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Control of meiotic divisions in oocytes : a novel role for cyclin B3

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Thèse de doctorat de Sorbonne Université

Ecole doctorale Physiologie Physiopathologie et Thérapeutique

Présentée par **Nora Bouftas**

Control of meiotic divisions in oocytes : a novel role for cyclin B3

Soutenue le 26 Septembre 2019

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Summary:

Throughout my PhD, I focused on the study of female meiotic cell cycle regulation and more specifically, the two meiotic divisions and chromosome segregation in oocytes. Meiosis is a tightly regulated process that must be completed in an orderly manner to obtain gametes with the correct number of chromosomes. The two divisions occurring during meiosis (called meiosis I and II) have different patterns of segregation. Importantly, S-phase must be suppressed between the two divisions. This leads to the creation of haploid gametes. All the steps of meiotic cell division are regulated by cyclins associated to their binding catalytic partners Cdks. Female mammalian meiosis presents a lot of challenges. It was shown that it is an error-prone process where errors in segregation occur creating aneuploid gametes. Aneuploidy occurs more frequently during female meiosis compared to male meiosis. In addition, incidence of aneuploidy increases in correlation with age. Understanding the mechanisms involved in the regulation of female mammalian meiosis is therefore essential. Progression through the meiotic cell divisions in mammalian oocytes (also named meiotic maturation) is characterized by two arrests: oocytes formed during embryonic development arrest at prophase I until hormonal stimulation induces resumption of meiosis. The second arrest is marked at metaphase II, also known as CSF arrest. This arrest is lifted only upon fertilization. During my PhD, I investigated the role of a unique cyclin, cyclin B3, through the use of cyclin B3 KO mice. I found that lack of cyclin B3-Cdk1 activity induces an arrest at metaphase I. This is due to high cyclin B1 levels and Cdk1 activity as well as inactive separase. APC/C activity was also affected since endogenous APC/C substrates were not efficiently degraded. Furthermore, the role of cyclin B3 was found to be conserved, as cyclin B3 from other species was able to rescue mouse cyclin B3 KO oocytes.

As I further explored the possible roles of cyclin B3, and with the use of *Xenopus* oocytes, I was able to show that cyclin B3 is able to inhibit CSF arrest. The mechanism of this inhibition is still being explored, but my recent data suggests that cyclin B3 is able to induce the degradation of the APC/C inhibitor Emi2. Furthermore, oocytes lacking cyclin B3 put a precocious CSF arrest into place, leading to the metaphase I arrest observed. Mimicking fertilization or inhibiting one of the components required for CSF arrest rescues this metaphase I arrest. Hence, my PhD work has shown that cyclin B3 is essential for female meiosis to correctly activate the APC/C in meiosis I and to prevent precocious CSF arrest in meiosis I instead of meiosis II.

Abbreviations

AMP	Adenosine MonoPhosphate
APC/C	Anaphase Promoting Complex/Cyclosome
Arpp19	cAMP-regulated phosphoprotein 19
Bub	Budding uninhibited by benomyl
CamKII	Calmodulin-dependent protein Kinase II
Cdc	Cell division cycle
Cdk	Cyclin dependent kinase
CKI	Cyclin dependent Kinase Inhibitor
CPC	Chromosomal Passenger Complex
CPEB	Cytoplasmic Polyadenylation Element Binding
CSF	CytoStatic Factor
C-ter	C-terminal
Dbox	Destruction box
Emi	Early mitotic inhibitor
GFP	Green Fluorescent Protein
GVBD	Germinal Vesicle Breakdown
GV	Germinal Vesicle
G phase	Gap
KD	Knockdown
KO	Knockout
Mad	Mitotic arrest deficient
MAPK	Mitogen Activated Protein Kinase
MCAK	Mitotic Centromere Associated Kinase
MCC	Mitotic Checkpoint Complex
MPF	M phase Promoting Factor
Mps1	Monopolar spindle 1
MTOC	MicroTubule Organizing Center
M phase	Mitosis or Meiosis
N-ter	N-terminal
PBE	Polar Body Extrusion
PKA	Protein Kinase A
Plk1	Polo like kinase 1
PP2A	Phosphatase 2A

p90Rsk	p90 Ribosomal S6 Kinase
Rec8	Recombination protein
RFP	Red Fluorescent Protein
SAC	Spindle Assembly checkpoint
Scc	Sister chromatid cohesion
SCF	Skp-Cullin-Fbox
Sgo	Shugoshin
Sr	Strontium
S phase	Synthesis
WT	Wild Type
XErp1	Xenopus Emi1-related protein 1
YFP	Yellow Fluorescent Protein

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Introduction

Aneuploidy

The cell cycle is a complex series of steps that produce all the cells of the body. The division of cell components occurs during the two types of M phase: mitosis and meiosis. Meiosis is the specialized type of cell cycle that forms the gametes that are capable of undergoing fertilization. Its proper regulation is needed to obtain a healthy embryo and offspring. Mistakes during meiosis and mitosis can lead to cells with the incorrect number of chromosomes making them aneuploid. It is known that aneuploidy can be an important characteristic of different cancers. Errors during meiosis can have devastating consequences on the future embryo.

Aneuploidies observed during meiosis are in majority due to errors during female meiosis. Only 1-2% of spermatozoa were found to be aneuploid compared to 10-30% of oocytes. This marked difference clearly shows that female meiosis is more error prone. The incidence of aneuploidy even increases exponentially with the increase of maternal age (**Fig. 1**) (MacLennan et al. 2015; Templado, Uroz, and Estop 2013; Nagaoka, Hassold, and Hunt 2012; Hassold and Hunt 2001). This was already observed in early studies (Penrose 1933). However, this is not the case in male meiosis. No correlation of increase of age and aneuploidy were found during male meiosis. This is probably due to the fact that the mechanisms that regulate correct meiotic progress are more robust in male germ cells that lead more frequently to germ cell loss rather than increase of the incidence of aneuploidy (Vrooman et al. 2014). In addition, male meiosis is shorter in timing compared to female meiosis where for humans, oocytes are arrested for years before they can resume meiosis and meiotic maturation is a very lengthy process.

The percentages of aneuploidies observed in correlation with women's age range from around 35% up to 80% in some studies (Capalbo et al. 2017; Hassold and Hunt 2001). Very few types of aneuploidies are viable in humans. It seems that embryos are very sensitive to gene dosage and addition or removal of a chromosome more often than not lead to embryonic lethality. Therefore, aneuploidy in somatic cells is very dangerous for the development of the future embryo. These types of aneuploidies are a major cause of miscarriage and it was observed that more than half of miscarriages during the first trimester are due to aneuploidy in somatic cells. However, there are a few viable aneuploidies due to a gain of a chromosome (trisomies). These include trisomy 13 (Patau

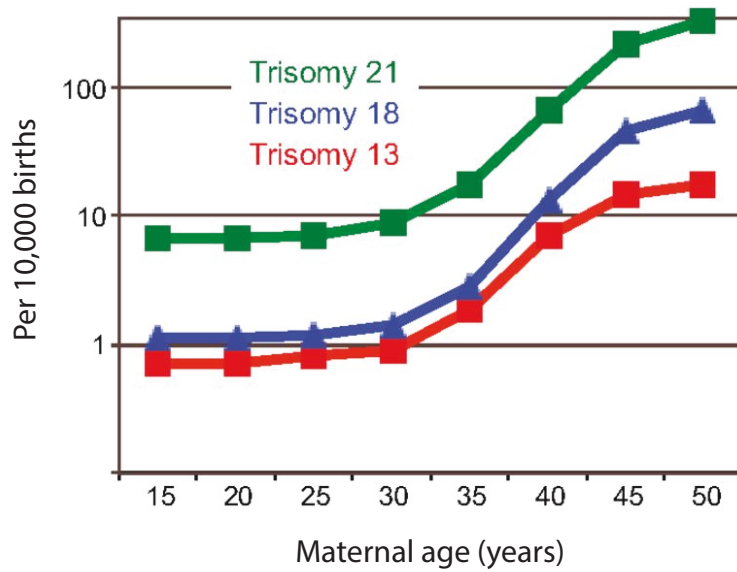


Figure 1: Increase of trisomies in relation to maternal age. The three trisomies indicated in this graph are the viable form of trisomy for humans. Number of trisomies per 10,000 births was recorded. The incidence of each trisomy significantly increases as maternal age increases. Adapted from Jones and Lane 2013.

syndrome), trisomy 18 (Edwards syndrome), and trisomy 21 (Down syndrome) (**Fig. 1**). The first two show graver consequences, as life expectancy is very low, with only 10% of individuals reaching the age of 10. This difference with trisomy 21 is probably due to the smaller size of chromosome 21 that contains fewer genes (Potapova and Gorbsky 2017). Loss or gain of sex chromosomes also presents more mild phenotypes in humans. Loss of the X chromosome in women (Turner syndrome) and gain of chromosome X in men (Klinefelter's syndrome) leads to infertility. Gain of the Y chromosome presents no obvious defects and individuals don't present issues with fertility. The Y chromosome has very few genes, and these genes are not needed for viability, which could explain this mild phenotype upon a supplemented gene copy (Potapova and Gorbsky 2017).

These findings are of concern in modern societies, as we know that today women have their first child at a later age. Aneuploidies during female meiosis can lead to spontaneous abortions, miscarriages, and congenital diseases. It is important to first understand how different mechanisms are put in place during female mammalian meiosis to then comprehend what goes wrong during human oocyte maturation.

1. Cell cycle

A) Steps of the cell cycle

The cell cycle is comprised of 2 Gap phases (G1 and G2), with a replication stage (Sphase), all three considered to constitute interphase, and finally the mitotic phase (M phase). After duplication of genetic material and cell growth occurring during interphase, the different components are divided into two cells at M phase (**Fig. 2**). Each phase has to be correctly completed and follow the previous phase to complete an orderly cell cycle.

During G1 phase, cells complete a growth phase that includes the synthesis of the components necessary to move on to the next phase, such as mRNA, proteins and ribosomes. At G1 entry, the cell must make a decision on whether it will begin a new cell cycle or not. The parameters of this decision depend on the cell type. This phase, like all others in the cell cycle, is controlled by a specific checkpoint that verifies the correct growth of the cell to analyze if it can move to S phase or halt at a quiescent G0 phase until conditions are more favorable. S phase is the replication stage, where DNA is replicated in order to have two copies of the cell's genetic information. This phase is also controlled by a

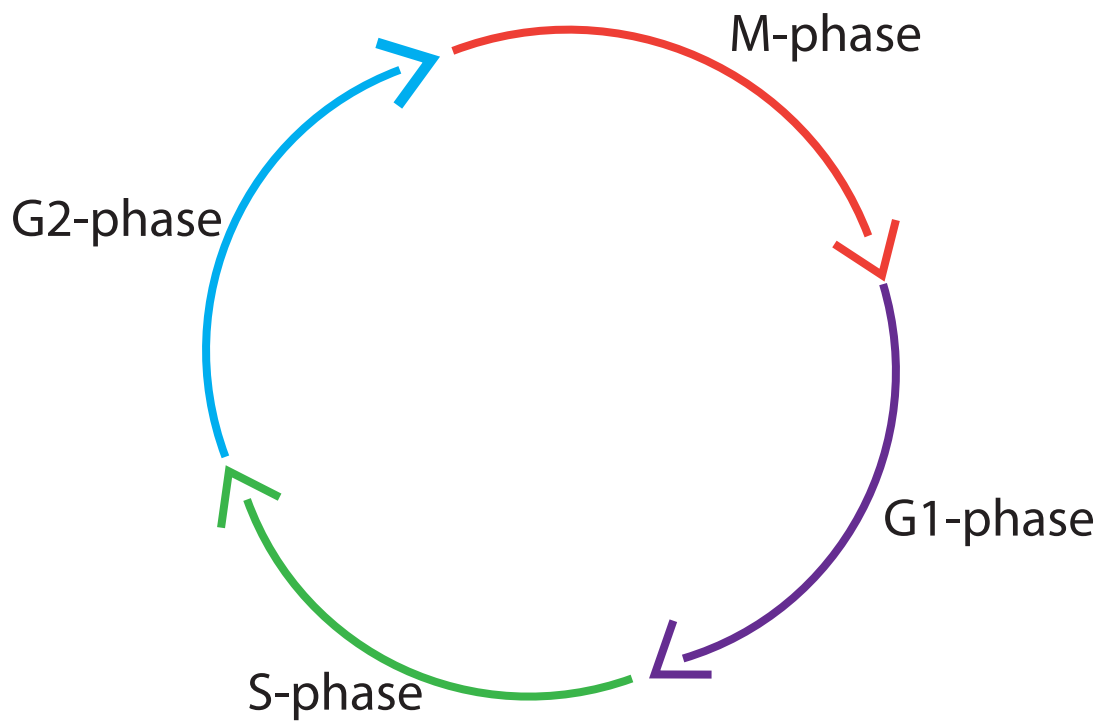


Figure 2: Different phases of the cell cycle. The cell cycle is comprised of four phases. Two Gap phases, G1 and G2 phase, as well as an S phase make up interphase. The fourth phase is the M-phase. Each phase must follow each other in a regulated and orderly manner. G1 phase allows growth of the cell and preparation for replication phase. During S phase, the genetic information of the cell is duplicated. G2 phase permits the cell to continue growing and synthesizing proteins and other components necessary for M-phase. Finally, during M-phase, DNA is divided and the cell gives rise to daughter cells.

checkpoint verifying that DNA synthesis occurred correctly and the DNA damage checkpoint. G2 phase follows right before the cell enters mitosis. At this stage the cell continues to grow and synthesize protein as it prepares to enter mitosis. The transition from G2 to M phase is controlled by a DNA damage checkpoint that verifies that the genetic material is not impaired. Correct completion of these steps allows the cell to transition to M phase. During M phase, the DNA segregates into daughter cells. There exist two types of M phase called mitosis and meiosis. Both of them are controlled by the Spindle Assembly Checkpoint (SAC) that delays M phase exit if errors are observed. Consequently, each step of the cell cycle is monitored through different checkpoints (Elledge 1996). It is primordial to regulate each step in order to avoid deregulation that can lead to numerous disorders.

B) Mitotic division

Mitosis is a cell division that occurs in all somatic cells. During mitosis, a mother cell divides its genetic material and components of the cell into two daughter cells. Mitosis is divided into 5 phases: prophase, prometaphase, metaphase, anaphase and telophase. Cells enter mitosis in prophase where chromosomes begin to condense to be prepared for segregation. During prometaphase, chromosomes are condensed, the nuclear envelope breaks down and the spindle begins to form. Importantly, at metaphase, the microtubules emanating from the spindle must be correctly attached to a structure in the centromeric region called the kinetochore. At this stage, sister chromatids are linked together by a ring-like structure called cohesin and attached to the spindle in a bipolar manner. The chromosomes are aligned at the metaphase plate. Anaphase is marked by the segregation of chromatids due to cleavage of the cohesin. This frees sister chromatids from each other and due to the spindle pull, sister chromatids are segregated to opposite poles. The next phase is telophase where the DNA begins to decondense once again and forms a new nuclear envelope around both clusters of DNA in each daughter cell. This process is ended by a final step of cytokinesis where the membranes separate and reform to produce two individual daughter cells (**Fig. 3**).

C) Meiosis: specialized cell cycle

The production of haploid gametes is achieved by a specialized cell cycle, meiosis. During meiosis, the cell must divide its DNA during two different divisions with only one preceding S

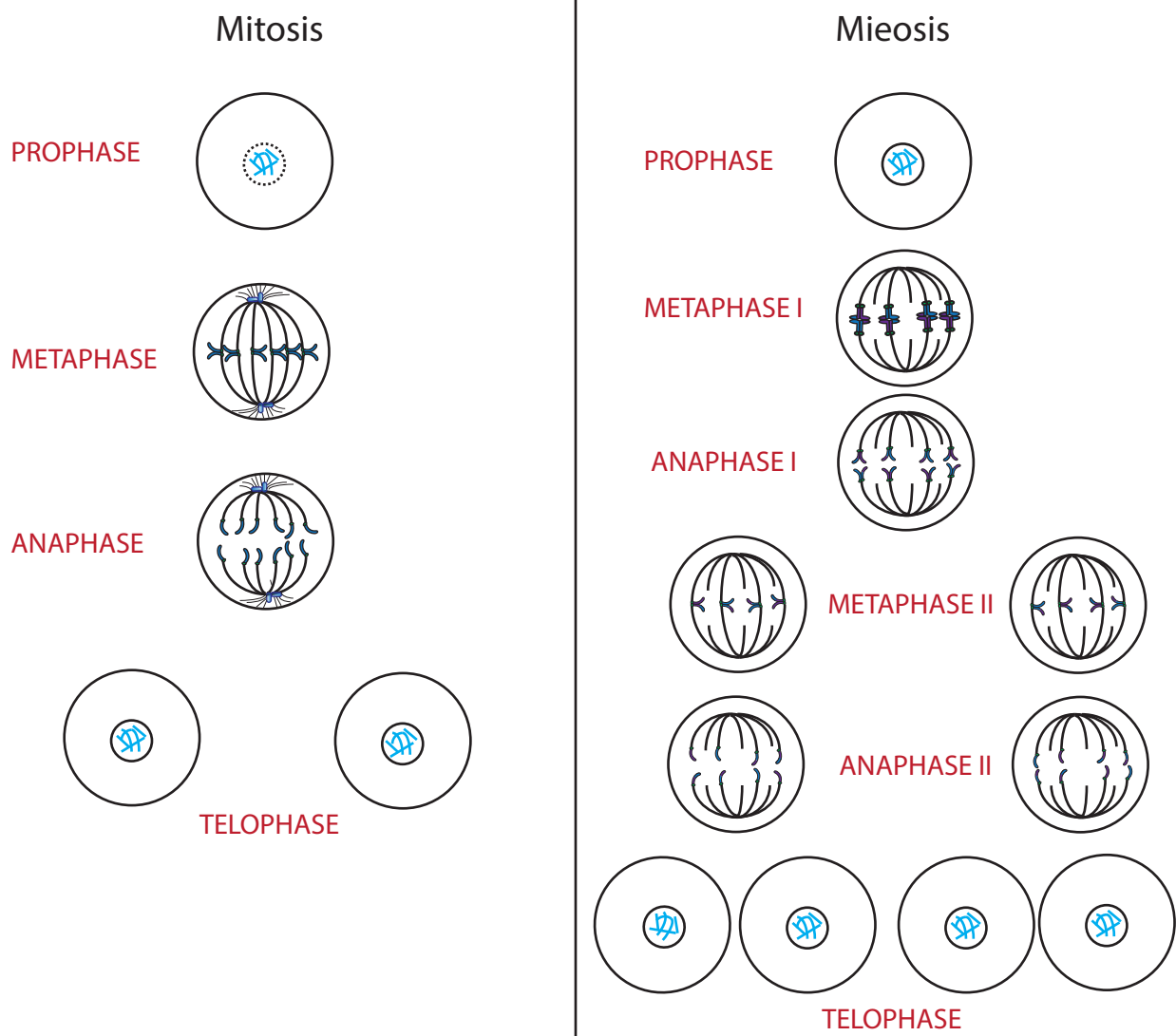


Figure 3: Mitosis vs. meiosis. Two types of M-phases exist, mitosis and meiosis. Both contain similar phases: prophase, prometaphase (not shown), metaphase, anaphase and telophase. Mitotic cells undergo one round of division while in meiosis there are two rounds of division with no intermediate S phase allowing the creation of haploid gametes. In meiosis I, chromosomes segregate while in meiosis II sister chromatids segregate.

phase. The phases described for mitosis are conserved and present during meiosis. Unlike mitosis, the challenge of the regulation of meiosis is to correctly achieve two different patterns of division resulting in a haploid gamete ready for fertilization. The first division requires segregation of homologous chromosomes while the second leads to sister chromatid segregation (**Fig. 3**). Female and male meiosis has a similar end goal: the creation of a euploid (with a correct number of chromosomes) gamete ready for fertilization. However, there are marked differences between the two divisions, especially for mammals. Male meiosis is less error prone than female meiosis. This could be due to the fact that male gametes (spermatozoa) are produced throughout their lifetime. This is not the case for mammalian oocytes. Male meiosis can take approximately 26 days in humans versus decades for women (S. Lane and Kauppi 2019). The stock of available oocytes is present since birth and therefore ages considerably. The oocyte is also of significant size and will be the host cell for the development of the future embryo. During female mammalian meiosis, the two cell divisions are asymmetric. Half of the genetic material is discarded into a smaller cell called the polar body. This asymmetry permits the oocyte to retain most of the nutrients and cytoplasmic components needed to complete meiotic maturation, fertilization and the next steps in embryonic development. In female mammalian meiosis, there are two main arrests observed during maturation; prophase I arrest and metaphase II arrest. The first must be maintained for up to decades and the second is vital for correct ploidy as it is only lifted at fertilization. Therefore, different and additional regulatory mechanisms are present for correct completion of female meiosis.

2. Regulation of the Cell cycle

A) Cyclin-Cdks of the cell cycle

Cdk (Cyclin dependent kinase) levels govern entry and exit from the different phases of the cell cycle. Cdks are serine/threonine kinases that regulate all steps of the cell cycle. More than 20 families of Cdks have been identified so far (Malumbres et al. 2009). They are characterized by a binding domain for cyclins, an ATP-binding catalytic pocket and a T-loop motif (Lim and Kaldis 2013). For Cdks to become active, they must be partnered with their regulatory subunits called cyclins. Cyclin-Cdk complexes can be formed and complete their roles in part due to the presence of different motifs found on cyclins. An example of this is the MRAIL motif found on the α helix of the cyclin box. This motif was first described in cyclin A-Cdk complexes where different observations were made. Mutating this motif was shown to disturb cyclin-Cdk binding at times and at other times abolish substrate recognition without affecting cyclin-Cdk binding, demonstrating the importance of these regulatory motifs (Bendris et al. 2011; Schulman, Lindstrom, and Harlow 1998; Jeffrey et al. 1995). Different cyclin-Cdk complexes need to be formed at different time points of the cell cycle to allow correct progression throughout the cell cycle (Murray 2004). The complex needed to progress through G1 phase is cyclin D-Cdk4/6. Entry into S phase necessitates the activity of cyclin E-Cdk2 while the rest of S phase is controlled by cyclin A-Cdk2. To successfully transition from G2 to M phase, cyclin A-Cdk1 must be involved. Finally, during M phase, cyclin B-Cdk1 seems to play the pivotal role (**Fig. 4**) (Satyanarayana and Kaldis 2009; Sánchez and Dynlacht 2005; Malumbres and Barbacid 2005). This does not mean that no other complexes are involved in the process. For example, depending on the species, more than one cyclin-Cdk complex is necessary for M phase. This is the case for female mammalian meiosis, which will be discussed in depth in other sections.

An additional regulator that influences the activity of cyclin-Cdk complexes is the CKIs (Cdk inhibitors). CKIs are present and play a role in the regulation through the inhibition of Cdk activity. Cyclins and Cdks were also found to have functions outside the regulation of the cell cycle. These include roles in transcription, metabolism, epigenetic regulation, neuronal functions and stem cell processes. These roles can be fulfilled by the activity of the complex, or by the individual subunits, demonstrating surprisingly independent roles from each other

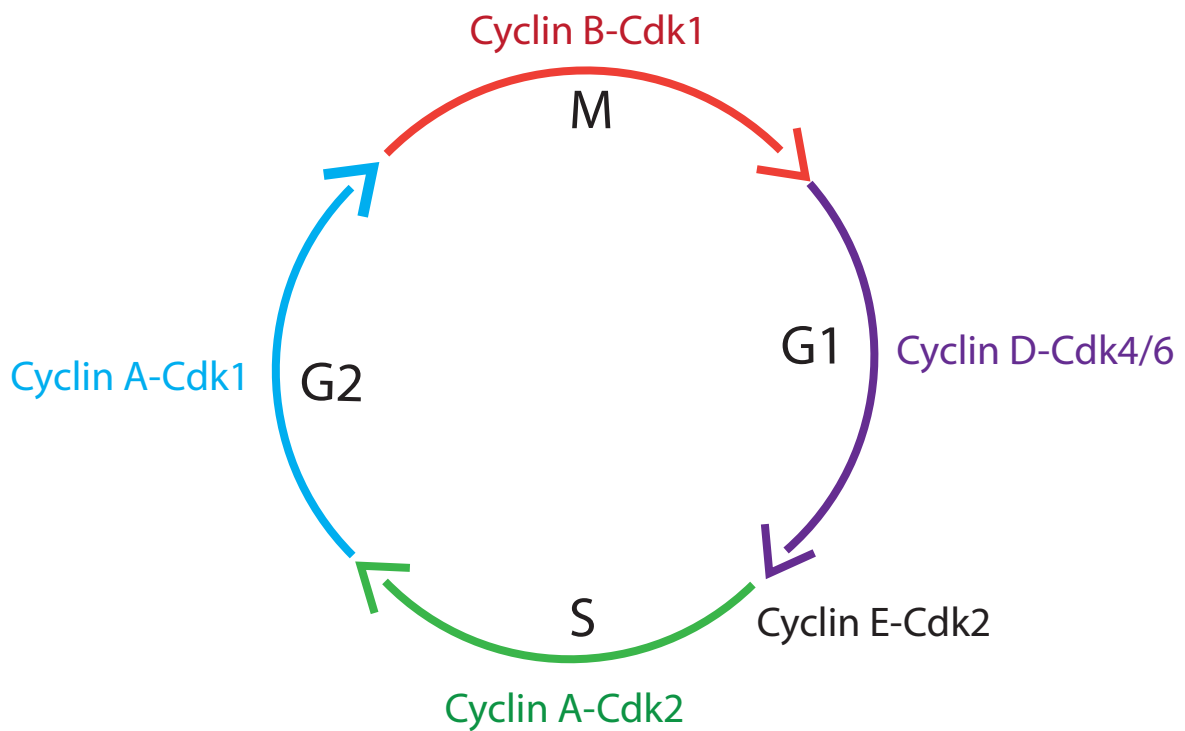


Figure 4: Cyclin-Cdk complexes drive the cell cycle. Different complexes are needed at different stages of this cycle. G1 phase progression is governed by cyclin D-Cdk4/6 while transition into S phase requires cyclin E-Cdk2. S phase is regulated with activity of cyclin A-Cdk2. Progression through G2 phase and into M-phase is controlled by cyclin A-Cdk1 and M phase needs cyclin B-Cdk1 activity. Each complex plays an important role in each phase to complete the cell cycle correctly.

(Lim and Kaldis 2013). It has been hypothesized that the only factor that drives different phases of the cell cycle is the levels and threshold of Cdk activity rather than specific interactions with different cyclins. This was hypothesized due to the fact that in fission yeast only one cyclin-Cdk complex was needed to complete the cell cycle (Gutiérrez-Escribano and Nurse 2015; Coudreuse and Nurse 2010; Fisher and Nurse 1996; Richardson et al. 1992). This idea doesn't seem to fit what is observed today particularly during female meiosis, where different M phase cyclins are essential for proper cell cycle progression.

B) M phase cyclins: mitotic and meiotic cyclins

M phase cyclins are present during mitosis and meiosis. Some play different roles while others are redundant or can compensate for each other. M phase cyclins are important regulators of Cdk1 or Cdk2 activity, making their regulation vital for correct progression through both types of M phase.

i) A type cyclins

There are two A-type cyclins in higher vertebrates called cyclin A1 and cyclin A2. Cyclin A1 was found to be required for male meiosis since cyclin A1 knockout (KO) male mice are viable but sterile, while females were both viable and fertile (van der Meer et al. 2004; Liu et al. 1998; R. Yang, Morosetti, and Koeffler 1997; Sweeney et al. 1996). Therefore, cyclin A1 plays an important role in male fertility and meiosis. On the other hand KO of cyclin A2 provokes embryonic lethality (Kalaszczynska et al. 2009). Cyclin A2 is needed at two different stages of the cycle, which separates this cyclin from other mammalian cyclins. It is necessary for both S phase and entry into mitosis (G2/M phase). Although it is not necessary for all tissues, it is a ubiquitously expressed cyclin. Cyclin A2 is able to bind to both Cdk1 and Cdk2 (Wolgemuth 2011). During mammalian meiosis, cyclin A2 is present in prophase I oocytes and in metaphase II arrested oocytes. It is degraded during prometaphase and reappears in meiosis II. Its exact role is still being defined as two different techniques, KO or antibody injection and overexpression experiments, seem to uncover conflicting roles (Q.-H. Zhang et al. 2017; Touati et al. 2012).

ii) B type cyclins

B-type cyclins are essential mitotic regulators. They are expressed temporally and secure the correct timing of each step of mitosis and meiosis. In mammals, three B-type cyclins have been studied: cyclin B1, cyclin B2 and cyclin B3. This is not the case in all other species. For

example, in amphibians such as *Xenopus*, four B-type cyclins are thought to be present in their cells, cyclin B1, B2, B4 and B5 (Hochegger et al. 2001). Our preliminary results suggest that cyclin B3 is also present in *Xenopus* oocytes and therefore that five B-type cyclins are present. Cyclin B1 is essential for mitosis and meiosis, while cyclin B3 was recently found to be essential for female meiosis. Although, cyclin B2 does play a non-negligible role in meiosis, cyclin B2 is not vital for mitosis and meiosis since cyclin B2 KOs are viable and fertile (although sub-fertile) (Daldello et al. 2019). Cyclin B1 is therefore the only B-type cyclin needed for mitosis. Cyclin B2 is not vital for either mitosis or meiosis but was shown to have a similar function to cyclin B1 and able to fulfill its function if expressed at its place (J. Li et al. 2018). Cyclin B3, the third B-type cyclin, can be considered as a separate type of cyclin. It shares characteristics with both A and B-type cyclins. Its particular characteristics will be further developed in its section (Part 6). It is mainly expressed in the germline in mammals. Each B-type cyclin contributes to correct meiotic maturation in mammals.

