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Summary

To ensure successful offspring ploidy, vertebrate oocytes must halt the cell cycle in meiosis II until sperm entry. Emi2 is essential to keep oocytes arrested until fertilization. Yet, how this arrest is implemented exclusively in meiosis II and not prematurely in meiosis I remained enigmatic. Using mouse and frog oocytes, we show here that cyclin B3, an understudied B-type cyclin, is essential to keep Emi2 levels low in meiosis I. Direct phosphorylation of Emi2 at an evolutionarily highly conserved site by Cdk1/cyclin B3 targets Emi2 for degradation. In contrast, Cdk1/cyclin B1 is inefficient in Emi2 phosphorylation providing a molecular explanation for the requirement of different B-type cyclins for oocyte maturation. Cyclin B3 degradation at exit from meiosis I enables Emi2 accumulation and thus, timely arrest in meiosis II. Our findings illuminate the evolutionarily conserved mechanisms controlling oocyte arrest for fertilization at the correct cell cycle stage, essential for embryo viability.

Introduction

The meiotic cell division is special, because two divisions – called meiosis I and II – have to be executed without intervening S-phase to generate haploid gametes (Bouftas and Wassmann, 2019; Petronczki et al., 2003). Additionally, the meiotic cell cycle program must be synchronized with the developmental program of gametogenesis such that key cell cycle events such as chromosome and sister chromatid segregation take place at the correct meiotic division (Lie et al., 2009; Ozturk, 2022; Petronczki *et al.*, 2003). Vertebrate female meiosis poses an additional challenge with the implementation of two cell cycle arrests: A first arrest in prophase of meiosis I, required for immature oocytes to grow and accumulate maternal components for successful embryo development, and a second called CSF- (Cytostatic factor-) arrest in metaphase of meiosis II, for mature oocytes to await fertilization (Masui and Markert, 1971; Ozturk, 2022). This second arrest is essential to prevent parthenogenetic divisions, i.e., embryo development without a paternal genome.

Emi2 (XErp1 in *Xenopus*) was identified as the key CSF component that prevents parthenogenesis by directly inhibiting the APC/C (Anaphase-Promoting Complex/Cyclosome) (Liu et al., 2006; Madgwick et al., 2006; Schmidt et al., 2005; Shoji et al., 2006; Tung et al., 2005). Fertilization triggers the destruction of Emi2 resulting in APC/C activation and hence, meiotic exit (Hansen et al., 2006; Liu and Maller, 2005; Rauh et al., 2005; Suzuki et al., 2010). However, how CSF-arrest is synchronized with progression through the two meiotic divisions to ensure that oocytes arrest in meiosis II and not meiosis I, remained enigmatic. The current model proposes two mechanisms to restrain Emi2 to meiosis II: dampened translation and degradation upon Cdk1/cyclin B-dependent

phosphorylation (Ohe et al., 2007; Takei et al., 2021; Tang et al., 2008; Tung et al., 2007). But how Emi2 would be destabilized by Cdk1/cyclin B specifically in meiosis I and not meiosis II, remained unclear.

Meiotic cell cycle progression is driven by cyclin-dependent kinases (Cdks) in association with cell-stage specific cyclins. Entry into M-phase is mediated by the synthesis of M-phase cyclins, i.e., cyclin A (cyclin A1 and A2) and cyclin B (cyclin B1 and B2). Irreversible exit from M-phase results from cyclin B degradation upon ubiquitination by the APC/C. A key open question is how the rapid activation of Cdk1 at M-phase entry is translated into a temporally precisely regulated sequence of phosphorylation events to ensure that the multitude of Cdk1 substrates is phosphorylated in the right order. This task becomes even more challenging for meiotic divisions, where exactly two waves of Cdk1 activation have to take place during two functionally distinct meiotic divisions. Differential substrate preference of distinct Cdk1/cyclin complexes has been proposed as a mechanism (Hochegger et al., 2008). In this respect, the most striking meiosis-specific role for a cyclin was recently discovered for the understudied B-type cyclin B3: Mutant mice harboring a genetic invalidation of *Ccnb3*, the gene coding for cyclin B3, are viable and males are fertile (Bouftas and Wassmann, 2019; Karasu et al., 2019; Karasu and Keeney, 2019; Li et al., 2019), indicating that cyclin B3 is not required in mitosis or male meiosis. Females, however, are sterile, with the vast majority of oocytes failing to enter meiosis II. Homozygous *Ccnb3*$^{-/-}$ knock-out mouse oocytes arrest at metaphase I in a spindle assembly checkpoint-independent manner with stabilized securin and cyclin B (Karasu *et al.*, 2019; Li *et al.*, 2019). Re-expression of wild-type (WT) mouse (*M.m.*) cyclin B3, but not of a kinase-dead hydrophobic patch mutant (MRL), rescued the metaphase I arrest demonstrating that Cdk1/cyclin B3 activity is essential for exit from meiosis I(Karasu *et al.*, 2019). Cyclin B3 is degraded at exit from meiosis I (Karasu *et al.*, 2019;

Li *et al.*, 2019), and translation is restrained to the first meiotic division, unlike cyclin B1 and B2 (Han et al., 2017). Yet, how cyclin B3 promotes anaphase I onset in oocytes, and why it is not required for second female meiosis as well as during male meiosis, has remained unknown.

Capitalizing on the amenability of mouse and frog oocytes to complementary experimental approaches, we show here that the key role of cyclin B3 consists of ensuring that CSF-arrest takes place only in meiosis II and not meiosis I. We demonstrate that Cdk1/cyclin B3 specifically phosphorylates Emi2 at an evolutionarily highly conserved site in meiosis I, initiating a multisite phosphorylation cascade, which involves Polo-like kinase 1 (Plk1) and ultimately results in efficient degradation of Emi2. Hence, vertebrate oocytes without cyclin B3 cannot complete the first meiotic division because they establish an Emi2-dependent cell cycle arrest already at metaphase I.

Results

Cyclin B3 prevents precocious CSF-arrest in mouse oocyte meiosis I

The phenotype of *Ccnb3*$^{-/-}$ mouse oocytes is reminiscent of the metaphase II arrest of mature oocytes (see Figure S1 for schematic presentation), so we wondered whether untimely CSF activity accounts for the observed metaphase I arrest. To test this hypothesis, *Ccnb3*$^{-/-}$ arrested oocytes were treated with strontium, an inducer of CSF release (O'Neill et al., 1991). Exit from meiosis I was examined by preparing chromosome spreads 1 hour after activation, when more than 50 % of strontium-treated oocytes had extruded a polar body (PB). Indeed, while all control-treated *Ccnb3*$^{-/-}$ oocytes displayed bivalents (connected pairs of homologous chromosomes), 74% of strontium-treated oocytes instead had segregated homologous chromosomes and contained dyads (pairs of sister chromatids), indicating successful progression through meiosis I (Figures 1A). If

Cdk1/cyclin B3 activity was required to suppress CSF-arrest in meiosis I, ectopic expression of cyclin B3 in meiosis II, when it is absent under physiological conditions (Han *et al.*, 2017), should abrogate the CSF-arrest of mature mouse oocytes. Indeed, expression of WT, but not MRL mutant, *M.m.* cyclin B3 in mature CSF-arrested metaphase II wildtype oocytes resulted in rapid second PB extrusion with sister chromatid separation (Figures 1B and 1C), in accordance with (Meng et al., 2020). From these data we concluded that Cdk1/cyclin B3 negatively regulates CSF activity.

The key protein mediating CSF-arrest in mouse oocytes is Emi2 (Madgwick *et al.*, 2006; Shoji *et al.*, 2006). Thus, if precocious CSF-activity accounts for the metaphase I arrest of *Ccnb3*$^{-/-}$ oocytes, knock-down of Emi2 should rescue meiosis I progression. This was indeed the case: Injection of Emi2 morpholino oligonucleotides (MO) into prophase I arrested *Ccnb3*$^{-/-}$ mouse oocytes rescued chromosome segregation and PB extrusion upon release into meiosis (Figure 1D). Consistent with Emi2 being required for entry into meiosis II (Madgwick *et al.*, 2006; Shoji *et al.*, 2006), these oocytes then failed to proceed into meiosis II. Instead, they decondensed their DNA and entered interphase. Nevertheless, the rescue of meiosis I allowed us to conclude that untimely Emi2 activity in meiosis I accounts for the metaphase I arrest observed in *Ccnb3*$^{-/-}$ oocytes.

