 16 h after GVBD (n = 57; Fig. 1 C). Live cell imaging confirmed normal metaphase I entry but delayed PBE in OoMastl−/− oocytes (Fig. 2 D, right; and Video 2). However, it is worth noting that the in vivo PBE rate of ovulated OoMastl−/− oocytes (97.03%, n = 236) was comparable with OoMastl+/+ oocytes (97.77%, n = 90) when collected at 16 h after human chorionic gonadotropin (hCG) treatment (Fig. 1 D). Thus, OoMastl−/− oocytes are capable of completing meiosis I after a delay. It is possible that oocytes complete meiosis I faster under more optimal in vivo conditions than those in more challenging in vitro cultures. Thus the differences in the timing of completing meiosis I between the control and mutant oocytes might be missed in vivo.

To study whether the delayed PBE rate in OoMastl−/− oocytes is caused by a delayed activation of APC/C during meiosis I, we monitored the timing of endogenous securin degradation, which mirrors APC/C activity during oocyte maturation (McGuinness et al., 2009). We found that in OoMastl+/+ oocytes, securin levels had already decreased at 9 h after GVBD (Fig. 1 E). In contrast, the securin level in OoMastl−/− oocytes still remained high at 9 h after GVBD but was decreased at 14 h after GVBD, suggesting a delayed degradation of securin in OoMastl−/− oocytes (Fig. 1 E). Thus, Mastl is required for the timely activation of APC/C that is needed for the completion of meiosis I in mouse oocytes.

We checked whether the suppressed APC/C activity in the OoMastl−/− oocytes was caused by spindle assembly checkpoint (SAC) activation because of possible subtle defects in spindle and/or chromosome alignment. As an indicator of the SAC status, we examined the localization of the checkpoint protein Mad2, which binds to unattached kinetochores but is released upon the formation of stable microtubule–kinetochore attachments at metaphase I (Wassmann et al., 2003). As shown...
Figure 1. Meiotic maturation of OoMastl<sup>−/−</sup> oocytes. (A) Comparison of the kinetics of GVBD after release from dbcAMP. The total numbers of oocytes used (n) are indicated. (B) Representative images of immunostaining for DNA, CREST, and spindle showing normal progression to metaphase I in OoMastl<sup>−/−</sup> oocytes. Oocytes were cultured for the indicated periods after GVBD and were fixed. 30 oocytes were analyzed for each time point. (C) Kinetics of PBE. Oocytes that had undergone GVBD within 2 h after release into dbcAMP-free M16 medium were selected (at time = 0) and cultured further. PBE was scored at 1-h intervals. The numbers of oocytes examined are indicated (n). (D) Comparable PBE rates of the ovulated oocytes collected at 16 h after hCG. The numbers of oocytes used (n) are shown. All of the experiments were repeated at least three times, and representative results are shown. (E) Western blots showing the timing of securin degradation and Cdc20 level during oocyte maturation. Lysate from 100 oocytes was loaded in each lane. The levels of β-actin were used as a control. The same β-actin panel is presented again in Fig. 3B, which shows the expression of other proteins of interest in the same cell lysates.

In Fig. S2 C, we detected Mad2 staining in virtually every kinetochore in both control and mutant oocytes at prometaphase I (3 h after GVBD). Subsequently, Mad2 was completely dissociated from kinetochores of OoMastl<sup>−/−</sup> oocytes at metaphase I (7 h after GVBD) when stable kinetochore–microtubule attachments were established, and this was similar to the OoMastl<sup>+/+</sup> oocytes (Fig. S2 C). Therefore, we propose that the activation of SAC in Mastl-null oocytes occurs normally as in the wild-type oocytes.
B [“PB1” and “N” in insets] and C). This was in sharp contrast to the typical bipolar MetII spindles and aligned chromosomes seen in the OoMastl+/+ oocytes (Fig. 2 A, inset). In 33% (n = 229) of the OoMastl−/− oocytes, residual central spindle microtubules were still visible between the decondensed chromatin in the PB1 and the nucleus (Fig. 2 B, left inset, ovulated oocytes 16 h after hCG). Moreover, live cell imaging revealed that after PBE, OoMastl−/− oocytes decondensed their chromatin and did not reform the MetII bipolar spindle (Fig. 2 D and Video 2). These results suggest that Mastl is essential for MII entry. The OoMastl−/− oocytes subsequently entered S phase after PBE (Fig. S2 D), and within 24 h of in vitro culture, most of the OoMastl−/− oocytes were cleaved into two cells and subsequently degenerated (not depicted).