C) MPF

MPF (M phase promoting factor) produces the kinase activity necessary for mitosis and meiosis. It is an essential component of the cell cycle. Experiments completed on the frog *Rana pipiens* have led to the discovery of the MPF. Injection of the cytoplasm of a metaphase arrested oocyte into prophase I arrested oocytes led to induction of maturation and resumption of meiosis. This component was therefore called the Maturation Promoting Factor or MPF (Masui and Markert 1971). This factor must be active and present for entry into mitosis and meiosis while its inhibition is necessary for the exit and entry into other steps of the cell cycle. Uncovering what makes up the MPF took many years after its discovery. Using *Xenopus* cell extracts, it was possible to determine that MPF is composed of a kinase and a subunit needed for its activity (Lohka, Hayes, and Maller 1988). It was also shown that the kinase was the same as one known to be essential for mitosis in *S. pombe* named Cdc2. In most species, the kinase is now called Cdk1 with its sub-unit cyclin B. These important findings led to the obtainment of a Nobel Prize in physiology or medicine by three groups (Nurse 1990; T. Hunt 1989; Hartwell et al. 1973). Cyclin B usually refers to both cyclin B1 and cyclin B2. Cyclin B2 is capable of playing the role of cyclin B1 at least in meiosis (J. Li et al. 2018). Today, due to its known function, MPF is mostly known as M-phase promoting factor. To allow the proper substrate phosphorylation during M phase, the MPF requires the

help of another kinase to impede the phosphatase activity, which dephosphorylates the substrates of cyclin B1-Cdk1. This additional kinase is believed to be Greatwall (or Mastl for mammals), which phosphorylates and therefore activates Arpp19 and ENSA. These proteins are needed to inhibit the activity of the phosphatase PP2A (Castro and Lorca 2018). This maintains the phosphorylations put in place by cyclin B1-Cdk1 and allows proper entry into mitosis and meiosis.

D) APC/C regulation and proteolysis by the 26S proteasome in mitosis

i) Mechanism of APC/C activity

Another important regulator of the cycle is the E3 ubiquitin ligase Anaphase Promoting Complex or cyclosome (APC/C). It targets substrates for their ubiquitination. The multi-ubiquitination of the substrates leads to their degradation by the 26S proteasome. The APC/C is composed of several subunits, between 15-21, depending on the species (Pines 2011). The APC/C is part of the RING family of ubiquitin ligases but unlike other Ring family ubiquitin ligases it does not complete on its own the ubiquitination of the selected substrates. Rather, it acts by linking the ubiquitin conjugating E2 co-enzymes to the substrate selected. This allows E2 to place a ubiquitin on the lysines of the substrates. The first E2 enzyme must initiate the ubiquitination. E2 C known as Ube2C/UbcH10 is recruited to the APC/C in proximity to the substrates. A second ubiquitin-conjugating enzyme completes the elongation process of the ubiquitination chain, E2 S (Ube2S) (Brown et al. 2016; Wickliffe et al. 2011; Williamson et al. 2009). Multiple ubiquitinations make the substrate susceptible to the 26S proteasome that in turn degrades the substrate. This system is the one used for degradation of substrates during part of the cell cycle by the APC/C. The regulation of the APC/C is primordial to provoke orderly degradation of its substrates. This orderly destruction of substrates controls the outcome of specific steps during the cell cycle (Fung and Poon 2005). For example, cyclin B1 and securin must be degraded in a timely manner for correct segregation and exit from M phase. There are primary sites on substrates that are recognized by the APC/C, called degrons. These are well established and studied and include D-box, KEN box, and ABBA box motifs. But these motifs do not alone determine how and when the substrates are recognized and targeted for degradation. Several components are taken into account when it comes to timing of substrate recognition and degradation (Davey and Morgan 2016).

ii) APC/C co-activators Cdh1 and Cdc20 in mitosis

The APC/C plays a role throughout certain cell cycle steps, targeting different substrates at different time points with its coactivators, Cdh1 or Cdc20 (Nakayama and Nakayama 2006). Through ubiquitylation by the APC/C, its substrates are then targeted for degradation by the 26S proteasome. The co-activators associated with APC/C recruit and bind substrates with the help of the sub-unit APC10 (da Fonseca et al. 2011; Buschhorn et al. 2011). There is an order in the activation of either co-activator in mitosis. Cdh1 is thought to have a more diverse range of substrates compared to Cdc20 that targets fewer substrates more specifically. Cdc20 is activated earlier in mitosis while Cdh1 is bound to APC/C activating it at mitosis exit and G1 phase (**Fig. 5**). Cdc20 is important for APC/C activity during mitotic exit. In mitosis, phosphorylation by Cdk1 promotes association of Cdc20 and APC/C (Kraft et al. 2003). On the contrary, Cdh1 is phosphorylated and inactivated by Cdk1. Once Cdk1 activity decreases, Cdh1 is no longer inhibited allowing it to activate the APC/C and target its substrates (**Fig. 5**). One of its substrates is Cdc20 that also contains a KEN box allowing its degradation and take over by Cdh1. APC/C^{Cdh1} is active until G1/S transition to prevent precocious cyclin accumulation and entry into S-phase (Watson et al. 2019; Yamano 2019; Alfieri, Zhang, and Barford 2017; Pines 2011).

It was shown that both Cdh1 and Cdc20 are vital to obtain viable and healthy mice. Cdh1 null mice were embryonically lethal due to placental defects leading to death (M. Li et al. 2008). Cdc20 null mice showed an even more precocious death arresting at the two-cell stage due to a prolonged metaphase arrest that led to embryonic lethality (M. Li, York, and Zhang 2007). Knockdown of Cdh1 in human and mouse somatic cells showed that although it is not needed for completion of mitosis, it is necessary for G1 phase and S-phase regulation (Sigl et al. 2009). However, knockdown of Cdc20 in HeLa cells led to mitotic arrest inducing cell death demonstrating its essential role in mitosis (Eichhorn et al. 2013). These studies suggest that the two co-activators can not substitute for each other in activating the APC/C and targeting substrates. Therefore, each one is essential and needed to progress through the cell cycle.

APC/C activity must be repressed at certain time periods during the cell cycle. APC/C activity is inhibited during S-phase and G2 phase to allow appropriate accumulation of cyclins. This is completed by the inhibitor Emi1, expressed during S-phase and degraded during mitosis (Reimann et al. 2001). Emi1 inhibits the APC/C by acting as a pseudo substrate and also

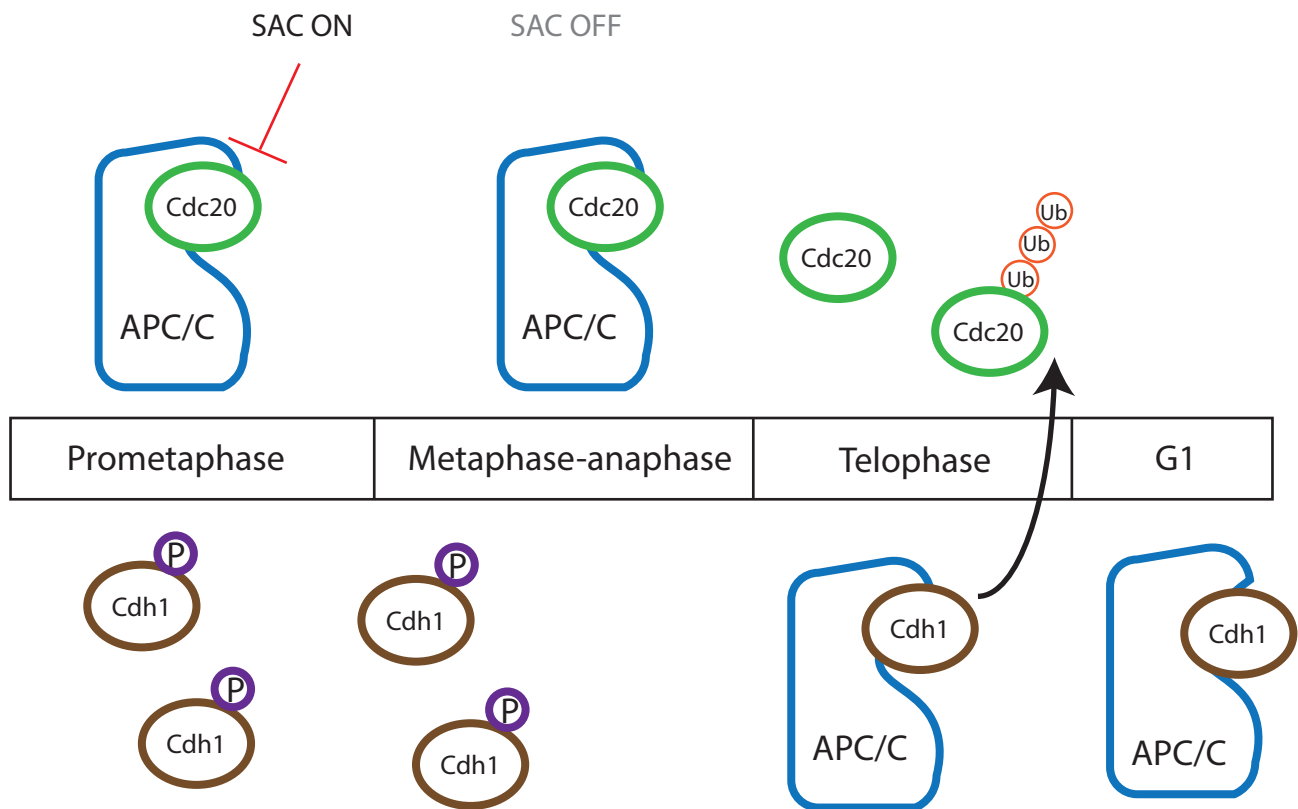


Figure 5: APC/C co-activators Cdh1 and Cdc20 during the cell cycle. During certain phases of the cell cycle, the APC/C plays an essential regulatory role by targeting substrates to 26S proteasome dependent degradation through ubiquitination. Co-activators that bind to the APC/C are needed for its activity at different time points. When cells enter mitosis, APC/C binds to its activator Cdc20. Its activity is inhibited by the Spindle Assembly Checkpoint (SAC). High Cdk1 activity during metaphase leads to the phosphorylation of another co-activator Cdh1 inhibiting its binding to the APC/C. At the metaphase-to-anaphase transition, APC/C^{Cdc20} is fully active targeting its substrates to degradation provoking a decrease of Cdk1 activity. This decrease allows Cdh1 to become active and bind to the APC/C. APC/C^{Cdh1} targets one of its substrates, Cdc20, for degradation. APC/C^{Cdh1} is then active at the end of mitosis and during the G1 phase.

through inhibition of the chain elongation by Ube2S. This allows deubiquitylating enzymes to remove the initial ubiquitins on the substrates preventing their degradation (W. Wang and Kirschner 2013). During the M phase, the APC/C is inhibited by the Spindle Assembly Checkpoint (SAC) allowing correct spindle formation, chromosome congression and kinetochore to microtubule (KT-MT) attachments. The SAC inhibits APC/C activity by sequestering Cdc20 through its binding to SAC components. This mechanism adds another fine-tuned regulation of APC/C substrate degradation (Sullivan and Morgan 2007; Musacchio and Salmon 2007).

3. Meiotic maturation in mouse oocytes

A) Summary of meiosis in mouse oocytes

Many studies on gamete aneuploidy and analyses of aging oocytes have shown that female mammalian meiosis is error prone. In mouse oocytes, the meiotic process is complex and tightly regulated. Oocytes are formed during embryonic development and are arrested in prophase I for prolonged periods of time. This stage can be recognized by the presence of a germinal vesicle (GV) in the oocyte. Therefore, this phase is also called the GV stage (**Fig. 6**). All the machinery needed for completing meiosis is present in the oocyte at this stage. A growth period of the oocytes in the ovaries is important for their abilities of completing successful meiotic maturation. During this growth period, the oocytes accumulate all the transcripts necessary to complete meiosis (Holt, Lane, and Jones 2013). The nutrients and transcripts accumulated as well as the important growth makes oocytes ready and competent to complete meiotic maturation. Smaller oocytes tend to not be able to complete meiosis even if they are adept to exit prophase I arrest and resume meiosis.

The long period of arrest might be one of the causes for the errors observed specifically in females. This arrest is lifted due to hormonal stimulation (starting from puberty for humans). Oocytes can then resume meiosis marked by germinal vesicle breakdown (GVBD). During prometaphase, chromosomes condense and the spindle begins to form. At metaphase, the bipolar spindle is fully formed holding chromosomes that are aligned at the equatorial plate. Chromosomes segregate during anaphase. The first polar body is extruded (PBE1) in an asymmetric manner with half of the genetic material discarded in it. The oocytes enter meiosis II immediately while preventing the start of a possible S phase. All oocytes then

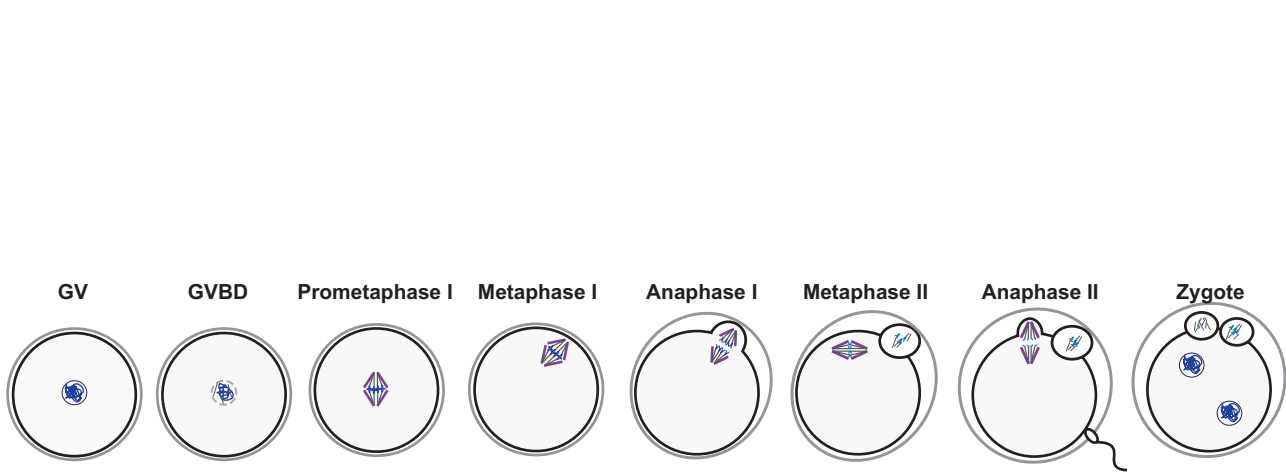


Figure 6: Mammalian meiotic maturation. Mouse oocytes are formed during embryonic development and arrest at prophase I also called germinal vesicle stage (GV). With hormonal stimulation, oocytes resume meiosis with germinal vesicle breakdown (GVBD). Chromosomes condense and the spindle forms during prometaphase and chromosomes align with a fully formed spindle at metaphase. During anaphase I, chromosomes segregate and the first polar body is extruded. Oocytes then immediately enter meiosis II and arrest at metaphase II also known as CSF arrest. Fertilization triggers anaphase II onset with chromatid segregation and the second polar body extrusion. The zygote can then form with the male and female pronuclei.

arrest at metaphase II, also known as CSF arrest. At this stage, the spindle is formed for a second time and the chromosomes stay aligned during the arrest. Fertilization provokes the lift of the metaphase II arrest and sister chromatid segregation. A second polar body is extruded (PBE2) and the paternal genetic material from the sperm enters the oocyte. The two pronuclei (female and male) fuse and form the zygote (**Fig. 6**) (Terret and Wassmann 2008). Following this, embryonic development will commence. If all of these steps are correctly completed, meiosis will give rise to euploid gametes and a healthy embryo.

B) Oogenesis: the development of the oocyte

Oocytes are formed during female embryonic development in mammals. At first, diploid primordial germ cells (PGCs) are found in embryos that later develop into the germline for both male and female mammals (H. Zhang et al. 2014). The development of the PGCs into fully formed gametes is controlled by different growth factors and cytokines. They must stay undifferentiated and avoid differentiating into somatic cells. These PGCs migrate to the developing ovaries (named genital ridges) in the female embryo, undergo multiple mitotic cycles and begin to express gamete specific genes leading to their differentiation. Then, they complete a final replication phase and begin meiosis and arrest at prophase. This final replication phase is a particular replication and is essential to put in place all the components that are necessary to correctly undergo the two meiotic divisions, namely loading cohesin onto the chromosomes and replicating chromosomes (Wear, McPike, and Watanabe 2016; McLaren 2003). At this stage oogenesis begins (MacLennan et al. 2015). Oocytes can then grow surrounded by their follicles that grow and develop resulting in preovulatory follicles (**Fig. 7**) (Piotrowska et al. 2018).

At the start of oocyte production during fetal development, a large stock of oocytes is created. The number of these immature oocytes is estimated at around 7 million (Thomson, Fitzpatrick, and Johnson 2010). The decrease of this stock continues as follicle formation proceeds. This process known as atresia is observed in both mice and humans (P. A. Hunt and Hassold 2008). The decrease continues after birth until puberty and this process is also observed with increase of age especially when the age of menopause is reached. Typically, only one oocyte is ovulated in women and fertilizable once a month. Therefore, very few of

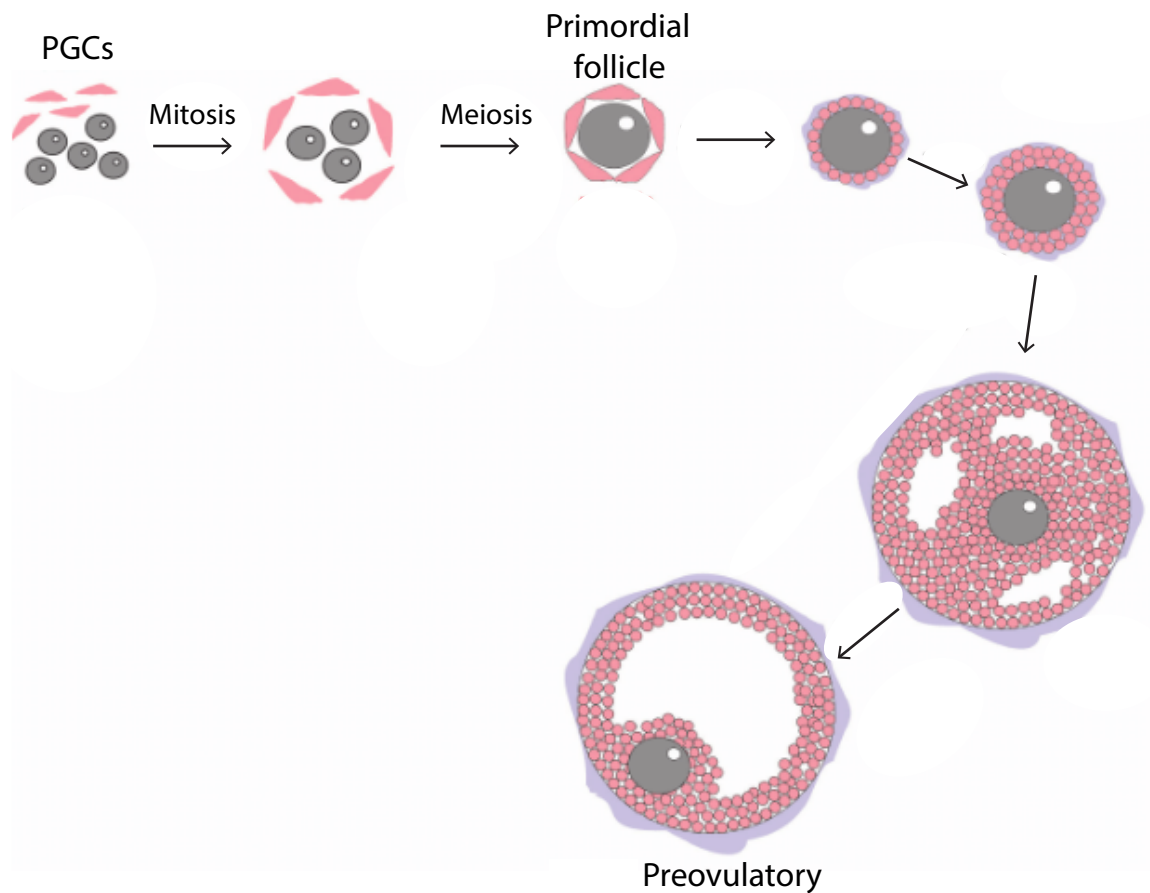


Figure 7: Oogenesis during mammalian development. During mammalian embryonic development, primordial germ cells (PGCs) are formed and later develop into germ cells. In female embryos, PGCs undergo several mitotic divisions and begin to differentiate to enter meiosis and form the primordial follicle. The oocytes arrest at prophase I following replication and recombination. They then enter a growth phase where they increase in size and development of their surrounding cells. When they complete their growth at the preovulatory stage, oocytes are sensitive to hormonal stimulation and are competent to resume meiosis. Adapted from Piotrowska *et al* 2018

the initial pool of immature oocytes are ovulated for both female mice and for women (Thomson, Fitzpatrick, and Johnson 2010).

During meiosis, prophase is divided into 4 stages: leptotene, zygotene, pachytene and diplotene. It is during these phases that recombination is established between paternal and maternal homologous chromosomes. Recombination is initiated by double strand breaks prompted by the endonuclease Spo11 (Ohkura 2015; Marston and Amon 2004). Crossing-over of some selected double strand breaks allows the recombination and higher genetic diversity increasing the variety within a population. Moreover, recombination sites called chiasmata are crucial for maintaining homologous chromosomes together during meiosis I (MacLennan et al. 2015).

C) Resumption of meiotic maturation

i) Prophase I arrest: GV stage

Vertebrate oocytes arrest at prophase I. For humans, this arrest can last up to decades. The main goal of the prophase I arrest is to maintain low Cdk1 activity (Adhikari and Liu 2014). In order to do so, a complex mechanism is put in place to ensure this arrest. The communication between the oocyte and the granulosa cells surrounding it is essential for GV stage maintenance. Indirectly regulated through contact with the granulosa cells, high cyclic adenosine 3', 5' –monophosphate (cAMP) levels are integral in keeping the oocytes in a GV stage. If the oocytes are separated from the granulosa cells, cAMP levels drop and oocytes resume meiosis. In order to keep a low level of Cdk1 activity, cAMP binds to the kinase Protein Kinase A (PKA) activating it. PKA can then phosphorylate its substrates such as Wee1/Myt1 kinases and the phosphatase family Cdc25 (Holt, Lane, and Jones 2013). In mouse oocytes, it is known that Cdc25B and Wee1B with Myt1 are essential during prophase I arrest. Wee1B with Myt1 phosphorylate Cdk1 on residues Thr14 and Tyr15 inhibiting its activity. On the other hand, Cdc25B dephosphorylates Cdk1 to activate it. During GV stage, high levels of PKA activate Wee1B/Myt1 kinases and inhibit Cdc25B. This allows Cdk1 activity level to stay low inhibiting GVBD (**Fig. 8**) (Jeong Su Oh, Han, and Conti 2010; Pirino, Wescott, and Donovan 2009; Y. Zhang et al. 2008).

Another regulatory mechanism of GV stage arrest is APC/C bound to its co-activator Cdh1. Knockdown of Cdh1 leads to accelerated GVBD underlining its importance (Rattani et al.

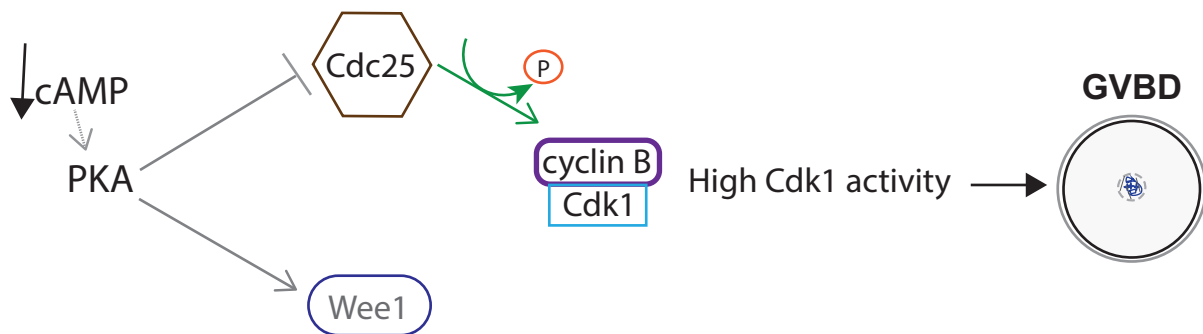
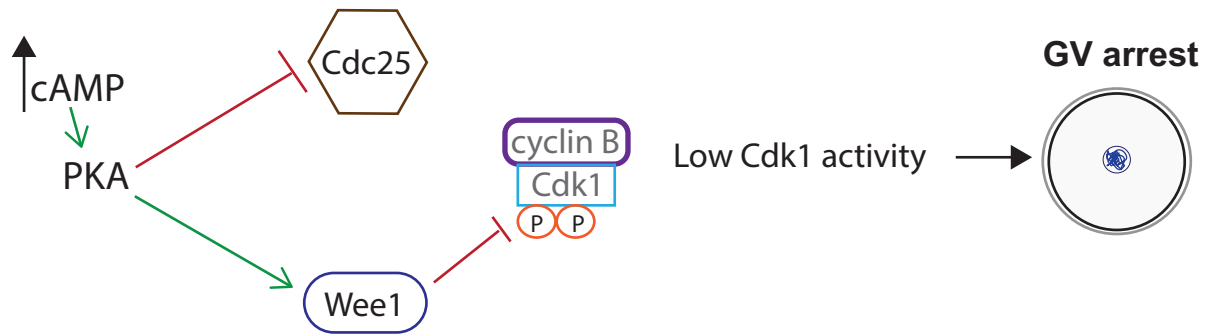


Figure 8: Prophase I arrest (GV) vs. resumption of meiosis (GVBD). At GV stage, oocytes are arrested at prophase I mainly through the inhibition of cyclin B-Cdk1 activity. Cyclic adenosine 3', 5'-monophosphate (cAMP) levels are high during GV stage which maintains PKA active. PKA maintains the inhibitory kinase Wee1 active and inhibits the Cdk1-activating phosphatase Cdc25. Wee1 inhibits Cdk1 activity through phosphorylation on two residues (Thr14 and Tyr15). This maintains low Cdk1 activity and arrest at GV stage. Hormonal stimulation induces a decrease in cAMP and PKA activity. Cdc25 is no longer inhibited and is able to dephosphorylate Cdk1 allowing increase of Cdk1 activity to trigger GVBD.

2017). Cyclin B1 levels must be correctly controlled in GV oocytes. Therefore, it is believed that cyclin B1 must be targeted for degradation to avoid possible increase of cyclin B1 leading to increase of Cdk1 activity and premature GVBD. APC/C^{Cdh1} itself is also regulated in GV mouse oocytes. Emi1 acts as an inhibitor of APC/C activity while the phosphatase Cdc14 promotes its activity. The localization of all of these components also plays a significant role. The inhibitory factors APC/C^{Cdh1} and Wee1B are found mainly in the nucleus while cyclin B1-Cdk1 and Cdc25B seem to be mainly cytoplasmic (Holt, Lane, and Jones 2013). Another recent study has shown that cyclin B2 could be the main cyclin needed for GVBD in mouse oocytes. Mouse oocytes devoid of cyclin B2 have a large delay in GVBD timing (Daldello et al. 2019; J. Li et al. 2018) while cyclin B1 KO oocytes enter meiosis at a regular timing with no significant delay (J. Li et al. 2018). Therefore, it seems that several regulatory pathways are put in place in mammalian oocytes to maintain this prolonged GV arrest to prevent any precocious resumption of meiosis.

ii) **Lift of prophase I arrest: GVBD**

The lift of the prophase I arrest is induced by hormonal stimulation. Once hormonal stimulation occurs, cAMP levels drop in the oocyte. This leads to a decrease in PKA activity and therefore less activation of Wee1B tipping the balance in favor of the phosphatase Cdc25B. More Cdk1 is activated through the dephosphorylation of inhibitory phosphorylation. In addition, prior to GVBD, a shift occurs where the cyclinB1/B2-Cdk1 complexes as well as Cdc25B translocate to the nucleus. More Cdc25B is present to activate Cdk1. Cyclin B1/2 translocation to the nucleus is thought to dominate over APC/C^{Cdh1} ubiquitination leading to an increase in cyclin B1/B2 levels and higher Cdk1 activity (Holt, Lane, and Jones 2013). All these factors lead to a surge in overall Cdk1 activity and therefore resumption of meiosis marked by the GVBD (**Fig. 8**). Following envelope breakdown, chromosomes begin to condense and the spindle begins to form as the oocyte transitions into the next steps of meiotic maturation.

D) Prometaphase: starting meiotic maturation

Once oocytes resume meiosis, cyclin B1-Cdk1 activity is essential to obtain a correct spindle and timely chromosome alignment. Following GVBD, the spindle begins to form and chromosomes condense. During meiosis I, a pair of homologous chromosomes are

connected each made up of two sister chromatids. Chromosomes are connected by their recombination sites that form the chiasmata, and sister chromatids by a ring-like protein complex named cohesin. These components are important to maintain a correct ploidy in the oocyte because they maintain chromosomes and sister chromatids together until anaphase I and II respectively. Cohesin is found on the arms and around the centromere. These two localizations determine the two patterns of segregation observed during meiosis. An important aspect is that during the first meiotic division arm cohesin is targeted for cleavage by the cysteine protease separase. Therefore, in meiosis I, the centromeric cohesin must be protected (see below) (Wassmann 2013).

i) Spindle formation and KT-MT attachments

The nucleation of microtubules from the centrosomes leads to the formation of the bipolar spindle during mitosis. The centrosomes are previously duplicated during S phase. The microtubules emanate from these centrosomes each made up of two centrioles and pericentriolar material. The microtubules begin to polymerize and search in the cytoplasm for chromosomes. They then attach to them at the kinetochores. This mechanism is known as “search and capture” (Heald and Khodjakov 2015; Kirschner and Mitchison 1986). In mammalian oocytes, the meiotic spindle differs from the mitotic spindle due to one key feature. No centrosomes are present in meiosis, meaning that another mechanism is needed. The spindle forms from a structure called acentriolar MicroTubule Organizing Centers (aMTOCs). They lack centrioles and microtubules emanate from them during meiosis as aMTOCs form clusters and are organized to form the two poles for the spindle (Dumont et al. 2007).

