Mouse Emi2 as a distinctive regulatory hub in second meiotic metaphase

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SUMMARY
The oocytes of vertebrates are typically arrested at metaphase II (mII) by the cytostatic factor Emi2 until fertilization. Regulatory mechanisms in *Xenopus* Emi2 (xEmi2) are understood in detail but contrastingly little is known about the corresponding mechanisms in mammals. Here, we analyze Emi2 and its regulatory neighbours at the molecular level in intact mouse oocytes. Emi2, but not xEmi2, exhibited nuclear targeting. Unlike xEmi2, separable N- and C-terminal domains of mouse Emi2 modulated metaphase establishment and maintenance, respectively, through indirect and direct mechanisms. The C-terminal activity was mapped to the potential phosphorylation target TxSxS, a destruction box (D-box), a lattice of Zn²⁺-coordinating residues and an RL domain. The minimal region of Emi2 required for its cytostatic activity was mapped to a region containing these motifs, from residue 491 to the C terminus. The cytostatic factor Mos-MAPK promoted Emi2-dependent metaphase establishment, but Mos autonomously disappeared from meiotically competent mII oocytes. The N-terminal Plx1-interacting phosphodegron of xEmi2 was apparently shifted to within a minimal fragment (residues 51-300) of mouse Emi2 that also contained a calmodulin kinase II (CaMKII) phosphorylation motif and which was efficiently degraded during mII exit. Two equimolar CaMKII γ isoform variants were present in mII oocytes, neither of which phosphorylated Emi2 in vitro, consistent with the involvement of additional factors. No evidence was found that calcineurin is required for mouse mII exit. These data support a model in which mammalian meiotic establishment, maintenance and exit converge upon a modular Emi2 hub via evolutionarily conserved and divergent mechanisms.

KEY WORDS: Emi2, Metaphase II, Mammalian meiosis, *Xenopus*

INTRODUCTION
The meiotic cell cycle of fertilizable oocytes in vertebrates is typically restrained at the second metaphase (mII) by a cytostatic factor (CSF) to prevent development without a paternal genome (parthenogenesis). The underlying mechanisms of mII arrest and exit are best understood in *Xenopus* and relatively poorly in mammals. In both, mII arrest correlates with the kinase activity of maturation promoting factor (MPF), a heterodimer of Cyclin B (CycB) and the cyclin-dependent kinase Cdc2 (Masui and Markert, 1971; Gautier et al., 1989; Gautier et al., 1990; Perry and Verlhac, 2008). MPF is active in both mitotic and meiotic cell cycles in vertebrates, but its prolonged stabilization by CSF is unique to mII and results in mII arrest.

Exit from mII occurs when CycB undergoes destruction box-(D-box)- dependent ubiquitylation by the anaphase-promoting complex, APC, an E3 ubiquitin ligase; this targets CycB for 26S proteasomal hydrolysis and eliminates MPF, thereby inducing metaphase exit (Glotzer et al., 1991; Peters, 2006). Arrest at mII is achieved by suspending APC activity, which is the function of CSF. One CSF responsible for this inhibition is the endogenous meiotic inhibitor 2, Emi2, the activity of which is essential for mII arrest as independently revealed in *Xenopus* (Schmidt et al., 2005) and the mouse (Shoji et al., 2006). Depletion of Emi2 from intact mouse oocytes causes mII release in a manner that requires the APC activator, Cdc20; one explanation of this is that Emi2 prevents Cdc20 from activating the APC (Shoji et al., 2006; Amanai et al., 2006).

*Xenopus* Emi2 (xEmi2) is stabilized during mII by phosphorylation from xMos to xMek to xMAPK to xRsk to xEmi2 (Sagata et al., 1989; Bhatt and Ferrell, 1999; Gross et al., 2000; Inoue et al., 2007; Nishiyama et al., 2007a) (Fig. 1). xRsk phosphorylates xEmi2 at S335, T336, S342 and S344. Phosphorylation at S335 and T336 facilitates the binding of protein phosphatase 2A (xPP2A), which in turn dephosphorylates phospho-residues at T545 and T551, and S213, T239, T252 and T267 (Wu et al., 2007b). Dephosphorylation of T545/T551 enhances binding of the xEmi2 C-terminal domain to the APC core component, xCdc27 (xAPC3) to inhibit the APC (Wu et al., 2007b) whereas dephosphorylation of the S213-T267 cluster stabilizes xEmi2 (Wu et al., 2007a). In *Xenopus*, xPP2A activity towards xEmi2 is thus stimulated by xMos via xRsk to promote mII arrest (Fig. 1).

In the mouse, *Mos null* oocytes fail to activate the MAPK pathway but nevertheless often arrest or pause at mII with MPF activity initially unaffected, or progress through mII and then ‘collapse’ back to mIII (Verlhac et al., 1996; Choi et al., 1996). Oocytes from *Mos-null* mice contain anomalously long, interphase-like microtubules during mI to mII and mII to mIII transitions (Verlhac et al., 1996). Emi2-depleted oocytes undergo aberrant cytokinesis (Shoji et al., 2006), but the relationship between Mos and Emi2 in the mammalian meiotic cell cycle remains unknown and meiotic coordination with spindle dynamics poorly understood.

Fertilization triggers an increase in the oocyte concentration of intracellular ‘free’ calcium, [Ca²⁺] (reviewed by Runft et al., 2002). In *Xenopus* oocyte extracts, this activates the Ca²⁺-dependent...
enzymes calmodulin kinase II (CaMKII) and calcineurin (CaN) (Fig. 1). It is unclear whether xCaN regulates the APC directly through xEmi2, with support both for (Nishiyama et al., 2007b) and against (Mochida and Hunt, 2007). Activated xCaMKII phosphorylates xEmi2 at threonine 195 (T195) of its canonical motif, RXST (Rust et al., 2005). xEmi2 phosphorylated at T195 is a favoured substrate for polo-like kinase, Pxl1 (the counterpart of mammalian Plk1), which then phosphorylates xEmi2 at S33/S38 in the phosphodegron motif DSGX3S, targeting xEmi2 for xPLK1 (Pxl1-) dependent proteasomal destruction (Schmidt et al., 2005; Rauh et al., 2005).