Establishment of CSF-arrest requires the activity of the Mos/MAPK pathway signaling to Emi2 (Wu and Kornbluth, 2008). Mos/MAPK activity steadily increases upon resumption of meiosis, being present from metaphase I until CSF-arrest in mouse oocytes (Verlhac et al., 1994; Verlhac et al., 1996). If oocytes without cyclin B3 were to establish a CSF-arrest in meiosis I instead of meiosis II, inhibition of the Mos/MAPK pathway with the MEK inhibitor UO126 (Favata et al., 1998) should override this premature arrest (Figure 2A). Indeed, similar to control metaphase II oocytes that exit CSF-arrest (Phillips et al., 2002), the majority of *Ccnb3*$^{-/-}$ oocytes treated with

UO126 from prometaphase I onwards, extruded a PB and segregated bivalent chromosomes into dyads indicating exit from meiosis I (Figure 2B-D). We also observed sister chromatid separation in UO126-treated *Ccnb3*$^{-/-}$ oocytes indicating in line with the reported requirement of MEK activity for the MII arrest that these oocytes failed to establish a CSF-arrest in meiosis II (Figure 2D). Accordingly, removal of MEK inhibitor immediately after first PB extrusion allowed *Ccnb3*$^{-/-}$ oocytes to progress into metaphase II with only dyads being present. Strikingly, these oocytes could then be activated with strontium to segregate sister chromatids, indicating that they had mounted a second CSF-arrest, now at the correct cell cycle stage (Figure 2E). Hence, loss of cyclin B3 can be rescued by interfering with the signaling pathway leading to untimely CSF-arrest in meiosis I.

Role of cyclin B3 is conserved in *Xenopus laevis* oocytes

The metaphase I arrest of *Ccnb3*$^{-/-}$ mouse oocytes can be rescued by expressing *Xenopus laevis* (*X.l.)* cyclin B3, indicative of an evolutionarily conserved function (Karasu *et al.*, 2019). This result opened up the exciting possibility that *Xenopus* oocytes can be used as a complementary model system for biochemical approaches not feasible in the mouse. To this end, we first raised antibodies against a fragment (aa 1-150, Ab-B3^{F}) and peptide (aa 6-26, Ab-B3^{P}) of *X.l.* cyclin B3 and confirmed their specificity by Trim-Away. This technique exploits the E3 ligase and cytosolic antibody receptor TRIM21 to target endogenous proteins bound to antibodies for proteasomal degradation (Clift et al., 2017; Clift et al., 2018). Western blot (WB) analysis using Ab-B3^{F} confirmed that cyclin B3 was depleted in oocytes injected with TRIM21 mRNA and the peptide antibody Ab-B3^{P}, but not control (Ctrl) Ab (Figure S2A). Unlike previously suggested based on WB data using a different antibody (Hochegger et al., 2001), cyclin B3 was expressed in prophase

I *Xenopus* oocytes (Figure 3A). Upon progesterone (PG) treatment these oocytes resumed meiosis as shown by reduced SDS-PAGE mobility of the APC/C subunit Cdc27, phosphorylation of MAPK (indicating activation of the Mos/MAPK pathway), and loss of inhibitory Cdk1 phosphorylation (a hallmark of Cdk1 activation) (Figures 3A and S1). As a macroscopic marker for successful meiotic resumption, we determined the percentage of oocytes displaying the characteristic white spot in their animal hemisphere, caused by pigment dispersal at the cell cortex as a consequence of germinal vesicle breakdown (GVBD, asterisk in Figure 3A marks the time when >80% of the oocytes had undergone GVBD). Cyclin B3 levels remained constant until about 1 hour post white spot appearance (4h post PG). The subsequent decline in its levels coincided with a transient downshift of Cdc27 marking the meiosis I to meiosis II transition (Figures 3A and S1). Thus, cyclin B3 is expressed in *X.l.* female meiosis I, degraded at meiosis I exit and does not re-accumulate in meiosis II.

Next, we assessed the consequences upon loss of cyclin B3 in *Xenopus* oocytes. Cyclin B3 was depleted from prophase I oocytes with Trim-Away (Figures 3B and S2B). Control and cyclin B3-depleted oocytes efficiently resumed meiosis upon PG stimulation. Thus, frog oocytes, like mouse oocytes, do not require cyclin B3 for efficient meiotic resumption (Karasu *et al.*, 2019; Li *et al.*, 2019) (Figure 3B). However, unlike control oocytes, cyclin B3-depleted *X.l.* oocytes were unable to exit meiosis I but remained arrested in metaphase I as judged by the presence of stable cyclin B2, persistently upshifted Cdc27, and failure of PB extrusion (Figures 3B, C and S2B). Thus, the phenotype of *Xenopus* oocytes depleted of cyclin B3 mimics that of *Ccnb3*$^{-/-}$ mouse oocytes. It is specific to cyclin B3 depletion because co-injection of *in-vitro* translated (IVT) Flag-tagged *X.l.* cyclin B3$^{\Delta 15}$ resistant to Trim-Away rescued the meiosis I arrest (Figures 3B, 3C, S2B, S2C)..

Crucially, depletion of Emi2 by co-injecting Emi2 MO, allowed cyclin B3 depleted oocytes to exit meiosis I, as judged by the downshift of Cdc27 and cyclin B2 degradation (Figures 3D, S2D, and S2E). As in the mouse, *Xenopus* Emi2 is required for entry into meiosis II (Ohe *et al.*, 2007; Tang *et al.*, 2008; Tung *et al.*, 2007) and hence, these oocytes subsequently entered interphase (constantly downshifted Cdc27 and no re-accumulation of cyclin B2, Figure 3D). Thus, in both *Xenopus* and mouse oocytes, cyclin B3 loss results in a precocious, Emi2-mediated CSF-arrest in meiosis I suggesting that the function of cyclin B3 is evolutionarily conserved in vertebrate oocytes.

Cyclin B3 targets Emi2 for Plk1-dependent manner degradation

To understand how cyclin B3 suppresses the function of Emi2 as CSF, we utilized *Xenopus* CSF egg extract prepared from metaphase II oocytes. Due to the absence of cyclin B3 in meiosis II (Figure 3A), ectopic cyclin B3 can be functionally analysed without interfering with endogenous cyclin B3. CSF extract supplemented with mRNA encoding *X.l.* Flag-cyclin $B3^{WT}$ was unable to maintain the CSF-arrest and exited meiosis II as indicated by cyclin B2 degradation (Figure 4A). Under these conditions, Emi2 was hyperphosphorylated followed by its degradation (see lambda phosphatase (λ) treated samples). Since Emi2 depletion is sufficient to activate the APC/C in CSF extract (Schmidt *et al.*, 2005), we concluded that cyclin $B3^{WT}$ induced meiotic exit by targeting Emi2 for degradation. Cyclin $B3^{MRL}$, unable to bind Cdk1 (Figure S3A), did not destabilize Emi2 and accordingly the extract maintained the CSF-arrest (Figure 4A). Thus, expression of WT *Xenopus* cyclin B3 in CSF extract results in the identical phenotype observed upon expression of

mouse cyclin B3 in mouse CSF-arrested oocytes (Figure 1C), i.e., override of the Emi2-mediated CSF-arrest.

Previous studies showed that MPF (Maturation-promoting factor) activity remains constant during *Xenopus* CSF-arrest despite ongoing cyclin B1 synthesis (Isoda et al., 2011; Wu et al., 2007b). At high MPF activity, Cdk1/cyclin B1 phosphorylates the N-terminus of Emi2 resulting in initial recruitment of Plk1, which then creates its own high affinity docking sites by phosphorylating Emi2 at T170 and T195 (Figure S3B). Subsequently, Plk1 targets Emi2 for degradation by $SCF^{\beta\text{-}TRCP}$ through phosphorylation of two phosphodegrons ($DSGX_3S^{38}$ and $DSAX_2S^{288}$). The drop in Emi2 levels and the resulting transient APC/C activation reduces MPF activity, tipping the balance in favor of PP2A-B56, which dephosphorylates the inhibitory sites on Emi2 causing its re-stabilization (Inoue et al., 2007; Isoda *et al.*, 2011; Nishiyama et al., 2007; Wu et al., 2007a; Wu *et al.*, 2007b). Hence, transitory phosphorylation of Emi2 controls the APC/C to maintain MPF activity at constant levels while cyclin B1 synthesis continues.

Based on these data, we hypothesized that cyclin B3 destabilizes Emi2 *via* this pathway. Indeed, endogenous Emi2 remained stable when cyclin $B3^{WT}$ was expressed in CSF extract treated with the Plk1 inhibitor BI2536 (Figure 4B). Next, we analysed the stability of ectopic IVT Myc-Emi2. Please note that we used Emi2 variants deficient in APC/C inhibition (ZBR) (Heim et al., 2018) to preclude that they interfere with CSF release. Mutation of the phosphodegron $DSGX_3S^{38}$ (DSG) strongly stabilized ectopic $Emi2^{ZBR}$, while the $DSAX_2S^{288}$ (DSA) mutation had only a minor effect (Figure 4C). The DSG/DSA double mutant was completely stable in CSF extract expressing *X.l.* cyclin $B3^{WT}$. Mutation of T170 (T170A), but not of T195 (T195A), also resulted in stabilization of

Emi2^{ZBR} (Figure 4C). During CSF-arrest, Plk1 itself phosphorylates T170 and this depends on Cdk1/cyclin B1 phosphorylation-dependent recruitment of Plk1 to Emi2's N-terminus (Figure S3B) (Isoda *et al.*, 2011). Crucially, Emi2^{ZBR} carrying non-phosphorylatable mutations at all four N-terminal Cdk1 sites (4 T/SP > 4A) was stable in presence of cyclin B3^{WT} (Figure S3C). Of these sites, mutation of T97 (T97A), an evolutionarily highly conserved site (Figure S3B), was sufficient to stabilize Emi2^{ZBR} (Figures 4C and S3C). Thus, Plk1 activity as well as T97, T170 and the two phosphodegrons present in *Xenopus* Emi2 are critical for its degradation triggered by cyclin B3.