During the formation of the spindle, it is important to correct wrong KT-MT attachments. These include lateral attachments, where KTs aren't attached to the end of the MT, but rather at the side. Meiosis I merotelic attachments, where each KT is attached to a MT coming from opposite poles, are also corrected (note that this configuration is not wrong in mitosis). To correct wrong attachments, a complex called the CPC (Chromosome passenger complex) made up of the proteins Aurora B/C, Incenp, Borealin and Survivin are present. The CPC induces destabilization of the KT-MT attachments leading to their detachments. This allows another MT to attempt a correct attachment on that KT (Carmena et al. 2012). As stated previously, the activity of cyclin B1-Cdk1 progressively increases throughout meiosis I

and reaches its peak activity around metaphase. This slow increase has been found to be important for correct KT-MT attachments, congression and alignment of chromosomes. It was shown that a decrease in Cdk1 activity led to a delay in stable KT-MT attachments while overexpression of cyclinB1, leading to higher Cdk1 activity, provoked a precocious stabilization of these attachments. As a consequence, errors in segregation occur in anaphase I due to lagging chromosomes (Davydenko, Schultz, and Lampson 2013).

ii) Spindle Assembly Checkpoint

Similarly to mitosis, the Spindle Assembly Checkpoint (SAC) plays an important role in regulating correct chromosome segregation. An active SAC delays anaphase to allow correct attachment of microtubules to chromosomes. Initially, the existence of the SAC during meiosis was debated. This was due to the fact that errors in segregation occur with such high incidence in human oocytes (20-30% in healthy women). Therefore, a possible explanation for these errors was thought to be an absence of a functional SAC. However, this is not the case. This mechanism is well characterized during meiosis I and its function is still debated in meiosis II. It was shown that the SAC is functional during meiosis I thanks to several studies. They have shown that in absence of SAC proteins, meiosis I is accelerated and aneuploidy occurs. If one of the core components of the SAC is depleted, chromosomes segregate precociously with untimely APC/C activation and accelerated degradation of cyclin B1 and securin even in absence of microtubules (due to nocodazole treatment). This clearly demonstrates the importance of the activity of the SAC during meiosis I (Touati et al. 2015; Hached et al. 2011; McGuinness et al. 2009; Niaux et al. 2007; Wassmann, Niaux, and Maro 2003).

As oocytes resume meiosis, the bipolar spindle begins to form as the chromosomes condense. The microtubules begin to attach to the chromosomes. The two sister kinetochores are oriented to the same pole in order to segregate together. This orientation is essential for correct segregation in meiosis I. When kinetochores are not attached, components of the SAC are recruited to the unattached kinetochore. The recruitment is done in a sequential manner with the kinase Mps1 first being recruited to the unattached kinetochores. Mps1 can then recruit Bub3 and Bub1 through phosphorylations. Finally BubR1 is recruited. These core components can then recruit the complex composed of Mad1-Mad2. Each of the components described here are essential for a functional SAC.

Mad2 exists in two conformations: open and closed. When Mad2 is not bound, it is in an open conformation. Once it binds to Mad1, Mad2 is converted to a closed conformation. Mad2 bound to Mad1 is then capable of converting other open Mad2 proteins into the closed conformation (M. Yang et al. 2008; Mapelli et al. 2007). This allows Mad2 to bind to Cdc20 (De Antoni et al. 2005) and furthermore, to Bub1 and Bub3 forming the mitotic checkpoint complex (MCC) (Sudakin, Chan, and Yen 2001). The MCC is a potent APC/C inhibitor leading to the inhibition of ubiquitination of APC/C substrates and therefore inhibition of anaphase onset (**Fig. 9**).

Interestingly, the SAC in meiosis seems to be less robust than in mitosis. In mitotic cells, one unattached kinetochore is sufficient to activate the SAC (Rieder et al. 1995). However, this does not seem to be case in meiosis. The APC/C substrates cyclin B1 and securin begin to be degraded during meiosis I before all kinetochores are correctly attached (S. I. R. Lane, Yun, and Jones 2012). This indicates that the SAC is not sensitive enough to detect incorrect attachments of some kinetochores. This could be due to the size of the cell with an important volume of the cytoplasm. In this case, the signal of the SAC on the kinetochores would be diluted in the cell and therefore too weak to be able to inhibit correctly the APC/C. Some studies have hypothesized that the size of a cell impacts the strength of the SAC. When the size of the cell is decreased, this leads to a more robust SAC while increasing the size induces an accelerated anaphase (Kyogoku and Kitajima 2017; Galli and Morgan 2016; Hoffmann et al. 2011). However, this does not seem to be the case for embryonic divisions in the mouse where the size of the cell doesn't correlate with the robustness of the SAC (Vázquez-Diez, Paim, and FitzHarris 2019). Therefore, the reasons for leaky SAC control in oocytes are still not clear.

E) Metaphase I to anaphase I transition

i) Cohesin, the glue that keeps chromosomes together

In both mitosis and meiosis, cohesin holds sister chromatids together. It is essential for many processes during the cell cycle and crucial for correct chromosome segregation. In mammals, this ring is composed of 4 sub-units; SMC1 and SMC3 (“structural maintenance of chromosomes”), one HEAT repeat domain sub-unit STAG1, STAG2 or STAG3 and a α -kleisin subunit (Brooker and Berkowitz 2014). The kleisin subunit is the target that is cleaved by

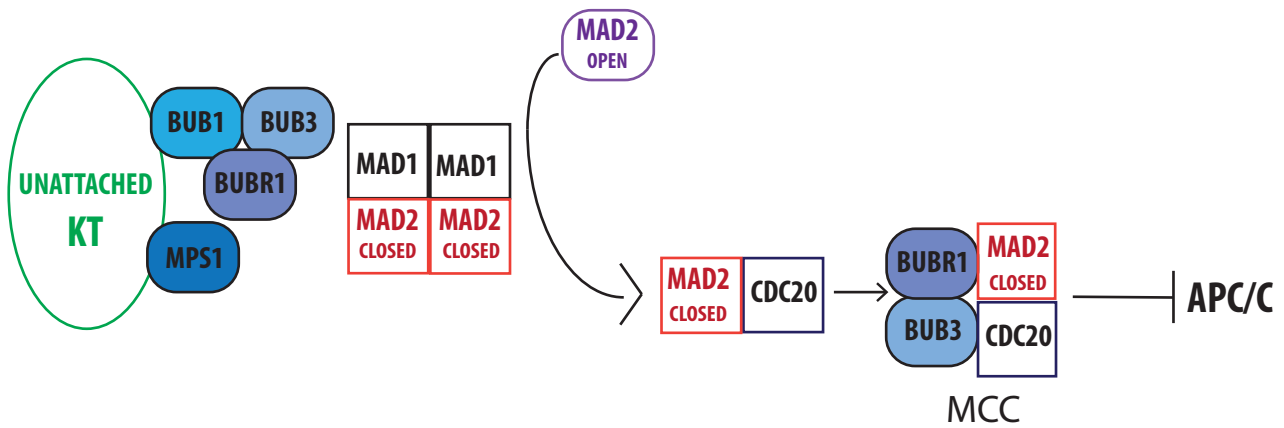


Figure 9: Active SAC inhibits APC/C^{Cdc20} activity during meiosis I. During mitosis or meiosis, the SAC is active when kinetochores (KTs) are unattached. This generates the recruitment of different proteins to the KT. Mps1 is recruited to the unattached KT and then recruits Bub1, Bub3 and BubR1. They can then recruit dimers of Mad1-Mad2. Mad2 protein is present in two conformations: closed when binding to Mad1 and open when free. Free open Mad2 can interact with the closed Mad2, become converted into closed Mad2 to bind to Cdc20 and form the MCC to inhibit the APC/C until KT's are correctly attached.

separase allowing opening of the ring and separation of chromosomes. There are subunits that are specific to either mitosis or meiosis. The meiotic α -kleisin subunits are Rad21L, present early in meiosis, (Herrán et al. 2011; Lee and Hirano 2011) as well as Rec8, while Scc1 is the kleisin present during mitosis (**Fig. 10**) (Petronczki, Siomos, and Nasmyth 2003). They are substrates of separase and are therefore cleaved by separase during anaphase (Petronczki, Siomos, and Nasmyth 2003). Cohesin is put in place during DNA replication. This implies that during meiosis, cohesin is present on the DNA since foetal development. During meiosis, like in mitosis, cohesin holds sister chromatids together. In addition, it also plays a role in stabilizing the chiasmata created during recombination that holds the homologous chromosomes together in meiosis I. Furthermore, in mice it was shown that there is no turnover of cohesin (Tachibana-Konwalski et al. 2010). Therefore, cohesin is holding chromosomes together for extended periods of times especially in mammals. It was suggested that an additional cause of high aneuploidy rates in female mammalian meiosis is cohesin fatigue. In older mice, the level of cohesin found in oocytes is significantly decreased compared to younger females (Jessberger 2012; Chiang et al. 2010; Lister et al. 2010). The decreased levels of cohesin in older mice could partly explain how aneuploidy rates increase with age.

ii) Two step removal of cohesin

A particularity of meiosis is the two different segregation patterns observed in MI and MII with no S phase in between. In meiosis I, cohesin holding together sister chromatids and stabilizing the physical connection between homologous chromosomes is present on both chromosome arms and the centromere. To correctly segregate chromosomes during meiosis I, separase must only cleave arm cohesin. Therefore, centromeric cohesin must be “protected” at this stage. Oocytes can then enter MII with sister chromatids still held together at the centromere. This allows correct bipolar attachment of sister kinetochores. Cohesin located at the centromere can then be “deprotected” in order to allow separase to cleave it inducing sister chromatid segregation at anaphase II (Wassmann 2013). These two patterns of segregation must therefore be tightly regulated.

1. Protection of centromeric cohesin

The arm cohesion removal occurs during the first meiotic segregation and depends on separase activity (Kudo et al. 2006; Terret et al. 2003). It was shown in yeast that in order to

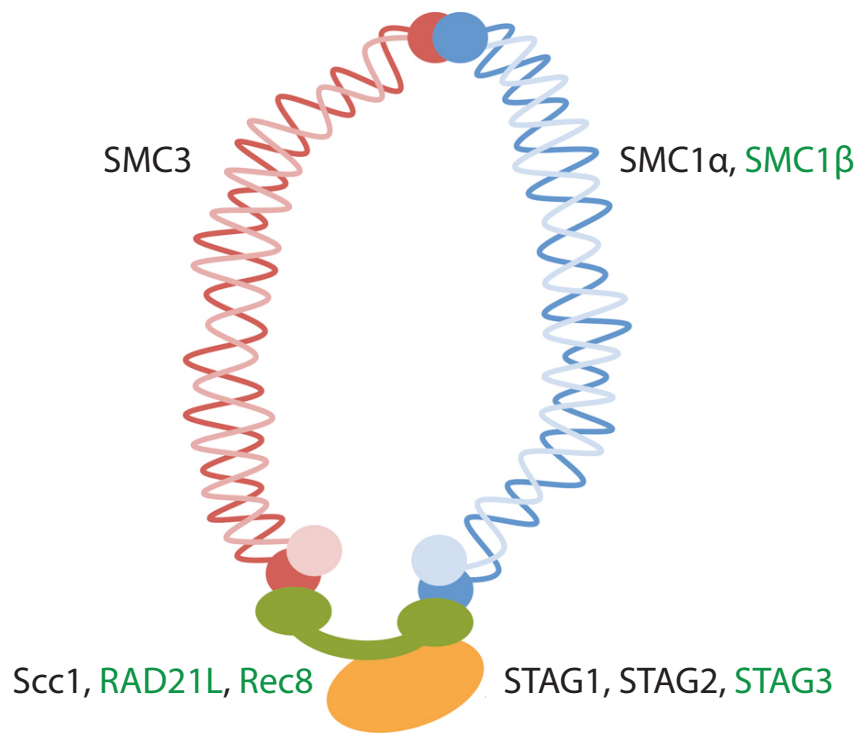


Figure 10: The cohesin complex and its subunits. Cohesin is a ring like complex that holds sister chromatids together during mitosis and meiosis. Its cleavage allows separation of the sister chromatids during anaphase. It is made up of SMC1 and SMC3 (structural maintenance of chromosomes), STAG1, STAG2, or STAG3 (HEAT repeat domain sub-unit) and a α -kleisin sub-unit. The sub-units expressed specifically during meiosis are in green. Adapted from Brooker and Berkowitz 2014

be cleaved, the cohesin sub-unit Rec8 must be phosphorylated. This phosphorylation is counteracted by the phosphatase PP2A that is recruited by the protein shugoshin (Sgo) at the centromere during meiosis I (**Fig. 11**) (Katis et al. 2010; Ishiguro et al. 2010; Riedel et al. 2006). Two isoforms of shugoshin are present in mammalian cells; Sgo1 and Sgo2. It was found that Sgo2 is essential for meiosis while Sgo1 seems to play a more crucial role in mitosis (Rattani et al. 2013; Lee et al. 2008; Llano et al. 2008; McGuinness et al. 2005). During mitosis a mechanism called the prophase pathway removes arm cohesion independent of separase. Sgo1 present at the centromeres protects cohesin from this form of cleavage to prevent precocious sister chromatid segregation (McGuinness et al. 2005). Sgo2 mutant mice are sterile underlining its importance during meiosis. Lack of Sgo2 leads to loss of centromeric cohesin protection and precocious sister chromatid segregation at anaphase I. This leads to random second segregation and aneuploid gametes (Llano et al. 2008). Furthermore, its exact localization in the centromeric region is also essential for correct protection and segregation. Recently, it was shown that the centromeric localization of Sgo2 and not at the pericentromere seems to be the primary pool of protective Sgo2. Sgo2 could have different roles during meiosis that could be distinguished by its exact localization (Yakoubi et al. 2017). It plays an important role in bi-orientation through its interaction with MCAK and inhibition of Aurora B/C. Sgo2 is also able to bind to Mad2 leading to inactivation of the SAC (Rattani et al. 2013). As previously stated, Sgo2 acts as a recruiting platform for PP2A. PP2A was also found to be crucial for the protection of centromeric cohesin. Inhibition of PP2A using Okadaic Acid (OA) provokes precocious sister chromatid segregation similarly to Sgo2 depletion (Mailhes et al. 2003). These findings indicate that without the protection of Sgo2-PP2A, separase is able to cleave all cohesin. This outcome must be avoided or it will lead to severe aneuploidies.

2. Deprotection: removing centromeric cohesin

The second important step during meiosis is the chromatid segregation during anaphase II. This is accomplished by separase that cleaves centromeric cohesin. Therefore, at this stage, centromeric cohesin must be “deprotected”. There are a few hypotheses on how this is done. One hypothesis is the tension-sensing model. In this model, the different KT-MT attachment in meiosis I and II determines how centromeric cohesin is deprotected only in meiosis II. Because sister kinetochores are attached in a bipolar way in meiosis II, meaning

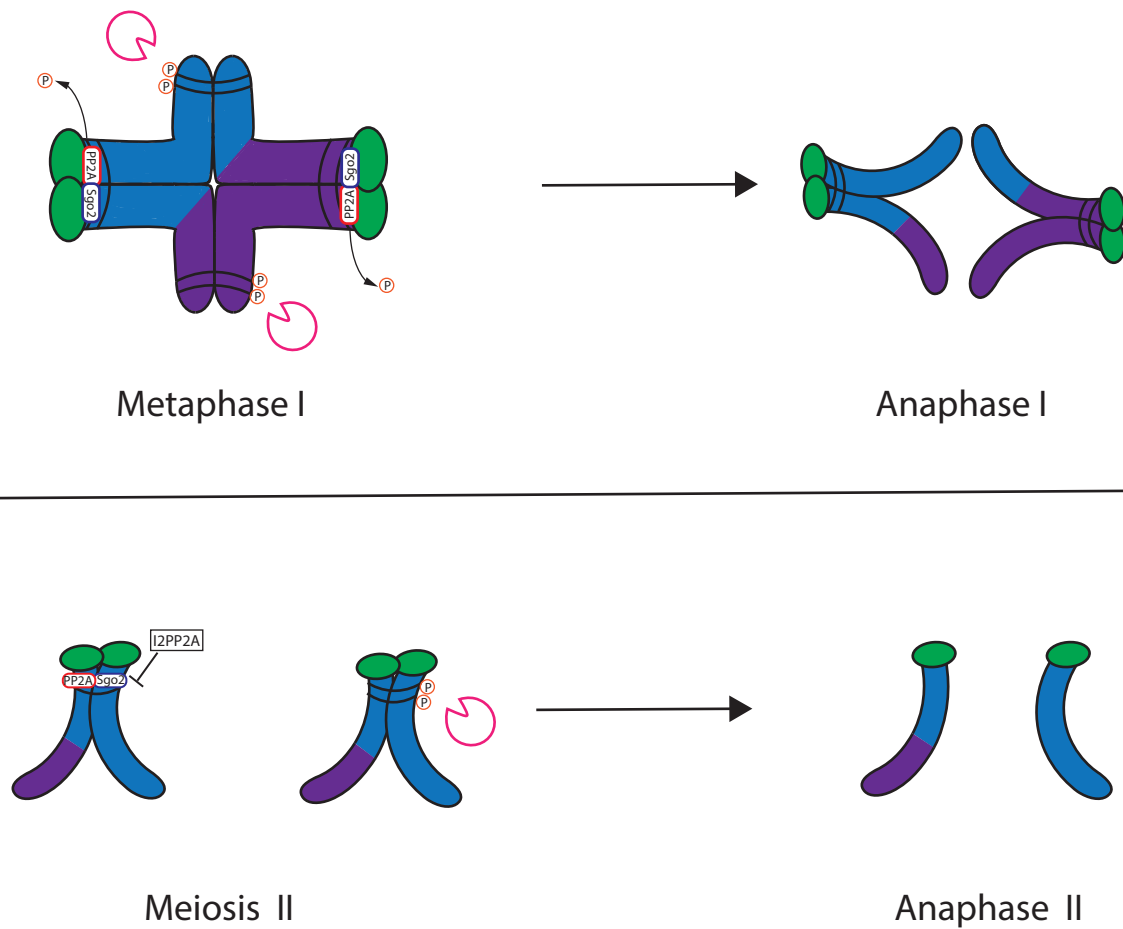


Figure 11: Protection and deprotection of centromeric cohesin during meiosis. Cohesin at the centromeres must be protected during meiosis I and “deprotected” during meiosis II. Cohesin, and more specifically Rec8, is believed to necessitate phosphorylation to be cleaved by separase. Rec8 is protected from phosphorylation by the complex Sgo2-PP2A. Sgo2 recruits PP2A to the centromere and PP2A dephosphorylates Rec8. This protects Rec8 from separase cleavage. At the arms, Rec8 is not protected leading to its phosphorylation and cleavage and chromosome segregation at anaphase I onset. During meiosis II, PP2A-Sgo2 should be inhibited to deprotect centromeric cohesin and allow its phosphorylation and cleavage. This inhibition is thought to be performed by I2PP2A. Once centromeric cohesin is deprotected, separase cleaves it at anaphase onset provoking sister chromatid segregation.

pulled away from each other, this physically dislocates Sgo2-PP2A away from the centromere making it accessible to phosphorylation and separase cleavage (Nerusheva et al. 2014; Lee et al. 2008; Gómez et al. 2007). A second model involves the protein SET/I2PP2A. SET/I2PP2A has several functions such as acting as a histone chaperone and interestingly as a PP2A inhibitor even though this role is highly controversial. It is found on the centromere and co-localizes with PP2A and Rec8. It was shown that SET/I2PP2A knockdown leads to defective sister segregation in anaphase II. When SET/I2PP2A is knocked down, an obvious lack of sister chromatid segregation is observed during anaphase II. Therefore, without SET/I2PP2A, centromeric cohesin stays protected (**Fig. 11**) (Chambon et al. 2013). The mechanism by which SET/I2PP2A deprotects centromeric cohesin is still unknown at this stage, and currently examined in the group.

iii) **Cyclin B1 vs Cyclin B2 regulation during meiosis I**

As evidenced by the KOs of cyclin B1 or cyclin B2, both cyclins share a lot of similarities but also differences in timing of expression. It was shown that when cyclin B1 is depleted only in oocytes, they are able to complete meiosis I similarly to control oocytes. This was found to be caused by an increase in cyclin B2 expression. *Ccnb1*^{-/-} oocytes therefore compensate by expressing more cyclin B2. This increase is sufficient for correct GVBD as well as normal anaphase I timing. Therefore, cyclin B2 is able to take over as part of the MPF when cyclin B1 is not present only during meiosis I. After meiosis I exit, oocytes fail to enter meiosis II because oocytes are not able to re-accumulate enough cyclin B2 and Cdk1 activity when cyclin B1 is absent. Exogenous expression of cyclin B2 at this time is sufficient to rescue this phenotype indicating that it is capable of fulfilling the function of cyclin B1 but that the regulation of its expression is different from that of cyclin B1. This difference in expression could explain why mice lacking cyclin B1 in oocytes are sterile (J. Li et al. 2018). On the other hand, *Ccnb2*^{-/-} mice are viable and fertile but do give a smaller number of litter of smaller size. In these oocytes, GVBD is hindered and asynchronous. They present other defects such as late anaphase onset and in a proportion of oocytes, failure to complete and exit meiosis I. There are nonetheless oocytes that are able to complete it and enter meiosis II. The defects observed in these oocytes are thought to be due to lower MPF activity. Unlike *Ccnb1*^{-/-} oocytes, lack of cyclin B2 doesn't induce an increase in cyclin B1 expression suggesting that

this would be the reason for the phenotype observed (Daldello et al. 2019). Therefore, what marks an important difference is the regulation of expression of both cyclins.

Translation plays a key role in regulating the different steps of meiosis. As previously shown through the KOs, the translation of cyclin B1 compared to cyclin B2 differs during meiotic maturation. Cyclin B2 seems to already be present at important levels in GV oocytes. In addition, cyclin B1 is also present but at lower levels. Cyclin B2 is present at GV and its levels remain somewhat constant throughout meiosis I. However, cyclin B1 experiences an important increase in protein levels as the oocytes resume meiosis. It is important for cyclin B1 levels to increase gradually leading to a paralleled increase of Cdk1 activity (Han et al. 2017). The activity of cyclin B1-Cdk1 allows correct timing in condensation of chromosomes, KT-MT attachments, and chromosome alignment at the metaphase plate. Cyclin B1 translation is governed by a different mechanism compared to cyclin B2. It depends not only on the length of the 3'UTR but also RNA binding protein CPEB1. Short 3' UTRs lead to translation of cyclin B1 during the GV stage while long or intermediate lengths lead to a decrease in translation at this stage but increase of translation after GVBD (Y. Yang et al. 2017; Han et al. 2017). The ribosome loading during meiosis is also an indication of translation rate. Ribosome loading was therefore different between cyclin B1 and cyclin B2. The ribosome loading stayed constant for cyclin B2 during meiosis I but increased after GVBD for cyclin B1 explaining the difference in expression between the two cyclins (Han et al. 2017).

F) Exit of meiosis I: Anaphase I

Before anaphase I, chromosomes are attached to the spindle and aligned at the metaphase plate. They then migrate to the cortex because of the F-actin network present in the oocyte. Since meiotic spindles lack astral microtubules, the connection with the rest of the oocyte is established by this network. The complex F-actin network is different in the cytoplasm, around the spindle and the cortex. This network orients the position of the spindle holding the chromosomes and allows it to migrate towards the cortex of the oocyte provoking the essential asymmetric division (Almonacid, Terret, and Verlhac 2014). Once the SAC is satisfied, the MCC complex disassembles and SAC components are no longer present at the

kinetochores. This allows all Cdc20 to bind to the APC/C. APC/C^{Cdc20} then can bind to its substrates to ubiquitinate them leading to their degradation.

The aim of anaphase is to induce separase activation through degradation of its inhibitors. Active separase can then cleave the cohesin found on chromosomes arms. During metaphase, cyclin B1 bound to Cdk1 exhibit their peak activity. High Cdk1 activity inhibits separase activity and therefore keeps oocytes in meiosis I (Gorr et al. 2006). Once enough cyclin B1 is degraded, Cdk1 activity decreases significantly allowing exit from meiosis I. The second major substrate to be degraded after targeted ubiquitination is securin (**Fig. 12**). Securin is a potent inhibitor of separase acting as a pseudo-substrate. Interestingly, although securin is a strong inhibitor of separase, securin KO mice are viable and fertile (Z. Wang, Yu, and Melmed 2001). Therefore, other mechanisms must be put in place to regulate separase. As previously stated, high cyclin B1-Cdk1 activity can inhibit separase. Using *Xenopus* extracts and *in vitro* assays in a purified system, Olaf Stemmann's lab was able to show that Cdk1 phosphorylates separase. This phosphorylated separase can then bind to the cyclin B1-Cdk1 complex inhibiting its activity. In this complex, separase is also able to inhibit Cdk1 (Gorr, Boos, and Stemmann 2005). Further studies have shown that a specific phosphorylation during mitosis on Ser1126 provokes aggregation of separase making it inactive irreversibly. However, when cyclin B1-Cdk1 binds to the phosphorylated separase, the inactivation is reversible (Hellmuth et al. 2015). This additional regulation of separase activity gives a possible reason why securin is not essential for viability. This mechanism has been observed in mouse oocytes as well. Injection of stable non-degradable cyclin B1 ($\Delta 90$ -cyclin B1) arrests oocytes in metaphase I with no chromosome segregation. Injection of the non-phosphorylatable separase by Cdk1 (PM-separase) induces chromosome segregation with no exit from meiosis I due to the absence of cyclin B1 degradation and decrease of Cdk1 activity (Touati et al. 2012). Therefore, in mouse oocytes, cyclin B1-Cdk1 activity can also inhibit separase activity through inhibitory phosphorylation. However the majority of separase in a WT cells is bound to securin (Konishi et al. 2018). Once securin and most of cyclin B1 is degraded, Cdk1 activity decreases and separase can be fully activated and cleave arm cohesin allowing chromosome segregation. Although degradation of both substrates happens gradually, these events occur concomitantly and after a certain threshold is exceeded, provoke a switch-like response with rapid onset of anaphase and segregation (**Fig. 12**).

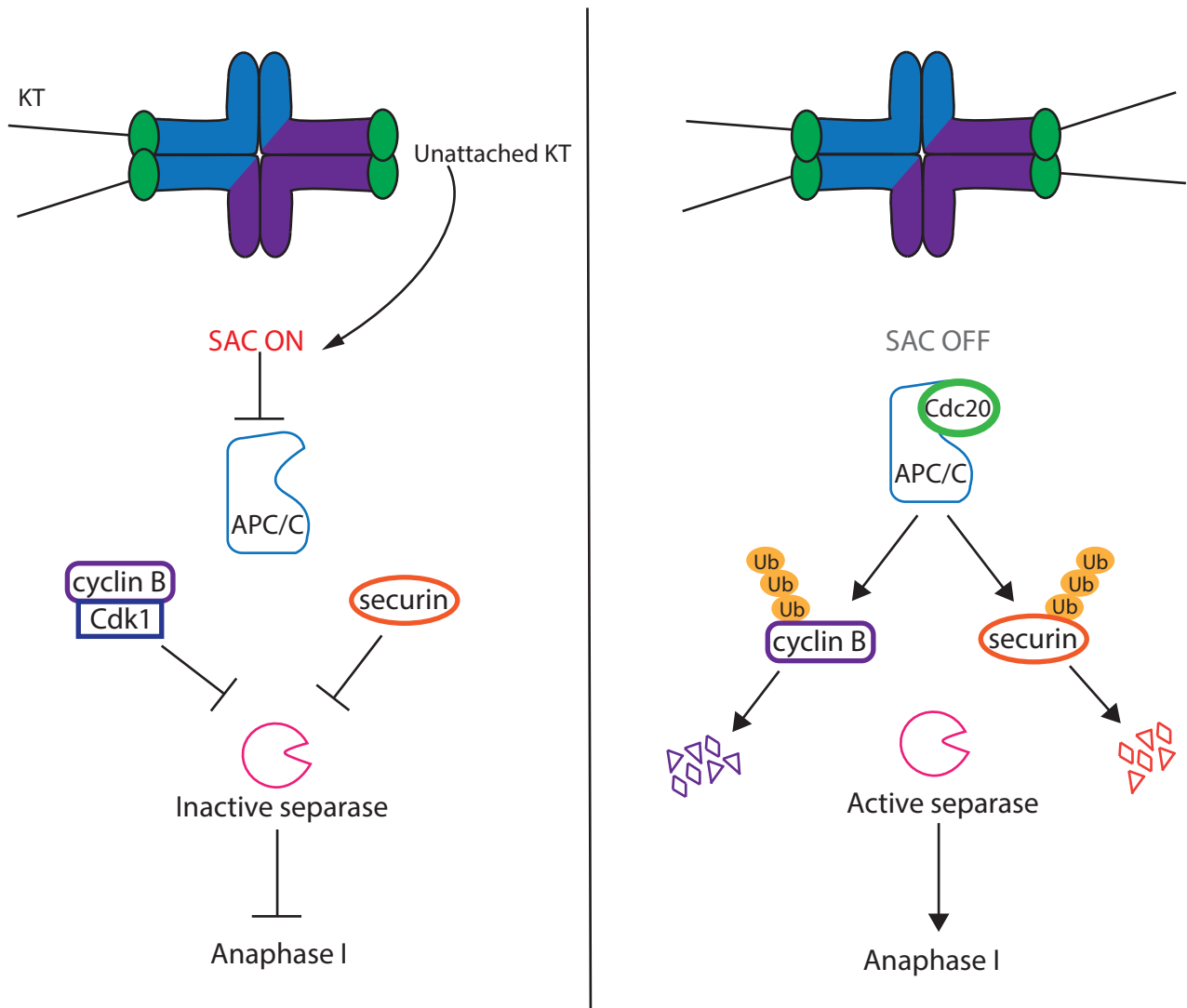


Figure 12: APC/C activity is necessary for anaphase onset during meiosis. During prometaphase, SAC is active due to unattached kinetochores. Active SAC inhibits the activity of APC/C^{Cdc20}. Securin is present and cyclin B1-Cdk1 activity is high at this stage leading to inhibition of separase and meiosis I exit. Once the SAC is satisfied, it is switched off leading to full APC/C^{Cdc20} activity. APC/C^{Cdc20} then targets its substrates securin and cyclin B1 to 26S proteasome dependent degradation by ubiquitinating them. This induces a decrease of Cdk1 activity and separase activation at anaphase onset allowing it to cleave arm cohesin inducing chromosome segregation.