These details await analysis in mammalian Emi2 but it already seems clear that mouse and Xenopus (x)Emi2 differ. The N-terminal Pxl1 phosphoryde does not have an N-terminal mouse Emi2 counterpart (Rauh et al., 2005; Perry and Verlhac, 2008). Moreover, Xenopus xRsk links the Mos-MAPK cascade to xEmi2 but mouse oocytes lacking Rsk (those of Rsk1-, Rsk2-, Rsk3 triple null mice) present a stable ml arrest (Dumont et al., 2005). Although it has recently been shown (Chang et al., 2009; Backs et al., 2010) that the γ isoform of CaMKII (CaMKIIγ) transmits the Ca2+ signal during mouse fertilization, the nature of this relay to Emi2 – whether, for example, it is direct – is unknown and mammalian Emi2 lacks the canonical RXXT/S CaMKII phosphorylation target used in xEmi2 (Rauh et al., 2005; Perry and Verlhac, 2008).

Here, we report a detailed molecular dissection of mammalian Emi2 and ml arrest. Xenopus and mouse Emi2s are functionally non-equivalent. Both N- and C-terminal Emi2 domains possess metaphase modulating activity, the latter through a potential TSS phosphorylation, D-box, Zn2+-binding motif and RL domain. Oocytes contain equimolar isoforms of CaMKIIγ, although neither directly phosphorylates Emi2 in vitro.

MATERIALS AND METHODS

Collection, culture and activation of oocytes
Eight- to 12-week-old B6D2F1; females (SLC, Shizuoka-ken, Japan) were superovulated by standard serial intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) followed 48 hours later by human chorionic gonadotropin (hCG). Oviductal ml oocytes were collected typically 12 to 14 hours after hCG injection and cumulus cells removed following hyaluronidase treatment as previously described (Yoshida et al., 2007; Suzuki et al., 2010). Fully grown germinal vesicle stage (GV) oocyte-cumulus cell complexes were collected from 8- to 10-week-old B6D2F1 females 44 to 48 hours after PMSG injection. After 1 hour of culture in Waymouth medium (Waymouth, 1959) supplemented with 10% (v/v) foetal calf serum (FCS), cumulus cells were displaced from associated GV oocytes by repeated pipetting in TaM medium (Miki et al., 2006) supplemented with 10% FCS and 150 μM isobutylmethylxanthine (IBMX). GV oocytes were held in TaM medium containing 150 μM IBMX until micromanipulation and IBMX washed out or incubation in IBMX-containing medium continued, depending on the experiment (see Fig. S1 in the supplementary material). Culture of ml oocytes was typically in kalium simplex optimized medium (KSOM) (Erbach et al., 1994). All oocyte and embryo culture was in humidified CO2 [5% (v/v) in air] at 37°C. Parthenogenetic activation was by incubating ml oocytes in Ca2+-free CZB (Chatot et al., 1989) supplemented with 10 mM SrCl2 in humidified CO2 [5% (v/v) in air] at 37°C for 1 hour. Oocytes were then washed in KSOM and incubation continued at 37°C.

Sperm preparation and microinjection
Sperm were demembranated as described previously, with minor modifications (Yoshida et al., 2007). Briefly, cauda epididymal spermatozoa from 12- to 30-week-old male B6D2F1; mice were triturated in nuclear isolation medium [NIM: 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na2HPO4, 1.4 mM KH2PO4, 3.0 mM EDTA (pH 7.45)] containing 1.0% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) at room temperature (25°C) for 1 minute and washed twice in ambient temperature NIM to give control demembranated heads – cHds. Where appropriate, sperm suspensions were incubated at 48°C for 30 minutes, with trituration after 15 minutes, to generate ‘inactivated’, iHd preparations. Sperm were mixed with 1 to 2 volumes of 15% (w/v) PVP360 (average Mr=360,000; Kanto Chemical, Tokyo, Japan) and microinjected as described (Yoshida and Perry, 2007), typically within 60 minutes of PVP mixing.

Immunological methods
Standard immunoblotting (IB) was with rabbit polyclonal anti-CycB1 (Santa Cruz Biotechnology, CA, USA), anti-Emi2 (Shoji et al., 2006), anti-Tub (α-tubulin; Abcam, MA, USA), rabbit monoclonal anti-phosphoErk1/2 (Cell Signaling Technology, MA, USA), rabbit polyclonal anti-MAPK1/2 (Cell Signaling), rabbit polyclonal anti-Mos (Santa Cruz), rabbit polyclonal anti-IP3R (Calbiochem, USA), rabbit polyclonal anti-Mos (Santa Cruz), goat polyclonal anti-CaMKIIγ (calmodulin kinase II γ; Santa Cruz), rabbit polyclonal anti-Cdc2 (Delta Bioslabs, CA, USA), rabbit polyclonal anti-phosphoY15Cdc2 (R&D Systems, MN, USA) or rabbit polyclonal anti-Tuba (alpha-tubulin; Abcam) primary antibodies, and anti-rabbit or -goat IgG (Invitrogen) secondary antibodies.