OoMastl−/− oocytes up-regulate Cdk1 activity normally during prometaphase I but fail to increase Cdk1 activity for the entry into MII

To investigate the activity of Cdk1 in OoMastl−/− oocytes throughout the maturation process, we performed in vitro kinase

In addition, we found that in contrast to OoMastl+/+ oocytes in which the level of Cdc20 was increased at 9 h after GVBD when the activity of APC/C is elevated, the level of Cdc20 in OoMastl−/− oocytes remained low at this time (Fig. 1 E). A previous study has shown that PBE is delayed when the amount of Cdc20 protein is limited in mouse oocytes (Jin et al., 2010), and this appears to be a similar phenomenon to what we observed in the Mastl-null oocytes. Thus, we hypothesize that Mastl might regulate APC/C activity directly during meiosis I by regulating the levels of Cdc20. Because the regulation of the multisubunit complex of APC/C in mouse oocytes is far from being completely understood, however, the possibility of other regulatory mechanisms being involved cannot be discounted.

OoMastl−/− oocytes cannot enter MII

Although OoMastl−/− oocytes completed meiosis I in vivo with normal cytokinesis (Fig. 1 D) and extruded morphologically normal PB1s (Fig. 2 B), confocal microscopy revealed that after PBE, OoMastl−/− oocytes contained distinct nuclei with decondensed chromatin in both the oocytes and the PB1 (Fig. 2, B [“PB1” and “N” in insets] and C). This was in sharp contrast to the typical bipolar MetII spindles and aligned chromosomes seen in the OoMastl+/+ oocytes (Fig. 2 A, inset). In 33% (n = 229) of the OoMastl−/− oocytes, residual central spindle microtubules were still visible between the decondensed chromatin in the PB1 and the nucleus (Fig. 2 B, left inset, ovulated oocytes 16 h after hCG). Moreover, live cell imaging revealed that after PBE, OoMastl−/− oocytes decondensed their chromatin and did not reform the MetII bipolar spindle (Fig. 2 D and Video 2). These results suggest that Mastl is essential for MII entry. The OoMastl−/− oocytes subsequently entered S phase after PBE (Fig. S2 D), and within 24 h of in vitro culture, most of the OoMastl−/− oocytes were cleaved into two cells and subsequently degenerated (not depicted).
which is caused by cyclin B1 degradation mediated by APC/C as has been previously (Madgwick et al., 2006; Jin et al., 2010). In contrast, the Cdk1 activity in Oo
Mastl
−/−

MII entry in oocytes remains high at this point (Fig. 3 A), which is likely caused by low APC/C activity (as shown in Fig. 1 E) in Oo
Mastl
−/−
oocytes. This result suggests that Mastl plays an essential role in triggering the activation of APC/C that is required for the down-regulation of Cdk1 activity and that this mediates the timely onset of anaphase I in mouse oocytes.

The activation of Cdk1 that is required for the entry into MII and the following arrest at MetII (Kubiak et al., 1992) was observed in Oo
Mastl+/+
oocytes at 14 h after GVBD and after further culture for up to 20 h (Fig. 3 A, top right). However, the Cdk1 activity in Oo
Mastl
−/−
oocytes failed to increase after PBE and remained at low levels throughout the extended culture period (Fig. 3 A, bottom right). This indicates that in the absence of Mastl, the rapid elevation in Cdk1 activity, which is needed for MII entry, was completely inhibited. As another indicator of Cdk1 activity, the level of phosphorylation of lamin A/C, an endogenous Cdk1 substrate (Peter et al., 1990; Haas and Jost, 1993), was decreased after PBE in Oo
Mastl
−/−
oocytes (Fig. 3 D, ovulated oocytes), which might directly trigger the reformation of the nuclear membrane in the mutant oocytes (Fig. 2 B).

We measured PP2A activity in the oocytes (as described in Materials and methods) and found that the PP2A activities in both Oo
Mastl+/+
and Oo
Mastl−/−
oocytes were comparable at the GV and GVBD stages (Fig. S3 A). Thus, our results suggest that Mastl-deficient oocytes are capable of phosphorylating Cdk1 substrates through normal up-regulation of Cdk1 activity; thus, the progression through prometaphase I in the Oo
Mastl−/−
oocytes appears to be normal.