G) Meiosis II

i) Entry into meiosis II

The principal step that must be taken to properly enter meiosis II is to re-accumulate cyclin B1 in order to have a return of Cdk1 activity to inhibit a possible transition into S phase. As stated previously, meiosis contains two segregations with no intermediate S phase. To avoid entry into S-phase after MI exit, Cdk1 must be activated rapidly. The rapid re-accumulation of cyclin B1 is helped by the fact that not all cyclin B1 must be degraded to exit MI and a small quantity is still present. Furthermore, it was suggested that the Mos-MAPK pathway is needed for a correct transition from MI to MII. In *Xenopus* oocytes, the presence and activity of Mos-MAPK allows stabilization of the APC/C inhibitor Emi2. Emi2 can then inhibit APC/C activity allowing re-accumulation of cyclin B1 (Tang et al. 2008; Ohe et al. 2007). This is not the only mechanism put in place to avoid entry into S-phase since Mos^{-/-} oocytes are able to enter meiosis II similarly to control oocytes (M. H. Verlhac et al. 1996; Hashimoto et al. 1994; Colledge et al. 1994). It would be of interest to take a closer look and investigate the mechanisms that control entry into meiosis II, an indispensable step during meiosis. Once chromosomes segregate with extrusion of the first polar body, the spindle must form again and is positioned parallel to the cortex where chromosomes align. The KT-MT attachment must be different compared to meiosis II. At this stage, the KT-MT attachments resemble the ones observed in mitosis pulling each kinetochore to opposite poles. Once again, these attachments are important for correct ploidy once sister chromatids segregate.

ii) Regulation of Metaphase II arrest: CSF pathway

Vertebrate oocytes arrest at metaphase II awaiting fertilization. The factor that provokes this arrest and is found in MII arrested oocytes is called CytoStatic Factor, CSF. Therefore, this metaphase II arrest is also called CSF arrest. The cytostatic factor inhibits APC/C activity allowing this arrest to persist (Perry and Verlhac 2008). CSF arrest was first described in frog oocytes. In 1971, Masui and Markert injected the cytoplasm of an MII arrested oocyte into dividing blastomeres of embryos. This induced a cell cycle arrest. To provide a stable metaphase II arrest, the egg has to maintain high Cdk1 activity and inhibit APC/C activity. This prevents any possible parthenogenic activation and embryo development in an oocyte lacking paternal DNA. To maintain the Cdk1 activity at the necessary threshold, the following regulations are put in place. Cdk1 itself is regulated through a loop of the inhibitory kinase

Wee1B (in mouse oocytes) and its activating phosphatase Cdc25A. Knockdown of Cdc25A leads to exit from meiosis II for an important proportion of oocytes with decondensation of chromosomes and formation of pronuclei. In Cdc25A depleted oocytes, both MPF and MAPK activity is decreased but Cyclin B1 is not degraded. This phenotype is rescued when oocytes are co-depleted for Cdc25A and Wee1B. Although Cdc25A seems to be the main isoform in metaphase II, the authors were not able to sufficiently knockdown Cdc25B and Cdc25C to conclude that they do not play an important role as well (J. S. Oh et al. 2013). The equilibrium between Cdc25A and Wee1B is essential to keep Cdk activity at an appropriate level. Therefore, maintaining high Cdk1 activity in CSF arrested oocytes is essential to prevent precocious exit from meiosis. Another factor controlling high MPF activity in CSF arrested oocytes is the level of cyclinB1. It was shown that the APC/C is still partially active in CSF arrested *Xenopus* oocytes. Therefore, a steady state of cyclin B1 is maintained with turn over of cyclin degradation and synthesis. These dynamic mechanisms are important to maintain this stable arrest. Interestingly, it was found that the threshold of Cdk1 activity must be tightly regulated during MII arrest (Yamamoto et al. 2005; Hochegger et al. 2001; Thibier et al. 1997). Tight regulation is essential when it comes to Cdk1 activity. It is very dynamic and participates in many feedback loops. An example being that Cdk1-cyclin B1 phosphorylates the APC/C leading to its activation. Another example is the phosphorylation of Emi2 achieved by high Cdk1 activity leading to its dissociation from APC/C and therefore APC/C activation.

1. Mos-MAPK pathway

MAPK is a serine threonine kinase involved in many cellular processes. It is involved in the MAPK pathways that functions through a series of phosphorylations: MAPKKK, MAPKK and MAPK. Mos, a known proto-oncogene protein, is a MAPKKK. During mouse meiosis, Mos-MAPK proteins are expressed after GVBD and are present throughout meiotic maturation until metaphase II. The Mos-MAPK pathway performs several tasks during meiotic maturation. For example, it is needed to establish a correct actin network in the oocyte (Chaigne et al. 2013). In *Xenopus* oocytes, Mos is present in GV stage oocytes. Studies have show that to initiate GVBD, Mos is able to activate MPF and MAPK (Haccard et al. 1995; Kosako, Gotoh, and Nishida 1994; Sagata et al. 1989). This doesn't seem to be the case for mouse oocytes. The protein expression of Mos begins after GVBD and is not essential for meiotic resumption as Mos KO oocytes are able to resume meiosis (Colledge et al. 1994;

Hashimoto et al. 1994; Paules et al. 1989). Nonetheless, the injection of Mos in GV oocytes does induce GVBD and therefore Mos is capable of promoting entry into meiosis (M.-H. Verlhac et al. 2000; Choi et al. 1996) However, I will be focusing on the role of this pathway in meiosis II.

The importance of MAPK for CSF arrest is its involvement in the Mos-MAPK pathway. This pathway plays an important role in stabilizing Emi2, the APC/C inhibitor in meiosis II. The components of this pathway include Mos, which phosphorylates Mek (or MAPKK). Mek in turn phosphorylates MAPK (**Fig. 13**). This pathway is conserved in *Xenopus* oocytes. In order to promote *Xenopus* Emi2 (XErp1) activation and stability, this pathway phosphorylates residues S335 and T336, which recruit the phosphatase PP2A important for XErp1 stability. PP2A phosphatase activity counteracts the destabilizing phosphorylation by kinases (Cdk1, Plx1, Ck1) (Hertz et al. 2016; Isoda et al. 2011; Nishiyama, Ohsumi, and Kishimoto 2007; Inoue et al. 2007; Gross et al. 2000, 1999; Bhatt and Ferrell 1999). A similar mechanism is observed for mouse oocytes with a phosphorylation of T337 that is important for Emi2 CSF activity. Therefore, the Mos-MAPK pathway is important in establishing Emi2 activity in meiosis II (Suzuki et al. 2010). However, one point that seems to differ is the kinase that links the Mos-MAPK pathway to Emi2. In *Xenopus* oocytes, the kinase p90rsk is the kinase that phosphorylates Emi2 to stabilize it upon Mos-MAPK activation. This does not seem to be the case in mouse oocytes as it was shown that a triple Rsk (Rsk 1, 2, and 3) KO establishes a standard metaphase II arrest (Dumont et al. 2005). Hence, the link between the Mos-MAPK pathway and Emi2 remains to be discovered in mammalian oocytes. The importance of Mos has also been shown through the study of Mos KO mice. In *mos*^{-/-} oocytes, oocytes enter meiosis II but do not maintain the CSF arrest, exit meiosis II and continue in a parthenogenic manner (M. H. Verlhac et al. 1996; Hashimoto et al. 1994; Colledge et al. 1994). During both meiotic divisions, *mos*^{-/-} oocytes present a spindle with longer microtubules and divide in a symmetrical manner with very large polar bodies, establishing yet another function for this pathway during meiosis (M. H. Verlhac et al. 1996). Additionally, inhibition of Mek using the inhibitor U0126 induces anaphase II onset of MII arrested oocytes. Anaphase II artificial onset in metaphase II arrested oocytes is also known as activation. Therefore, Mek inhibition induces activation of MII arrested oocytes. This was defined by the observation of cytokinesis through polar body extrusion and pronuclear formation. Inhibition of Mek led to a rapid inactivation of MAPK 1h after treatment. This inactivation usually takes 5h during

Metaphase II

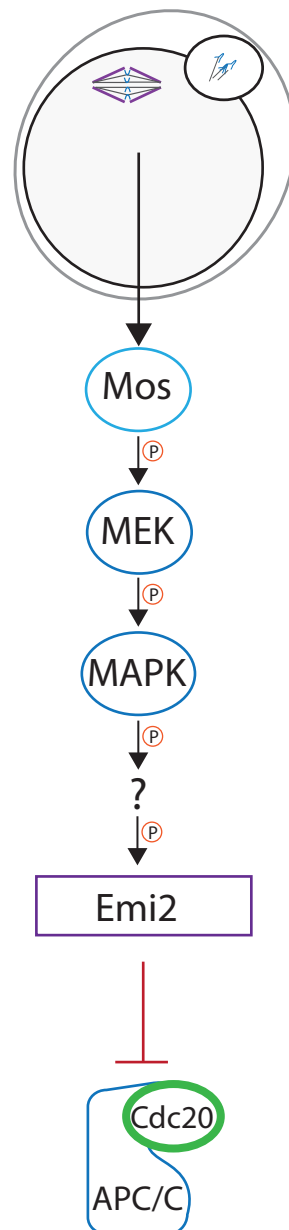


Figure 13: Metaphase II arrest is partly controlled by the Mos-MAPK pathway. After exit from meiosis I, oocytes enter meiosis II and arrest at metaphase II (also called CSF arrest). This arrest must be established rapidly and maintained until fertilization occurs. A pathway is put in place composed of different kinases. Mos first phosphorylated the MAPKK, MEK, which then phosphorylates MAPK. In *Xenopus* oocytes, a fourth kinase is needed, p90Rsk. The identity of this fourth kinase is still unknown in mouse oocytes. This pathway stabilizes the APC/C inhibitor Emi2. Emi2 activity is necessary to establish and maintain inhibition of the APC/C and CSF arrest.

normal fertilization. Inhibition of the pathway also led to inactivation of the MPF in a similar manner to fertilization. Treatment with an inhibitor of Cdk, Roscovitine, provoked a similar phenotype of parthenogenetic activation. It also induced inactivation of MAPK. Both types of inhibition presented similar phenotypes to *mos*^{-/-} oocytes (Phillips et al. 2002). Therefore, in order to obtain a suitable CSF arrest, Mos activity is necessary and its expression must be correctly regulated.

2. Regulation of Emi2, a potent APC/C inhibitor

Emi2, known also as XErp1 in *Xenopus* oocytes, was first characterized in *Xenopus* oocytes. Emi2 is an Fbox protein composed of different protein structures important to its function; a Dbox motif thought to be a competitor of cyclin B1 for binding to the APC/C, a zinc-binding domain (ZBD) that inhibits APC/C ubiquitin ligase activity and an RL tail (needed for its binding to the APC/C) (Suzuki et al. 2010). In female meiosis, Emi2 is necessary not only to maintain an MII arrest but also to enter meiosis II correctly (**Fig. 13**). Emi2 is present in mouse oocytes and seems to present a similar function to Xerp1 (Shoji et al. 2006). It was shown through morpholino injection, that oocytes lacking Emi2 do not properly enter meiosis II. After polar body extrusion, DNA quickly decondenses, no MII spindle is formed and a nucleus begins to form (Madgwick et al. 2006; Shoji et al. 2006). Therefore, oocytes are not able to enter meiosis II. This is due to a lack of re-accumulation of cyclin B1 (Madgwick et al. 2006). H1 kinase activity is significantly reduced and additional cleavages were also observed (Shoji et al. 2006). Injection of Emi2 or stable cyclin B1 right after polar body extrusion rescued this phenotype (Madgwick et al. 2006). Therefore, Emi2 is necessary for the entry and establishment of meiosis II.

Overexpression of Emi2 and more specifically just its C-term (aa 251-Cter) induces an MI arrest in mouse oocytes demonstrating that ectopic expression of Emi2 is sufficient to inhibit the APC/C in meiosis I (Suzuki et al. 2010; Shoji et al. 2006). This C-term is essential for Emi2's CSF activity and contains the MAPK phosphorylation motif, the PP2A interacting domains, the Dbox, the ZBR domain and RL tail (**Fig. 14**). This fragment of Emi2 was found to co-immunoprecipitate with both Cdc20 and Cdc27 (a component of the APC/C) (Suzuki et al. 2010). Through different studies mainly on *Xenopus* oocytes, it is proposed that Emi2 inhibits the APC/C by binding to it and also inhibiting Cdc20 from binding to the APC/C (Sako et al. 2014; Tang et al. 2010; Ohe et al. 2010; Nishiyama, Ohsumi, and Kishimoto 2007). The first

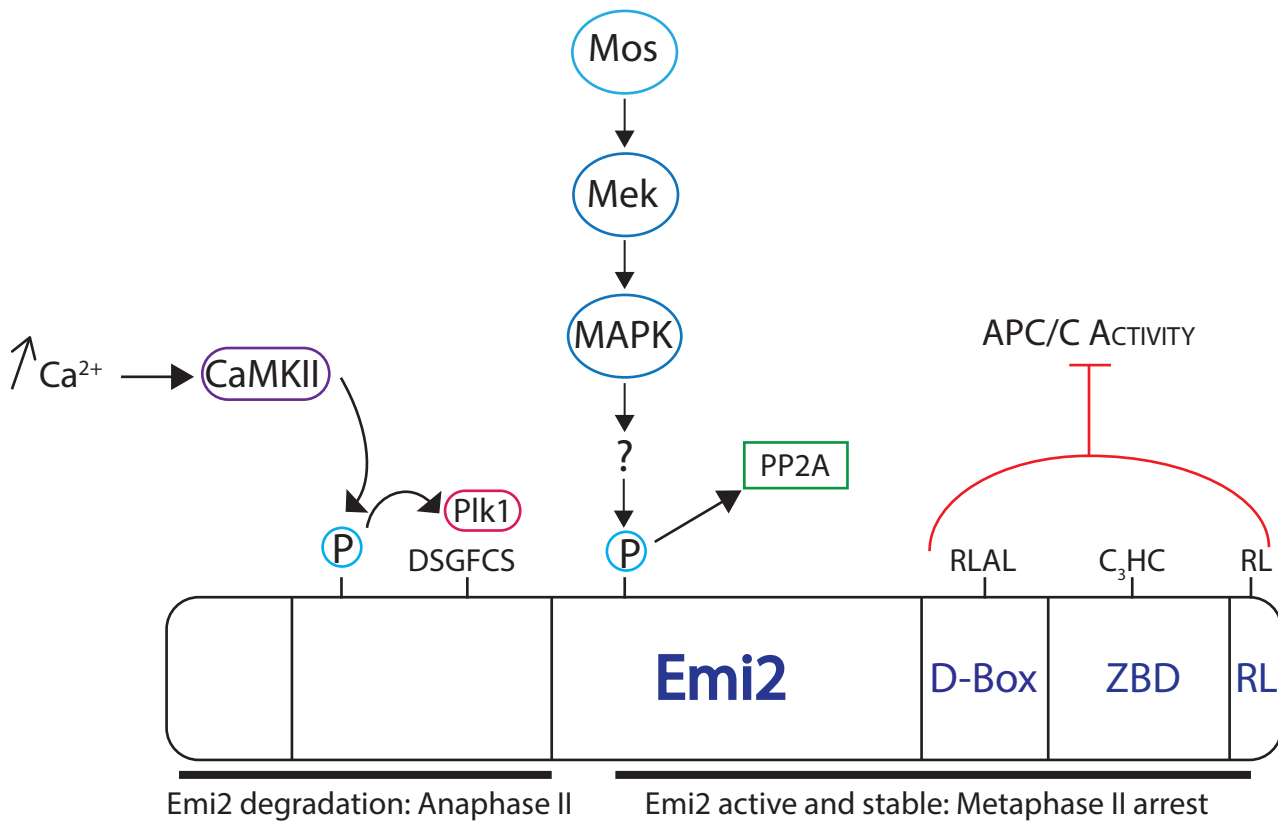


Figure 14: Emi2 regulation during metaphase II arrest. Emi2 activity is closely regulated by different proteins. Its activity as an APC/C inhibitor is completed mainly by its C-term that comprises a D-box, a zinc binding domain (ZBD) and a RL tail. The Mos-MAPK pathway phosphorylates Emi2. This phosphorylation allows recruitment of PP2A essential for Emi2 stabilization. Once fertilization occurs, increase of calcium in the cytoplasm activates the kinase CaMKII, which phosphorylates Emi2 recruiting Plk1 to Emi2. Both these steps are essential for destabilization and degradation for Emi2 leading to MII exit.

study of Emi2 in mouse oocytes showed that Emi2 could interact with Cdc20 through immunoprecipitation (Shoji et al. 2006).

The regulation of Emi2 concerns different pathways present to ensure persistent CSF arrest. Its goal is to inhibit APC/C activity, maintaining high MPF activity. At the entry of meiosis II, the activation of the Mos-MAPK pathway leads to the phosphorylation of Emi2 (**Fig. 13**). As mentioned above, the direct link between this pathway and Emi2 is unknown in mouse oocytes. In *Xenopus*, where XErp1/Emi2 was first characterized, this phosphorylation is achieved by the kinase p90Rsk. The phosphorylation of XErp1/Emi2 on residues S335 and T336 by p90Rsk leads to the recruitment of the phosphatase PP2A. PP2A is essential in maintaining the MII arrest by counteracting destabilizing phosphorylation on Emi2 by Cdk1 (**Fig. 14**). The importance of PP2A in mouse oocytes was shown through its inhibition during meiosis II. Inhibiting this phosphatase using Okadaic Acid and a dominant negative mutant, leads to release from CSF arrest. This lift is characterized by degradation of the APC/C substrates due to destabilization of Emi2 achieved by Cdk1 phosphorylation (Chang et al. 2011). In *Xenopus*, it is known that Cdk1 does indeed phosphorylate Emi2 to make it more unstable which is then counterbalanced by Mos-MAPK recruited PP2A. During CSF arrest, cyclin B1 levels are dynamic but controlled to maintain Cdk1 activity at the necessary threshold. This limits Cdk1 activity to not override PP2A's role in stabilizing Emi2 and maintaining MII arrest. This mechanism is also shown to be present at the transition MI-MII and important for correct exit of MI and entry into MII. Emi2 is expressed at the end of MI when Cdk1 activity is high leading to its destabilization. Once anaphase occurs, Cdk1 activity drops allowing PP2A activity to take over and stabilize Emi2 to then inhibit APC/C and allow re-accumulation of its substrates (Tang et al. 2008). These pathways once again highlight the dynamic nature of establishment and maintenance of CSF arrest.

Emi2 KO mice were generated to investigate its role during male meiosis. Both male and female mice were viable but sterile. This demonstrates the important role of Emi2 not only in female meiosis but also for male fertility. However, the role of Emi2 differs in male meiosis. Spermatocytes lacking Emi2 do not progress past diplotene stage in prophase due to low Cdk1 activity (Gopinathan et al. 2017). Therefore, its role is important at a different meiotic time point.

H) Exit from meiosis II and fertilization

The completion of female mammalian meiosis is achieved after fertilization. When the sperm comes into contact with the egg and both gametes fuse, it releases phospholipase C zeta (PLC ζ), which then induces waves or oscillations of Ca²⁺ in the egg (Sanders and Jones 2018; Jones 2005). The waves of calcium induce the activation of a kinase calmodulin-dependent protein kinase II (CamKII) (Rauh et al. 2005; Lorca et al. 1993). This kinase is able to play on two pathways; destabilization of Emi2 and activation of Wee1B. It was shown that CamKII phosphorylates Wee1B provoking its activation, which leads to inhibition of Cdk1 activity (**Fig.15**) (Jeong Su Oh, Susor, and Conti 2011). In *Xenopus* oocytes, the phosphorylation of Emi2 by CamKII recruits polo-kinase 1 (Plx1, Plk1 in mammals). Plx1 then phosphorylates Emi2 on the DSGX₃S motif, which is recognized by \square Trcp-dependent proteasomal degradation (Schmidt et al. 2005; Rauh et al. 2005). This mechanism seems to be conserved in mouse oocytes as well. A non-canonical phosphorylation site of CamKII was found to be important for Emi2 degradation. Mutation of the site T176 to A inhibited the degradation of Emi2 after Sr²⁺ activation (Suzuki et al. 2010). Using X-ray crystallography and biochemical assays, this same site and an additional one, T152, was shown to be necessary for Plk1 recruitment to Emi2 PBD (polo box domain) (**Fig. 14**). Inhibition of this interaction domain led to a compromised MII exit (Jia et al. 2015). Plk1 is consequently recruited to Emi2 by CamKII phosphorylation and this interaction is necessary for Emi2 degradation and MII exit (Madgwick et al. 2006). In addition, removal of Cdc20 in MII oocytes inhibits exit from meiosis II when artificially activated or fertilized. The co-activator Cdc20 is hence essential for proper APC/C activity in MII exit (Shoji et al. 2006). At exit of meiosis II, it was shown that Emi2 is degraded before cyclin B1. This degradation is, as shown in *Xenopus*, independent of the APC/C (Madgwick et al. 2006). This degradation mechanism also seems to be conserved between *Xenopus* and mouse. Mutations on the phosphodegron DSGXnS inhibits Emi2 degradation, which hints at a \square Trcp-dependent proteasomal degradation (Suzuki et al. 2010). Another component in *Xenopus* was shown to play a role in destabilization of Emi2 and exit from MII arrest, calcineurin. However, no data so far shows that this is also the case for mouse oocytes. Thus, for correct metaphase II exit, inhibition of Emi2 through calcium dependent CamKII activity leads to degradation of Emi2 and activation of APC/C^{Cdc20}, which targets cyclin B1 and securin to degradation, releasing separase from

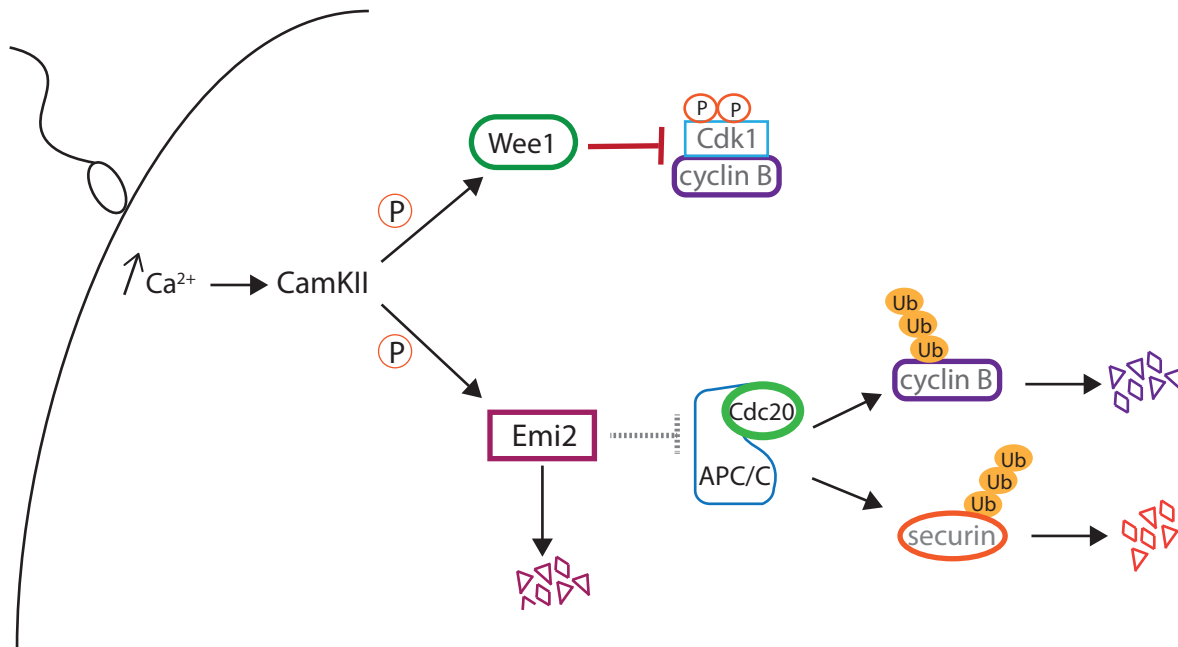


Figure 15: Fertilization triggers anaphase II onset. Fertilization of the MII arrested oocyte induces an increase of Ca^{2+} waves in the cytoplasm. This increase activates CamKII, which, in turn phosphorylates Wee1 activating it. Wee1 can then inhibit Cdk1 activity through its inhibitory phosphorylation. Concomitantly, CamKII phosphorylates Emi2 to target it for degradation. APC/C becomes active and targets its substrates, securin and cyclin B1, for degradation. All these steps lead to a decrease in Cdk1 activity and meiosis II exit.

inhibition (**Fig. 15**). Sister chromatids are then able to segregate and the fertilized egg can then begin to form a pronucleus.

4. APC/C activity during meiosis

A) Co-activators Cdh1 and Cdc20 in meiosis

Contrary to mitosis, the APC/C binds first to its co-activator Cdh1 during prophase to maintain GV arrest and then prometaphase for a gradual increase in Cdk1 activity, followed by binding to Cdc20 during metaphase needed for exit from meiosis I. Both Cdh1 and Cdc20 seem to be present in meiosis II. Cdc20 is active at fertilization degrading its specific substrates containing a Dbox. Then, after second polar body extrusion Cdh1 becomes active once Cdk1 activity decreases, targeting its substrates harboring a KEN box and/or a Dbox (Chang, Levasseur, and Jones 2004). In order to study the role of Cdh1 in meiosis, a conditional KO was created in mice. The depletion of Cdh1 in germ cells had an effect on both male and female fertility. Male KO mice are sterile due to defects in both mitosis preceding meiosis and during meiosis that led to apoptosis. This means that no mature spermatozoa were found in male KO mice. Female KO mice were sub-fertile with ovarian failure that was observed by the age of 5 months. Overall, these issues were found to be due to the fact that Cdh1 is necessary to complete appropriate prophase (Holt et al. 2014).

In order to study the role of Cdc20, Jin *et al* decided to generate a hypomorphic mutant of Cdc20 due to the embryonic lethality of the full mutant. This technique produced mice with lower levels of Cdc20. These levels did not have an observable effect on somatic cells since adults were healthy and comparable to control mice. This suggests that the low level of Cdc20 was sufficient to carry out its function during mitosis. However, female fertility was strongly impacted. These mutants produced very few to no offspring. Taking a closer look, they observed issues with segregation during meiosis. Although oocytes could be fertilized, they harbored an abnormal count of chromatids or chromosomes. This was due to a high number of lagging chromosomes in meiosis I. This created around 90% of aneuploid oocytes. After fertilization, these aneuploid embryos never reached the blastocyst stage (Jin et al. 2010). This phenotype is probably due to inefficient degradation of APC/C substrates such as cyclin B1 and securin leading to delayed metaphase and incomplete segregation.

The KO Cdh1 doesn't inform us about its role during meiotic maturation since it is needed to complete prophase at earlier stages. Injection of Cdh1 morpholino in GV oocytes permitted further analysis of the roles of APC/C^{Cdh1} vs APC/C^{Cdc20}. Oocytes depleted for Cdh1 had chromosomes that do not congress during metaphase. During anaphase I, lagging chromosomes are observed as well as non-disjunction of chromosomes. The knockdown also provoked accelerated meiosis I and PBE due to precocious degradation of cyclin B1 and securin. Cdh1 was found to be important for Cdc20 degradation during early prometaphase. The levels of Cdc20 were increased at an earlier time point in Cdh1 knockdown oocytes. APC/C^{Cdh1} seemed to preferentially target Cdc20 for degradation over cyclin B1 and securin. The degradation of Cdc20 is thought to be necessary to avoid precocious APC/C^{Cdc20} activity. When Cdc20 is depleted in oocytes, no PBE is observed and meiosis I was not completed. Both knockdowns indicate that regulated synthesis of Cdc20 during meiosis is important. APC/C^{Cdh1} targets Cdc20 for degradation at the start of meiosis to allow the gradual accumulation of cyclin B1-Cdk1. Higher levels of Cdk1 inhibit Cdh1 through phosphorylation. This then permits re-synthesis of Cdc20. These findings underline the importance of protein synthesis during meiotic maturation (Reis et al. 2007). The importance of these co-activators is obviously vital for development of a healthy adult and is needed in both mitosis and meiosis. Aberrant expression of either can lead to important defects. Accordingly, studies have shown that overexpression of Cdc20 is observed in several cancers (Wu et al. 2013).