Immune complex detection, IB of oocytes and early embryos, and immunoprecipitation (IP) from NIH 3T3 cell extracts, were performed essentially as described previously (Itoh et al., 1998; Shoji et al., 2006). NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gemini-Bioscience) and penicillin/streptomycin (Sigma). Lipofectamine-mediated transfection (Invitrogen) of NIH 3T3 cells used 0.4 μg of each construct in pcDNA-neomycin plasmid DNA (Shoji et al., 2006) per six-well plate. Constructs encoded bacterial alkaline phosphatase (BAP) or Emi2 (or deletions thereof) as C-terminal fusions to (FLAG) for Fig. 4B, or mCherry (control) or Emi2-mCherry for Fig. 4F. Cells were collected 48 hours after transfection, and ~5×106 cells lysed in 300 μl incubation buffer [150 mM NaCl, 2 mM Na2EDTA, 100 mM PMSE, 5 mM DTT, 50 mM Tris (pH 7.5)] supplemented with 0.05% (w/v) NP-40, on ice for 10 minutes. Where appropriate, TPEN was added to cells at a final concentration of 100 μM 1 hour prior to harvesting. Lysates were cleared by centrifugation at 20,000 g at 4°C for 30 minutes. Cleared lysates (~5%) were stored for analysis by SDS-PAGE, and
the remainder incubated with 20 μl of anti-Cdc27 (clone AF3.1, Sigma) or anti-DeRred (Clontech Laboratories, CA, USA) antibody overnight at 4°C and immunoprecipitates (IPs) incubated with protein A-agarose beads ([2.5 μl of 50% (v/v) suspension] for 1 hour at 4°C, washed three times with ice-cold incubation buffer, and resuspended in a sample buffer. To generate FLAG IPs, lysates were applied to a mouse anti-FLAG M2 affinity matrix (Sigma) overnight with agitation at 4°C, unbound material removed, the matrix washed five times in 150 mM NaCl, 0.01% (v/v) NP-40, 10% (v/v) glycerol, and bound protein eluted at 4°C for 2 hours in a solution containing 5 mg/ml 3× FLAG peptide (Sigma) in 1 M NaCl, 0.5 M Tris (pH 7.5). Immunoblotting was with mouse anti-FLAG [1:1000 (v/v)] overnight and anti-mouse IgG secondary antibody [1:100,000 (v/v)] for 1 hour. Approximately 50% of each eluate was analyzed by SDS-PAGE. Labelled protein detection was performed as described previously (Shoji et al., 2006).

Preparation and injection of cRNA and siRNA

The different injection protocols adopted for in this work are illustrated in Fig. S1 in the supplementary material. Constructs containing PCR-generated Emi2 or Mos cDNA Nhel-Xhol fragments cloned into pCI-Neo-mCherry (Shoji et al., 2006) were used to produce cRNA-encoded mCherry fusions at the C terminus of Emi2. Venus constructs were similarly constructed using a pCI-Neo-Venus scaffold, either as Nhel-Xhol (β-Catenin) or Nhel-Xbal (Emi2, Bora) fragments (see Fig. S11A in the supplementary material). For the transfections of Fig. 4B, (FLAG)-BAP or -Emi2 cDNA (i.e. encoding FLAG fused at the N terminus) were cloned into pNEBR-X1-Hygro (New England Biolabs, MA, USA) as Xhol-NotI fragments. For transfections in Fig. 4F, mCherry or Emi2-mCherry cDNA were cloned as Xhol-NotI fragments into pNEBR-X1-Hygro and the AAT preceding the start codon in Emi2-mCherry altered to ACC by site-directed mutagenesis. Site-directed mutagenesis was performed in the Quick-Change system (Strategene, CA, USA). cRNAs were synthesized in vitro from linear plasmid DNA template and 5'-capped and polyadenylated in the same reaction using an mSCRIPT mRNA Production System (Epicentre Biotechnologies, WI, USA) according to the instructions of the manufacturer. cRNAs were dissolved in nuclease-free distilled water, quantified and stocked in aliquots at −80°C. Double-stranded siRNAs (iGENE Therapeutics, Tsukuba, Japan) were designed as described previously (Amanai et al., 2006) and stored in aliquots at −80°C.

RNA stock solutions were diluted with sterile DEPC-treated water to the desired concentration and 5–10 pl injected at default concentrations of 0.5 to 1.0 mg/ml (‘Lo’) or occasionally at higher concentrations (2.0 to 2.5 mg/ml; ‘Hi’) in Fig. 4G and Fig. S4 in the supplementary material, for cRNA and 25 μM for siRNA within 1 hour of thawing, either through a fine needle (tip inner diameter ≤1 μm) into GV oocytes in BMM-containing M2 medium, or via a piezo-actuated micropipette (tip inner diameter 6–7 μm) into mII oocytes in M2 medium. Where appropriate, post-injection levels of cRNA were confirmed by semi-quantitative PCR (qPCR; not shown).

PCR

Ratiometric quantification of mRNAs (qPCR) was essentially as described previously, with cDNA prepared by Superscript (Invitrogen, CA, USA) from RNA from sperm or GV or mII oocytes (Amanai et al., 2006; Shoji et al., 2006) using the following primer pairs (5’ to 3’): H2afz, GGCTATCCCACCCCTGCTATCTG and TTCCTGTTGCTCTTTCTCCCG; H3βα, CGCAGCAACAGGTTTAC and CACGGTTTTTTCATGCATTGAC; CaMKIIb, TCTTCCGACAGCACCAACACAAC and GGGTCACAG - ACCACCATTGAGG and GGTCGCACATCTTCGTGTAGGACTC; CaMKIIg, TGAAGGTGACCAAGGGTGGC and GTTTCCG; CaMKIId, CaMKIIe, CaMKIIh, or CaMKIIi were translated as Myc-His fusions in vitro using a TNT rapid coupled transcription/translation (IVTT) system (Promega) charged with 2 μg template DNA, and purified using the MagZ Protein Purification System (Promega, Cat. No. V8830). As a positive control, we employed preparation of bovine brain CaMKII containing all four isoforms (Upstate Biotechnology, NY, USA). CaMKII kinase assays were performed using the CaMKII Assay Kit (Upstate) as instructed by the manufacturer. Where appropriate, 9 μl of IVTT-purified mouse Emi2 or xEmi2 plus 1 μl of 400 ng/μl CaM replaced 10 μl CaM in the CaMKII substrate cocktail (500 μM autophosphorylation peptide plus 40 ng/μl CaM). Where both CaM and Emi2 substrates were present in the same reaction, 10 μl of IVTT-purified (x)Emi2 and 10 μl of CaM in the CaMKII substrate cocktail (500 μM autophosphorylation peptide plus 40 ng/μl CaM) were added to give a final volume of 60 μl. As a negative control, ADBI buffer was included instead of CaMKII and/or Emi2. To visualize phosphorylation, 14 (out of 50) μl of each reaction was subjected to SDS-PAGE, fixed in a mixture of 30% (v/v) methanol plus 10% (v/v) acetic acid for 10 minutes, incubated in crack-proof solution for 10 minutes and dried prior to autoradiography using a BAS-2500 image detection system.