Cyclin B1 and B3 confer distinct Cdk1 substrate preference *in vitro*

Endogenous cyclin B1 was not able to complement for loss of cyclin B3 in mouse and frog oocytes, resulting in the observed metaphase I arrest. Furthermore, only addition of IVT WT *X.l.* cyclin B3, but not *X.l.* cyclin B1, induced rapid Emi2 degradation in CSF extracts and CSF release (Figures 4D and S3D). These observations suggest that Cdk1/cyclin B3 and Cdk1/cyclin B1 differ in their substrate specificity with only the former being able to efficiently phosphorylate Emi2 for fast degradation. To address this, we performed *in vitro* kinase assays using Flag-tagged cyclin B3 or cyclin B1 immunoprecipitated from CSF extract and an MBP-tagged Emi2 fragment (aa 1-200) as substrate. The respective MRL mutants served as negative controls. WB analyses of the IP fractions confirmed that Cdk1 was efficiently co-precipitated with the respective WT, but not MRL mutants (Figure S3E). Since T97 was most critical for degradation of Emi2 (Figures 4C and S3C), Emi2$^{1-200}$ carried non-phosphorylatable mutations in the other three N-terminal Cdk1 sites to specifically detect T97 phosphorylation. Indeed, Cdk1/cyclin B3^{WT}, but not Cdk1/cyclin B1^{WT}, efficiently phosphorylated Emi2$^{1-200}$ (Figure 4E, upper panel, lanes 17-21 and 7-11). We confirmed that Cdk1/cyclin B3 and Cdk1/cyclin B1 were equally active using histone H1 as generic Cdk1 substrate (Fig. 4E, lower panel). Notably, Emi2 with an additional mutation at T97 (T97A) was not

detectably phosphorylated by Cdk1/cyclin B3^{WT} (lanes 22 – 27). From these data we concluded that cyclin B3 and cyclin B1 distinguish themselves in their substrate specificity: Cdk1/cyclin B3 and not Cdk1/cyclin B1 can efficiently phosphorylate Emi2 on T97 *in vitro*.

Cyclin B3 destabilizes Emi2 in *Xenopus* meiosis I oocytes

A key corollary of our hypothesis is that in absence of cyclin B3 endogenous Emi2 should accumulate prematurely during meiosis I in intact oocytes. To test this, we immunoblotted for Emi2 in control- or cyclin B3-depleted oocytes released with PG. Since all samples were treated with λ-phosphatase to correctly determine Emi2 levels and thus, all phosphorylation-dependent meiotic markers were lost, successful meiotic resumption was quantified using the appearance of the white spot. In control-depleted oocytes, Emi2 was not detected until 5h post PG treatment (Figure 5A). Note, that at 4h post PG all oocytes had already undergone GVBD. In cyclin B3-depleted oocytes however, low levels of Emi2 were already detectable 3h after PG treatment, at a time when just ~50% of oocytes had resumed meiosis I (Figure 5A). Premature Emi2 accumulation in early meiosis I was partially suppressed by co-injecting IVT Flag-cyclin B3$^{\Delta 15}$ (Figure 5A). We repeated the experiment but omitted the phosphatase treatment so that the timing in Emi2 accumulation can be correlated with the upshift of Cdc27 at entry into meiosis I. Indeed, Emi2 accumulated 1 hour earlier in cyclin B3-depleted oocytes compared to control oocytes, and relative to the initial upshift of Cdc27 (Figure S4A). Premature Emi2 accumulation was rescued by IVT Flag-cyclin B3$^{\Delta 15}$. Note, that Emi2 did not accumulate to the same levels under rescue conditions because ectopic cyclin B3 was not completely degraded at exit from MI preventing full accumulation of Emi2. To further corroborate this finding, we analysed the stability of IVT Myc-Emi2^{ZBR} in cyclin B3-depleted and control oocytes. Indeed, loss of cyclin B3 drastically stabilized Emi2^{ZBR} (Figure 5B).

In line with our data from CSF-extract, mutation of T170, T97, or the two phosphodegrons also resulted in significant stabilization of Emi2^{ZBR} in otherwise untreated oocytes undergoing meiosis I (Figures 5C and S4B). Altogether, we concluded that cyclin B3 prevents untimely CSF-arrest in *Xenopus* meiosis I oocytes by targeting Emi2 for degradation.

Cyclin B3 destabilizes Emi2 through a conserved mechanism

Finally, we asked whether the degradation mechanism is conserved between frog and mouse. Since *X.l.* cyclin B3 (419 aa) is only one-third the size of mouse cyclin B3 (1396 aa), but still capable of rescuing *Ccnb3*$^{-/-}$ mouse oocytes (Karasu *et al.*, 2019), we first tested whether *X.l.* cyclin B3 can induce the degradation of mouse Emi2 in *Xenopus* CSF extracts. As for *Xenopus* Emi2, we used a mouse Emi2 mutant (IVT Myc-Emi2^{ZBR}) deficient in APC/C inhibition. Indeed, expression of *X.l.* cyclin B3^{WT}, but not MRL mutant, destabilized mouse Emi2^{ZBR} in CSF extract (Figure 6A). Crucially, mutation of T86 (T86A), corresponding to T97 in *X.l.* Emi2 (Figure S3B), stabilized mouse Emi2^{ZBR} (Figure 6A). Furthermore, both T152 (T170 in *X.l.* Emi2) and the only phosphodegron present in mouse Emi2 (DSGX$_2$S^{279}) were equally essential for its degradation. These findings indicate that mouse and frog Emi2 indeed share the same mechanism of cyclin B3-dependent degradation.

Cyclin B3 targets Emi2 in CSF-arrested mouse oocytes

Next, we turned to mouse oocytes. However, due to the low number of oocytes obtained per mouse and low extract yield per oocyte, endogenous Emi2 can be only scarcely detected by WB precluding the possibility to reliably quantify Emi2 levels. Thus, we analysed the degradation of YFP-tagged Emi2^{ZBR} variants in CSF-arrested oocytes by live imaging. Endogenous cyclin B3 is not translated in mouse CSF oocytes (Han *et al.*, 2017), allowing us to investigate the effect of

ectopic *M.m.* cyclin B3 on Emi2^{ZBR}-YFP stability. Of note, we used stable *M.m.* cyclin B3 (ΔDbox) to ensure that protein levels were sufficient to efficiently target ectopic Emi2. Indeed, Emi2^{ZBR} was degraded in CSF oocytes co-injected with mRNA encoding *M.m.* cyclin B3$^{\Delta Dbox}$, but not the corresponding MRL mutant (Figure 6B). Oocytes expressing cyclin B3$^{\Delta Dbox}$, but not the MRL variant, underwent anaphase II, indicating that endogenous Emi2 was also degraded upon expression of *M.m.* cyclin B3$^{\Delta Dbox}$. Crucially, T86A Emi2^{ZBR}-YFP was stable when co-expressed with *M.m.* cyclin B3$^{\Delta Dbox}$ in CSF oocytes (Figures 6C and S5A). Additionally, mutation of T152 and the phosphodegron stabilized Emi2^{ZBR}-YFP indicating that Cdk1/cyclin B3 in concert with Plk1 targets Emi2 for degradation. To further corroborate the function of Plk1 in Emi2 degradation, we investigated if CSF release induced by cyclin B3 expression depends on Plk1 activity by treating oocytes with BI2536 , and this was indeed the case (Figure S5B).

Mouse cyclin B3 prevents CSF-arrest in meiosis I through an evolutionary conserved mechanism

Finally, we asked whether cyclin B3 controls Emi2 stability in its physiological context, i.e., mouse oocyte meiosis I. First, we analysed Emi2^{ZBR}-YFP stability in WT oocytes progressing through meiosis I. Emi2^{ZBR}-YFP was efficiently degraded in meiosis I, while the T86A variant was stabilized (Figure 7A). To confirm that degradation of Emi2^{ZBR} during meiosis I in WT oocytes was mediated by cyclin B3, we analysed *Ccnb3*$^{-/-}$ oocytes expressing either WT or MRL mutant *M.m*. cyclin B3. Emi2^{ZBR}-YFP was efficiently degraded in *Ccnb3*$^{-/-}$ oocytes expressing WT cyclin B3, but almost completely stable in MRL mutant expressing oocytes (Figure 7B) demonstrating that Cdk1/cyclin B3 destabilizes Emi2 during meiosis I in mouse oocytes.