B) Substrates during meiotic maturation

As mentioned above, the APC/C with their co-activators recognize specific motifs of their substrates; D-boxes, KEN boxes, ABBA box (rarer motifs including the O-, G-, A- and CRY boxes). During meiotic maturation, APC/C targets several different substrates. The degradation of cyclin B1 and securin through their ubiquitination is necessary to exit MI and MII. APC/C does discriminate between different forms of cyclin B1 or securin and has a temporal regulation when it comes to an order of substrate degradation (Levasseur et al. 2019; Konishi et al. 2018; Hellmuth et al. 2014). The temporal choice of substrates is important for correct progression through meiosis to avoid precocious exit or delayed meiotic maturation. APC/C is regulated to have a temporal and sequential effect on its substrates discriminating between the different substrates available in the cell.

This all plays a role in establishing correct segregation and ploidy. APC/C^{Cdh1} seems to play an important role during the GV arrest in mouse oocytes since cyclin B1 or B2 synthesis does occur during the prophase I arrest. At this stage, APC/C^{Cdh1} is active in order to keep cyclin levels low and subsequently maintaining low Cdk1 activity to maintain the arrest in parallel to Wee1B inhibitory phosphorylation. Once Cdk1 activity increases sufficiently during MI, Cdh1 becomes inactive and Cdc20 takes over as APC/C co-activator. Although SAC inhibits most APC/C activity by blocking the interaction with Cdc20, a proportion of Cdc20 escapes this inhibition and some APC/C activity is still observed before the SAC is satisfied and inactive (Holt, Lane, and Jones 2013). It was shown that during meiosis, there is a difference in timing of cyclin B1 degradation depending on whether it is free or bound to cdk1. In meiosis, cyclin B1 is in excess compared to Cdk1 and this difference in quantity is important for correct progression through meiosis I. Cyclin B1 bound to Cdk1 seems to be protected from degradation during prometaphase, while free cyclin B1 is degraded. This equally suggests that not all APC/C is inhibited by the SAC, allowing the degradation of excess cyclin B1 (Levasseur et al. 2019). Therefore, hidden motifs and localization do seem to be contributors of which substrate is targeted and the order of degradation. In mitotic cells, it was shown that cyclin B1 that is localized closest to chromosomes represents the pool that inhibits separase unlike cytoplasmic cyclin B1. The cytoplasmic pool is preferentially targeted by the APC/C while cyclin B1 closer to the chromosomes is degraded at a slower rate (Konishi et al. 2018). Post-translational modification of substrates can also affect substrate selection. In mitotic cells, securin bound to separase is protected by PP2A that dephosphorylates destabilizing phosphorylations. However, free securin is not dephosphorylated making it more of an efficient substrate of the APC/C leading to its degradation. Therefore, securin protected by the binding of separase and post-translational modifications is degraded later during mitosis (Hellmuth et al. 2014). These two forms of regulation of degradation of cyclin B1 or securin have not yet been studied in mouse oocytes.

5. *Xenopus* as an alternative model system

Findings in *Xenopus* oocytes have been discussed throughout the introduction. Regulatory mechanisms in meiosis have been extensively explored and studied using *Xenopus* oocytes. Furthermore, many of these findings are conserved in mouse oocyte maturation (**Fig. 16**). The study of meiotic mechanisms using *Xenopus* oocytes as an alternative model has many benefits. First, the majority of the different processes during meiotic maturation are conserved between *Xenopus* and mouse oocytes. *Xenopus* oocytes also undergo the two vital arrests, prophase I arrest and CSF arrest. One major benefit of using it as a second model is the fact that *Xenopus* oocytes contain a much higher quantity of protein compared to mouse oocytes. This makes it a great model to use for biochemical techniques such as western blots and immunoprecipitation. Oocytes are quite large (1-1,3 mm) and are easily manipulated with possibilities of morpholino, protein and RNA injection. Another advantage for using *Xenopus* oocytes is the fact that adult females produce a large number of oocytes, hundreds, compared to 30-50 in mice depending on the genetic background. This provides us with a lot of material to work with. In the second part of my results section, I will be using *Xenopus* oocytes as a tool to determine the molecular mechanisms controlling the cyclin that my PhD work focuses on, namely cyclin B3.

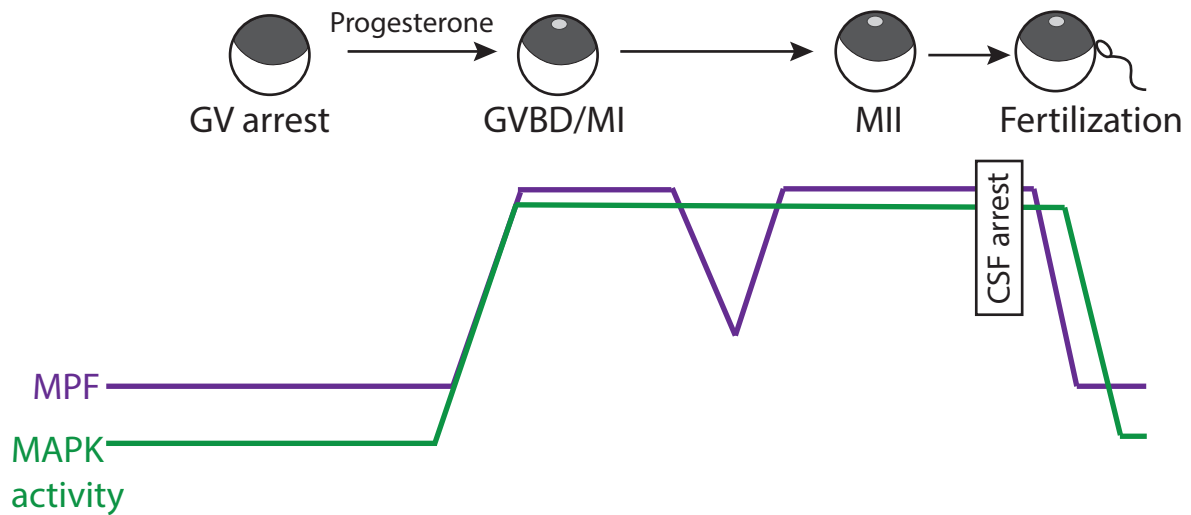


Figure 16: Xenopus oocyte meiotic maturation. Similarly to mouse oocytes, Xenopus oocytes are arrested at GV stage with low MPF and MAPK activity. Oocytes of the correct size resume meiosis after addition of progesterone. This induces an increase of both MPF and MAPK activity. GVBD is marked by the appearance of a white spot on the pigmented part of the oocyte. Oocytes exit meiosis I as seen by the decrease of MPF activity and enter meiosis II with no S phase. Like mouse oocytes, they arrest at metaphase II (CSF arrest). They then exit this arrest once they are fertilized leading to a drop in both MPF and MAPK activity. Adapted from Tunquist and Maller 2003

6. Cyclin B3

A) Characteristics of the structure of Cyclin B3

As previously stated, cyclin B3 is a B type cyclin that in mammals seems to have a specific meiotic role in females. Cyclin B3 was first discovered in chicken cells. It shares particularities with both A and B type cyclins showing similarities in protein sequence with both types of cyclins and nuclear localization in interphase like A-type cyclins. Mouse cyclin B3 protein was found to share 34,6% identity with cyclin A1, 34,5% with cyclin A2 and 34% identity with cyclin B1. The similarity is located mostly in the cyclin box (**Fig. 17**). There are some similarities also found in the N-term mostly at the destruction box while what is in between these two areas shows an important lack of conservation (Malumbres and Barbacid 2005). More extensive study must be done to further understand what in these sequences gives a different function to cyclin B3 compared to A and B type cyclins. It is interesting to note that in some studies, cyclin B3 is considered as a type of cyclin separate from A and B type cyclins (J. C. Lozano et al. 2012). Like other cyclins, cyclin B3 has a cyclin box as well as a Dbox. Its structure is conserved in different species. Cyclin B3 seems to share more protein sequence similarities with cyclin B3 from other species than with A or B type cyclins. For example, mouse cyclin B3 shares 51,2% identity in protein sequence with zebrafish, 39,5% with *C. elegans*, 42,4% with *Drosophila* cyclin B3 and 50,6% with *Xenopus* cyclin B3. The similarity in protein sequence is once again mostly located at the cyclin box and some in the N-term. The importance and role of the section of cyclin B3 that is not conserved is still unknown and characteristics of the sequence must be explored (**Fig. 18**). However, an interesting change is observed in placental mammals. In these species, cyclin B3 undergoes an abrupt 3-fold increase due to an extension of an exon, exon 8. This makes the size of cyclin B3 in placental mammals significantly larger than in other species: 1396 aa for mouse compared to 403 aa in chicken, 385 aa in *C. elegans* and 575 aa for *Drosophila* (**Fig. 18**). However, the consequence of this extension on its role is still unknown and must be further investigated. This extension covers the section between the N-term and the cyclin box. The extended exon in human cyclin B3 shows mostly repetitive amino acid sequences. There seems to be no specific structures such as tertiary structures in this extension. Whether this has an effect on the role is unknown. The lack of specific structures and repetitive sequences indicates that the non-conserved area of exon 8 is unstructured which may be beneficial for protein-protein



Figure 17: Cyclin B3 compared to A and B type cyclins. Cyclin B3 is compared to cyclin A1, cyclin A2, cyclin B1 and cyclin B2. The % in identity of the protein sequence compared to cyclin B3 is shown next to each cyclin. The N-term of the cyclin box is in purple and the C-term is in pink. The similarities in protein sequence is located in the cyclin box. Cyclin B3 can be considered as a separate type of cyclin close to both A-type and B-type cyclins but considerably different in sequence. Adapted from Malumbres and Barbacid 2005.

interactions. Therefore, this extension was hypothesized to provide new unknown functions through novel protein-protein interactions (J. C. Lozano et al. 2012).

In order to fulfill its role, a cyclin must partner up with a Cdk. In different organisms, the capability of cyclin B3 to bind to a Cdk has been explored. When first discovered in chicken cell, through immunoprecipitation, it was found that cyclin B3 can form a complex *in vivo* with Cdk1. Furthermore, with transfection of cyclin B3 in HeLa cells, they were able to observe that cyclin B3 forms a complex both with Cdk1 and Cdk2 similarly to cyclin A (Gallant and Nigg 1994). In human cells, cyclin B3 was found to co-immunoprecipitate with Cdk2. However, there was no real kinase activity detected with this complex (J.-C. Lozano et al. 2002). In both *C. elegans* and *Drosophila*, cyclin B3 is capable of forming a complex with Cdk1 although the possibility of Cdk2 binding to cyclin B3 was not investigated in these organisms (Deyter et al. 2010; van der Voet et al. 2009; Jacobs, Knoblich, and Lehner 1998).

B) Role and conservation in different organisms

Cyclin B3 is conserved evolutionarily in several species from *C. elegans* to humans. However, it is not expressed the same way in all these organisms. In human, the only detectable cyclin B3 was found in testis, although through RT-PCR it is found in 8 tissues overall (J.-C. Lozano et al. 2002). In *Xenopus*, although RNA of cyclin B3 exists, there was not enough to detect the protein and therefore, it was believed that it plays no major role in the *Xenopus* (Hochegger et al. 2001). In *C. elegans*, cyclin B3 is vital for development and is found in oocytes and embryos (Deyter et al. 2010; van der Voet et al. 2009). *Drosophila* cyclin B3 is present during meiosis and mitosis although it is only necessary for female meiosis (Jacobs, Knoblich, and Lehner 1998). In mice, the mRNA levels seem to be highest in testis and developing ovaries. The expression of mammalian cyclin B3 was pronounced during male prophase, and to a lesser extent, fetal developing ovaries (Nguyen et al. 2002). Therefore, cyclin B3 was thought to have a role in early meiotic prophase. However, as explained below, we could show that this is not the case.

The role of cyclin B3 has been investigated in a few different species. Overexpression of cyclin B3 during male meiosis leads to abnormal progression through spermatogenesis (Refik-Rogers, Manova, and Koff 2006). This suggests that cyclin B3 must be tightly regulated to obtain normal mature spermatozoa. In mouse oocytes, this role has been studied by using a knockdown technique. Using this method, the authors determined that oocytes lacking

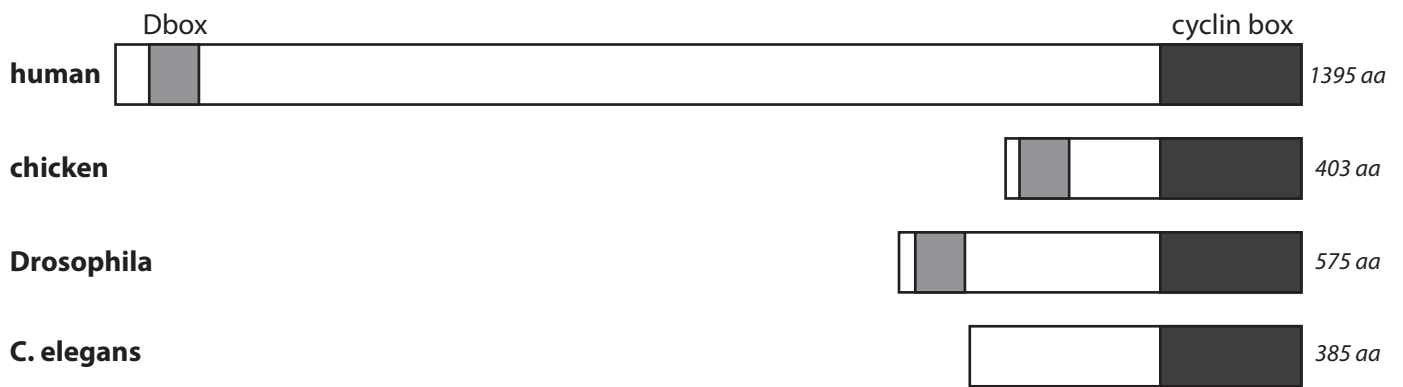


Figure 18: Cyclin B3 size from different species. In most species, cyclin B3 contains both a Dbox and a cyclin box. Most of the similarity in sequence is found in the cyclin box being conserved from *C. elegans* to humans. Human cyclin B3 is much larger in size compared to other cyclin B3 being 1395aa long in humans compared to 575aa long in *Drosophila*. This difference in size is due to an extension of an exon (exon 8) and seems to be specific to placental mammals. Adapted from Nguyen et al 2002

cyclin B3 arrest at metaphase I independently from the SAC (T. Zhang et al. 2015). However, this study was not sufficient to investigate the overall effect of cyclin B3 depletion on the female mouse. But, it does indicate that cyclin B3 seems to play an important role during female meiotic maturation in mice.

This role has been further examined in *C. elegans*. Cyclin B3 is vital for embryo development. A depletion of cyclin B3 through knockdown is lethal in this organism. During meiosis, the knockdown oocytes go through meiosis similarly to the control but an important proportion present abnormalities starting from anaphase II. At this stage in cyclin B3 depleted eggs, sister chromatids do not segregate (Deyter et al. 2010; van der Voet et al. 2009). Once the zygote enters mitosis, more defects are observed. In order to form a correct embryo, nuclear envelope breakdown (NEBD) must occur synchronously and both nuclei have to migrate towards each other to fuse. However, when cyclin B3 is knocked down, these steps are asynchronous and delayed. Finally, as the first mitosis progresses, the first metaphase is very delayed and chromosomes do not separate. This does not prevent other steps to occur such as cleavage furrow, spindle and centrosome disassembly. DNA was also at times cut when placed where the furrow forms. Furthermore, the metaphase delay is also observed in the small percentage of oocytes that complete anaphase II correctly. Depletion of SAC components does partially rescue the phenotype described (Deyter et al. 2010). Therefore, this mechanism seems to be partly dependent on the SAC contrarily to what was observed in mouse oocytes.

Cyclin B3 is also important in *Drosophila* oocytes. Although cyclin B3 is important for female fertility in *Drosophila*, it is dispensable for mitosis and male meiosis. This might be due to the case that it seems to act in a redundant way with cyclin B1. For *Drosophila*, cyclin B1 is dispensable for mitosis and only a double knockdown of both cyclin B1 and cyclin B3 induces embryonic lethality (Jacobs, Knoblich, and Lehner 1998). Cyclin B3 was found to be important to progress through anaphase in both meiosis I and meiosis II. This shows once again that it could have a role in promoting APC/C activity. It does have a redundant role with cyclin A where they are needed to inhibit DNA replication after meiosis I (Bourrouh et al. 2016).

Cyclin B3 was also shown to play a role in *Ciona intestinalis*. In this organism, cyclin B3 seems to have a different function than that observed in the other models studied. So far, cyclin B3 was mostly suggested to have a role in promoting anaphase. However, in ascidians, it seems

to play a role in the regulation of timely zygotic genome activation (ZGA). During zygote development, the organism must at some point transition from the maternal to the zygotic genome. In this study, they found that depletion of cyclin B3 induced an untimely and precocious activation of the ZGA (Treen et al. 2018). The role of cyclin B3 in ascidian seems related to transcription, providing another possible function of this cyclin.

Although cyclin B3 is not necessary for mitosis and is poorly expressed in somatic cells, its ectopic expression can lead to cell abnormalities. This is the case found in a certain type of sarcoma, Ewing-like sarcoma. These cancer cells express a fusion protein BCOR-CCNB3. BCOR is a transcriptional repressor that is ubiquitously expressed. Mutations of BCOR as well as other fusions with other proteins are found in different types of cancers. It was shown that the truncated form of cyclin B3 separated from its fusion partner could lead to the sarcoma phenotype observed (W.-S. Li et al. 2016; Cohen-Gogo et al. 2014; Pierron et al. 2012). Thus, abnormal presence and activity of cyclin B3 can alter normal cell cycle progression leading to aberrant and severe phenotypes.

7. Project

Thanks to numerous studies on meiotic models it has become clear that regulation of meiosis is quite different from that of mitosis. This is mainly due to the fact that there are significant differences between the two types of M phase such as the pattern of segregation. During meiosis, two divisions with no intervening S-phase takes place with chromosome segregation in meiosis I and sister chromatid segregation in meiosis II. As established, meiosis, especially mammalian female meiosis, is prolonged beginning in fetal life up to adulthood. Furthermore, the completion just of meiosis I lasts several hours (7h-11h in mouse, 24h-36h for humans). Mammalian oocytes must also mark two important arrests for long periods of time in a stable manner, namely the prophase I arrest and metaphase II arrest, as previously explained. Therefore, it is clear that meiosis demands additional regulation to correctly complete the two meiotic divisions.

The aim of my PhD project was to characterize the role of cyclin B3 during female meiotic maturation. This particular cyclin was thought to mainly have a role in germline prophase. It is expressed in both human and mouse mainly in the germline during prophase and more abundantly in testes compared to developing ovaries. However, through the use of cyclin B3 KO mice, it has become clear that cyclin B3 only plays a primordial role in female meiosis. It doesn't seem to be vital for either mitosis or male meiosis. Unexpectedly, cyclin B3 does not regulate entry into meiosis I, but occupies an essential role in meiosis I, to prevent precocious establishment of CSF arrest. Importantly, this role of cyclin B3 seems to be conserved in oocytes of different organisms. I have investigated the effect of the lack of cyclin B3 on the different stages of female meiosis. The role of cyclin B3 and the possible mechanism it utilizes to regulate female meiosis will be discussed and detailed in the following chapters.

Results

PART I: Cyclin B3 promotes anaphase I onset in oocyte meiosis

Cdk activity determines the entry and exit of the different phases of the cell cycle. This is performed with its regulatory subunit, cyclins. Different cyclin-Cdk complexes are formed and oscillate during the cell cycle. This activity is partly regulated by timely degradation of the cyclins. The APC/C, an E3 ubiquitin ligase, targets its substrates, such as M-phase cyclins, for degradation. Cyclin B1-Cdk1 complex is essential for mitosis and meiosis. Increased activity of cyclin B1-Cdk1 is necessary for resumption of meiosis while inactivation of Cdk1 and significant degradation of cyclin B1, due to APC/C activity, allows anaphase I onset and exit from meiosis I. Such as outlined in the introduction, cyclin B2 was also found to play a role in entry into meiosis and is able to fulfill the function of cyclin B1 during meiosis if expressed with the correct timing.

Cyclin B3 is a particular type of cyclin. It is considered to be a B-type cyclin due to similarity in sequence identity but in chicken cells, cyclin B3 was mostly found in the nuclear fraction like A-type cyclins. In *C. elegans* and *Drosophila*, cyclin B3 binds to Cdk1. It is necessary for *Drosophila* female meiosis as lack of cyclin B3 leads to sterility in females. It promotes anaphase II in *C. elegans* and is necessary for embryonic development in a SAC dependent manner. Cyclin B3 in mouse was found to be significantly expressed in testis and was thought to have a role in early prophase I. Moreover, knockdown of cyclin B3 in mouse oocytes using RNAi interrupted meiotic maturation. However, the role of cyclin B3 was not further elucidated.

For my PhD, I studied the role of cyclin B3 by using a KO created through CRISPR-Cas9 in collaboration with Scott Keeney and former PhD student Mehmet Erman Karasu from the Memorial Sloan Kettering Cancer Center. First, male meiosis was investigated due to the strong expression of cyclin B3 found in testis in the group of our collaborators. They showed that surprisingly male KO mice present no irregularities, with normal meiosis. Female cyclin B3 KO mice were found to be sterile. Mating with WT males did not lead to any pregnancies and no pups were born. This is the main phenotype observed as tissues and organs did not show any anomalies. Cyclin B3 in mice seems to be essential for female fertility and dispensable for mitosis and male meiosis. Hence, we decided to investigate female meiosis in these mice.

Looking closely at female meiosis, ovaries removed from 2-3 month old mice were examined. Primordial and growing follicles seem to be similar to control ovaries. This indicates that steps preceding prophase I arrest, such as recombination, have occurred normally. If this were not the case, we would expect to observe a decrease of the follicles. Oocytes withdrawn from ovaries were matured *in vitro* and polar body extrusion was analyzed. We found that, unlike control oocytes, the majority of cyclin B3 KO oocytes do not extrude polar bodies. However, this phenotype is not fully penetrant as a very small proportion of oocytes (less than 10%) are able to exit meiosis I with polar body extrusion.

To further investigate these oocytes, we injected H2B-RFP and Tubulin-GFP in GV oocytes and analyzed them through live imaging. Control oocytes show a spindle fully formed with aligned chromosomes at metaphase. These oocytes then go through anaphase I with chromosome segregation and first polar body extrusion. The spindle is reformed and oocytes arrest at metaphase II. Cyclin B3 KO oocytes also reach metaphase with a fully formed spindle and aligned chromosomes but do not exit meiosis I with no chromosome segregation or polar body extrusion. They do not go through metaphase-to-anaphase transition.

To verify if there is indeed a lack of chromosome segregation in cyclin B3 KO oocytes, chromosome spreads were performed. In metaphase I, homologous chromosomes are physically linked at their recombination sites (chiasmata) and the two sister chromatids making up a chromosome are held together by the ring complex cohesin at the centromeres and arms. This conformation was observed on chromosome spreads at BD+6h for both control and KO oocytes. At metaphase II arrest, at BD+16h for mouse oocytes, chromosomes have segregated with cleavage of arm cohesin by separase giving way to sister chromatids held together by cohesin only at the centromeres. This is what can be seen in control spreads at BD+16h. KO oocytes at this time point still have homologous chromosomes in a meiosis I conformation proving chromosomes do not segregate.

Finally, to take a closer look at the spindle and KT-MT attachment, whole oocytes immunofluorescence (IFs) was done with stable spindle staining, Hoechst staining for chromosomes and CREST for kinetochores. IFs were performed at metaphase I and II for control and KO oocytes. Both control and KO spindle was formed properly in a bipolar barrel shape. Chromosomes are aligned and correctly attached in both conditions with sister kinetochores oriented in a monopolar manner. In meiosis II, control oocytes contain a fully

formed spindle with aligned chromosomes. Sister chromatids are as expected held together by cohesin at the centromere. The microtubules are attached in a similar way as in mitosis, with kinetochores oriented in a bipolar manner. For KO oocytes, the spindle and the chromosomes are in the same conformation as in meiosis I and stably attached. Through these different techniques, we were able to determine that cyclin B3 seems to be needed for anaphase onset and that lack of this cyclin leads to a metaphase I arrest.

Since cyclin B3 KO oocytes are arrested at the first metaphase, we decided to verify if separase was active. As previously stated, separase activity is needed for chromosome and chromatid segregation as it cleaves the kleisin subunit of the cohesin complex. To analyze separase activity, a sensor previously established in the lab was used. This sensor is made up of H2B-mCHERRY that drives the sensor to the chromosomes fused to Scc1-YFP, which is a mitotic substrate of separase. Chromosomes are then colored by colocalizing mCherry and YFP, but once separase becomes active at anaphase, it cleaves Scc1-YFP resulting in a change to only mCherry (red) on the chromosomes. This is the case for control oocytes where, at anaphase, separase becomes active giving off only red color on the chromosomes. KO oocytes do not show any cleavage of the YFP signal away from chromosomes therefore strongly suggesting that separase is not active.

Inactive separase in these oocytes suggested that the APC/C could also be inactive allowing some of its substrates, securin and cyclin B1, to inhibit separase activity. Endogenous substrate degradation was investigated through western blots. Starting with securin, samples of control oocytes were collected in early meiosis I and at anaphase. The same was done with KO oocytes with additional time points after anaphase onset timing. Securin in control oocytes is present early in meiosis I and is degraded fully at anaphase onset. A similar profile is observed for cyclin B1 for control oocytes, although it is not fully degraded. For KO oocytes, securin and cyclin B1 are both present in early meiosis I and are still observed at later time points. Securin is slightly decreased at later time points while cyclin B1 seems to increase at these time points. The high levels of cyclin B1 in KO oocytes suggests that these oocytes have high CDK1 activity. To analyze this, a H1 kinase assay was performed at similar time points since most of the H1 kinase activity is due to cyclin B1-Cdk1 activity. Control oocytes show high kinase activity at metaphase I and a striking decrease at anaphase. KO oocytes show continual high kinase activity at metaphase I and at the timing of anaphase onset. This correlates with high cyclin B1 observed in the western blot. Since

securin levels do decrease while kinase activity is maintained at a high level, inhibition of Cdk1 activity could lead to a rescue of the phenotype, allowing exit from meiosis I. In order to investigate this, KO oocytes were treated with the Cdk inhibitor roscovitine. These oocytes were able to extrude the first polar body and segregate chromosomes indicating that this metaphase I arrest seems to be due to high cyclin B1-Cdk1 activity. To test if the APC/C is indeed partially active as suspected due to partial degradation of endogenous securin, KO and control oocytes were injected with exogenous securin-YFP and cyclin B1-GFP. Both of these exogenous substrates were degraded in both KO and control oocytes confirming that the APC/C is indeed partially active.

A metaphase arrest with high cyclin B1-Cdk1 activity could be due to an active SAC. Mad2 staining at kinetochores showed no difference between control and KO oocytes with the signal decreasing at late metaphase in both conditions. KO oocytes were also treated with reversine to override any possible SAC activity. This treatment did not lead to a rescue, and the oocytes remained arrested at metaphase I. Therefore, this arrest is not due to an active SAC.

Cyclin B3, like known APC/C substrates, contains a Dbox motif. It was also shown to be degraded in a sequential manner after cyclin B1 during mitosis in *Drosophila*. Exogenous cyclin B3-RFP was co-injected with securin-YFP or cyclin A2-GFP in WT oocytes to compare degradation patterns. Cyclin B3-RFP is degraded later than both securin-YFP and cyclin A2-GFP making it a potential late APC/C substrate during meiosis I. Δ Dbox cyclinB3-RFP was also injected in WT oocytes. The deletion of the Dbox inhibited degradation of cyclin B3 making it stable and confirming that it is indeed an APC/C substrate.

By expressing and purifying from insect cells, we were able to show that cyclin B3 does co-purify with Cdk1 and is able to exhibit kinase activity. Mutation on a motif located in the cyclin box, MRAIL, important for binding of cyclin-Cdk complexes to their substrates, abolishes the kinase activity of cyclin B3-Cdk1 even though binding is not altered. Therefore, cyclin B3 forms a complex with Cdk1 exhibiting kinase activity. To confirm that this kinase activity is essential for cyclin B3 function in meiosis I, KO oocytes were injected with WT cyclin B3, cyclin B3 with the MRAIL mutation (cyclin B3 MRL mutant), and the stable cyclin B3 Δ Dbox cyclinB3. Both WT cyclin B3 and Δ Dbox cyclinB3 rescue the KO phenotype with polar body extrusion and chromosome segregation. In addition, degradation of cyclin B3 is not essential for meiosis I exit. Cyclin B3 MRL mutant injection does not induce anaphase

onset and KO oocytes remain arrested. This was confirmed by chromosome spreads where chromosomes have not segregated. Chromosome spreads of cyclin B1 injected into cyclin B3 KO oocytes also showed that cyclin B1 can not rescue the KO phenotype. Cyclin B3 with Cdk1 activity is essential for its function in meiosis I.

Mammalian cyclin B3 is much larger in size compared to other metazoans due to an exon extension. It was therefore possible that cyclin B3 from other species can not compensate for mouse cyclin B3 function. However, injection of *Xenopus*, zebrafish, or *Drosophila* cyclin B3 in cyclin B3 KO mouse oocytes provokes polar body extrusion rescuing the KO phenotype. This shows that although it differs in some aspects they are functionally complementary. Therefore, this data indicates that cyclin B3 has a specific role in meiosis I, different from that of cyclin B1, suggesting that Cdk1 activity might have substrate specificities depending on which cyclin sub-unit binds to it.