Nevertheless, Cdk1 activity in Oo
Mastl+/+
oocytes sharply decreased at the time of anaphase I onset (9 h after GVBD), which is caused by cyclin B1 degradation mediated by APC/C as has been shown previously (Madgwick et al., 2006; Jin et al., 2010). In contrast, the Cdk1 activity in Oo
Mastl−/−
oocytes cannot be increased after PBE (14 h) in Oo
Mastl−/−
oocytes (bottom right). [B] Western blots showing the dynamics of p-Cdk1 (Y15) during oocyte maturation. Lysate from 100 oocytes was loaded in each lane. The levels of β-actin and Cdk1 were used as controls. The same β-actin panel is presented in Fig. 1 E, which shows the expression of other proteins of interest in the same cell lysates. [C] Comparable PP1 phosphorylation (indicating inhibition) at GVBD in Oo
Mastl−/−
and Oo
Mastl+/+
oocytes. [D] Expression of Mastl, cyclin B1, p-Cdk1 (Y15), Cdk1, phospho-lamin A/C (S22), and lamin A/C in ovulated oocytes. β-Actin was used as the loading control. Lysate from 100 oocytes was loaded in each lane.

assays. From the GV stage to prometaphase I (3 h after GVBD) and metaphase I (6 h after GVBD) stages, both Oo
Mastl+/+
and Oo
Mastl−/−
oocytes displayed similar increases in Cdk1 activity (Fig. 3 A, left of the dashed line). Consistent with the similar levels of Cdk1 activity, both Oo
Mastl+/+
and Oo
Mastl−/−
oocytes displayed similar levels of inhibitory phosphorylation on Cdk1 (Y15) at the GV stage and subsequent dephosphorylation after GVBD (Fig. 3 B). We also found that during prometaphase I the endogenous Cdk1 substrate, PP1, was phosphorylated on residue T320 to a similar degree in both Oo
Mastl+/+
and Oo
Mastl−/−
oocytes (Fig. 3 C). These results demonstrate that Mastl-deficient oocytes are capable of phosphorylating Cdk1 substrates through normal up-regulation of Cdk1 activity; thus, the progression through prometaphase I in the Oo
Mastl−/−
oocytes appears to be normal.

We measured PP2A activity in the oocytes (as described in Materials and methods) and found that the PP2A activities in both Oo
Mastl+/+
and Oo
Mastl−/−
oocytes were comparable at the GV and GVBD stages (Fig. S3 A). Thus, our results suggest that mouse oocytes progress through prometaphase I without suppressing PP2A activity and, therefore, that Mastl regulation of PP2A does not play a major role during prometaphase I.

Nevertheless, Cdk1 activity in Oo
Mastl+/+
oocytes sharply decreased at the time of anaphase I onset (9 h after GVBD),
To determine the reasons of the low Cdk1 activity in OoMastl−/− oocytes after PBE, we measured the levels of cyclin B1. In fact, the levels of cyclin B1 in OoMastl−/− oocytes were higher compared with OoMastl+/+ oocytes (Fig. 3 D, ovulated oocytes), and this seems to be the result of low APC/C activity in OoMastl−/− oocytes (Fig. 1 E). However, the inhibitory phosphorylation of Cdk1 at Y15 was significantly increased in OoMastl−/− oocytes after PBE in both in vitro–cultured oocytes (Fig. 3 B, 14 h after GVBD) and in ovulated oocytes (Fig. 3 D). The increased inhibitory phosphorylation of Cdk1 in OoMastl−/− oocytes is most likely the reason for the low activity of Cdk1 after PBE (Fig. 3 A).

Collectively, our results indicate that Mastl depletion in mouse oocytes results in Cdk1 inactivation through its inhibitory phosphorylation at MII entry, but not during meiosis I. We propose that during MII entry, the suppression of PP2A activity by Mastl might prevent the dephosphorylation of Wee1B at its inhibitory sites and prevent the dephosphorylation of Cdc25B at its activating sites (Pal et al., 2008; Vigneron et al., 2009), both of which promote the activation of Cdk1.