In mouse oocytes, expression of a small Emi2 fragment (aa 80-115) was shown to induce a metaphase I arrest by an unknown mechanism (Suzuki *et al.*, 2010). Intriguingly, Emi2$^{80\text{-}115}$ lacks the APC/C inhibitory domain (Figure S6A) and was only able to provoke an arrest when endogenous Emi2 was present (Suzuki *et al.*, 2010). We therefore hypothesized that ectopic Emi2$^{80\text{-}115}$, containing T86, outcompetes the phosphorylation of endogenous Emi2 by Cdk1/cyclin B3, resulting in stabilization of endogenous Emi2, and hence, the observed metaphase I arrest. To test our hypothesis, we first expressed Emi2$^{80\text{-}115}$-RFP in WT prophase I oocytes that were released into meiosis I. Indeed, these oocytes largely failed to exit meiosis I as indicated by the failure of first PB extrusion (Figure 7C). Intriguingly, expression of T86A mutated Emi2$^{80\text{-}115}$-RFP at comparable levels (Figure S6B) did not interfere with exit from meiosis I confirming our hypothesis that T86 phosphorylation of Emi2$^{80\text{-}115}$ is critical for the observed dominant-negative effect of the fragment. If the metaphase I arrest was due to competition between Emi2$^{80\text{-}115}$ and endogenous Emi2 for T86 phosphorylation by cyclin B3, increasing cyclin B3 levels should suppress the arrest. Indeed, co-expression of WT, but not MRL mutant, cyclin B3 allowed *Ccnb3*$^{-/-}$ oocytes to overcome the arrest caused by Emi2$^{80\text{-}115}$ expression, as determined by using PB extrusion as readout for exit from meiosis I (Figure 7D). In conclusion, also in mouse oocytes cyclin B3 in association with Cdk1 destabilizes Emi2 in meiosis I, a prerequisite for cell cycle progression.

Discussion

Emi2 destabilization in meiosis I

We identified cyclin B3 as the key protein that interferes with establishment of precocious CSF-arrest in female meiosis I by keeping Emi2 levels below the threshold critical for APC/C inhibition.

Based on our data, we propose a model where Emi2 degradation is initiated by direct phosphorylation of T86/T97 (mouse/frog) by Cdk1/cyclin B3 (Figure 7E). Thereby, Cdk1/cyclin B3 serves as priming kinase for Plk1 triggering the recruitment of Emi2 to Plk1, leading to phosphorylation of T152/T170 (mouse/frog) to create the previously identified docking site for Plk1 (Hansen *et al.*, 2006; Jia et al., 2015). Plk1 then phosphorylates Emi2 at its phosphodegron(s) resulting in its efficient degradation via the E3 ligase $SCF^{\beta\text{-}TRCP}$. To compare, in *Xenopus* oocytes at fertilization in meiosis II, Emi2 phosphorylation on T195 (another Plk1 docking site) by calcium/calmodulin dependent protein kinase II (CaMKII) primes Emi2 for Plk1, which then targets it for degradation by phosphodegron phosphorylation (Liu and Maller, 2005; Rauh *et al.*, 2005). Thus, Cdk1/cyclin B3 and CaMKII feed in the same Plk1-dependent degradation pathway resulting in fast and efficient Emi2 destruction, although with two completely different signaling inputs, i.e., activation of Cdk1/cyclin B3 during meiosis I versus calcium-triggered activation of CaMKII at fertilization.

By identifying the functional relationship between cyclin B3 and Emi2, our study provides the molecular explanation why *Ccnb3*$^{-/-}$ knockout mice are viable with infertile females (Bouftas and Wassmann, 2019; Karasu *et al.*, 2019; Karasu and Keeney, 2019; Li *et al.*, 2019). However, it does not explain why *Ccnb3*$^{-/-}$ knockout males are fertile even though Emi2 is expressed in spermatocytes (Gopinathan et al., 2017; Shoji *et al.*, 2006). Notably, spermatocytes – in contrast to oocytes – depend on Emi2 for progression through meiosis I as loss of Emi2 causes an arrest in early diplotene of male prophase I due to reduced Cdk1 activity (Gopinathan *et al.*, 2017). Based on the dependency of spermatocytes on Emi2 for progression through meiosis I, we speculate that

the activity and/or stability of Emi2 must be differentially regulated in spermatocytes compared to oocytes, allowing male germ cells to progress through meiosis I without cyclin B3.

The functional relationship between cyclin B3 and Emi2 we identified provides the molecular explanation why the expression of a short N-terminal fragment of mouse Emi2, lacking any APC/C inhibitory elements, causes a metaphase I arrest in mouse oocytes, but only when endogenous Emi2 is present (Suzuki *et al.*, 2010). As suggested by our data (Figures 7C and 7D), this fragment, comprising T86, titrates cyclin B3 away from endogenous Emi2 resulting in its stabilization and hence, APC/C inhibition and the observed metaphase I arrest.

Expression of cyclin B3 exclusively in meiosis I

A central pillar of the mechanism underlying Emi2 accumulation in meiosis II is the concurrent absence of cyclin B3. This meiosis I-exclusive expression of cyclin B3 allows the two meiotic divisions to be functionally distinct such that oocytes are only able to install CSF-arrest at metaphase of meiosis II. At exit from meiosis I, cyclin B3 – like the canonical B-type cyclins B1 and B2 – is targeted for degradation in a destruction-box and APC/C-dependent manner (Bouftas 2019). Importantly, however, and in contrast to cyclin B1 and B2, cyclin B3 does not re-accumulate in meiosis II (Figure 3A) (Han *et al.*, 2017). What makes cyclin B3 unique in that it is only expressed in meiosis I? In *Xenopus* oocytes, regulation of cyclin B3 translation is distinct from cyclin B1 (Pique et al., 2008): While cyclin B1 mRNA is translationally repressed in prophase I arrested oocytes and strongly activated upon PG stimulation, cyclin B3 mRNA, on the contrary, seems to be weekly repressed already during the arrest and not significantly activated upon PG treatment. Thus, translation of cyclin B3 mRNA ensures that cyclin B3 is present at sufficiently levels early in meiosis I, but does not re-accumulate following its destruction at exit from meiosis

I. Using loading of *Ccnb3* transcripts onto polysomes as readout for active translation, it has likewise been shown in mouse oocytes that cyclin B3 translation is strictly limited to meiosis I, while cyclin B1 and B2 mRNAs are efficiently loaded during both meiotic divisions (Han *et al.*, 2017). Yet, the specific regulatory elements in cyclin B3 mRNA limiting its expression exclusively to meiosis I are unknown and further studies are needed to understand the underlying mechanisms.

Cdk1/cyclin B3 substrate specificity for Emi2

Once oocytes progress into meiosis II, Emi2 becomes stabilized due to the absence of cyclin B3. In *Xenopus* CSF-arrested oocytes, Cdk1/cyclin B1 phosphorylates Emi2 at multiple N-terminal sites that result in Emi2 inactivation as well as destabilization *via* Plk1-mediated phosphorylation of the DSG/DSA phosphodegrons (Isoda *et al.*, 2011; Wu *et al.*, 2007a). Notably, T97 of *X.l.* Emi2, which we show here to be critical for Cdk1/cyclin B3 mediated degradation of Emi2, is part of the reported N-terminal Cdk1/cyclin B1 sites. This raises the important question of how oocytes can maintain the CSF-arrest with high Cdk1/cyclin B1 activity when at the same time Cdk1/cyclin B1 targets Emi2 for degradation.

As shown by our *in vitro* results (Figure 4E), T97 of *X.l.* Emi2 is a much better substrate of Cdk1 associated with cyclin B3 than with cyclin B1 demonstrating that cyclin B3 and B1 must distinguish themselves in their substrate specificities. While the *in vivo* situation in oocytes may differ from the *in vitro* situation, the phenotypes we observed argue against the idea of Emi2 being an optimal Cdk1/cyclin B1 substrate. First, the metaphase I arrest in mouse and frog oocytes upon loss of cyclin B3 was not rescued by endogenous cyclin B1 still present (this study and (Karasu *et al.*, 2019)). Second, even ectopically expressed cyclin B1 failed to complement the lack of

endogenous cyclin B3 (Karasu *et al.*, 2019). Third, supplementing CSF extract with cyclin B3, but not cyclin B1, triggers rapid and efficient degradation of Emi2 resulting in meiotic exit. Slow and inefficient degradation of Emi2 in the presence of Cdk1/cyclin B1 matches the proposed thermostat system, which transiently destabilizes Emi2 to keep MPF activity constant despite ongoing cyclin B1 synthesis during CSF-arrest (Isoda *et al.*, 2011). In sum, we conclude that differential substrate specificity of cyclin B3 and B1 is the reason why Emi2 is efficiently destabilized by cyclin B3 in meiosis I, while being stable in the presence of cyclin B1 in meiosis II.