ARTICLE

Cyclin B3 promotes anaphase I onset in oocyte meiosis

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Meiosis poses unique challenges because two rounds of chromosome segregation must be executed without intervening DNA replication. Mammalian cells express numerous temporally regulated cyclins, but how these proteins collaborate to control meiosis remains poorly understood. Here, we show that female mice genetically ablated for cyclin B3 are viable—indicating that the protein is dispensable for mitotic divisions—but are sterile. Mutant oocytes appear normal until metaphase I but then display a highly penetrant failure to transition to anaphase I. They arrest with hallmarks of defective anaphase-promoting complex/cyclosome (APC/C) activation, including no separase activity, high CDK1 activity, and high cyclin B1 and securin levels. Partial APC/C activation occurs, however, as exogenously expressed APC/C substrates can be degraded. Cyclin B3 forms active kinase complexes with CDK1, and meiotic progression requires cyclin B3-associated kinase activity. Cyclin B3 homologues from frog, zebrafish, and fruit fly rescue meiotic progression in cyclin B3-deficient mouse oocytes, indicating conservation of the biochemical properties and possibly cellular functions of this germline-critical cyclin.

Introduction

Eukaryotic cell division depends on oscillations of CDKs associated with specific cyclins (Morgan, 1997; Malumbres et al., 2009; Uhlmann et al., 2011; Fisher et al., 2012). In vertebrate somatic cells, progression from G1 into S phase, G2, and mitosis depends on the cyclin D family, followed by the cyclin E, A, and B families (Morgan, 1997). Ordered CDK activity likewise governs progression through meiosis: chromosome condensation, congression, and alignment require a rise in cyclin B1-CDK1 activity, then anaphase onset is driven by inactivation of cyclin B1-CDK1 by the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets substrates for degradation (Heim et al., 2017). Cyclin B1-CDK1 activity then reaccumulates, without DNA replicating again, while cyclin B1-CDK1 activity is low between meiosis I and II (Petronczki et al., 2003; El Yakoubi and Wassmann, 2017). Apart from cyclin B2, which can compensate for loss of cyclin B1 (Li et al., 2018a), the roles of specific cyclins in determining orderly meiotic progression remain poorly understood in mammalian oocytes.

Particularly enigmatic is cyclin B3, which forms a family distinct from other cyclins based on sequence alignments, but which contains structural motifs characteristic of both A- and B-type cyclins (Nieduszynski et al., 2002; Gunbin et al., 2011). Chicken cyclin B3 shows nuclear localization when ectopically expressed in HeLa cells, similar to A-type cyclins (Gallant and Nigg, 1994), but its orthologues cluster more closely with B-type

cyclins based on amino acid sequence (Nieduszynski et al., 2002; Gunbin et al., 2011). Cyclin B3 is conserved across metazoans (Lozano et al., 2012). *Ciona intestinalis* cyclin B3 counteracts zygotic transcription (Treen et al., 2018). In *Xenopus laevis*, no cyclin B3 protein was detected in oocytes or the first embryonic divisions (Hochegger et al., 2001), but whether cyclin B3 has a role at these stages has not been addressed. *Drosophila melanogaster* cyclin B3 is dispensable for mitotic divisions and male fertility but is essential for female fertility (Jacobs et al., 1998). In flies, cyclin B3 promotes anaphase onset in early embryonic divisions (Yuan and O'Farrell, 2015), and loss of cyclin B3 perturbs exit from meiosis I (Jacobs et al., 1998). Moreover, *Drosophila* cyclin B3 is degraded depending on the APC/C in mitosis, with later timing than cyclin A and B (Sigrist et al., 1995; Yuan and O'Farrell, 2015). Unlike in *Drosophila* (Yuan and O'Farrell, 2015), in *Caenorhabditis elegans* embryos, cyclin B3 appears to promote anaphase onset in meiosis II and mitosis via the spindle assembly checkpoint (SAC; van der Voet et al., 2009; Deyter et al., 2010). *C. elegans* and *Drosophila* cyclin B3 associate with CDK1 and support kinase activity in vitro (Jacobs et al., 1998; van der Voet et al., 2009).

Cyclin B3 protein is larger in placental mammals because of extension of a single exon (Lozano et al., 2012). Mouse cyclin B3 was proposed to promote recombination in male meiosis because its mRNA is present early in prophase I (Nguyen et al.,

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2002; Refik-Rogers et al., 2006). Prolonged cyclin B3 expression perturbed spermatogenesis and cyclin B3 interacted with CDK2, although no kinase activity was detected (Nguyen et al., 2002; Refik-Rogers et al., 2006). In females, cyclin B3 was speculated to govern meiotic initiation because its mRNA is up-regulated as oogonia cease proliferation and enter prophase I (Miles et al., 2010). RNAi-mediated knockdown of cyclin B3 by ~70% in cultured oocytes perturbed meiosis I progression (Zhang et al., 2015). However, the molecular basis of the progression defect was not defined, and RNAi off-target effects could not be excluded, so cyclin B3 function remained unclear.

To sum up, cyclin B3 is implicated in female meiosis and early embryogenesis in different organisms, but its function is poorly understood, particularly in mammals. To gain insights into its potential roles, we generated mice with a targeted mutation in *Ccnb3*, the gene encoding cyclin B3. Mutant mice are viable, males are fertile, and oocytes enter meiosis. Strikingly, however, most mutant oocytes cannot progress beyond metaphase I, so *Ccnb3*-deficient female mice are sterile. Our data suggest that cyclin B3 fine-tunes APC/C activity toward meiotic substrates in oocyte meiosis I, at least in part as a partner enabling CDK1 catalytic activity, and that the biochemical function of cyclin B3 is conserved in vertebrates.

Results

Female mice are sterile after genetic ablation of *Ccnb3*

Ccnb3 spans 62 kb on the X chromosome and gives rise to a 4.1-kb mRNA of 14 exons encoding a 157.9-kD protein. CRISPR/Cas9 genome editing generated a 14-bp deletion at the 3' end of the 2.7-kb-long exon 7, causing a frameshift and premature stop codon upstream of the cyclin box in exons 9–13 (Fig. 1 A). Immunoprecipitation/Western blotting of extracts from *Ccnb3*^{-Y} mutant testes yielded no signal for either full-length cyclin B3 or the predicted truncation product (1,090 amino acids, ~123 kD; Fig. 1 B), indicating that the mutation is a null or severe loss-of-function allele.

Surprisingly, cyclin B3-deficient males were fertile, with no detectable meiotic abnormalities (the male phenotype will be described elsewhere). Heterozygous females had normal fertility, and homozygous mutant females were born in Mendelian frequencies (105 +/- [25.7%]: 100 -/- [25.5%]: 102 +/Y [25%]: 101 -/Y [24.8%] offspring from *Ccnb3*^{+/-} females crossed with *Ccnb3*^{-Y} males). Cyclin B3-deficient animals displayed no gross abnormalities in major organs and tissues (Materials and methods), so cyclin B3 is dispensable in most if not all nonmeiotic cells.

Strikingly, however, mutant females were sterile. No pregnancies were observed, and no pups were born from *Ccnb3*^{-/-} females ($n = 9$) bred with wild-type males for two rounds of mating. Thus, whereas cyclin B3 is dispensable for male meiosis, it is essential for female fertility.

Cyclin B3 is required for the metaphase-to-anaphase I transition in oocytes

Mammalian female meiosis initiates during fetal development (Herbert et al., 2015; El Yakoubi and Wassmann, 2017). Oocytes

complete chromosome pairing and recombination before birth, and then enter a prolonged arrest (the dictyate stage) and form primordial follicles, which are the resting pool of germ cells that will be recruited later for ovulation. *Ccnb3*^{-/-} ovary sections showed abundant primordial and growing follicles at 2–3 mo of age and were quantitatively and morphologically indistinguishable from controls (Fig. 1 C). Mutations compromising oogonial development, meiotic initiation, or recombination cause drastically reduced or absent follicles (Di Giacomo et al., 2005; Baltus et al., 2006; Li and Schimenti, 2007; Bolcun-Filas et al., 2014; Jain et al., 2018). The normal number of follicles in *Ccnb3*^{-/-} females therefore implies that cyclin B3 is dispensable for germ cell divisions before meiosis and for early events of meiotic prophase I.

We next tested the ability of mutant oocytes to enter meiosis I and to divide. Germinal vesicle (GV) stage oocytes from adult *Ccnb3*^{-/-} mice and age-matched controls from the same breedings (*Ccnb3*^{+/-}) were cultured in vitro. Germinal vesicle breakdown (GVBD; corresponding to nuclear envelope breakdown in mitosis) occurred with normal timing (within 90 min) and efficiency in *Ccnb3*^{-/-} oocytes (Fig. 1 D); thus, cyclin B3 is dispensable for entry into meiosis I. At ~7–10 h in culture after GVBD, depending on the mouse strain, oocytes extrude a polar body (PB), indicating execution of the first division and exit from meiosis I (Holt et al., 2013; El Yakoubi and Wassmann, 2017). *Ccnb3*^{-/-} mice had a highly penetrant defect: oocytes from most mutant mice failed to extrude a PB (71 of 79 mice; Fig. 1 D). These findings indicate that cyclin B3 is required for progression through meiosis I and that meiotic progression failure is the cause of female infertility. Because penetrance of meiotic arrest was mouse specific (i.e., most mice yielded only arrested oocytes), we focused on the large majority of mice in which no PB extrusion was observed. (For every *Ccnb3*^{-/-} mouse, a fraction of oocytes was matured in the incubator and left untreated to verify that no PB extrusion occurred; see Materials and methods.) Possible reasons for incomplete penetrance are addressed in the Discussion.

To further characterize arrest, we followed meiotic progression by live imaging. GV oocytes were injected with mRNA encoding histone H2B fused to RFP and β -tubulin fused to GFP to visualize chromosomes and spindles, respectively. Whereas control oocytes separated their chromosomes and extruded PBs ~8 h after GVBD, *Ccnb3*^{-/-} oocytes remained in a metaphase I-like state, with a spindle that had migrated to the cortex and chromosomes aligned at the spindle midzone (Fig. 2 A and Videos 1 and 2).

Hypothesizing that mutant oocytes were failing to transition to anaphase I, we predicted that homologous chromosomes would still be conjoined in arrested oocytes. Chromosome spreads from controls had the expected 20 bivalent chromosomes (homologue pairs connected by chiasmata) before the first division (6 h after GVBD) and pairs of sister chromatids juxtaposed near their centromeres afterward (metaphase II arrest, 16 h after GVBD; Fig. 2 B). In contrast, *Ccnb3*^{-/-} oocytes had intact bivalents with no homologue separation throughout the arrest in culture, indicating a metaphase I arrest (Fig. 2 B).

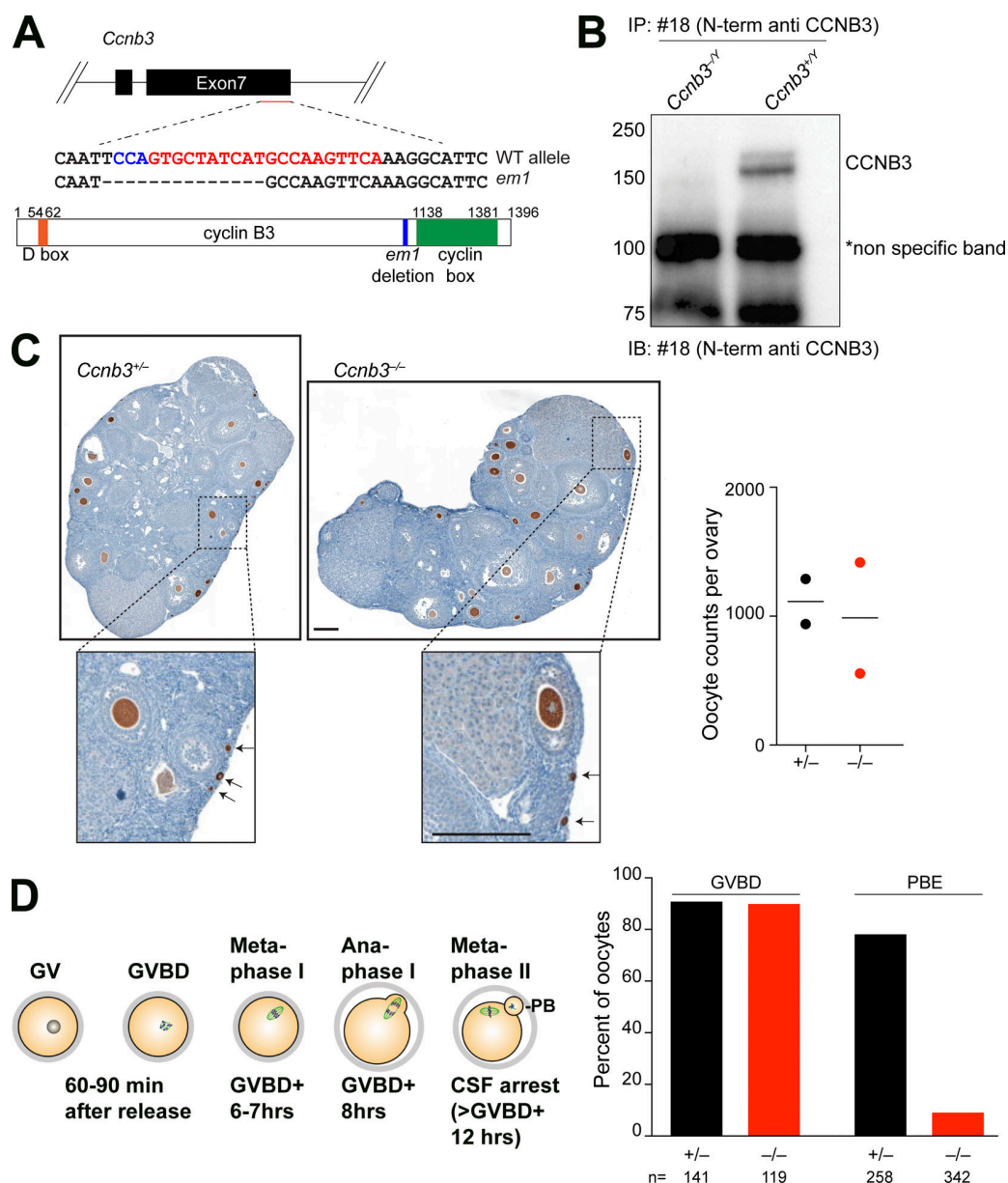


Figure 1. Generation of *Ccnb3*^{-/-} mice reveals a requirement for cyclin B3 in female meiosis. (A) Targeted mutation of *Ccnb3*. Top: Exons 6 and 7 are shown as black rectangles. A portion of the sequences of the wild-type allele and *em1* mutant allele are shown, with guide RNA position in red and the complement of the protospacer adjacent motif (PAM) sequence in blue. Bottom: Schematic of cyclin B3 protein. (B) Immunoprecipitation and Western blot analysis of adult testis extracts using an anti-cyclin B3 monoclonal antibody. (C) Apparently normal folliculogenesis and oocyte reserves in *Ccnb3*-deficient females. Left: PFA-fixed, anti-MVH-stained ovary sections from 3-mo-old animals. Zoomed images show presence of primary/primordial follicles indicated by black arrows. Scale bars, 500 μ m. Right: Oocyte counts from two 3-mo-old females of each genotype. PFA-fixed ovaries were sectioned completely and stained with anti-MVH. Stained oocytes were counted in every fifth ovary section and summed. Each point is the count for one ovary of one animal; means are 1,114 for *Ccnb3*^{+/+} and 987 for *Ccnb3*^{-/-}. (D) The scheme illustrates progression through the meiotic divisions until metaphase II arrest in oocytes of the mouse strain used. The graph on the right shows percentages of mature oocytes of the indicated genotypes that underwent GVBD within 90 min in culture after release, and oocytes that extruded PBs. n: total number of oocytes counted in >10 independent experiments (GVBD: 141% *Ccnb3*^{+/+}: 90.78%; 119 *Ccnb3*^{-/-}: 89.91%; PB extrusion (PBE): 258 *Ccnb3*^{+/+}: 78.13%; 342 *Ccnb3*^{-/-}: 9.06%).

Arrest was not due to failure in forming spindles or stable kinetochore-microtubule interactions, because metaphase I spindles displayed no obvious morphological defects and cold-stable microtubule fibers were comparable to control oocytes in metaphase I (Fig. 2 C). Microtubule fibers attached to kinetochores were clearly visible, so cyclin B3 is not

required for kinetochore-microtubule attachments (Fig. 2 C). Spindles in *Ccnb3*^{-/-} oocytes retained the barrel shape characteristic of metaphase I even at late time points 16 h after GVBD, when control oocytes had progressed to metaphase II, with elongated spindles and more focused spindle poles (Fig. 2 C).

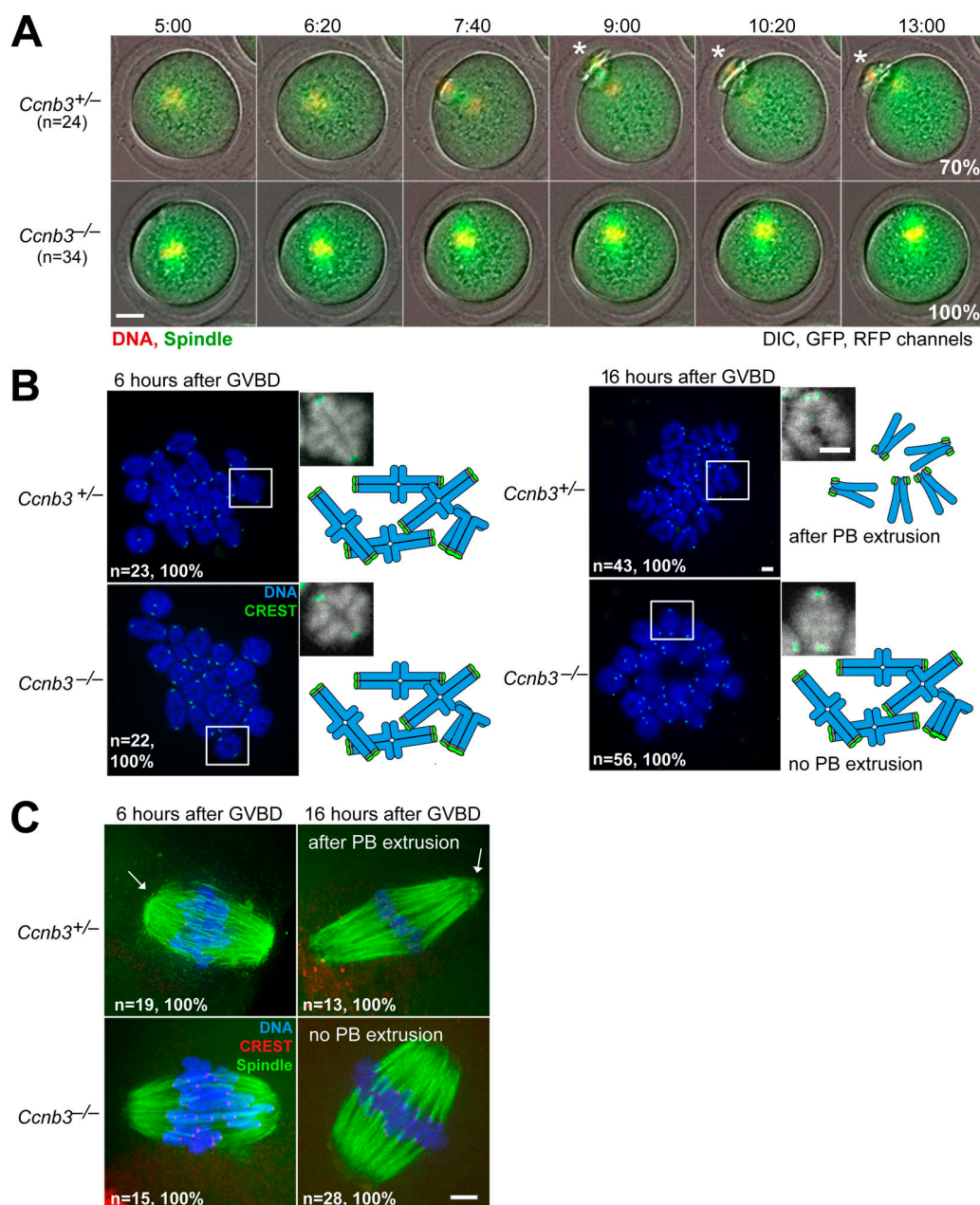


Figure 2. Cyclin B3 is required for the metaphase-to-anaphase I transition in oocytes. (A) Live-cell imaging of meiotic maturation. β -Tubulin-GFP and H2B-RFP mRNA, used to visualize spindle and chromosomes, were injected into GV-stage oocytes, which were then induced to enter meiosis I. Selected time frames are shown, with overlay of DIC, GFP, and RFP channels of collapsed z-sections (11 section, 3- μ m steps) from a representative video. Time after GVBD is indicated as hours:minutes. n: number of oocytes analyzed. Percentage of oocytes of the observed phenotype from two independent experiments is indicated. White asterisks indicate PBs. Scale bar, 20 μ m. Related to Videos 1 and 2. (B) Chromosome spreads 6 h (corresponding to metaphase I) and 16 h (corresponding to metaphase II in controls) after GVBD. Kinetochores were stained with CREST (green) and chromosomes with Hoechst (blue). Insets show typical chromosome figures observed; for better visualization, chromosomes are shown in grayscale. Schematics of metaphase I bivalents or metaphase II univalent chromosomes are shown to aid interpretation. Scale bar, 5 μ m. n: number of oocytes analyzed in three independent experiments. (C) Whole-mount immunofluorescence staining of cold-treated spindles. Microtubules were stained with anti-tubulin antibody (green), kinetochores with CREST (red), and chromosomes with Hoechst (blue). Spindle poles typical of metaphase I and metaphase II are indicated with arrows in controls. Scale bar, 5 μ m. n: number of oocytes analyzed in three independent experiments.

Arrest in *Ccnb3*^{-/-} oocytes is accompanied by incomplete degradation of APC/C substrates

Separase is a cysteine protease that cleaves the kleisin subunit of cohesin complexes to dissolve sister chromatid cohesion at

anaphase onset in mitosis and meiosis (Stemmann et al., 2006). Before anaphase, separase activity is restrained in part by binding to its inhibitory chaperone, securin, and by high cyclin B-CDK activity (Stemmann et al., 2006). In oocytes,

APC/C-dependent degradation of securin and cyclin B1 activates separase, which cleaves the meiotic kleisin REC8, allowing homologous chromosomes to separate (Herbert et al., 2003; Terret et al., 2003; Kudo et al., 2006). To follow separase activity in live oocytes, we expressed a separase activity sensor (Nikalayevich et al., 2018), similar to one used in cultured mitotic cells (Shindo et al., 2012). The sensor harbors two fluorescent tags separated by a fragment of mitotic kleisin RAD21 as a separase substrate; this ensemble is fused to histone H2B to localize it to chromosomes. Cleavage by separase leaves only the H2B-attached fluorophore on chromosomes (Fig. 3 A). Sensor cleavage occurs only under conditions when endogenous REC8 is removed from chromosome arms (Nikalayevich et al., 2018). In control oocytes, the fluorescent signal on chromosomes changed abruptly from yellow to red upon anaphase onset (Fig. 3 B, top row, compare 6:20 to 6:40). In contrast, both fluorophores remained chromatin-associated throughout arrest in *Ccnb3*^{-/-} oocytes (Fig. 3 B, bottom row), indicating that separase cannot be activated and explaining why mutant oocytes cannot separate homologous chromosomes and extrude PBs.

Failure to activate separase was potentially due to a failure to degrade cyclin B1 and securin, so we analyzed each protein by Western blotting (Fig. 3 C). In control oocytes, levels of both proteins decreased precipitously at anaphase onset (compare 8:30 to 3:30 after GVBD). In *Ccnb3*^{-/-} oocytes, in contrast, cyclin B1 levels did not decrease, even at late times. In fact, cyclin B1 levels were consistently higher during arrest (8:30 and later) than in prometaphase (3:30). Securin levels decreased between 3:30 and 8:30 after GVBD, but remained higher than in the controls. Thus, although securin levels drop partially, both proteins are inappropriately stabilized at the time the meiosis I division would normally be performed.

Next, we asked whether cyclin B1 stabilization led to elevated kinase activity in extracts from cultured oocytes. Phosphorylation of histone H1 in vitro is mostly attributable to CDK1 plus cyclin B1 (Kubiak et al., 1992; Morgan, 1997; Malumbres et al., 2009; Fig. 3 D). Control oocytes showed high H1 kinase activity in metaphase I (6:00 after GVBD) and a drop in kinase activity when they extruded PBs (8:00 after GVBD; Ledan et al., 2001). *Ccnb3*^{-/-} oocytes showed high kinase activity in metaphase I (6:00 after GVBD), but no drop in kinase activity was observed at the time when normal cells extruded PBs (8:00 after GVBD; Fig. 3 D). These findings suggest that persistence of high cyclin B1 levels in the mutant maintains high CDK1 activity.

Because endogenous securin decreased slightly in *Ccnb3*^{-/-} oocytes (Fig. 3 C), whereas cyclin B1 and H1 kinase activity did not (Fig. 3, C and D), we asked whether inhibiting CDK activity at the normal time of anaphase I onset would suffice for *Ccnb3*^{-/-} oocytes to undergo anaphase I and PB extrusion. Indeed, the CDK inhibitor roscovitine (Meijer et al., 1997) rescued PB extrusion and chromosome segregation, suggesting that meiotic arrest is mainly due to persistently elevated cyclin B1-CDK1 activity (Fig. 3 E).

The results thus far led us to conclude that *Ccnb3*^{-/-} oocytes arrest in metaphase I with aligned bivalents, bipolar spindles, high securin and cyclin B1 protein levels, and little or no separase activity. In oocytes, degradation of cyclin B1 and securin

depends on ubiquitination by the APC/C (Herbert et al., 2003; Terret et al., 2003). Hence, our results suggested that without cyclin B3, the APC/C is not sufficiently activated, but because endogenous securin levels did decrease slightly, we suspected that the APC/C might be partially active. To test this, we asked whether exogenously expressed securin and cyclin B1 were also stabilized by live imaging of oocytes injected with mRNAs coding for either protein fused to fluorescent tags (Herbert et al., 2003; Kudo et al., 2006; Niaux et al., 2007; Gui and Homer, 2012; Lane et al., 2012; Rattani et al., 2014). Surprisingly, cyclin B1-GFP and securin-YFP were both efficiently degraded in *Ccnb3*^{-/-} oocytes (Fig. 3 F). Securin-YFP was degraded with kinetics and final extent similar to the controls, whereas cyclin B1-GFP appeared to be degraded slightly more slowly and was higher in *Ccnb3*^{-/-} specifically at the time controls underwent PB extrusion (Fig. 3 F). Thus, even though both exogenous substrates were more efficiently degraded than their endogenous counterparts, the differences between the two substrates were similar in both contexts (i.e., securin was degraded to a greater extent than cyclin B1 in the absence of cyclin B3). These results indicate that the APC/C indeed becomes partially active in the mutants, but further suggest that cyclin B3 specifically promotes degradation of endogenous APC/C substrates. This may be by modulating APC/C specificity, per se, or by regulating the substrates or their localization to affect their ability to be ubiquitinated.

Timely APC/C activation depends on satisfaction of the SAC (Holt et al., 2013; Touati and Wassmann, 2016), so we asked whether inappropriate SAC activity was the reason for arrest in *Ccnb3*^{-/-} oocytes. Kinetochores recruitment of SAC components, such as MAD2, is a readout for SAC activation (Wassmann et al., 2003; Gui and Homer, 2012; Lischetti and Nilsson, 2015). Similar to controls, MAD2 was recruited to unattached kinetochores early in meiosis I and was not abnormally retained in metaphase I in *Ccnb3*^{-/-} oocytes (Fig. 4 A). We further treated *Ccnb3*^{-/-} oocytes with reversine, which inhibits the SAC kinase MPS1 (Santaguida et al., 2010). Reversine overcame nocodazole-induced arrest in control cells as previously shown (Touati et al., 2015) but did not rescue metaphase arrest caused by cyclin B3 deficiency (Fig. 4 B). We conclude that prolonged SAC activation is not the reason for metaphase I arrest and failure to efficiently degrade endogenous APC/C substrates.