Computer methods

Student’s t-tests were applied to comparative unpaired analyses. Data for each experiment were collected on at least 2 days. Emi2 protein sequences for mouse and species orthologues were obtained from Ensemble (http://www.ensemble.org/index/html) and the multiple alignments shown in Fig. 3A and Fig. 4C optimized via the CLUSTALW algorithm (http://align.genome.jp).
RESULTS

Mos is required for Emi2 to establish, but not maintain mII arrest.

In *Xenopus*, the classical cystostatic factor, xMos, stabilizes xEmi2 via xMAPK (Inoue et al., 2007), but roles for Mos in establishing and maintaining mII arrest in mammals have not been clearly delineated. To address this, we injected mouse germinal vesicle stage (GV) oocytes with complementary RNA (cRNA) encoding Mos-mCherry and cultured them for 24 hours in the phosphodiesterase inhibitor, IBMX, followed by in vitro maturation (IVM) for 16 hours (the IB24 protocol; see Fig. S1A in the supplementary material). Injected oocytes underwent 97.6±2.4% mI arrest, whereas the corresponding value dropped to 38.5±3.6% for the dominant-negative, MosS3A/S105A-mCherry and 35.7±14.3% for mCherry alone (Fig. 2A; see Fig. S2A in the supplementary material). Mos overexpression in IB24 oocytes induced the appearance of active, phospho-MAPK, but this was markedly reduced by the MAPKK (Mek) inhibitor U0126 (Fig. 2B), which also reduced the establishment of mII arrest by Mos (Fig. 2A). In corroborating earlier work relating Mos-MAPK to mouse cytostatic arrest (Verlhac et al., 1996), these findings confirm the fidelity of the IVM system.

The relationship between Mos and Emi2 in mammals was next investigated by co-injecting GV oocytes with *siEmi2* and *Mos-mCherry* cRNA in the IB24 system. Only 5.9±3.9% (n=52) of oocytes arrested at mI (Fig. 2A; see Fig. S2A in the supplementary material), suggesting that metaphase establishment by Mos requires Emi2. The *Xenopus* xMos-xMAPK pathway enhances xEmi2 stability and activity by phosphorylating S335 and T336 (Inoue et al., 2007). To test whether T336 of xEmi2 corresponds to T327 in the mouse, we injected *Emi2T327A-mCherry* cRNA in the si16 system (see Fig. S1C in the supplementary material). High concentrations of *Emi2T327A-mCherry* induced mI arrest (Fig. 2C), but only weakly compared with the same amount of cRNA encoding full-length Emi2-mCherry (compare Fig. 3D with ‘T327A Lo’ in Fig. 2C). This mirrors the result of a similar functional assay using xEmi2T336A in *Xenopus* extracts (Inoue et al., 2007), consistent with an analogous role for T327 in mouse Emi2 to T336 in xEmi2.

Few data thus far distinguish between the role of Mos in the establishment versus the maintenance of mII arrest. We investigated this in aging oocytes, which from 18 to >36 hours post-hCG retained the morphological features of mII, although metaphase plate cortical protuberances eventually decreased in
size and number (Fig. 2E). The spindles of Venus-tuba
transgene-expressing mII oocytes elongated and occasionally
became decoupled from the cortex during aging (see Fig. S2B in
the supplementary material) (Webb et al., 1986). Immunoblotting
showed that levels of meiotic exit signalling proteins changed
little; phospho-MAPK exhibited a pronounced decline after only
32 hours, the inactive Cdc2 modification Y15-phospho-Cdc2
was undetectable, and H1 kinase activity, a marker of MPF (i.e.
mII) persisted (see Fig. S2C in the supplementary material). By
contrast, Mos levels declined markedly and were undetectable
by ~28 hours post-hCG (Fig. 2D; see Fig. S2C in the
supplementary material). Exposure to the parthenogenetic agent,
SrCl2 activated >95% of oocytes up to 32 hours post-hCG, and
~50% thereafter. The proportion undergoing aberrant cytokinesis
following SrCl2 treatment exhibited a pronounced increase after
32 hours (Fig. 2E).

These findings suggest that Mos is not sufficient in itself to
establish mouse meiotic metaphase but requires Emi2, to which it
signals via MAPK. The disappearance of Mos during mII oocyte
aging correlates with aberrant spindle behaviour in oocytes that are
competent to exit mII, indicating that Mos is not required for mII
maintenance.

**Xenopus and mouse Emi2s are not interchangeable**

These results raise the possibility that mammalian and Xenopus
Emi2s are also functionally distinct. Species alignment of predicted
Emi2 sequences conserves multiple regulatory elements of xEmi2
(Fig. 3A) but it is not known whether they function outside
Xenopus. To address this, we compared xEmi2 and mouse Emi2
behaviour in mouse oocytes.

Mouse GV oocytes were injected with cRNA encoding mCherry
and subjected to IVM for 16 hours (referred to as IVM16; see Fig.
S1B in the supplementary material). Of these
mCherry
-injected
controls, 90±1.9% (n = 61) underwent normal meiotic progression
and arrested at mII, whereas those injected with Emi2-mCherry
cRNA precociously arrested at mI (see Fig. S3A in the supplementary material). Emi2 localized to mI spindles (Fig. 3B);
no first polar body (Pb) extrusion was observed by videomicroscopy (not shown). Segments of Emi2 (residues 1-250 and 251-C-terminus) localized nebulously to spindles (see Fig. S3B in the supplementary material). When GV (i.e. nuclear) breakdown was suspended by IBMX, Emi2-mCherry was efficiently targeted to the GV, localizing to a nuclear domain outside the nucleolus-like body (Bouniol-Baly et al., 1999; Fig. 3C; see Fig. S3C in the supplementary material) and to nuclei in NIH 3T3 cells (not shown), even though Emi2 lacks a consensus nuclear localization motif (Lee et al., 2006). Similar GV localization was exhibited by Emi2251-Cter – but not by the N-terminal Emi2 domain of Emi21-250-mCherry (see Fig. S3C in the supplementary material).