To investigate whether the suppressed Cdk1 activity and failure of MII entry in OoMastl−/− oocytes resulted from elevated PP2A activity, we measured PP2A activity in oocytes immediately after PBE. We found that PP2A activity in OoMastl+/+ oocytes was very low at this point, but PP2A activity in the OoMastl−/− oocytes was about nine times higher compared with OoMastl+/+ oocytes (Fig. 3 B). These results indicate that the deletion of Mastl in mouse oocytes results in a dramatic elevation in PP2A activity specifically after the completion of meiosis I, and this prevents the entry into MII. Based on these findings, we suggest that Mastl-mediated suppression of PP2A plays an essential role in the rapid activation of Cdk1 and the phosphorylation of Cdk1 substrates required for MII entry. However, Mastl-mediated suppression of PP2A is not required for the entry and progression of meiosis I in mouse oocytes. At the moment, the identity of the specific PP2A isoform that is regulated by Mastl in mouse oocytes remains unclear because of the lack of specific antibodies against the B55 subunits. It would also be of interest to measure the PP2A activity using a known physiological substrate of Cdk1 during mouse oocyte maturation.

**Cdk1 activation and PP2A inhibition drive OoMastl−/− oocytes into MII**

We tested whether pharmacological activation of Cdk1 or inhibition of PP2A could lead to MII entry in OoMastl−/− oocytes. Treatment with the Wee1 inhibitor PD166285 (Li et al., 2002; Hashimoto et al., 2006) led to reduced levels of Cdk1 (Y15) phosphorylation and dramatically increased H1 kinase activity in OoMastl−/− oocytes (Fig. 4 A). This resulted in the breakdown of the nuclear membranes and chromosome condensation in all of the oocytes (n = 33), and 73% of the oocytes formed bipolar MII spindles (Fig. 4, B and C; and Fig. S3 E). Because PP2A activity is higher in OoMastl−/− oocytes after PBE (Fig. S3 B), we also treated the OoMastl−/− oocytes after PBE with the protein phosphatase inhibitor okadaic acid (OA; 50 nM; Cohen et al., 1989; Chang et al., 2011; Tay et al., 2012). Inhibition of phosphatases resulted in 42% (n = 121) of the OoMastl−/− oocytes forming bipolar spindles with aligned chromosomes (Fig. 4, B and C; and Fig. S3 E). Oocytes that failed to form MII spindles after culture with OA still contained nuclei (Fig. S3 E, OA). The OA treatment also led to an increase in Cdk1 activity and decreased Cdk1 phosphorylation on residue Y15 (Fig. 4 A). Finally, we found that the combination of both inhibitors led to the breakdown of the nuclear membrane in all the oocytes and had a moderate additive effect, with 86% of the OoMastl−/− oocytes forming bipolar spindles with aligned chromosomes (Fig. 4, B and C). This suggested that both Cdk1 and PP2A are involved in an interdependent manner during MII entry.
Ensa is absent in OoMastl/−/− oocytes. We measured Ensa and Arpp19 protein levels in oocytes after PBE to investigate their involvement in the failure of OoMastl/−/− oocytes to enter MII. Similar levels of Arpp19 were expressed in OoMastl/−/− and OoMastl+/− oocytes, but the Ensa protein could not be detected in OoMastl/−/− oocytes (Fig. 5 A and Fig. S3 C) despite comparable levels of Ensa mRNA (Fig. 5 B). We found that the Ensa protein levels were increased after GVBD in OoMastl+/− oocytes but Ensa was absent in OoMastl/−/− oocytes (Fig. S3 C). A previous study in Xenopus egg extracts suggested that Mastl phosphorlylates and activates Ensa, leading to PP2A inhibition (Mochida et al., 2010), but our data indicate the possibility that the translation or stability of Ensa is regulated by Mastl in mouse oocytes. We hypothesize that Mastl might be essential for the stability of Ensa through its phosphorylation.

Recent studies have shown that Mastl/Gwl kinase is essential for the initiation and progression of prometaphase during mitosis by suppressing the antagonizing activity of PP2A (Gharbi-Ayachi et al., 2010; Mochida et al., 2010; Domingo-Sananes et al., 2011; Mochida and Hunt, 2012; Álvarez-Fernández et al., 2013). In our study, however, we found that the Mastl-deficient oocytes entered meiosis I with normal timing and efficiency and progressed through prometaphase and metaphase I normally. These oocytes completed meiosis I with a delay in PBE as the result of insufficient APC/C activation. Moreover, we demonstrated that the Mastl-deficient oocytes failed to proceed to MII and instead entered interphase, which was caused by a failure to increase Cdk1 activity and was associated with an increase in PP2A activity.