Counting the meiotic divisions

In conclusion, our work elucidates the long-standing question how vertebrate oocytes "count" the meiotic divisions, to arrest in meiosis II and not precociously, in meiosis I, for fertilization and successful embryo development. Our results demonstrate the essential and oocyte-specific role cyclin B3 occupies, by targeting the CSF-factor Emi2 for degradation through preferential substrate phosphorylation. Because all components for CSF-arrest are already functional in meiosis I, cyclin B3 ensures timely oocyte arrest for fertilization in meiosis II. Loss of cyclin B3 in mice can result in triploid embryos upon fertilization or intracytoplasmic sperm injection (Chotiner et al., 2022; Li *et al.*, 2019). Notably, mutations of *Ccnb3* in human oocytes have been associated with recurrent triploid pregnancies and miscarriage (Fatemi et al., 2020; Rezaei et al., 2021). Our work indicates that this phenotype is due to untimely CSF-arrest in oocytes devoid of functional cyclin B3. Future work will address how cyclin B3 substrate preference for Emi2 is brought about, and how cyclin B3 presence is restrained to meiosis I.

Limitations of the study

Our work demonstrates that cyclin B3 targets Emi2 for phosphorylation in meiosis I and thereby prevents precocious CSF-arrest, but the details of the Emi2 degradation mechanism in meiosis I are still unknown and require further study. We think that cyclin B3's essential role in oocyte meiosis is conserved throughout the vertebrate kingdom, however, this still has to be verified in oocytes of other species, including humans. Triploid pregnancies observed in humans and mice indicate that fertilization is possible in absence of cyclin B3, but the *in vivo* significance of cyclin B3's role in mammalian oocytes and embryos still has to be further clarified.

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Author contributions

Mouse oocyte experiments were performed by NB with help from DC. Double rescue experiments in Fig 2 were done by RM, FP performed initial experiments in *Xenopus* oocytes, both worked under the supervision of NB. Mouse husbandry and genotyping were done by DC. Experiments on *Xenopus* oocytes and with CSF egg extract were performed by LS, MH, MW, CK, JM, RD, HAA,

PW, and AH. Cyclin B3 antibody was generated by MB. *In vitro* kinase assays were performed by RD. Figures were prepared by NB, LS, MH, RD, AH, TUM and KW. The manuscript was written by TUM and KW with input from all authors. Supervision, funding acquisition and project administration were done by TUM and KW.

Declaration of Interests

The authors declare no competing interests.

Main figure legends

Figure 1. Cyclin B3 counteracts CSF-arrest in mouse oocytes

(A) Above, scheme of experimental setting. Below, chromosome spreads of *Ccnb3*$^{-/-}$ oocytes that were left untreated, or 1 hour after strontium treatment in metaphase I (7h30 after resumption of meiosis I). Chromosomes are stained with Hoechst (grey), centromeres with CREST (green). Quantification shows % of oocytes with dyads.

(B) Above, scheme of experimental setting. Below, chromosome spreads of CSF-arrested oocytes injected with mRNA encoding WT or MRL mutant M.m. cyclin B3. Quantification shows % of oocytes with sister chromatids.

(C) Time-lapse imaging of CSF-arrested oocytes injected as in (B) and incubated with SiR-DNA to visualize chromosomes. Quantification shows % of oocytes that exit CSF-arrest. Arrow indicates anaphase II.

(D) Control or Emi2 Morpholino (MO) knockdown *Ccnb3*$^{-/-}$ oocytes were used for time-lapse acquisitions using SiR-DNA. Scheme and quantification of oocytes that exit meiosis I are shown on the right. Arrow indicates anaphase I.

All error bars indicate means ± SD of the indicated number of oocytes from at least 3 independent experiments, indicated as dots. Scale bars: A/B: 10µm, C/D: 50µm

See also Figure S1.

Figure 2. Metaphase I arrest in *Ccnb3*$^{-/-}$ oocytes depends on Mos-MAPK pathway

(A) Scheme of experimental setting to rescue meiosis I (1, panel B-D) and II (2, panel E) in *Ccnb3*$^{-/-}$ oocytes.

(B, C) Oocytes were treated with UO126 in prometaphase I, 4h after resumption of meiosis and subjected to time-lapse imaging in presence of SirDNA. (B) Quantification shows % of oocytes extruding a PB. Error bars indicate means ± SD of the indicated number of oocytes from at least 3 independent experiments, indicated as dots. (C) Anaphase I onset is indicated with an arrow head (hours after resumption of meiosis I are specified).

(D) Oocytes treated as in (B) were analysed by chromosome spreads after overnight incubation. Chromosomes are stained with Hoechst (grey), centromeres with CREST (green).

(E) Oocytes were treated with UO126 as in (B), washed with medium without UO126 15 min after PB extrusion, and fixed for chromosome spreads after activation with strontium were indicated (+Str).

Scale bars: C: 50µm, D/E: 10µm, n indicates number of oocytes analysed from three independent experiments for each condition.

Figure 3. Loss of *Xenopus* cyclin B3 causes CSF-arrest in meiosis I

(A) *Xenopus* prophase I oocytes were treated with progesterone (PG) and at indicated time points immunoblotted for *X.l.* cyclin B3 (cycB3), Cdc27, inhibitory and activating phosphorylation of Cdk1 (ppCdk1) and MAPK (pMAPK), respectively. Asterisk marks the time when >80% of oocytes showed a white spot, indicating meiosis resumption. p150 served as loading control.

(B) Prophase I oocytes were injected with Flag-TRIM21 mRNA and control (Ctrl) or cyclin B3 peptide (Ab-B3^{P}) antibody. Where indicated, empty or Flag-cyclin B3$^{\Delta 15}$ IVT was co-injected. 18

hours later, oocytes were treated with PG to induce meiotic resumption and at indicated time points analysed by WB. See also Figure S2B.

(C) Oocytes treated as in B were microscopically analysed. Error bars indicate means ± SD of the indicated number of oocytes from 3 independent experiments (n), indicated as dots. Scale bars: 10μm

(D) Prophase I oocytes were injected with Flag-TRIM21 mRNA and control Ab or Ab-B3^{P} together with control MO or *Xenopus* Emi2 MO. 18 hours later, oocytes were treated with PG and immunoblotted. See also Figure S2D. Arrowheads point to the 5- and 6-hour time points post PG, where Cdc27 is transiently down-shifted in control-depleted oocytes, but constantly upshifted in cyclin B3-depleted oocytes expressing Emi2. In the absence of Emi2, cyclin B3-depleted oocytes fail to enter MII as evidenced by constantly down-shifted Cdc27. (**A**)-(**D**) All experiments were performed in triplicate and representative WB analyses are depicted.

See also Figure S1.

Figure 4. *Xenopus* cyclin B3 targets Emi2 for degradation in CSF extracts

(A) At the indicated time points after supplementing CSF extract with mRNA encoding Flag-tagged wildtype (WT) or MRL mutant (MRL) *X.l.* cyclin B3, extract samples were treated with lambda phosphatase (λ) were indicated, and immunoblotted. Tubulin served as loading control.

(B) CSF extract treated with 20μM BI2536 or DMSO was supplemented with *X.l.* Flag-cyclin B3^{WT} mRNA and samples were immunoblotted at indicated time points.

(C) CSF extract was supplemented with IVT Myc-Emi2^{ZBR} variants and samples were immunoblotted at indicated time points after adding *X.l.* Flag-cyclin B3^{WT} mRNA.

(D) IVT *X.l.* Flag-cyclin $B1^{WT}$, -cyclin $B1^{MRL}$, or -cyclin $B3^{WT}$ was added to CSF extract and at indicated time points samples were immunoblotted. Asterisk marks unspecific band.

(E) *In vitro* kinase assay using Flag-tagged WT or MRL mutant of *X.l.* cyclin B1 (cycB1) or cyclin B3 (cycB3) expressed in CSF extracts followed by anti-Flag immunoprecipitation. MBP/His-tagged Emi2 (aa 1-200) with S43, S73, and S157 mutated to A was used as substrate. Where indicated, the fragment contains an additional mutation at T97 (T97A). See also Figure S3E. (**A**)-(**E**) All experiments were performed in triplicate and representative analyses are depicted.

Figure 5. *Xenopus* oocytes lacking cyclin B3 prematurely accumulate Emi2

(A) *Xenopus* prophase I oocytes were injected with Flag-TRIM21 mRNA and Ctrl Ab or Ab-$B3^{P}$ and empty or *X.l.* Flag-cyclin $B3^{\Delta 15}$ IVT. 18 hours later, PG was added, and oocytes were immunoblotted at indicated time points. All samples were treated with lambda phosphatase (λ). The percentage of oocytes undergoing meiotic resumption upon PG treatment was quantified (see numbers below p150 WB). p150 serves as loading control. Red box highlights time points to compare, to appreciate Emi2 accumulation in oocytes lacking cyclin B3. IgG HC: injected Ab heavy chain

(B) Prophase I oocytes were injected with Flag-TRIM21 mRNA and Ctrl Ab or Ab-$B3^{P}$ and 18 hours later with IVT Myc-$Emi2^{ZBR}$. One hour later, oocytes were treated with PG and immunoblotted at indicated time points. All samples were treated with lambda phosphatase (λ).