These experiments did not distinguish between the metaphase-imposing activities of Emi2-mCherry and native Emi2. To address this, we employed the si16 system of RNAi (see Fig. S1C in the supplementary material) to deplete native Emi2, by injecting siEmi2-5'UTR#1 (Fig. 3D; see Fig. S3D in the supplementary material) or #2 (not shown), which are specific to native Emi2 mRNA (Shoji et al., 2006; Amanai et al., 2006). As expected (Shoji et al., 2006), almost none (1.9±1.9%) of the oocytes injected with mCherry mRNA in the si16 system exhibited mI arrest, whereas mouse Emi2-mCherry mRNA caused 95.6±2.6% mI arrest (Fig. 3D), showing that the Emi2 knock-down phenotype had been rescued.

If xEmi2 is mechanistically equivalent to mouse Emi2, it should have similar properties. However, in contrast to Emi2, xEmi2-mCherry was excluded from the GV of IBMX-treated oocytes, suggesting that it lacked a nuclear targeting signal (Fig. 3C). When native Emi2 was depleted in the si16 system, xEmi2-mCherry elicited metaphase arrest (mI and mII) at a lower efficiency than that achieved by its mouse counterpart, Emi2-mCherry (Fig. 3D; see Fig. S3D in the supplementary material). These findings indicate that mouse and Xenopus Emi2s are not functionally interchangeable and that they participate in distinct regulatory mechanisms. We therefore sought a mechanistic dissection of mouse Emi2 in vivo.

A regulatory N-terminal domain complements the principal zinc-dependent C-terminal cytostatic activity of mouse Emi2

Injecting GV oocytes with cRNA encoding either Emi21-250 or Emi2251-Cter as mCherry fusions induced ~100% mI arrest following IVM16 in a manner analogous to full-length Emi2-mCherry (Fig. 4A; see Fig. S4 in the supplementary material). Analogous to xEmi2, functional MAPK-dependent phosphorylation, D-box and ZBR motifs all reside in Emi2251-Cter (Fig. 3A), so Emi21-250 was not expected to possess cytostatic activity. In mitotic cells, full-length Emi2 and Emi2251-Cter co-complexed with APC subunits Cdc20 and Cdc27, as judged by co-immunoprecipitation (Fig. 4B). We did not observe interactions between Emi21-250 and Cdc27, but occasionally detected an interaction between Emi21-250 and Cdc20 (Fig. 4B).

Emi2251-Cter retained the ability to induce mI arrest in si16 oocytes but Emi21-250 did not (Fig. 4A; see Fig. S4 in the supplementary material), suggesting mechanisms that are, respectively, independent of and dependent on native Emi2.

We wished to define more specifically the Emi2 N-terminal cytostatic function. Residues 1-100 induced ml arrest in 42.3±3.6% (n=26) of IVM16 oocytes (Fig. 4A), suggesting that residue 100 lies within the cytostatic N-terminal region. Consistent with this, Emi280-115 induced 100% IVM16 ml arrest, but did not in si16 oocytes (Fig. 4A; see Fig. S4 in the supplementary material). Residues 80-115 of ectopically expressed Emi2 are therefore not inherently sufficient for metaphase establishment but enhance native Emi2-dependent ml arrest; the region is conserved among mammals (Fig. 4C).

The Emi2 C terminus contains a putative ZBR, but although a single ZBR mutant of Xenopus xEmi2 lacked cytostatic activity in cell-free extracts, its stability was not reported (Schmidt et al., 2005). To address the relationship between Emi2 and Zn2+ in mlI maintenance, we injected mI oocytes with cRNA and exposed them ~4 hours later to the highly specific Zn2+ chelator, N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), which has been shown to induce meiotic exit (Suzuki et al., 2010). Injection with cRNA encoding Emi2-mCherry, but not mCherry or Emi2C573A-mCherry (a mutant within its putative ZBR; see below), reduced the efficiency of TPEN-induced mlI exit (Pb2 extrusion; Fig. 4D). Injection of cRNA encoding ZBR mutants in the si16 system generally produced mCherry fluorescence intensities lower than Emi2-mCherry controls (see Fig. S5 in the supplementary material), even though qPCR confirmed higher mCherry fluorescence in oocytes (average, 1.23±0.38; control [Emi2-mCherry]=1.0±0.0). To compensate, more cRNA was injected and the resultant mCherry epifluorescence level related to its corresponding ability to impose metaphase arrest (Fig. 4E; see Fig. S6 in the supplementary material). These experiments indicate a pronounced contribution from ZBR residues C573, C591, C601 to Emi2 cytostatic activity in vivo.

Pre-treatment with TPEN had no discernable effect on Emi2 complex formation with the APC core subunit, Cdc27 in NIH 3T3 cells (Fig. 4F), consistent with separable APC- and Zn2+-binding domains (Miller et al., 2006; Tang et al., 2010). We also found that whereas Emi2551-Cter was unable to impose metaphase arrest in si16 oocytes, Emi2491-Cter did so efficiently; the results were mirrored in the IVM16 system (Fig. 4G; see Fig. S7A in the supplementary material). This lead us to investigate the candidate D-box RXXL, which lies between 491 and 551. The double mutant Emi2R511A,L514A-mCherry induced mlI arrest only weakly in si16 oocytes (22.5±2.5%, n=29), even when expressed at high levels (Fig. 4G; see Fig. S7A in the supplementary material), indicating that R511 and/or L514 are required by Emi2-mCherry for full cytostatic activity.

These studies predict autonomous meiotic regulatory activity in the Emi2 C terminus. Indeed, when cRNA encoding Emi2551-Cter was injected into mlI oocytes, ~100% underwent meiotic exit (Fig. 4H; see Fig. S7B in the supplementary material). This was probably not due to Zn2+ sequestration by the ZBR, as Emi2551-CterC573A also efficiently induced meiotic exit (Fig. 4H). Recently, an RL domain at the C terminus has been shown in Xenopus to mediate APC binding (Ohe et al., 2010). Accordingly, cRNA encoding Emi2551-CterR511A,L514A, in which the RL domain had been deleted, failed to elicit mlI exit (Fig. 4H). However, Emi2491-Cter, which contains the RLAL D-box in addition to the ZBR and RL domain (Fig. 3A), only modestly (13.3±13.3%, n=30) induced mlI exit (Fig. 4H), suggesting that it contained information not present in Emi2551-Cter that tended to impose mlI.