Our results suggest that in mammalian oocytes Mastl activity is essential for entry into MII. In contrast, Mastl is not required for meiosis I entry but becomes important at the end of meiosis I for the activation of APC/C. Because PP2A has been shown to inhibit APC/C-dependent degradation of securin in HeLa cells (Hellmuth et al., 2014), it might be possible that increased PP2A activity in Mastl-null oocytes inhibits APC/C and delays the onset of anaphase I and PBE. Previous experiments have shown that the dual-specificity phosphatase Cdc14B negatively regulates the resumption and progression of meiosis in an APC/C-dependent manner (Schindler and Schultz, 2009). In the absence of Mastl, Cdc14B might act to counteract Cdk1 and thereby inhibit the transition from prophase to M phase.

The prometaphase of oocyte meiosis I is a lengthy process that takes around 6–8 h in mice (Polanski et al., 1998). This is in sharp contrast to the <30 min required for prometaphase in mitotic cells (Rieder et al., 1994). Our findings indicate that progression to metaphase I in mouse oocytes does not require Mastl-mediated PP2A suppression. Our data raise the possibility that the sustained PP2A activity causes the slow increase in Cdk1 activity and at the same time dephosphorylates Cdk1 substrates, which slows the progression of meiosis I.

In summary, Mastl is required in mouse oocytes for the timely activation of APC/C at the exit of meiosis I and is essential for the reactivation of Cdk1 needed for entry into MII. This study indicates a switch in the mechanisms that regulate Cdk1 activity between the two meiotic divisions in mouse oocytes and provides the starting point for a more detailed exploration of the mechanisms behind Mastl’s role in mammalian meiosis.

Materials and methods

Generation of Mastl conditional knockout mice

The MastlFlox mouse strain was generated essentially as described previously for Cdk1Flox mice (Diril et al., 2012). Mouse genomic DNA harboring the Mastl locus was isolated from the BAC clone pBAC6.6.P23-280K (Invitrogen). Using recombinase techniques (Lee et al., 2001), the loxP recombination sites and a neomycin-selection cassette were introduced flanking the fourth coding exon of the mouse Mastl genomic locus. The 5′ homology arm comprised 3,690 bp (Mus musculus strain C57BL/6) GRCm38.p2 assembly, Chr2:23,149,361-23,145,671), whereas the 3′ homology arm included 5,899 bp (23,138,962-23,144,861). The resulting targeting vector [PKB926] was linearized by NotI digestion, and embryonic stem (ES) cells were electroporated. After positive and negative selection with Geneticin and ganciclovir, respectively, genomic DNA of surviving ES cell colonies was screened for homologous recombination by Southern hybridization using 5′ and 3′ probes (M. musculus strain C57BL/6) GRCm38.p2 assembly, Chr2:23,133,493-23,131,502, 150,719-150,726). PCR genotyping primers sequences can be found in Table S1. After multiple rounds of crossing, their oocytes (OoMastl+/−) were electroporated with free access to water and food. Illumination was on between 6 am and 6 pm. Experimental protocols were approved by the regional ethical committee of the University of Gothenburg, Sweden, and by the Institutional Animal Care and Use Committee at the Biological Research Centre mouse facility at Biopolis, Singapore.