Exogenously expressed cyclin B3 is an APC/C substrate in meiosis

Like other M-phase cyclins, cyclin B3 has a destruction box (D-box; Nguyen et al., 2002), a motif for APC/C-dependent ubiquitination (Yuan and O'Farrell, 2015; Fig. 1 A). In mitosis, exogenous cyclin B3 is degraded after cyclin B1 (Nguyen et al., 2002), so we asked about cyclin B3 stability in oocytes. mRNA encoding cyclin B3-RFP was injected into wild-type oocytes together with mRNA for either securin-YFP or cyclin A2-GFP, and oocytes were followed by live imaging. Cyclin B3-RFP accumulated during meiosis I, then was degraded abruptly just before PB extrusion. Importantly, cyclin B3 was degraded after both cyclin A2 and securin (Fig. 5, A and B), showing temporally ordered degradation of APC/C substrates in oocyte meiosis I, at

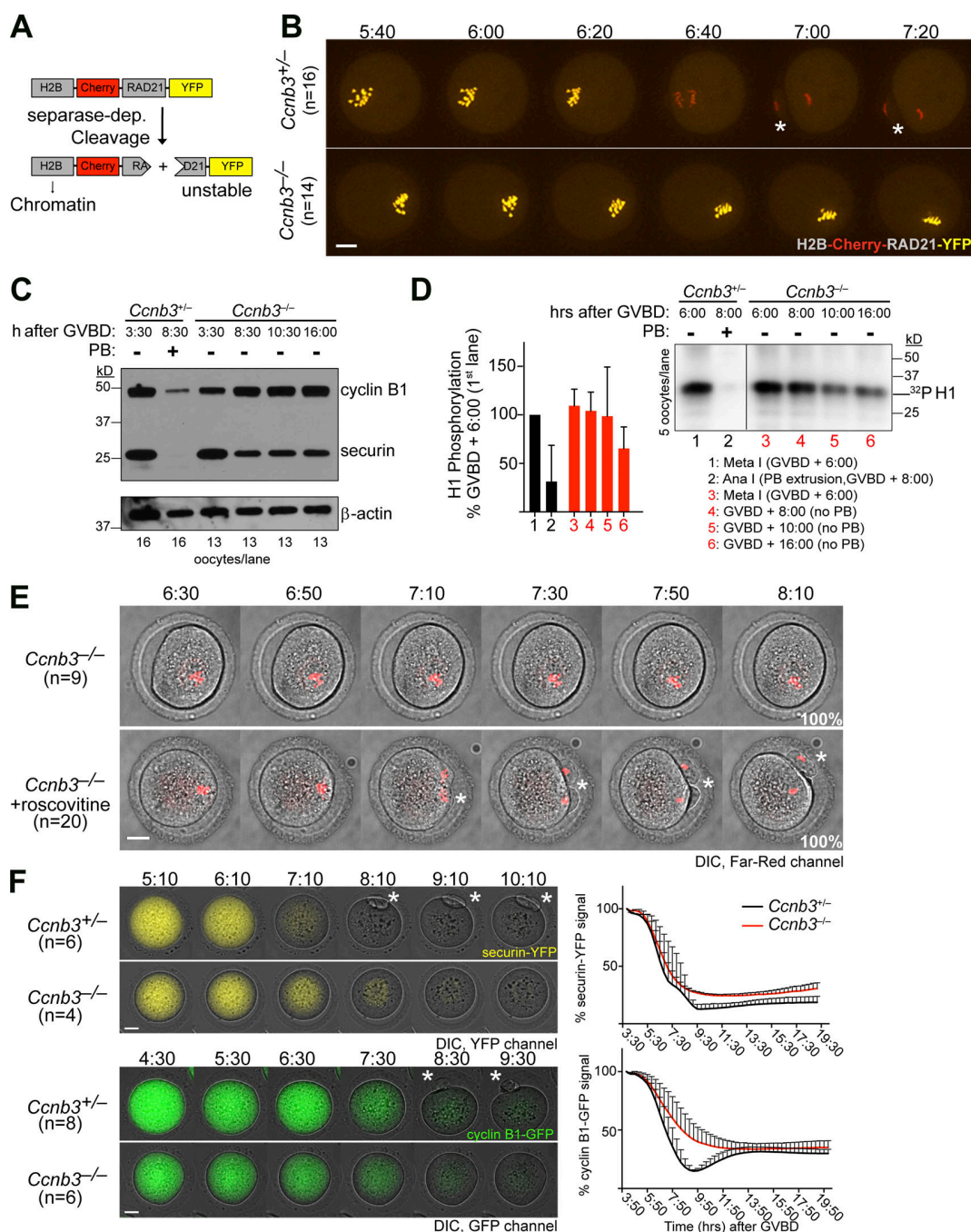


Figure 3. Cell cycle arrest in *Ccnb3*^{-/-} oocytes is associated with incomplete APC/C activation. (A) Schematic of separase activity sensor. See text for details. **(B)** Failure to activate separase in the absence of cyclin B3. Separase activity sensor mRNA was injected into GV oocytes, which were released into meiosis I and visualized by spinning-disk confocal microscopy. Selected time frames of collapsed z-sections (11 sections, 3- μ m steps) from a representative video are shown. Time points after GVBD are indicated as hours:minutes. Scale bar, 20 μ m. White asterisks indicate PBs. n: number of oocytes from three independent experiments. **(C)** Western blot analysis of cyclin B1 and securin during oocyte maturation, at the time points, indicated as hours:minutes after GVBD. β -Actin served as the loading control. The number of oocytes used and the presence or absence of a PB are indicated. Two mice of each genotype were used per experiment. The data shown are representative of results from two independent experiments. **(D)** Total cyclin B-CDK1 activity during oocyte maturation, at the time points indicated as hours:minutes after GVBD. Histone H1 was used as a substrate. Five oocytes were used per kinase reaction; presence or absence of a PB is indicated. The graph shows quantification of phosphate incorporation from three independent experiments, and error bars indicate SD (means \pm SD; lane 1: 100 [used for normalization]; lane 2: 31.27 \pm 37.28; lane 3: 109.34 \pm 17.09; lane 4: 104.05 \pm 19.33; lane 5: 98.67 \pm 50.56; lane 6: 65.40 \pm 22.12). **(E)** CDK inhibition rescues meiosis I division. Oocytes were incubated with siR-DNA to visualize chromosomes. In metaphase I, 6 h 20 min after GVBD, oocytes were treated with 0.2 mM roscovitine (final concentration), where indicated, and the video was started. Selected time frames of collapsed z-sections (11 sections, 3- μ m steps) of DIC far-red channel from a representative movie of *Ccnb3*^{-/-} oocytes with or without roscovitine treatment are shown. The asterisk indicates chromosome segregation in anaphase I. Time points after GVBD are indicated as hours:minutes. Scale bar: 20 μ m. n: number of oocytes

from three independent experiments. (F) Degradation of exogenous APC/C substrates. Securin-YFP (top) or cyclin B1-GFP (bottom) mRNA was injected into GV oocytes. Stills from representative videos are shown. Time points after GVBD are indicated as hours:minutes. Scale bar, 20 μ m. n: number of oocytes from two independent experiment; white asterisk indicates PB extrusion (PBE). Fluorescence intensities (mean \pm SD) were quantified from the indicated number of oocytes imaged (securin-YFP: 4 *Ccnb3*^{-/-}, 6 *Ccnb3*^{+/-}; cyclin B1-GFP injections: 6 *Ccnb3*^{-/-}, 8 *Ccnb3*^{+/-}).

least for exogenous proteins. No reaccumulation of cyclin B3-RFP was observed in meiosis II. Deleting the D-box stabilized cyclin B3-RFP (Fig. 5 C; there is no obvious KEN-box or ABBA motif; Davey and Morgan, 2016). These findings support the conclusion that cyclin B3 is an APC/C substrate in oocytes.

Cyclin B3-associated kinase activity is required for progression through oocyte meiosis I

To test whether mouse cyclin B3 supports CDK1 kinase activity as for *C. elegans*, *Drosophila*, and chicken proteins (Gallant and Nigg, 1994; Jacobs et al., 1998; van der Voet et al., 2009), we expressed and purified from insect cells cyclin B3 N-terminally tagged with maltose binding protein (MBP) and hexahistidine (^{MBPHis}cyclin B3) plus untagged or HA-tagged CDK1. Both CDK1 and CDK1-HA copurified with ^{MBPHis}cyclin B3 on amylose resin (Fig. 6 A, left, lanes 2 and 3), and both cyclin B3-CDK1 complexes displayed kinase activity toward histone H1 in vitro (Fig. 6 A, right, lanes 2 and 3).

To investigate this kinase activity, we mutated the conserved MRAIL motif on the cyclin fold (Jeffrey et al., 1995; Schulman et al., 1998). MRAIL mutations can prevent binding of cyclin-CDK complexes to substrates without affecting cyclin-CDK interactions (Schulman et al., 1998) or can disrupt cyclin-CDK interaction (Bendris et al., 2011). Mutating the methionine, arginine, and leucine residues of the MRAIL motif to alanine (cyclin B3-MRL mutant) did not affect copurification of CDK1 (Fig. 6 A, left, lanes 5 and 6) but eliminated kinase activity (Fig. 6 A, right, lanes 5 and 6). Furthermore, purification of wild-type cyclin B3 without coexpressed CDK1 yielded detectable kinase activity, presumably attributable to cyclin B3 association with one or more endogenous insect cell CDKs, but the MRL mutant gave no such activity (Fig. 6 A, right, lanes 1 and 4). Thus, cyclin B3 can form an active kinase complex with CDK1, suggesting in turn that CDK1 is a physiological partner. We also conclude that the MRL mutant permits CDK1 binding but abolishes associated kinase activity.

We further tested whether oocyte division requires cyclin B3-associated kinase activity. To this end, we first live-imaged *Ccnb3*^{-/-} oocytes injected with mRNA encoding wild-type or D-box-deleted (Δ Dbox) cyclin B3. Both constructs efficiently rescued PB extrusion (Fig. 6 B). Rescue of MI division by the Δ Dbox mutant demonstrated that cyclin B3 degradation is dispensable for anaphase I, and also led us to infer that exogenously expressed cyclin B3 was a quantitatively minor contributor to total CDK1 activity, because high CDK1 should prevent anaphase I. To confirm this idea, we examined H1 kinase activity in synchronized oocytes before and after PB extrusion in rescue experiments. Indeed, *Ccnb3*^{-/-} oocytes rescued by expression of wild-type or Δ Dbox cyclin B3 did not show elevated H1 kinase activity in anaphase I (Fig. 6 C, compare lanes 3, 7, and 9). Interestingly though, we found less H1 kinase activity in

metaphase I oocytes expressing Δ Dbox cyclin B3 (Fig. 6 C, lanes 4, 6, and 8), suggesting that cyclin B1-associated CDK1 kinase activity might be partially down-regulated by stable cyclin B3.

Separation of homologous chromosomes was also rescued upon cyclin B3 expression (Fig. 6 D), confirming that meiotic arrest is due to absence of cyclin B3 per se and establishing feasibility of structure/function experiments via complementation. In striking contrast to wild-type cyclin B3, the MRL mutant was unable to rescue meiosis I division, PB extrusion, or chromosome segregation (Fig. 6, B and D), indicating that cyclin B3-CDK complexes (likely CDK1) bring about anaphase I onset through their kinase activity toward yet unknown substrates. Note that, unlike cyclin B3, exogenous cyclin B1 did not rescue PB extrusion (Fig. 3 F) or homologous disjunction (Fig. 6 D).

Cyclin B3-CDK1 substrates are likely distinct from cyclin B1-CDK1 substrates, perhaps owing to different substrate specificity or accessibility. Furthermore, even though cyclin B3-CDK1 kinase activity is required for PB extrusion, its effect is probably locally restricted and does not lead to a detectable overall increase of CDK1 activity, at least as measured on histone H1 as a substrate.

Functional conservation of vertebrate cyclin B3 in meiosis I

It was previously reported that *X. laevis* oocytes have cyclin B3 mRNA but not detectable protein, from which it was inferred that cyclin B3 plays no role in *X. laevis* oocyte meiosis (Hochegger et al., 2001). Furthermore, cyclin B3 is much larger in mammals than in nonmammalian vertebrates owing to the extended exon 7 (Lozano et al., 2012), suggesting that the mammalian protein might be functionally distinct. To explore this possibility, we tested for interspecies cross-complementation by injecting mRNAs encoding either *X. laevis* or zebrafish (*Danio rerio*) cyclin B3 into mouse *Ccnb3*^{-/-} oocytes. Remarkably, heterologous expression of cyclin B3 from either species efficiently rescued PB extrusion (Fig. 7 A). Live imaging showed no obvious defect in chromosome alignment or anaphase I onset in oocytes rescued with *X. laevis* cyclin B3 (Fig. 7 B). The extended exon 7 in mouse cyclin B3 is thus dispensable to promote anaphase I onset. We further infer that cyclin B3 biochemical functions are conserved across vertebrates, in turn raising the possibility that cyclin B3 promotes the oocyte meiosis I division throughout the vertebrate lineage. Even more remarkably, *Drosophila* cyclin B3 was also able to rescue (Fig. 7 A), suggesting conservation of biochemical properties throughout metazoa.

Discussion

Whereas male fertility is unaffected by reduction (Tang et al., 2018) or complete loss (unpublished data) of cyclin B3, mutant

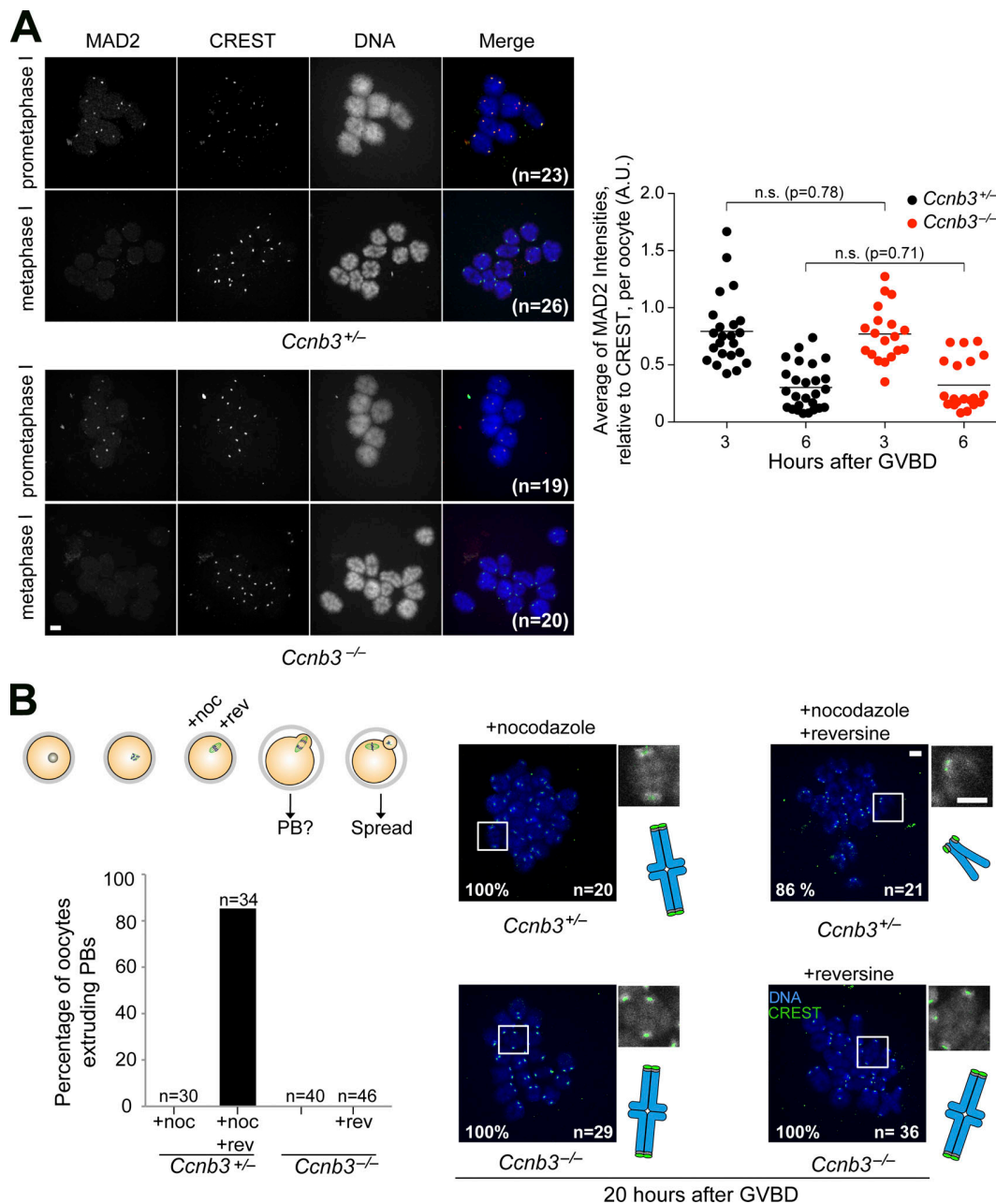


Figure 4. **SAC activation is not the cause of metaphase I arrest in *Ccnb3*^{-/-} oocytes.** (A) Left: Chromosome spreads were prepared 3 h (early prometaphase I) and 6 h (metaphase I) after GVBD, then stained with Hoechst (blue), CREST (green), and anti-MAD2 (red). Scale bar, 5 μ m. Right: Quantification of MAD2 signal intensity relative to CREST; each point is the mean relative intensity averaged across centromeres in an oocyte. n: number of oocytes from three independent experiments. P values are from *t* tests. Number and means of oocytes stained 3 h after GVBD: 23 *Ccnb3*^{+/-} (mean: 0.794) and 19 *Ccnb3*^{-/-} (mean: 0.77); 6 h after GVBD: 26 *Ccnb3*^{+/-} (mean: 0.302) and 20 *Ccnb3*^{-/-} (mean: 0.325). (B) Oocytes were treated with reversine to override a potential SAC arrest. Control oocytes were treated with nocodazole from 6 h after GVBD onward, whereas *Ccnb3*^{-/-} oocytes were allowed to arrest without nocodazole. 6 h 40 min after GVBD, reversine was added, PB extrusion was scored visually (graph on the right, n: number of oocytes scored per genotype. 0% of oocytes extruded PBs, except oocytes treated with nocodazole and reversine, which extruded PBs in 85.29% of oocytes analyzed), and all oocytes were spread 20 h after GVBD. Kinetochores were stained with CREST (green) and chromosomes with Hoechst (blue). Insets show typical chromosome figures observed. Scale bar, 5 μ m. n: the number of spreads from three independent experiments that allowed to unambiguously distinguish bivalents from univalents; percentage of oocytes with same phenotype is indicated.

females are sterile because cyclin B3 promotes degradation of APC/C substrates and thus triggers anaphase onset in the first meiotic division in oocytes. Similar findings were obtained with a different *Ccnb3* mutation (Li et al., 2018b Preprint). Our findings

definitively establish a crucial role for mouse cyclin B3 in the female germline.

The requirement in anaphase I onset is similar to *Drosophila* oocytes (Deyter et al., 2010; Yuan and O'Farrell, 2015). It was

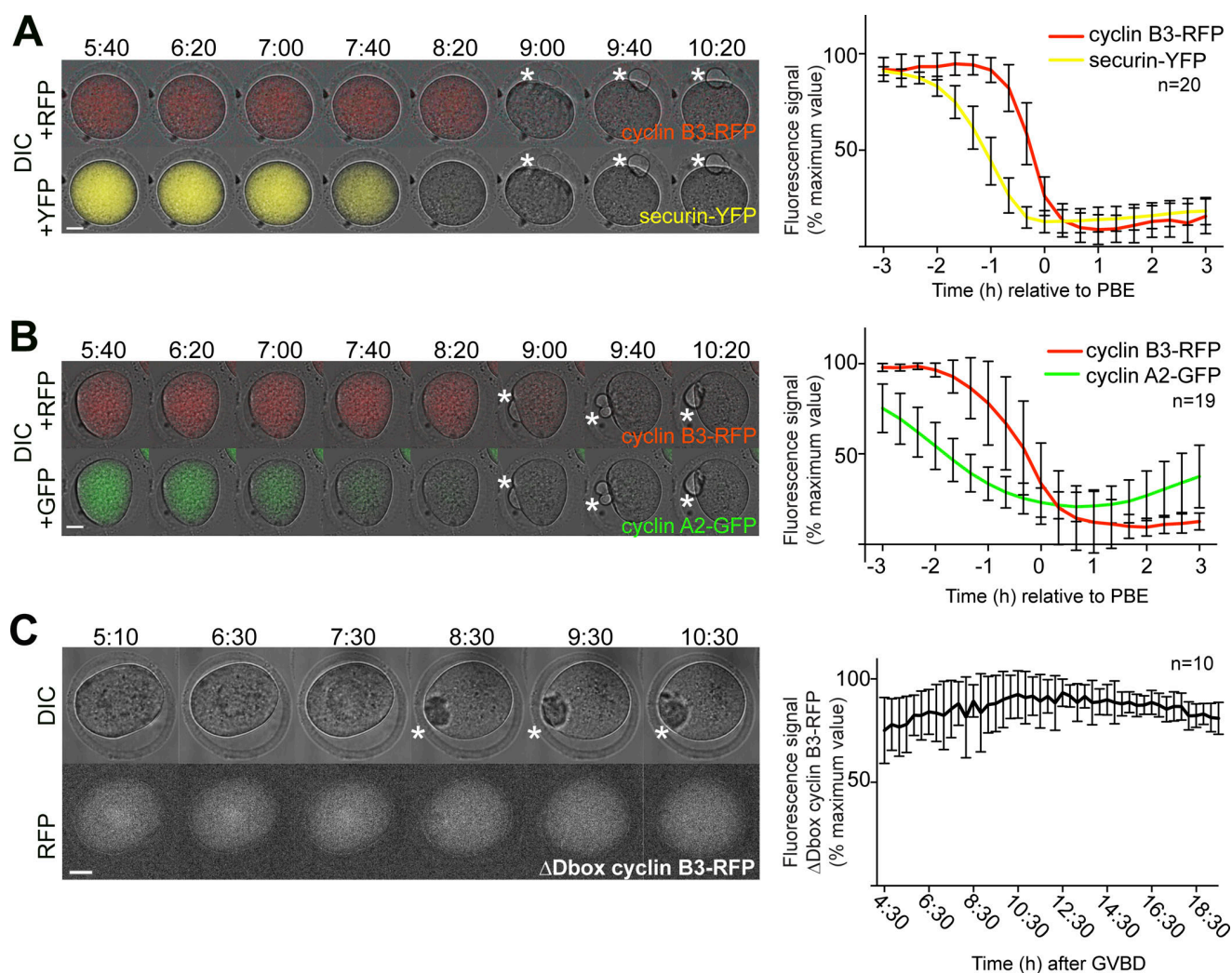


Figure 5. Ordered degradation of APC/C substrates in oocyte meiosis I. (A and B) Cyclin B3 was degraded after securin or cyclin A2. mRNA encoding cyclin B3-RFP and either securin-YFP (A; 20 oocytes) or cyclin A2-GFP (B; 19 oocytes) were co-injected into GV oocytes of CD-1 mice. Each panel shows selected frames from a video of a representative oocyte, with an overlay of DIC and RFP channels in the top rows, and DIC and YFP or GFP channels in the bottom rows. **(C)** The D box of cyclin B3 is required for degradation. CD-1 GV oocytes (10 oocytes) were injected with mRNA encoding cyclin B3-RFP with the D box deleted (Δ Dbox). Frames from a representative video are shown, with the DIC channel on top and RFP at the bottom. All panels: Time points after GVBD (BD) are indicated as hours:minutes. Scale bars, 20 μ m. White asterisks: PBs. Quantification of fluorescence intensities is shown on the right (mean \pm SD of the indicated number of oocytes from three independent experiments).

proposed that cyclin B3 regulates APC/C activity during the rapid mitotic cycles of the early *Drosophila* embryo (Yuan and O'Farrell, 2015). Our data are consistent with a similar role in mouse oocyte meiosis I. Interestingly, however, exogenous APC/C targets are degraded efficiently in cyclin B3-deficient oocytes, but endogenous substrates are not, to our knowledge, the first such discrepancy observed. What distinguishes exogenous from endogenous substrates is not clear. One possibility is that expression timing (starting upon mRNA injection into GV oocytes) places exogenous proteins at a disadvantage for binding to essential partners, e.g., of securin to separase and cyclin B1 to CDK. It is also possible that fluorescent protein tags interfere slightly with this binding. Notably, free securin is degraded earlier than separase-bound securin, which is stabilized by maintenance in a nonphosphorylated state through association

with phosphatase PP2A (Hellmuth et al., 2014). Securin phosphorylation enhances its affinity for the APC/C (Holt et al., 2008; Hellmuth et al., 2014), and cyclin B3 may control this step. Regardless of the source of the distinction, our data clearly demonstrate that APC/C becomes at least partially active on time, but may not be sufficiently active to robustly provoke degradation of all of its targets.

Alternatively, cyclin B3 may play a repressive role in translation of APC/C substrates after meiosis I entry. After GVBD, cyclin B1 and cyclin B2 mRNA association with polysomes is regulated in a distinct manner in mouse oocytes, resulting in translation of cyclin B1 transcripts but not cyclin B2 (Han et al., 2017). Translational control is crucial because oocytes contain stockpiles of mRNAs required for early embryo development, which are not required during oocyte meiosis (Yang et al., 2017).

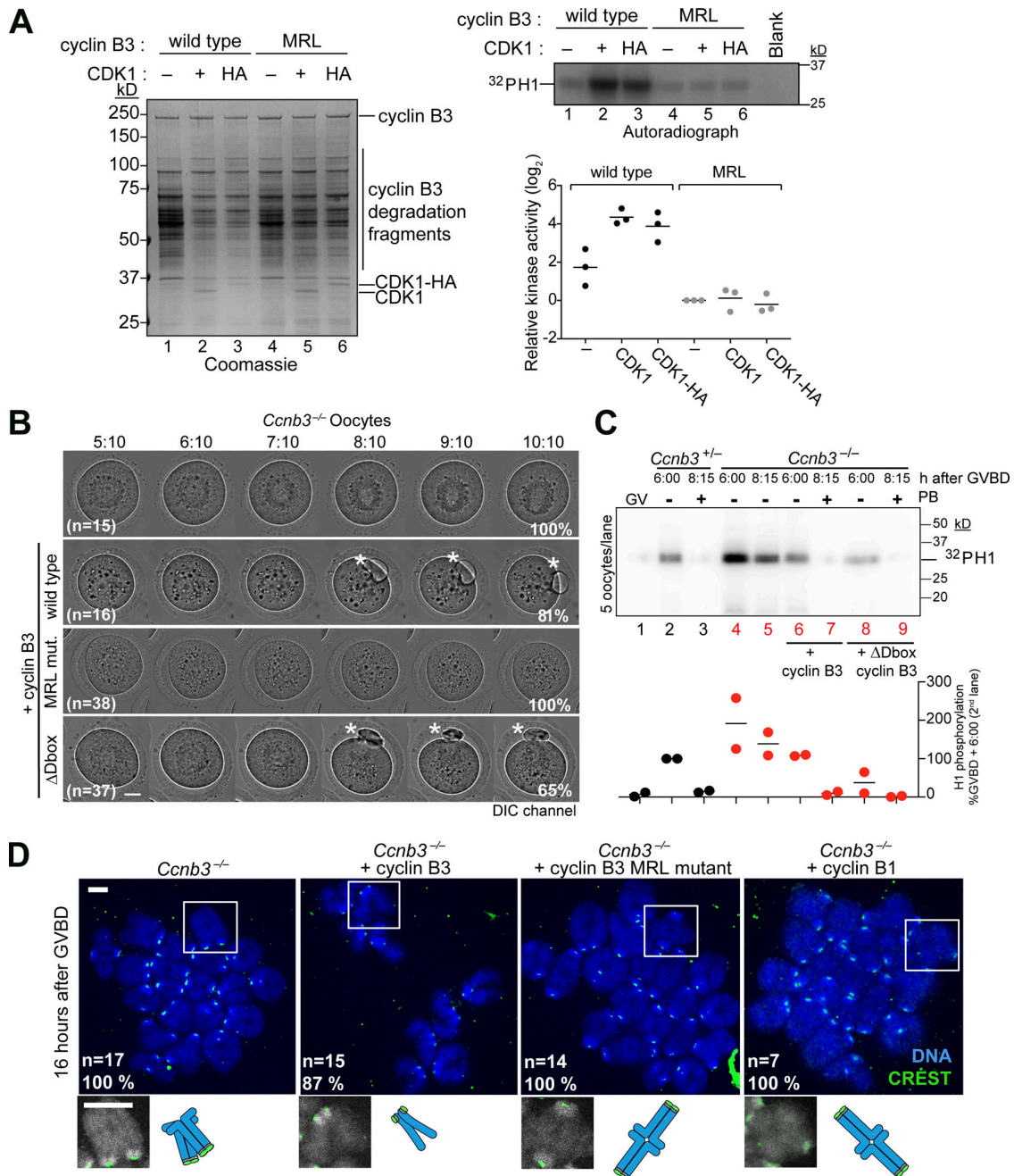


Figure 6. Only cyclin B3 that can support in vitro kinase activity can rescue *Ccnb3*^{-/-} oocytes. (A) Affinity purification of cyclin B3–CDK1 complexes. ^{MBP}His cyclin B3 or ^{MBP}His cyclin B3 MRL mutant were expressed in insect cells alone or coexpressed with either untagged or HA-tagged CDK1. Left: The eluates from purification on amylose resin were separated on SDS-PAGE and stained with Coomassie. Right: Representative autoradiograph (top) and quantification (bottom) from histone H1 kinase assays. In the graph, values in each experiment ($n = 3$) were normalized to the signal from the ^{MBP}His cyclin B3 MRL sample (lane 4 in the autoradiograph); lines indicate means (lane 1: 1.733; lane 2: 4.351; lane 3: 3.878; lane 4: 0 [used for normalization]; lane 5: 0.121; lane 6: -0.202). **(B)** Rescue of *Ccnb3*^{-/-} oocytes by expression of exogenous cyclin B3. *Ccnb3*^{-/-} oocytes were sham injected or injected with the indicated cyclin mRNA, then released into meiosis I. Frames of representative videos are shown. Times after GVBD are indicated as hours:minutes, and percentages of oocytes of the shown phenotypes are indicated. Scale bar, 20 μ m. White asterisks: PBs. n: number of oocytes from three independent experiments. **(C)** Total cyclin B–CDK1 activity during oocyte maturation, in control (lane 1–3), and *Ccnb3*^{-/-} (lanes 4–9, labeled in red) oocytes expressing wild-type cyclin B3 (lanes 6 and 7) or Δ Dbox cyclin B3 (lanes 8 and 9), at the time points indicated as hours:minutes after GVBD. Oocytes extruding PBs are indicated. Histone H1 was used as a substrate. A representative example from two independent experiments is shown above; quantification of both experiments is shown below (³²P-H1 signal normalized to the signal in lane 2; points are values from each experiment; lines indicate means: lane 1: 6.61; lane 2: 100 [used for normalization]; lane 3: 14.38; lane 4: 191.58; lane 5: 138.53; lane 6: 109.88; lane 7: 9.59; lane 8: 37.36; lane 9: 1.5). **(D)** Representative chromosome spreads 16 h after GVBD. Chromosomes were stained with Hoechst (blue) and kinetochores with CREST (green). Insets show typical chromosome figures observed (chromosomes are shown in grayscale). Scale bar, 5 μ m. n: number of oocytes from three independent experiments. Schematics of metaphase I bivalents or metaphase II univalent chromosomes are shown to aid interpretation.