Collectively, these data support the conclusion that residues 491 to the C terminus represent the (near) minimal region of Emi2 required for it to establish and maintain metaphase arrest. Meiotic stabilization by Emi2 involves its D-box (residues 511-514), ZBR (573–613) and RL domain (640-641) and possibly an indirect mechanism involving a novel N-terminal domain (80-115).
The regulation of metaphase exit and Emi2 degradation

We next investigated signalling processes implicated in mII exit to test how they converged on Emi2. The calmodulin (CaM) inhibitor, CGS9343B inhibited Pb2 emission and ectopic Emi2 degradation following parthenogenetic activation (see Fig. S8A,B in the supplementary material). CaM-dependent enzymes calcineurin (CaN, also called PP2B) and calmodulin kinase II (CaMKII) mediate Xenopus mII exit and it has been suggested that they achieve this by regulating xEmi2 degradation (Rauh et al., 2005;...
However, mouse oocytes lacked the CaN catalytic A subunit above a detection limit of ~700 fg/oocyte (43 nM; see Fig. S8C in the supplementary material), 10- to 100-fold lower than CaN concentrations in other tissues (Sharma et al., 1979; Stewart et al., 1983). ICSI-induced mII exit was slowed but not prevented by a combination of the CaN inhibitors CsA, FK506 or CaN-inhibitory peptide (see Fig. S8D in the supplementary material). Thus, in contrast to Xenopus, we found no evidence that CaN is essential for mouse mII exit, suggesting that the principal effect of CGS9343B was indirectly to reduce CaMKII activity.

In keeping with recent reports (Chang et al., 2009; Backs et al., 2010), GV and mII oocytes contained mRNA for CaMKII \( \gamma \) (CaMKII \( \gamma \)), but not \( \alpha \), \( \beta \) or \( \delta \) (a, b and g) in sperm (sp) or oocytes (GV and mII). Immunoblotting (centre) with anti-CaMKII\( \gamma \) reveals two isoforms, \( \gamma 3 \) and \( \gamma J \), in mII oocytes, the variant sequences of which are shown below. IVTT, coupled in vitro transcription/translation. (B) SDS-PAGE of in vitro assays to measure phosphorylation of mouse Emi2 (m) and xEmi2 (X) produced in vitro, by CaMKII\( \gamma \) mII oocyte isoforms (\( \gamma 3 \) and \( \gamma J \)) produced in vitro (A) or a mixture of all CaMKII isoforms from bovine brain (brain). AC, positive control autocamtide peptide. Red arrowheads indicate autophosphorylated CaMKII monomers. (C) Data summary of ectopic Emi2 degradation following oocyte activation. mII oocytes injected with cRNA encoding mCherry fused to Emi2, or the Emi2 mutations indicated were exposed to 10 mM SrCl\( _2 \) (see Fig. S1D in the supplementary material) and fluorescence levels recorded 1 hour after SrCl\( _2 \) withdrawal. Complete data sets are presented in Fig. S10A,B in the supplementary material. (D) Fluorescence images (left) and histograms for mII oocytes injected with cRNA encoding xEmi2-mCherry (xEmi2), without (top) or with exposure to SrCl\( _2 \) as in C. Recordings were made at the times indicated after oocytes had been exposed to SrCl\( _2 \) for 1.5 hours. Scale bar: 50 \( \mu \)m. Values above columns show oocyte numbers. (E) Fluorescence levels in mII oocytes injected with cRNA encoding Emi2-mCherry and 4 hours later treated with 10 mM SrCl\( _2 \) (+Sr\( ^{2+} \), broken lines) or not treated (−Sr\( ^{2+} \), solid lines). Oocytes were from the hybrid B6D2F1 (black plots, wt) or homozygous null Trcpb1–/– mutants (red).

Nishiyama et al., 2007b). However, mouse oocytes lacked the CaN catalytic A subunit above a detection limit of ~700 fg/oocyte (43 nM; see Fig. S8C in the supplementary material), 10- to 100-fold lower than CaN concentrations in other tissues (Sharma et al., 1979; Stewart et al., 1983). ICSI-induced mII exit was slowed but not prevented by a combination of the CaN inhibitors CsA, FK506 or CaN-inhibitory peptide (see Fig. S8D in the supplementary material). Thus, in contrast to Xenopus, we found no evidence that CaN is essential for mouse mII exit, suggesting that the principal effect of CGS9343B was indirectly to reduce CaMKII activity.

In keeping with recent reports (Chang et al., 2009; Backs et al., 2010), GV and mII oocytes contained mRNA for CaMKII\( \gamma \) (CaMKII\( \gamma \)) but not the three other isoforms; we also found CaMKII\( \gamma \) transcripts in spermatozoa (Fig. 5A). Mature mII oocytes contained two CaMKII\( \gamma \) mRNA variants, encoding the previously reported 67 kDa \( \gamma 3 \) (\( \gamma 3 \)) isoform (Chang et al., 2009) and the J isoform (\( \gamma J \)), not previously described in the mouse, containing a predicted 21 amino acid variable domain insertion (AAL69956.1; Fig. 5A) (Gangopadhyay et al., 2003). CaMKII\( \gamma 3 \) and \( \gamma J \) isoforms were approximately equimolar in mII oocytes (Fig. 5A). Injecting a mixture of active bovine brain CaMKII\( \gamma \) isoforms into mII oocytes did not induce mII exit (not shown), leading us to challenge Xenopus and mouse (x)Emi2 proteins with active bovine brain CaMKII\( \gamma \) or mouse oocyte CaMKII\( \gamma 3 \) and \( \gamma J \) isoforms in a phosphorylation assay in vitro. All CaMKII preparations tested phosphorylated a control substrate and exhibited autophosphorylation, indicative of holoenzyme activity (Fig. 5B). However, there was little or no phosphorylation of either mouse or Xenopus (x)Emi2 (Fig. 5B). The absence of detectable Emi2 phosphorylation in vitro is unlikely to be because CaMKII functions as a non-CaMKII\( \gamma \) hetero-multimer, as neither was phosphorylated by the cocktail of neuronal CaMKII isoforms (Fig. 5B). We therefore investigated whether this lack of phosphorylation in vitro might reflect a predisposing requirement for protein kinase
C (PKC), which has also been implicated in oocyte activation (Halet, 2004). Combining CGS9334B with the PKC inhibitor chelerythrine markedly inhibited mII exit (see Fig. S8E in the supplementary material), but chelerythrine alone only modestly inhibited Emi2-mCherry degradation in mII oocytes (see Fig. S8F in the supplementary material) suggesting that PKC does not potentiate CaMKII signalling that might lead to Emi2 degradation.