Figure 5. Absence of Ensa protein expression in OoMastl/−/− oocytes. (A) Expression of Mastl, Ensa, and Arpp19 in ovulated OoMastl+/− and OoMastl/−/− oocytes. Lysate from 100 oocytes was loaded in each lane. (B) Relative mRNA levels of Ensa and Arpp19 in ovulated oocytes were determined by real-time PCR. The mRNA levels in the OoMastl+/− oocytes were set as 1.0, and the fold changes in OoMastl/−/− oocytes are shown. Error bars represent SDs.
Reagents, antibodies, and immunological detection methods
Rabbit pAbs against Mastl were raised using an N-terminal 6-His-tagged peptide fragment from the mouse Mastl protein (residues 461–694, PBK9008) as the antigen using a published protocol (Berthet et al., 2003). Rabbit pAbs against Arpp19 were raised using an N-terminal GST-tagged fusion protein of mouse Arpp19 (residues 25–112, PBK1506) as the antigen using a published protocol (Berthet et al., 2003). Mouse mAbs against Securin (jasmin, M2 and M6 media, diubitylcyCLC–dbCAMP), pregnant mouse serum gonadotropin (PMSG), HCG, hyaluronidase, DAPI, mouse monoclonal anti–a-tubulin–FITC, and mineral oil were purchased from Sigma-Aldrich. OA and PD166285 were obtained from Abcam. Human pAbs against (Thr320), phospho–lamin A/C (Ser22), lamin A/C, cyclin securin and rabbit pAb against Cdc20 were from Abcam. Mouse mAbs against α-tubulin–FITC, and mineral oil were purchased from Cell Signaling Technology. Mouse mAbs against β-tubulin–FITC, and mineral oil were purchased from Santa-Cruz Biotechnology, Inc. Rabbit pAbs against ENSA, phospho-PY1 (Thr320), phospho–lamin A/C (Ser22), lamin A/C, cyclin securin and rabbit anti-Mad2 antibody was obtained from Covance. Mouse mAbs against Cdk1 and HA were purchased from Santa-Cruz Biotechnology, Inc. Rabbit pAbs against Ensa, phospho-PY1 (Thr320), phospho–lamin A/C (Ser22), lamin A/C, cyclin securin and rabbit anti-Mad2 antibody was obtained from Covance. Mouse mAbs against Cdk1 and HA were purchased from Selleck Chemicals. Western blots were performed according to the instructions of the suppliers of the different antibodies and visualized using the ECL Prime Western Blotting Detection System (GE Healthcare). Alkaline phosphatase treatment of the different antibodies and visualized using the ECL Prime Western Blot-ern blots were performed according to the instructions of the suppliers of the different antibodies and visualized using the ECL Prime Western Blotting Detection System (GE Healthcare).

Immunofluorescence and confocal microscopy
To determine the localization of Mastl protein in mouse oocytes, wild-type oocytes at GV, GVBD, and MetII stages were fixed in 4% PFA. After the fixed oocytes were permeabilized with 0.5% Triton X-100 at RT for 20 min, they were blocked with 1% BSA-supplemented PBS for 1 h and incubated at 4°C with Mouse anti-α-tubulin (Roche) overnight, followed by an incubation with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) for 1 h at RT. OoMastl+/− oocytes were used as negative controls.

Fluorescence live imaging of oocyte maturation
Capped RNAs were synthesized in vitro with the 17 mMessage mMachine kit (Ambion) using linearized plasmids provided by M. Anger (Veterinary Research Institute in Brno, Brno, Czech Republic) and purified with the RNAeasy Mini kit (QiAGEN). RNAs prepared at 150 ng/µl and 350 ng/µl for H2B-mCherry (for DNA visualization) and Map7-EGFP (for spindle visualization, respectively), were mixed in a 1:1 ratio and injected with an IM-6 (Narishige) into GV-stage oocytes placed in M2 medium supplemented with 0.2 mM 3-isobutyl-1-methylexanthine (IBMX; Sigma-Aldrich) at RT. Injected oocytes were cultured in M16 medium for 6 h in an incubator at 37°C and 5% CO2. Time-lapse live epifluorescence imaging was performed with a DML6000 B (Leica) equipped with an HCX PL FLUOTAR 60×/0.8 objective with the following band pass emission filters (in nm): 385–470 (DAPI), 505–530 (Alexa Fluor 488 and FITC), and 585–615 (Alexa Fluor 594). Z sections were analyzed and projected into one picture using the LSM image browser (Carl Zeiss). The images were then assembled by Illustrator CS2 (Adobe).

Histone H1 kinase assay
Histone H1 kinase activity assays were performed using five oocytes in a 10-µl reaction volume based on a published protocol (Kudo et al., 2006). In brief, the reaction was performed in a buffer containing 25 mM HEPES, pH 7.4, 15 mM MgCl2, 20 mM EGTA, 0.05% NP-40, 0.1 mM ATP, 100 mM PMSF, 1 mM DTG, 1 µg histone H1 (Roche), and 3 µCi γ[32P] ATP (PerkinElmer) and incubated at 37°C for 30 min. Samples were boiled in SDS sample buffer and then separated on a 15% SDS–polyacrylamide gel. The radioactive signal was exposed by placing the gels on an FLA 3000 phosphorimager (Fujiﬁlm).