(C) Prophase I oocytes were injected with indicated IVT Myc-$Emi2^{ZBR}$ variants and at indicated time points after PG addition analysed by immunoblotting. Tubulin served as loading control. Where indicated (λ), samples were treated with lambda phosphatase. Experiments were performed

as duplicate (B) or triplicate (A and C) and representative WB analyses are depicted. Asterisk marks unspecific band.

Figure 6. Cyclin B3 can target Emi2 for degradation in CSF-arrested mouse oocytes

(A) *Xenopus* CSF extract was supplemented with IVT *M.m.* Myc-Emi2^{ZBR} variants. At indicated time points after adding WT or MRL mutant *X.l.* Flag-cyclin B3 mRNA, samples were treated with lambda phosphatase (λ) and immunoblotted. Tubulin served as loading control. The experiment was performed in triplicate and a representative WB is depicted.

(B) Scheme illustrating the experimental setting and representative images showing time-lapse imaging of mouse WT CSF-oocytes injected with mRNA encoding Emi2^{ZBR}-YFP and either cyclin B3$^{\Delta Dbox}$ or cyclin B3$^{\Delta Dbox\ MRL}$. SiR-DNA was used to visualize chromosomes. Anaphase II is indicated with an arrow. Scale bar: 50μm.

(**C**) Quantifications of the indicated Emi2^{ZBR}-YFP mutants co-injected with cyclin B3$^{\Delta Dbox}$ into CSF-arrested oocytes. Acquisitions in the YFP channel (1z section) were done every 20 minutes. All oocytes analysed underwent anaphase II within 4 hours. (C): n = number of oocytes from at least 3 independent experiments, error bars indicate means ± SEM. See also Figure S5.

Figure 7. Cyclin B3 targets Emi2 in mouse oocyte meiosis I

(A) The scheme above illustrates the experimental setting. Prophase I-arrested oocytes were injected with Emi2^{ZBR}-YFP or Emi2$^{ZBR\ T86A}$-YFP and induced to resume meiosis I. YFP fluorescence was quantified every 30min.

(B) *Ccnb3*$^{-/-}$ prophase I oocytes were injected with mRNA encoding Emi2^{ZBR}-YFP and either WT or MRL mutant *M.m.* cyclin B3. Following release, the YFP signal was quantified every hour.

(C) WT prophase I oocytes were injected with WT or T86A Emi2$^{80\text{-}115}$-RFP mRNA. Following release, first PB extrusion was quantified.

(D) *Ccnb3*$^{-/-}$ prophase I oocytes were injected with WT Emi2$^{80\text{-}115}$-RFP and either WT or MRL mutant *M.m.* cyclin B3 mRNA and quantified as in (C).

(**E**) Model of how cyclin B3 prevents CSF-arrest in oocyte meiosis I. Degradation of *Xenopus* (*X.l.*) Emi2 is mediated via two phosphodegrons, while mouse (*M.m.*) Emi2 has only one phosphodegron. For further details, see text.

(A-D): n = number of oocytes from at least 3 independent experiments, error bars indicate means ± SEM for A, and B, and ± SD for C and D.

See also Figure S6.

STAR METHODS

Resource availability

Lead contact

Further information and requests for resources should be directed and will be fulfilled by the lead contact Katja Wassmann (katja.wassmann@ijm.fr).

Materials availability

All unique reagents generated in this study are available from the lead contact without restriction. This study did not generate new mouse lines.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. Raw data from Figures 3, 4, 5, 6 and Figures S2, S3 and S4 were deposited on Mendeley at doi: 10.17632/htx484fv8p.1.

Experimental Model and subject details

Animals

Mice were kept in a temperature, humidity and light controlled enriched environment with ad libitum access to food and water, at the animal facility of UMR7622 (authorization B75-05-13) and the IBPS (authorization A75-05-24), according to French regulations and under reviewing from the ethical committee at the institute. Animals were not subjected to any invasive procedures. Adult female CD-1 (Swiss) mice were obtained from Janvier, France. *Ccnb3*$^{-/-}$ and *Ccnb3*$^{+/-}$ mice (C57BL/6/JRj background strain) were bred locally at the animal facilities. Genotyping was done as previously described (Karasu et al., 2019).

Xenopus laevis frogs were bred and maintained under laboratory conditions at the animal research facility, University of Konstanz, and all procedures performed were approved by the Regional Commission, Freiburg, Germany (35-9185.81/G-17/121 and 35-9185.81/G-17/120).

Method details

Mouse oocyte culture

To harvest mature prophase I oocytes, female mice were sacrificed by cervical dislocation by certified personnel at 8-16 weeks of age. Ovaries were dissected and oocytes recovered by mouth-pipetting follicles through a narrow glass pipet. Prophase I oocytes were cultured at 38° C in drops of M2 or M16 medium (homemade) supplemented with dbcAMP (dibutyryl cyclic AMP, Sigma Aldrich Merck, D0260) and covered with mineral oil (Sigma-Aldrich Merck, M8410). To release oocytes from prophase I arrest, they were washed three times in drops of M2 or M16 medium. Oocytes were resynchronized visually at GVBD (Germinal vesicle breakdown), and only oocytes undergoing GVBD within less than 90 minutes were used. *In vitro* culture conditions were controlled by verifying whether extrusion of the first polar body took place within 7,5 to 9 hours after GVBD in control oocytes. *Ccnb*$^{+/-}$ and *Ccnb3*$^{-/-}$ oocytes were always harvested in M2 medium for activation experiments. To inhibit MEK, UO126 (Promega, V1121) was added at a final concentration of 50 mM to M2 medium 4 hours after resumption of meiosis. Oocytes were maintained in UO126-containing M2 medium, unless stated otherwise. Wash-out of UO126 was done within 15 minutes after PB extrusion through three wash-steps in M16 medium without UO126. To induce release from CSF-arrest with Strontium, *Ccnb3*$^{-/-}$ oocytes at 7,5 hours after GVBD were put into M16 medium without CaCl2 (homemade) for 20-30 minutes, and then transferred into activation medium (M16 medium without CaCl2 containing 100mM Strontium

chloride, Sigma-Aldrich Merck 204463) for 1 hour. Exactly the same procedure for activation was used to activate UO126 treated *Ccnb*$^{+/-}$ and *Ccnb3*$^{-/-}$ oocytes after wash-out and overnight incubation. CSF-arrested *Ccnb3*$^{+/-}$ oocytes were used as controls for the activation procedure. Note: the medium change from M2 to M16 for activation with Strontium was necessary for optimal activation in oocytes of this strain background. However, the change of culture medium and activation was not compatible with concurrent live imaging, as oocytes of the C57BL/6/JRj background strain are much more sensitive to culture conditions and light exposure than oocytes of CD-1 (Swiss) mice.

Mouse oocyte microinjection

M.m. Cyclin-B3 pRN3 plasmids for *in vitro* transcription have been published (Karasu et al., 2019), Emi2 plasmids were PCR cloned into pRN3 for transcription from the T3 promoter. ZBR was mutated (C573A) with Pfu PCR mutagenesis kit. This plasmid was used as a template for the other Emi2 constructs, generated with Q5 site directed mutagenesis kit. Emi2 80-115 aa-RFP was generated with In-Fusion HD cloning kit (Ozyme). Primer sequences for cloning are available upon request.

mMessage machine kit (Invitrogen, AM1348) and RNAeasy purification columns (Qiagen, 74104) were used to obtain capped mRNA for microinjection into prophase I or CSF-arrested oocytes, using Eppendorf micromanipulators, Femto Jet, self-made microinjection pipettes (PN-31; Narishige) and constant flow settings. After injection, oocytes were left to recover in the incubator for 1-2 hours, unless otherwise specified. To study Emi2 stability in cyclin-B3-injected oocytes, Emi2 encoding mRNA was injected at least 30 minutes prior to cyclin-B3 mRNA. For MO knock-down of Emi2, Emi2 morpholinos (5’ ATTGCTTCCTGCTCTGTGGCTGGCT 3’) were injected

into prophase I oocytes around 18 hours prior to release. Knock-down efficiency was verified by controlling for DNA decondensation and entry into interphase after first PB extrusion (Madgwick et al., 2006). For inhibition of Plk1, BI2536 was added at 50nM 1 hour before cyclin-B3 mRNA injection. Efficiency of Plk1 inhibition was controlled for by checking inhibition of anaphase I in control oocytes treated from GV onwards (Touati et al., 2015).