To address whether CaMKII transduces the Ca$^{2+}$ signal at fertilization to degrade mouse Emi2, mature mII oocytes were injected with cRNA encoding truncation mutant Emi2-mCherry fusions, followed by Sr$^{2+}$ exposure [mII(Sr)] or ICSI [mII(ICSI); see Fig. S1D,E in the supplementary material]. Taking fluorescence decrease as a meter of degradation, Sr$^{2+}$ induced loss of Emi2$^{1-300}$ and Emi2$^{50-300}$, but not Emi2$^{1-250}$ or Emi2$^{251-300}$-mCherry (Fig. 5C; see Fig. S9, S10 in the supplementary material). Thus, residues 50-300 are apparently required for Ca$^{2+}$-dependent degradation of mouse Emi2, but not the region corresponding to the Plx N-terminal phosphodegron in xEmi2 (Fig. 5A). Analogous to Xenopus, residues 50-300 contain a candidate, non-canonical CaMKII phosphorylation target, T176 (Fig. 3A); full-length xEmi2 was efficiently degraded in response to Sr$^{2+}$ in mouse oocytes (Fig. 5D). Mouse Emi2$^{T176A}$-mCherry did not undergo Sr$^{2+}$-induced degradation in mII oocytes (Fig. 5C). A similar phenotype was exhibited by D274A and S275N mutants, which lay within a consensus phosphodegron, DSGXnS (Fig. 5C); the corresponding phosphodegron is located differently in xEmi2, and is essential for destruction downstream of Ca$^{2+}$-dependent xCaMKII phosphorylation (Rauth et al., 2005).

Finally, we investigated the activity responsible for Emi2 removal in response to fertilization-induced Ca$^{2+}$ mobilization. The degradation of Emi2 is highly selective in that Ca$^{2+}$ mobilization does not result in the targeting of Ca$^{2+}$/Plk1-regulated substrates, Emi1, β-Catenin or Bora for destruction (see Fig. S10, Fig. S11A in the supplementary material). The pathway responsible did not require the APC, as depletion of its activator, Cdc20, by RNAi (Amani et al., 2006) had little, if any, effect on Emi2 degradation (see Fig. S11B in the supplementary material). Moreover, degradation of Emi2 occurred efficiently in the oocytes of gene-targeted mice lacking the E3 ubiquitin ligase SCF component Trepb1 (Fig. 5E), implying that Trepb1 is not essential.

**DISCUSSION**

This work reveals in intact mouse oocytes, essential domains and residues, protein interactions and degradative mechanisms required by Emi2 to establish, maintain and exit from meiotic metaphase (Fig. 6). We also show that the establishment of metaphase by Mos requires Emi2 and suggest that they are linked by MAPK, as in Xenopus (Inoue et al., 2007). This begs the question of precisely how, as Xenopus MAPK signals to xEmi2 via xRsk, but Rsk is dispensable in the mouse (Dumont et al., 2005). It was therefore important to establish whether mouse Emi2 possessed a functional site corresponding to the Xenopus xRsk phosphorylation target. It does: T327 (which corresponds to the xRsk target, T336 in Xenopus) is essential for mouse Emi2 cytostatic activity. This suggests that Mos-MAPK signals to Emi2 either directly or via a similar pathway to that in Xenopus, omitting Rsk and passing through an unknown intermediate.

Our work implies that, once established, Mos is not required to maintain mammalian mII in that it autonomously disappears during mouse mII oocyte aging. Rather, the disappearance of Mos occurs during an increase in spindle abnormalities (Webb et al., 1986; Verlhac et al., 1996) (Fig. 2). Loss of active MAPK also correlates with spindle lengthening (see Fig. S2B,C in the supplementary material), implicating the mouse oocyte spindle regulators MISS and Doc1R, both of which are MAPK substrates that do not mediate mII arrest (Lefebvre et al., 2002; Terret et al., 2003). It is therefore possible that, after mII arrest is established, the principal role of Mos is to maintain spindle integrity, possibly in conjunction with spindle regulators such as MISS and Doc1R.

Mouse Emi2 and Xenopus xEmi2 possess distinguishable activities in mouse oocytes; xEmi2 did not fully compensate for mouse Emi2 in meiotic regulation and localization patterns of xEmi2 were different from those of mouse Emi2. Differences may (in part) reflect the temperatures at which mouse and Xenopus fertilization occurs: 36.9°C and ~23°C, respectively. Species alignment of Emi2 sequences reveals both conservation and variation of domains and amino acid residues shown to be important for xEmi2 function (Fig. 3A), suggesting that non-conserved regions have evolved in different species to perform these functions.

It has already been shown that mouse Emi2 has the ability to arrest meiotic oocytes at metaphase (Shoji et al., 2006). When expressed in GV oocytes, this cytostatic activity was found principally to reside in a large C-terminal segment (Emi2$^{251-300}$), which contains domains that are important for Emi2 regulation by analogy to Xenopus, including PP2A-interacting domains, a D-box, ZBR and RL domain (Fig. 3A) (Schmidt et al., 2005; Wu et al., 2007a; Nishiyama et al., 2007a; Ohe et al., 2010). We provide evidence here and in complementary work (Suzuki et al., 2010), that Zn$^{2+}$ is required for mII arrest, working through Emi2; we identify residues that contribute to the putative Zn$^{2+}$-coordinating lattice needed for Emi2 cytostatic activity. Taken together, these results suggest that a Zn$^{2+}$-containing functional Emi2 ZBR is essential for it to inhibit the APC in vivo.