PP2A activity assay
PP2A activity was measured with the PP2A Immunoprecipitation Phospho- assay Kit (EMD Millipore). In brief, PP2A was immunoprecipitated from lysates of oocytes (400 oocytes for each time point) in lysis buffer containing 20 mM imidazole–HCl, pH 7.0, 2 mM EDTA, 2 mM EGTA, 0.1% NP-40, and freshly added protease inhibitors (Roche). The PP2A protein was pull-down using anti-PP2A antibodies (clone 1D6, EMD Millipore).
lysates were prepared in EBN buffer (80 mM (PKB1483) plasmids were transfected into HEK293 cells using Lipofectamine Arpp19 proteins, siRNA, and pDNA vectors, and the sequences were verified. For expressing the Ensa and HA tags by PCR, the cDNAs were cloned into pBOBI mammalian expression vectors (EMD Millipore) for 20 min with constant shaking at 8°C. For 10 min. After neutralization for 10 min in 100 mM Tris-HCl, pH 8.0, and a second fixation with 4% PFA for 20 min at RT, oocytes were blocked overnight at 4°C with 1% BSA-supplemented PBS. Oocytes were labeled with Bdz mouse mAb (clone MoBu1-1; 1:200 dilution) and Alexa Fluor 488 conjugate (Life Technologies) for 2 h at RT. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the oocytes were costained with DAPI (1 µg/ml in PBS). Finally, the oocytes were mounted on glass slides with DABCO-containing mounting medium and examined by laser-scanning confocal microscopy (iZM 700 Inverted).

Real-time quantitative PCR

The reactions were performed in triplicate. Threshold cycle (Ct) values were applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems). The reactions were performed using primers 5'-CCAGCGAGGAGGCC-3' and 5'-AGTGGCTTGTGCTGCTGC-3' and for Arpp19 using primers 5'-CCGCAACCTTTGAGGGAAAG-3' and 5'-CTTCCAGGCTTTGCTCCAA-3'. An ABI 7500 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems). The reactions were performed in triplicate. Threshold cycle (Ct) values were obtained, and the ΔΔCt method was used to calculate the fold changes. All of the values were normalized to Gapdh.

Overexpression of HA-Ensa and HA-Arp19 proteins in HEK293 cells

Mouse Ensa and Arpp19 cDNAs were amplified by RTPCR from RNA extracted from mouse embryonic fibroblasts. After the introduction of N-terminal HA tags by PCR, the cDNAs were cloned into pBnBI mammalian expression vectors and the sequences were verified. For expressing the Ensa and Arpp19 proteins, pBnBI-HA-Ensa (PKB1481) and pBnBI/HA-Arp19 (PKB1483) plasmids were transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells lysates were prepared in EBN buffer (80 mM β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM MgCl2, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, and protease inhibitors [20 µg/ml each of leupeptin, chymostatin, and pepstatin; E18, E16, and E10; EMD Millipore]) for 20 min with constant shaking at 1,200 rpm. Lysates were centrifuged for 30 min at 18,000 g at 4°C, and supernatants were snap-frozen in liquid nitrogen and stored at −80°C.

Statistical analysis

All experiments were repeated at least three times. For comparisons between groups, the differences were calculated with Student’s t test, and a difference was considered to be significant if P < 0.05.

Online supplemental material

Fig. S1 shows the phosphorylation and localization of Mastl in mouse oocytes and generation of Mastl conditional knockout mice. Fig. S2 shows that although the Mastl conditional knockout females are infertile, the spindle, chromosome condensation, and SAC activation in the oocyte are normal and Mastl-null oocytes enter S phase after PBE. Fig. S3 shows the PP2A activities and Ensa protein levels during oocyte maturation. Table S1 lists PCG genotyping primers. Video 1 shows the normal MI entry in an OoMastl−/− oocyte. Video 2 shows the failure of MI entry in an OoMastl−/−/− oocyte by live imaging. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.2014060033.DC1.

We are thankful to Eileen Souton and Susan Reid (National Cancer Institute—Frederick, Frederick, MD) for help in generating the Mastl−/− mouse and to the staff at the University of Gothenburg core facility for cellular imaging for assistance with confocal microscopy. The authors declare no competing financial interests.

Submitted: 9 June 2014
Accepted: 25 August 2014

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