Live imaging of mouse oocytes

Mouse oocytes were imaged under temperature-controlled conditions at 38°C on an inverted Zeiss Axiovert 200M microscope equipped with an EMCCD camera (Evolve 512, Photometrics), a Yokogawa CSU-X1 spinning disc, a nanopositioner MCL Nano-Drive, an MS-2000 automated stage (Applied Scientific Instrumentation) and piloted by Metamorph software, using a Plan-APO (63x/1.4 NA) oil objective (Zeiss). A final concentration of 1µM SirDNA (far-red DNA labeling probe, Spirochrome, SC007) was added to the culture medium 0,5-1 hour prior to imaging. At the indicated time points 11, 12 or 15 z-sections (3µm spacing) were acquired in the 640nm channel (Far-red) for SiRDNA imaging, and 1 z-section for 491nm channel for YFP, and the DIC image. Acquisitions for quantifications in Figure S5B were done on a Nikon Eclipse TE2000-E inverted microscope and PrecisExite High Power LED Fluorescence, a Prime sCMOS camera and a Plan APO (20x/0.75NA) objective. 1 z-section was acquired in the RFP channel for quantifications. Stills of movies were mounted with Fiji software, contrast and brightness was adjusted equally to all conditions being compared.

Chromosome spreads

In short, zona pellucida-free mouse oocytes (tyrode's acid treatment) were fixed in 0,65% - 1% paraformaldehyde, 0.15% Triton X-100 and 3mM DTT (Merck Sigma-Aldrich) at the indicated

time points on microscopic slides (Chambon et al., 2013). Centromeres were stained with human CREST serum auto-immune antibody (Immunovision, HCT-100, at 1:50) and donkey anti-human Alexa Fluor 488 (709-546-149, Jackson Immuno Research, 1:200) secondary antibody, chromosomes with Hoechst 33342 (Invitrogen) at 50µg/ml. Slides were mounted with AF1 Citifluor mounting medium (Biovalley, AF1-100) or Vectashield (Eurobio H-1000), and examined with an inverted Zeiss Axiovert 200M spinning disc microscope as described for live imaging, using a 100X/1,4 NA oil objective. 6 z-sections with 0,4µm interval were taken. Images were mounted with Fiji software, contrast and brightness was adjusted equally to all conditions being compared.

CSF extract preparation

3-14 days before the experiment, female Xenopus laevis frogs were injected with 20 U hCG subcutaneously into the dorsal lymph sack. 1 day (16-18 h) before the experiment, frogs were injected with 500U hCG to induce ovulation. The frogs were then placed into small tanks containing MMR (5mM Na-HEPES, 0.1mM EDTA, 0.1mM NaCl, 2mM KCl, 1mM MgCl2, 2mM CaCl2; pH 7.8). At the day of the experiment, laid eggs were collected, avoiding eggs with irregular pigmentation and apoptotic ones. Eggs of sufficient quality were washed with MMR and incubated in dejelling solution (2% cysteine, 0.1M KCl, 1mM MgCl2, 0.1mM CaCl2, pH 7.8) for up to 7 minutes, depending on oocyte surface state. Subsequently, eggs were washed with CSF-XB (100mM KCl, 1mM MgCl2, 0.1mM CaCl2, 50mM Sucrose, 10mM K-HEPES, 5mM EGTA, 1mM MgCl2, pH 7.7). Next, eggs were transferred to centrifuge tubes containing CSF-XB and Cytochalasin B (100µg/ml). The eggs were then compacted in a two-step centrifugation with the first step at 200 g for 1 minute and the second step at 650g for 1 minute. After removing residual buffer, eggs were lysed in a final centrifugation step at 16,500g for 10 minutes. From the resulting

layers, the CSF-extract layer was taken with a syringe and mixed with Cytochalasin B (10µg/ml). From the resulting CSF-extract, a sample was taken and mixed with sperm nuclei to check for integrity of metaphase II arrest via the chromatin shape. Thereby, the DAPI stained chromatin appears as compacted fibers during the arrest and forms nuclei-like structures in interphase. A second sample was mixed with sperm nuclei and CaCl2 (0.6mM) to confirm the ability of the extract to release from metaphase II arrest. The extract was used at the day of preparation and kept on ice until used.

Surgery and handling of prophase I arrested Xenopus laevis oocytes

To obtain oocytes arrested in prophase I, female Xenopus laevis frogs were anesthetized in Tricain solution (1g in 1L of MMR (5mM Na-HEPES, 0.1mM EDTA, 0.1mM NaCl, 2mM KCl, 1mM MgCl2, 2mM CaCl2; pH 7.8). To maintain a constant pH, 1g of NaHCO3 was added. The lack of reflexes was tested after 15 minutes to ensure proper anesthesia. Subsequently, skin and muscle were cut in a curved shape at either the left or right side of the abdomen. The ovary was partially removed by pulling with forceps and cutting with scissors. The removed parts were stored in MBS plus CaCl2 (88mM NaCl, 1mM KCl, 1mM MgSO4, 5mM HEPES, 2.5mM NaHCO3, 0.7mM CaCl2, pH 7.8). Muscle and skin were sewed separately and during recovery from anesthesia, the frog was placed in a separate tank with MMR, ensuring that the nose was above water. For microinjection, oocytes were then roughly separated in bundles of 15-20 oocytes and treated with 1mg/ml Liberase (Roche) to remove the surrounding tissue.

Xenopus laevis oocyte injection and rescue experiments

For depletion of endogenous cyclin-B3, Trim21 mRNA (see section mRNA preparation for Xenopus oocytes) and anti-cyclin-B3 (Ab-B3P) antibody were injected into stage-VI oocytes.

Here, 7ng of Trim21 mRNA and 20ng of antibody were used per oocyte. As control, unspecific rabbit IgG (20ng) were injected. For rescue experiments, IVT cyclin-B3Δ15, not recognized by the antibody was co-injected (1.2 nl IVT per oocyte).

The oocytes were incubated overnight at 19°C in MBS medium (88mM NaCl, 1mM KCl, 1mM MgSO4, 5mM HEPES, 2.5mM NaHCO3, 0.7mM CaCl2, pH 7.8). Then, they were transferred to OR2 medium (82.5mM NaCl, 2.5mM KCl, 1mMCaCl2, 1mM MgCl2, 1mM Na2HPO4, 5mM HEPES, pH 7.8) containing 5µg/ml progesterone to induce meiotic resumption. From one pool, oocytes were taken every hour until the appearance of the white spot in the animal hemisphere of the oocyte marking GVBD, then every 30 minutes oocytes were collected from the same pool. For Emi2 depletion via Morpholino oligonucleotides (MO) (GENE TOOLS, LLC) a mixture of Emi2 MO2 (AGATTTGCCATCTCTTGTTTCTT) and Emi2 MO3 (TGTGCCATCTCTTGTTTCTTTCTTC) was used. 9.2 pmol of each MO per oocyte were injected. As control the same amounts of inverse MO2 (TTCTTTGTTCTCTACCGTTTAGA) and inverse MO3 (CTTCTTTCTTTGTTCTCTACCGTGT) were injected.

IF staining of Xenopus oocytes

For IF staining, oocytes were transferred to a fixation solution (100mM KCl, 3mM MgCl2, 10mM HEPES, 0.1% Triton, 0.1% glutaraldehyde, 3.7% formaldehyde, pH was adjusted to 7.8 before addition of Triton, glutaraldehyde and formaldehyde) and incubated overnight with slow shaking at 4°C. Then oocytes were bleached in 10% H2O2 in methanol for 20 h at room temperature exposed to light. Afterwards all incubation steps were done at 4°C in the dark (well-plates covered with aluminum foil). Oocytes were washed and blocked by incubating them 3 times for 1 h in AbDil buffer (0.1% Triton X-100, 2% BSA, 0.1% NaN3, in PBS). Next, oocytes were placed for 40h at 8°C in AbDil buffer containing FITC-labeled anti-tubulin antibody (1µg/ml final

concentration) and Hoechst 33342 (1µg/ml final concentration). Oocytes were washed 4 times for 1 h by incubation in PBST on a shaker with gentle agitation. Finally, oocytes were mounted on glass slides in mounting solution (100mM KCl, 3mM MgCl2, 10mM K-HEPEs, pH 7.8, 0.1% Triton X-100, 50% glycerol).

CSF time-course experiments

To analyze the stability of ectopic Emi2 in CSF extract, IVT reactions of the different Emi2 constructs were diluted 1:50 in CSF extract and incubated for 20 minutes at 20°C. Then, cyclin-B3 mRNA was added (either WT or MRL mutated) to CSF extract (final concentration: 90ng/µl) and samples were taken every 20 minutes up to 80 minutes. Where indicated, samples were treated with lambda phosphatase (NEB), for details see section "phosphatase treatment". Finally, samples were mixed 1:10 with 1.5x Laemmli buffer (90mM Tris, 5% (w/v) SDS, 15% (w/v) glycerol, 12.5% (w/v) ß-Mercaptoethanol and Bromphenol blue) and heated for 10 minutes at 95°C.