A fragment of Emi2 from 551-Cter elicited mII exit with or without the ZBR mutation, C573A (Fig. 4H; see Fig. S7B in the supplementary material). This fragment contains a C-terminal RL...
motif shown in *Xenopus* to mediate Emi2-APC binding, the overexpression of which causes native Emi2 to dissociate from the APC, causing meiotic progression (Ohe et al., 2010). A similar mechanism is likely in the mouse, as Emi2<sub>551-Cter</sub> failed to induce mII exit (Fig. 4H). In contrast to 551-Cter, Emi2 residues 491-Cter, which also contain the D-box and RLAL, only weakly induced mII exit (Fig. 4H). The low activity relative to 551-Cter is probably not because the D-box turned the fragment into an APC substrate; expression levels were comparably high (see Fig. S7B in the supplementary material). Rather, we favour a model in which the D-box, when combined with ZBR and RL domains, effectively inhibits the APC, consistent with the behaviour of xEmi2 (Tang et al., 2010). We tested this in the IVM16 and si16 systems and found that, unlike 551-Cter, which possessed only limited cytostatic activity, the APC, consistent with the situation in *Xenopus* (Wu et al., 2007b; Tang et al., 2010) and indicates that in mammals a region of Emi2 from a position between residues 491 and 551 to the C terminus is sufficient to establish and maintain mII arrest.

Unexpectedly, an N-terminal fragment, Emi2<sub>1-250</sub>, also exhibited metaphase-inducing activity, as evidenced by its ability to induce mII arrest in GV oocytes (Fig. 4A; see Fig. S4 in the supplementary material). This activity required native Emi2, implying an indirect mechanism. Deletion mutagenesis mapped the activity to the region (residues 80-115) that lacks consensus phosphorylation or other regulatory sites (Marchler-Bauer et al., 2009) but is conserved among several species, including mammals (Fig. 4C). It has been suggested (Wu and Kornbluth, 2008) that smooth mI-to-mII transition relies on Emi2 degradation in mI, and it is possible that Emi2<sub>80-115</sub> is required for mII inactivation and/or degradation of Emi2, such that ectopically expressed 80-115 interferes with this negative regulation.

Separable modules of mouse Emi2 orchestrate mII metaphase establishment and exit. In *Xenopus*, it has been shown that S33/38 is essential for xEmi2 degradation during mII exit, but although there are signs that this degron (DSGXnS) is conserved in lizards and the platypus, there is no conserved motif in the N-terminal region of Emi2 from most other species, including eutherian mammals (Fig. 3A). *Xenopus* possesses the more C-terminal sequence, DSAFHS, that is involved in degradation independently of mII exit (Nishiyama et al., 2007a) and this position corresponds to 258DSGFC579 in the mouse; we find that D274 and S275 are essential for Ca<sup>2+</sup>-dependent mouse Emi2 degradation (Fig. 5C; see Fig. S10B in the supplementary material). Consistent with this, the 258DSGFC579 motif is located among residues 51-300, which approximately define the minimal region of Emi2 sufficient for Ca<sup>2+</sup>-dependent degradation (Fig. 5C). This suggests that the motif has become a Ca<sup>2+</sup>-dependent phosphodegron in the mouse and other species in which it is conserved (Fig. 3A) and that the position of this Emi2 phosphodegron has shifted during evolution. The reasons for this transposition will remain unknown until the function of the N-terminal domain of Emi2 becomes clear. The evolutionary retention of an N-terminal extension in mammals, even though it is no longer required for mII exit, implies that it serves one or more additional roles, such as that played by residues 80-115 or even roles that do not directly modulate the cell cycle.

We found no evidence that CaN is essential for mII exit in the mouse: a cocktail of inhibitors did not prevent meiotic exit and the CaN catalytic subunit was undetectable in mII oocytes. This implies that in mammals the Ca<sup>2+</sup> signal is primarily transduced by CaMKIγ to downregulate Emi2 activity (Chang et al., 2009; Backs et al., 2010). We identified two isoforms of oocyte CaMKIγ, present in equimolar amounts, raising the question of whether CaMKIγ mediates dual regulation of Emi2 or only one of the isoforms is essential. In other cell types, CaMKII splice variants exhibit distinctive protein interactions that influence CaMKII activity directly and/or by influencing subcellular targeting (Bayer et al., 1999; Hudmon and Schulman, 2002; Gangopadhyay et al., 2008). CaMKIγ splice variants that affect protein binding include G-2, which associates specifically with the PP2C-type phosphatase SCP3; the G-2 isoform shares a variant region with the γ isoform present in oocytes (Gangopadhyay et al., 2003; Gangopadhyay et al., 2008). Interactions have been shown to modulate CaMKII activity through allosteric mechanisms that promote substrate phosphorylation in vivo but only poorly in vitro (e.g. Lu et al., 2003). This may explain why CaMKIγ did not phosphorylate Emi2 in vitro, with the clear implication that one or more accessory proteins are required. One goal of future work will be to validate this and identify the adaptor molecule(s) responsible.

The machinery responsible for Ca<sup>2+</sup>-induced Emi2 degradation in mouse oocytes is highly specialized. Neither Emi1, β-Catenin nor Bora – which undergo Plk- and Ca<sup>2+</sup>-dependent proteosomal degradation in response to cell cycle progression – were degraded in oocytes treated with SrCl<sub>2</sub> (see Fig. S11A in the supplementary material). Emi2 degradation was not sensitive to the removal of Cdc20; Cdc20 is an APC activator required for mII exit (see Fig. S11B in the supplementary material) (Shoji et al., 2006). This is consistent with the situation in *Xenopus*, in which xEmi2 degradation requires βTrcp, a component of the E3 ubiquitin ligase, SCF (Tung et al., 2005). Using mII oocytes from gene-targeted females that lack one of the two mouse βTrcp isoforms, Trcpb1, we provide strong evidence that Trcpb1 is dispensable for Emi2 degradation (Fig. 5E). As Trcpb1<sup>−/−</sup> null female mice exhibit normal fertility (Nakayama et al., 2003), it is possible either that Trcpb1 plays no physiological role in Emi2 removal during fertilization, or that Trcpb2 and/or additional E3 ubiquitin ligases can efficiently compensate when it is absent.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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