Phosphatase treatment

To facilitate the analysis of total protein levels, samples were treated with Lambda Protein Phosphatase (New England BioLabs). We found that for efficient dephosphorylation of CSF extract samples, conditions were slightly different from manufacturer's instructions. Here, CSF samples were mixed 1:1 with phosphatase solution containing 1 volume PMP buffer, 1 volume MnCl2 buffer, 0.1 volume H2O and 0.1 volume lambda phosphatase. Then samples were incubated for 45 minutes at 30°C, and then mixed 1:5 with 1.5 x Laemmli buffer (90mM Tris, 5% (w/v) SDS, 15% (w/v) glycerol, 12.5% (w/v) ß-Mercaptoethanol and Bromphenol blue) and heated for 10 minutes at 95°C.

SDS gels and Western Blot

For Western Blot samples, CSF extract was diluted 1:10 in 1.5x Laemmli buffer (90mM Tris, 5% (w/v) SDS, 15% (w/v) glycerol, 12.5% (w/v) ß-Mercaptoethanol and Bromphenol blue). Samples were heated at 95°C for 10 minutes and stored at -20°C until they were analyzed by immunoblotting. Per lane, 5µl samples were loaded corresponding to 0.5µl CSF extract.

Stage-VI oocytes were frozen in liquid nitrogen and stored at -80°C until lysis. 5µl of lysis buffer per oocyte (Mammalian Cell-PE LBTM Buffer (G-Biosciences) with 1x complete Protease inhibitor (Roche) were added, then scratched 10-15 times over a rack until a homogenous lysate formed. Then, the lysate was centrifuged 15 minutes at 20,000g at 4°C. From the resulting layers, the clear middle one was mixed 1:1 with 3x Laemmli buffer or underwent lambda phosphatase treatment with the same conditions as for CSF samples with the exception that here 5µl phosphatase solution were used for 18µl lysate. 5µl sample were loaded per lane to SDS gels, corresponding to half an oocyte.

For detection of Emi2, Cdc27 and cyclin-B3 8% SDS gels were used. For cyclin-B2, 10% SDS gels were used. The gels were run at 25mA for small gels (15 lanes) or 60mA for big gels (30 lanes). The proteins were then transferred to nitrocellulose membranes by wet blot. For small gels, the blot was run for 1h at 120V or 2h for big gels in wet blot buffer (25mM Tris, 0.19M glycine, 0.01% SDS and 20% methanol).

After blotting, membranes were blocked in 5% milk PBST or 3% milk TBST (for cyclin-B3) for 30 minutes at room temperature. The primary antibodies were incubated in the indicated dilutions overnight at 4°C. Before incubation of the HRP-coupled secondary antibodies (Dianova), the membranes were washed 3 times for 5 minutes in TBST (for cyclin-B3) or PBST (for all others). The secondary antibody solution was removed by washing 3 times for 5 minutes in TBST or PBST. For the detection of signals, membranes were incubated for 15sec in ECL solution (SuperSignal

west Pico PLUS, Thermo Scientific), then the signal was detected with LAS-3000 (GE Healthcare Life Sciences).

IVT preparation

Emi2 constructs were added as products from *in vitro* transcription and translation reactions. IVT reactions were done with the TNT® SP6 High-Yield Wheat Germ Protein Expression System (Promega) according to the manufacturer's instructions. For stability tests of ectopic Emi2 constructs, IVTs were added at a 1:50 dilution to CSF extract.

mRNA preparation for Xenopus oocytes

For expression of cyclin-B3 or Trim21, mRNA was added to CSF extract. The mRNA was prepared with the mMESSAGE mMACHINE® T7 Ultra Kit (Ambion) according to the manufacturer's instructions. For testing Emi2 stability after expression of cyclin-B3 mRNA, 4.4µg of mRNA was added to 50µl CSF extract.

IP of cyclin-B3 WT or MRL for kinase assays

Immunoprecipitation was performed with dynabeadsTM Protein G (Invitrogen). Beads were washed four times with PBST and once with PBS. Next, Flag antibody (Sigma-Aldrich) diluted in PBS was added and incubated overnight at 4°C rotating. Two-fold excess of beads were used for antibody binding compared to binding capacity described in the producer protocol. For example, for 100µl CSF-extract, 73.7µl beads were incubated with 9µg antibody. Before IP of Flag-tagged cyclin-B3, beads were washed three times with CSF-XB buffer.

For expression of Flag-cyclin-B3 WT or MRL, mRNA was added to CSF extract (final mRNA concentration: 40ng/µl) to validated CSF extract (see CSF extract preparation) and incubated for

30 minutes at 20°C. Through the addition of 100µM MG262 (Biomol GmbH) and IVT of a C-terminal fragment of Emi2 (aa 491-651, T545A, T551A; 1:20 diluted), maintenance of MII arrest was ensured. For immunoprecipitation, CSF extract was diluted 1:5 with CSF-XB containing 1x PhosStop (Roche), 1x complete Protease inhibitor (Roche), and 0.375µM MG132 (Cayman). The IP took place for 90 minutes at 4°C rotating. The supernatant was discarded, and the beads were washed four times with PBS with 150mM NaCl and 0.5% Tween20 and two times with PB-buffer (1x PBS, 1% Glycerol, 20mM EGTA, 15µM MgCl, 100mM DTT, 1x complete, 1x PhosStop). Beads were maintained on ice until the start of the kinase assay.

Kinase assay

For the kinase assay, purified fragments of Emi2 were diluted to a final concentration of 0.08mg/ml in PB-buffer (see section IP of cyclin-B3 WT or MRL for kinase assays) with 34.1µM ATP and 0.15µCi/µl [α-^{33}P]-ATP. For kinase assays using histone H1 as substrate, ATP and [α-^{33}P]-ATP were used at 44.9µM and 0.2µCi/µl, respectively. The zero-minute sample was taken previously to the assay start. The assay was started by adding recombinant Emi2 or histone H1 to the beads. Samples were taken at indicated time points. The assay took place at 25°C and mixed at 1,200 rpm. The kinase reaction was stopped by re-suspending the sample with 3x Laemmli buffer, followed by heat denaturation.

Cloning of *Xenopus laevis* cDNAs

The Xenopus laevis cyclin-B3 short isoform (Karasu et al., 2019) was cloned via PCR (Phusion polymerase kit, all primer sequences are available upon request) into pCS2 vectors containing either N-terminal Myc or Flag tag. The sequence is identical to NCBI NM_001085892.2. For the MRL construct, M190, R191, and L19A were mutated to alanine via PCR, the PCR reaction was

digested with DpnI and subsequently transformed into *E. coli*. The different Emi2 constructs were cloned using a Morpholino-resistant Emi2 template by mutagenesis PCR with subsequent DpnI digestion. Emi2 constructs were cloned into PCS2 vectors containing either N-terminal Myc or Flag tag.

For time-course experiments, Emi2 constructs with mutations in the ZBR region (C583A) were used to prevent Emi2 from interfering with APC/C activity during the release from metaphase II arrest. Additional mutations were added to this construct with the oligos available upon request, except for mutation of N-terminal XErp sites 4T/SP to Alanine (S43A, S73A, T97A, S157A), where the DNA sequence containing the mutated sites was ordered from Thermofisher, cut with restriction enzymes EcoRV Hf (NEB) and FseI (NEB) and ligated with T4 ligase into pCS2 vector containing the residual Emi2 sequence. For DNA amplification, the constructs were transformed into *E. coli* Turbo (NEB) and DNA was recovered with Macherey-Nagel NucleoBond Xtra Midi kit.

Mouse Emi2 was cloned via PCR into a pCS2 Vector containing an N-terminal Myc-tag with oligos available upon request. The plasmid was used in a new PCR reaction generating the mutation of C573A (ZBR). This plasmid was used as template for PCR reactions introducing further mutations in the Emi2 sequence with Phusion polymerase.

Quantification and statistical analysis

Quantification of Western Blot signals

Western Blots were quantified using GelAnalyzer software (GelAnalyzer 19.1) (www.gelanalyzer.com) by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc). The signal intensities for the protein of interest were normalized to the respective loading controls and the zero hour time-point value was set to 1. If not stated otherwise quantifications were done from 3

independent replicates and standard deviations were indicated. All information on replicate numbers is indicated in the corresponding figure legend.

Quantifications of Emi2-YFP in mouse oocytes

For fluorescence intensity signal measurement, a circle of 60 × 60 pixels was placed on each oocyte, and another circle placed next to the oocyte for background values. For each oocyte, oocyte values were normalized after background subtraction relative to the first value. Measurements were done with Fiji software using original, untreated acquisitions. GraphPad Prism 8 software was used for statistical analysis and at least 3 independent experiments were performed for each condition. The statistical tests applied are indicated in the corresponding figure legends.

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