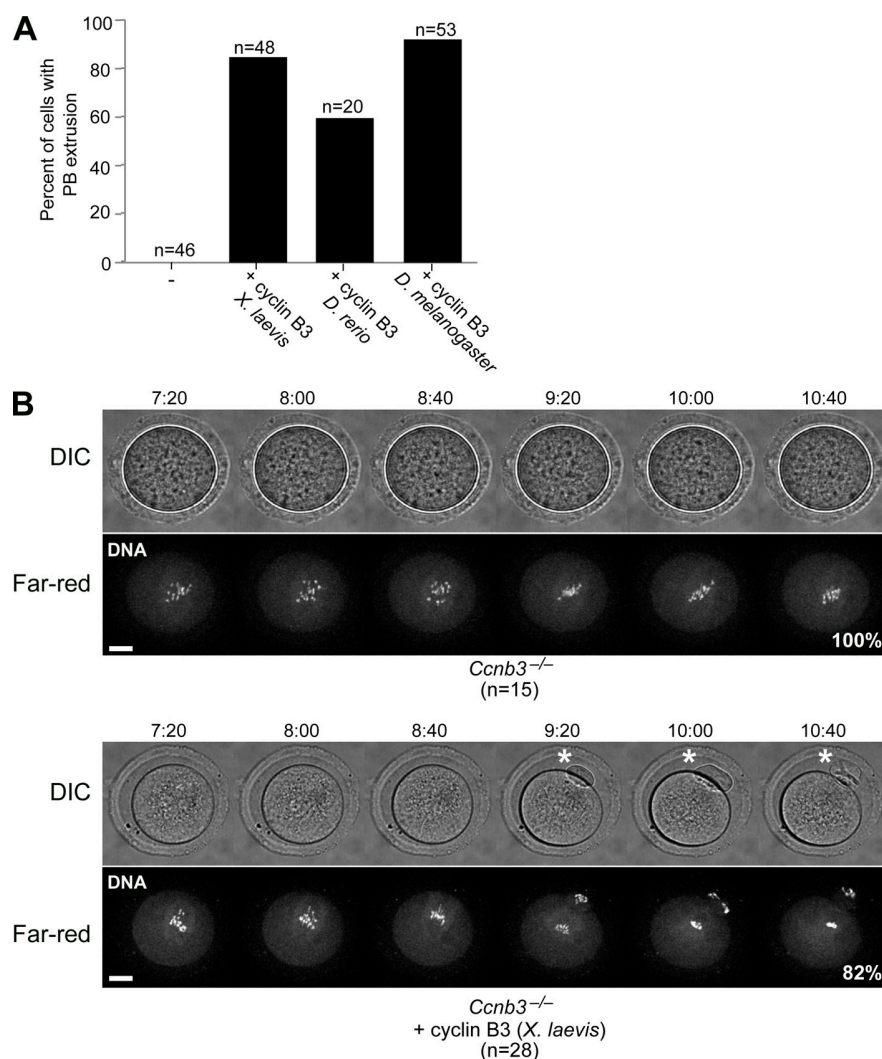


Figure 7. Interspecies cross-complementation of *Ccnb3*^{-/-} oocytes. (A) *Ccnb3*^{-/-} oocytes were injected with the indicated mRNA, induced to enter meiosis I, and scored for PB extrusion. n: number of oocytes from three independent experiments; number of oocytes analyzed and percentage of PB extrusion: 46 *Ccnb3*^{-/-} sham injected oocytes (0% PBs), 48 *Ccnb3*^{-/-} oocytes injected with mRNA coding for *X. laevis* cyclin B3 (85.41%), 20 *Ccnb3*^{-/-} oocytes with *D. rerio* cyclin B3 mRNA (60%), and 53 *Ccnb3*^{-/-} oocytes with *D. melanogaster* cyclin B3 mRNA (92.45%). (B) Selected time frames of collapsed z-sections (12 sections, 3- μ m steps) from a representative spinning disk confocal movie of *Ccnb3*^{-/-} sham-injected oocytes, and *Ccnb3*^{-/-} oocytes injected with *X. laevis* cyclin B3 mRNA. Before live imaging, oocytes were incubated with siR-DNA. Top panel shows the DIC channel and bottom panel shows siR-DNA staining in far-red. Time points after GVBD are indicated as hours:minutes. Scale bar: 20 μ m. White asterisks: PBs. n: number of oocytes from three independent experiments.

In principle, the failure of *Ccnb3*^{-/-} mutants to fully down-regulate steady-state cyclin B1 and securin levels could mean that cyclin B3-CDK1 represses translation of cyclin B1, securin, and/or other key cell cycle regulators. However, we think this is unlikely, as translation of transcripts coding for cyclin B1 and MOS is induced, not repressed, in a CDK1-dependent manner after GVBD (Han et al., 2017). Nevertheless, we cannot exclude a more indirect role of cyclin B3 in translational control.

We propose that cyclin B3 tips the balance from accumulation of cyclin B1-CDK1 activity toward APC/C-dependent degradation of cyclin B1 and progression into anaphase. Without cyclin B3, the APC/C is active, but endogenous cyclin B1 is not at all, and securin is not efficiently targeted for degradation. Consistent with this view, rescue of *Ccnb3*^{-/-} oocytes by artificially down-regulating cyclin B1-CDK1 activity with roscovitine indicates that sufficient separase is liberated from its inhibitory interaction with securin. Cyclin B3 in mammalian oocytes appears dispensable for turning off the SAC, unlike in *C. elegans* (Deyter et al., 2010). Cyclin B3-CDK1 complexes may foster full APC/C activity directly by modifying APC/C substrates, APC/C subunits, APC/C activators (CDC20 and CDH1), or some

combination (Alfieri et al., 2017). The robustness of the arrest is comparable to that observed when maintaining high cyclin B1 levels (e.g., with nondegradable cyclin B1; Herbert et al., 2003; Touati et al., 2012), but not SAC activation, which is transient (Touati and Wassmann, 2016).

Nevertheless, cyclin B3 is not strictly essential, in that some mice (~10% of those assayed thus far) harbored oocytes able to progress through meiosis I. This incomplete penetrance may reflect subtle variation (perhaps from strain background) in either the degree of APC/C activation or the threshold of APC/C activity needed for progression. Put another way, cyclin B3-deficient oocytes may occasionally achieve just enough APC/C activity during a critical time window to cross a threshold needed for the switch-like transition into anaphase I.

In budding and fission yeast, sequential substrate phosphorylation during cell cycle progression is thought to be primarily via changes in overall CDK activity as opposed to substrate specificities of different CDKs (Stern and Nurse, 1996; Coudreuse and Nurse, 2010; Bouchoux and Uhlmann, 2011; Uhlmann et al., 2011). Accordingly, the cell cycle can be driven by a single CDK-cyclin fusion complex in *S. pombe* (Coudreuse

and Nurse, 2010; Swaffer et al., 2016). In mouse, CDK1 is the only CDK essential for mitotic cell cycle progression per se (Santamaría et al., 2007). Loss of cyclin B2 does not affect viability or fertility, indicating that cyclin B1 is sufficient (Brandeis et al., 1998). It may thus seem surprising that cyclin B3 has such a specific role in oocyte meiosis that cannot be replaced by cyclin B1. Hence, cell cycle progression in meiosis appears to be specifically dependent on individual CDK complexes. This conclusion is further supported by the fact that loss of CDK2 does not affect somatic cells but leads to sterility due to failure to enter meiosis I (Ortega et al., 2003). Cell type specificity is also seen for cyclin A2, which is required in hematopoietic stem cells, fibroblasts, and female meiosis, but not in other tissues (Kalaszczynska et al., 2009; Touati et al., 2012; Zhang et al., 2017).

Overall, our data show that cyclin B3 is an M-phase cyclin with a singular role in oocyte meiosis, probably due to the specificities of meiotic cell cycle regulation in this huge cell. A key challenge now will be to determine whether APC/C activity or substrate accessibility is directly dependent on cyclin B3-CDK activity, and to define relevant phosphorylation targets needed for proper meiotic progression.

Materials and methods

Animals

Mice in the Keeney laboratory were maintained under regulatory standards approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee. Mice in the Wassmann laboratory were maintained according to current French guidelines in a conventional mouse facility (UMR7622), with authorization C 75-05-13. All experiments were subject to ethical review and approved by the French Ministry of Higher Education and Research (authorization no. B-75-1308). For this study, females were either purchased at 7 wk of age (CD-1 mice, Janvier Lab France) and used at 8–16 wk of age for experiments or bred in our animal facilities and used at 8–16 wk of age (*Ccnb3* mouse strain). Except for genotyping, the mice had not been involved in any procedures before experimentation, and they were given ad libitum access to food and water supply. The animals were housed under a 12-h light/12-h dark cycle according to the Federation of European Laboratory Science Associations.

Generation of *Ccnb3* knockout mouse strain, husbandry, and genotyping

Endonuclease mediated (*em*) allele was generated by the MSKCC Mouse Genetics Core Facility. Exon 7 of *Ccnb3* (National Center for Biotechnology Information [NCBI] gene ID: 209091) was targeted by guide RNA (C40-TGAACTTGCCATGATAGCAC). (Exon numbering used here is from the current accession. The targeted exon corresponds to exon 8 in the numbering used by Lozano et al. [2012].) Guide RNA was cloned into pDR274 vector for in vitro transcription. In vitro transcribed guide RNA (10 ng/ μ l) and Cas9 (20 ng/ μ l) were microinjected into pronuclei of CBA/J \times C57BL/6J F2 hybrid zygotes using conventional techniques (Romanienko et al., 2016).

Genomic DNA from founder animals were subjected to PCR by using the following primers: CCN3B-A, 5'-GAGTATTAGCAC TGAGTCAGGGAC-3', and CCN3B-B, 5'-GGAATACCTCAGTTT CTTTTCGAC-3'. T7 endonuclease I digestion was performed to identify the animals carrying indels. Because male animals have one copy of the X-linked *Ccnb3* gene, T7 assay on males was performed in the presence of wild-type genomic DNA.

To define the molecular nature of indels, genomic DNA from T7-positive animals was amplified using the primers indicated above. Amplified PCR fragments were used for TA Cloning (TA Cloning Kit with pCR 2.1 vector; Invitrogen). 10 white bacterial colonies were selected, inserts were sequenced, and the mutations were characterized as deletions, insertions, or substitutions. The *em1* allele, an out-of-frame deletion, was chosen to generate the *Ccnb3^{em1Sky}* line. After two rounds of backcrossing to C57BL/6J, animals were interbred to generate homozygous and heterozygous female animals and hemizygous male animals. Primers for genotyping were GT4-F, 5'-TGTTGATGAAGAGGA ATTTTTCAAATCATTCT-3', and GT4-R, 5'-TTCTTTTGACC CAGAGTTGACTTAAAG-3'. The amplified PCR product was subjected to *Bsr*I enzyme digestion (NEB). A *Bsr*I site is lost in the *em1* allele. Because *Ccnb3* is X-linked, no *Ccnb3^{+/+}* females can be obtained through crosses yielding homozygous *Ccnb3^{-/-}* mice. Therefore, we bred *Ccnb3^{-Y}* males with *Ccnb3^{+/+}* females to obtain *Ccnb3^{-/-}* and *Ccnb3^{+/-}* females to use as experimental mice and littermate controls, respectively.

Oocyte harvesting and in vitro culture

GV-stage oocytes were collected from ovaries of euthanized CD-1 mice or *Ccnb3^{+/+}* and *Ccnb3^{-/-}* mice aged 8–16 wk. Ovaries were transferred to drops of homemade M2 medium at 38°C and dissected to obtain oocytes. Oocytes were collected and isolated from follicular cells with mouth pipetting using torn-out Pasteur pipettes. For all microinjection experiments, ovaries and oocytes were incubated in drops of commercial M2 medium (Sigma-Aldrich) and kept at GV stage by the addition of dibutyl cyclic AMP (dbcAMP; Sigma-Aldrich) at 100 μ g/ml final concentration. Oocytes undergoing GVBD within at most 90 min after ovary dissection or release from dbcAMP were used for experiments. For all experiments using *Ccnb3^{-/-}* mice, for every *Ccnb3^{-/-}* mouse at least five mature GVBD-stage oocytes were left untreated and released in parallel to control oocytes to ascertain that no PB extrusion occurred. In the rare cases where PB extrusion occurred in *Ccnb3^{-/-}* oocytes, the experiment was aborted and not taken into account (this was especially important for the rescue experiments in Figs. 3 E, 4 B, 6 [B–D], and 7 [A and B]). Where indicated, roscovitine was added 6:20 after GVDB at a final concentration of 0.2 mM, nocodazole at 6:00 after GVBD at a final concentration of 400 nM, and reversine at 6:40 after GVBD at a final concentration of 500 nM. Because it is soluble in oil, reversine was also added to the oil covering the media drops.

Histology

Ovaries from *Ccnb3^{+/+}* and *Ccnb3^{-/-}* adult mice were fixed overnight in 4% PFA at 4°C. Fixed tissues were washed in water and stored in 70% ethanol at 4°C. Fixed tissues were submitted to

the Molecular Cytology Core Facility (MSKCC) for embedding in paraffin and sectioning of the embedded tissue (whole ovary) as 8- μ m sections and preparation of slides with ovary sections. The slides were then subjected to immunohistochemistry as follows. The immunohistochemical staining was performed at the Molecular Cytology Core Facility (MSKCC) using a Discovery XT processor (Ventana Medical Systems). Ovary sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), and antigen retrieval was performed with CC1 buffer (Ventana Medical Systems). Sections were blocked for 30 min with Background Buster solution (Innovex), followed by avidin-biotin blocking for 8 min (Ventana Medical Systems). Sections were then incubated with anti-VASA (ab13840; Abcam; 0.17 μ g/ml) for 5 h, followed by 60-min incubation with biotinylated goat anti-rabbit (PK6101; Vector Labs) at 1:200 dilution. The detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Thermo Fisher Scientific). Immunohistochemistry slides were scanned using Panoramic Flash 250 with a 20 \times /0.8 NA air objective. Images were analyzed using Panoramic Viewer Software (3DHistech). To count oocytes, every fifth section on the slide was analyzed, and the number of oocytes in primordial, primary, secondary, and antral follicles were noted and summed.

Histological examination of somatic tissues

Gross histopathological analysis of major organs and tissues was performed by the MSKCC Laboratory of Comparative Pathology Core Facility for the following mice: two 2-mo-old *Ccnb3*^{-/-} males, one 2-mo-old *Ccnb3*^{+/-} male, two 5-mo-old *Ccnb3*^{-/-} females, and two 5-mo-old *Ccnb3*^{+/-} females. Histological examination of the following tissues was performed: diaphragm, skeletal muscle, sciatic nerve, heart/aorta, thymus, lung, kidneys, salivary gland, mesenteric lymph nodes, stomach, duodenum, pancreas, jejunum, ileum, cecum, colon, adrenals, liver, gallbladder, spleen, uterus, ovaries, cervix, urinary bladder, skin of dorsum and subjacent brown fat, skin of ventrum and adjacent mammary gland, thyroid, parathyroid, esophagus, trachea, stifle, sternum, coronal sections of head/brain, vertebrae, and spinal cord. Tissues were fixed in 10% neutral buffered formalin, and bones were decalcified in formic acid solution using the Surgipath Decalcifier I (Leica Biosystems) for 48 h. Samples were routinely processed in alcohol and xylene, embedded in paraffin, sectioned (5 μ m), and stained with hematoxylin and eosin. Examined tissues and organs were grossly normal.

Extract preparation and Western blotting

To analyze endogenous protein levels, oocytes were cultured as described above. Oocytes were harvested at the indicated time points, washed in PBS to remove proteins from the medium, and then placed on the wall of the Eppendorf tube and snap-frozen in liquid nitrogen. Later, 7.5 μ l of 1 \times Laemmli lysis buffer was added to the tube, and samples were boiled for 5 min at 100°C.

For Western blotting, samples were separated on 4–12% Bis-Tris NuPAGE precast gels (Life Technologies) at 150 V for 70 min. Proteins were transferred to PVDF membranes by wet

transfer method in Tris-glycine-20% methanol, at 120 V for 40 min at 4°C. Membranes were blocked with 5% nonfat milk in PBS-0.1% Tween (PBS-T) for 30 min at room temperature on an orbital shaker. Blocked membranes were incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. Membranes were washed with PBS-T for 30 min at room temperature, then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Membranes were washed with PBS-T for 15 min and the signal was developed by ECL Plus Perkin Elmer or ECL Prime GE Healthcare.

To analyze cyclin B3 protein levels from mouse testis extracts, dissected testes were placed in an Eppendorf tube, frozen on dry ice, and stored at -80°C. The frozen tissue was re-suspended in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP-40) supplemented with protease inhibitors (Roche, Mini tablets). The tissue was disrupted with a plastic pestle and incubated by end-over-end rotation for 15 min, at 4°C. After brief sonication, samples were centrifuged at 15,000 rpm for 15 min. The cleared lysate was transferred to a new tube and used for immunoprecipitation. Whole-cell extract was precleared with protein G beads by end-over-end rotation for 1 h at 4°C. Anti-cyclin B3 monoclonal antibody (10 μ g; Abmart; clone number 19584-IR1-3/C500_140905, raised against the epitope SNMEKEFILDIPNK [amino acids 110–123]) was then added to the precleared lysates and incubated overnight with end-over-end rotation, at 4°C. Protein G beads were added to the tubes and incubate for 1 h with end-over-end rotation, at 4°C. Beads were washed three times with RIPA buffer, resuspended in 1 \times NuPAGE LDS sample buffer (Invitrogen) with 50 mM DTT, and boiled for 5 min to elute immunoprecipitated proteins.

Primary antibodies were used at the following dilutions to detect proteins: mouse anti-securin (1:300; ab3305; Abcam), mouse anti-cyclin B1 (1:400; ab72; Abcam), mouse anti- β actin (1:1,000; 8H10D10; CST), mouse anti-cyclin B3 (1:500; Abmart), and rat anti-tubulin α (1:1,000; MCA78G; Bio-Rad). Secondary antibodies were used at the following dilutions: goat anti-mouse IgG (H+L)-HRP (1:10,000; 1721011; Bio-Rad), and goat anti-rat IgG-HRP (1:10,000; AP136P; Millipore).

Plasmids

Mouse *Ccnb3* mRNA was amplified by PCR from a whole-testis cDNA library and cloned into pRN3-RFP vectors using the In-Fusion cloning kit (Clontech). *X. laevis* cyclin B3 cDNA (clone ID: 781186; NCBI ID: [379048](https://doi.org/10.1083/jcb.201808091)) was purchased from Dharmacon and used as a template for PCR amplification. *D. rerio* and *D. melanogaster* cyclin B3 (NCBI ID: [767751](https://doi.org/10.1083/jcb.201808091) and [42971](https://doi.org/10.1083/jcb.201808091), respectively) were amplified by PCR from cDNA from *D. rerio* embryo or the S2 cell line, respectively. Amplified products for *X. laevis*, *D. rerio*, and *D. melanogaster* were cloned into pRN3-GFP vector using the In-Fusion cloning kit (Clontech). To generate *Ccnb3* MRL mutation, overlapping primers with specific mutation were used to amplify PCR product from the wild-type template pRN3-cyclin B3-RFP, the template was digested by *DpnI* treatment, and the PCR product was used to transform *Escherichia coli* DH5 α competent cells. To generate the Δ D-box mutation, PCR primers that delete the D-box were used to amplify the *Ccnb3* plasmid, and the

resulting PCR product was phosphorylated and ligated. The primer list can be found as Table S1. Plasmids for in vitro transcription of cyclin B1-GFP, securin-YFP, histone H2B-RFP, cyclin A2-GFP, and β -tubulin-GFP were used (Brunet et al., 1998; Herbert et al., 2003; Terret et al., 2003; Tsurumi et al., 2004; Touati et al., 2012).

Expression and purification of recombinant proteins

Vectors for expression of cyclin B3 (wild-type or MRL mutant) N-terminally tagged with maltose-binding protein and 6 \times histidine were generated by cloning in pFastBac1-MBP6XHis. Wild-type *Ccnb3* was amplified from pRN3-cyclin B3-RFP and the MRL mutant was amplified from pRN3-cyclin B3 MRL-RFP. The plasmid was digested with *SspI* restriction endonucleases. By using In-Fusion cloning kit (Clontech) the PCR products were cloned into *SspI* linearized plasmid. Viruses were produced by a Bac-to-Bac Baculovirus Expression System (Invitrogen) following the manufacturer's instructions. Baculovirus expressing CDK1 and CDK1-HA were generously supplied by R. Fisher (Desai et al., 1992).

To express ^{MBPHis}cyclin B3 and ^{MBPHis}cyclin B3 MRL alone, *Spodoptera frugiperda* Sf9 cells were infected with virus at a multiplicity of infection (MOI) of 3. To express ^{MBPHis}cyclin B3-CDK1 or CDK1-HA and ^{MBPHis}cyclin B3 MRL-CDK1 or CDK1-HA complexes, Sf9 cells were infected with both viruses at an MOI of 3 and 2, respectively. Cells from two 150-mm plastic dishes per infection (each containing $\sim 3 \times 10^7$ cells) were harvested 48 h after infection by centrifugation at 1,500 rpm for 5 min and then washed with ice-cold PBS. Cell pellets were re-suspended in 1.7 ml of ice-cold lysis buffer (25 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, and 100 μ M leupeptin; L2884; Sigma-Aldrich), 1 \times complete protease inhibitor tablet (Roche), and 0.5 mM PMSF. Cells were lysed by sonication and centrifuged at 15,000 rpm for 30 min, at 4°C. The cleared extract was moved to a 2-ml Eppendorf tube and mixed with 300 μ l slurry of amylose resin (E802L; NEB) preequilibrated with lysis buffer. After 1 h at 4°C with end-over-end rotation, the amylose resin was centrifuged at 300 *g* for 1 min at 4°C. Resin was washed briefly with ice-cold lysis buffer before transfer on Bio-Spin chromatography columns (7326008; Bio-Rad). The resin on the column was washed five times with ice-cold wash buffer (lysis buffer plus 10% glycerol). To elute the proteins from the column, the resin was incubated with 100–200 μ l ice-cold elution buffer (wash buffer plus 10 mM maltose [M5885; Sigma-Aldrich]). All steps during the purification were done at 4°C.

Histone H1 kinase assay with recombinant proteins expressed in Sf9 cells

Eluates from the amylose affinity purification step were used for histone H1 kinase assays. Reactions were performed in 10 mM Hepes-NaOH, pH 7.4, 75 mM NaCl, 1 mM DTT, 10 mM MgCl₂, 100 μ M ATP (11140965001; Roche), 1 μ Ci [γ -³²P] ATP, and histone H1 (5 μ g/ μ l; 10223549001; Sigma-Aldrich). 10 μ l reaction mix was added to 10 μ l eluate from the amylose resin and incubated for 30 min at room temperature. To terminate reactions, 5 μ l of 4 \times SDS sample buffer was added to each reaction, and samples were boiled for 5 min before loading on 12% NuPage

Bis-Tris NuPAGE precast gels (Life Technologies). Samples were subjected to electrophoresis at 150 V for 60 min. The gel was then vacuum dried for 45 min at 80°C. Radiolabeled species were imaged using a Fuji phosphorimager and analyzed by Image Gauge software.

Histone H1 kinase assays using oocyte extracts

Kinase assays to determine endogenous kinase activity were performed on aliquots of five oocytes at the indicated stages of meiotic maturation (Kudo et al., 2006). In short, oocytes were lysed in 3 μ l of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal [Sigma-Aldrich], 10% glycerol, and 2 mM EDTA, supplemented with protease inhibitors [Mini tablets; Roche]) on ice for 20 min before adding 6 μ l kinase assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT, 2.5 mM EGTA, and 150 μ M ATP) supplemented with 3 μ Ci of [γ -³²P]ATP (Perkin Elmer) per sample. Histone H1 (0.5 μ g per sample; Millipore) was used as a substrate. Kinase reactions were incubated 30 min at 30°C, denatured, and analyzed by SDS-PAGE. The gel was fixed and dried before being exposed and scanned to detect incorporation of [γ -³²P]ATP into the substrate, using a Typhoon FLA9000 phosphorimager (GE Healthcare Life Sciences). Scans were analyzed by ImageJ (National Institutes of Health).

Microinjection and live imaging

In vitro transcription of all mRNAs was done using the Ambion mMessage Machine kit according to the manufacturer's instructions. mRNAs (1 to 10 pM) were purified on RNeasy purification columns (Qiagen). GV stage oocytes were microinjected with mRNA on an inverted Nikon Eclipse Ti microscope. Microinjection pipettes were made using a magnetic puller (PN-30; Narishige). Oocytes were manipulated with a holding pipette (VacuTip; Eppendorf), and injection was done using a FemtoJet Microinjector pump (Eppendorf) with continuous flow. Injection of the oocytes was done on a Tokai Hit temperature controlled glass plate at 38°C. Live imaging of oocytes in Fig. 3 (B and E) and Fig. 7 B and Videos 1 and 2 was performed on an inverted Zeiss Axiovert 200M microscope coupled to an EMCCD camera (Evolve 512; Photometrics), combined with an MS-2000 automated stage (Applied Scientific Instrumentation), a Yokogawa CSU-X1 spinning disc, and a nanopositioner MCL Nano-Drive and using a Plan-APO (40 \times /1.4 NA) oil objective (Zeiss). Images of chromosomes were acquired with 11 z-sections of 3 μ m, unless otherwise mentioned. For live imaging in Fig. 2 A; Fig. 3 F; Fig. 5, A–C; and Fig. 6 B, a Nikon eclipse TE 2000-E inverted microscope with motorized stage, equipped with PreciSxite High Power LED Fluorescence (LAM 1: 400/465, LAM 2: 585), a Märzhäuser Scanning Stage, a CoolSNAP HQ2 camera, and a Plan APO (20 \times /0.75 NA) objective was used. One section in z was acquired for all videos except videos for Fig. 2 A where 11 z-sections of 3 μ m were acquired. Both microscopes were controlled by Metamorph software, and all live imaging was done in commercial M2 medium (Sigma-Aldrich), covered by mineral oil (embryo certified; Sigma-Aldrich) at 38°C. All images were stacked and assembled using ImageJ. Quantifications were done on untreated images. For figures, brightness was adjusted

with the same parameters per experimental condition with ImageJ.

Chromosome spreads and immunofluorescence

Oocytes were rinsed in successive drops of Tyrode's acid solution to remove their zona pellucida. For chromosome spreads, oocytes were fixed at room temperature using a spread solution (1% PFA, 0.15% Triton X-100, and 3 mM DTT; Sigma-Aldrich), at the time points indicated. For whole-mount immunofluorescence, chambers were coated with concanavalin A (Sigma-Aldrich) in M2 polyvinylpyrrolidone (0.1 mM; Merck-Millipore) medium, and oocytes were placed in the chambers and centrifuged at 1,400 rpm for 13 min at 38°C. Oocytes were then placed in a cold treatment solution (20 mM Hepes-NaOH and 1 mM MgCl₂, pH 7.4) on top of ice (4°C) for 4 min to remove unstable microtubules. After cold treatment, oocytes were incubated in a formaldehyde fixation solution (BRB80 medium with 0.3% Triton X-100 and 1.9% formaldehyde [Sigma-Aldrich]) at 38°C for 30 min. Primary antibodies were used at the indicated concentrations: human CREST serum autoimmune antibody against centromere (1:100; HCT-0100; Immunovision), rabbit polyclonal anti-MAD2 (1:50; Wassmann and Benezra, 1998), and mouse monoclonal anti- α -tubulin (DM1A) coupled to FITC (1:100; F2168; Sigma-Aldrich). Secondary antibodies were used at the following concentrations: anti-human Alexa Fluor 488 (1:200; A11013; Life Technologies), anti-human Cy3 (1:200; 709-166-149; Jackson ImmunoResearch), anti-rabbit Cy3 (1:200; 711-166-152; Jackson ImmunoResearch). Hoechst 3342 (H21492; Invitrogen) at 50 μ g/ml was used to stain chromosomes, and AF1 Cityfluor mounting medium was used.

Image acquisitions of fixed oocytes

An inverted Zeiss Axiovert 200M microscope as described above for live imaging was used to image chromosome spreads and for whole-mount immunofluorescence acquisitions, using a 100 \times /1.4 NA oil objective coupled to an EMCCD camera (Evolve 512; Photometrics). Six z-sections of 0.4 μ m were taken for spreads, and 11 z-sections of 1 μ m for whole-mount oocytes. Stacks acquired with Metamorph software were assembled in ImageJ. Quantifications were done on untreated acquisitions. For figures, brightness was adjusted with the same parameters per experimental condition, using ImageJ.

Quantification of fluorescent signals

For the quantification of MAD2 fluorescence signal, the intensity was calculated for each kinetochore using an 8 \times 8-pixel square on the kinetochore (where CREST signal is located). The same sized square was used adjacent to the MAD2 fluorescence to measure background, which was subtracted from the MAD2 signal. The MAD2 fluorescence intensity was normalized to the CREST signal of the same kinetochore. For the cyclin B3-RFP, Δ Dbox cyclin B3-RFP, cyclin A2-GFP, cyclin B1-GFP, and securin-YFP quantifications, a 150 \times 150-pixel circle was placed in the center of each oocyte for the fluorescence intensity signal measurement, and another was placed adjacent to the oocyte to measure background. For each oocyte, background-subtracted values were normalized relative to the highest

value. All measurements were done using ImageJ on untreated acquisitions.

Statistical analysis

For statistical analysis, GraphPad Prism 7 was used. For the comparison of independent samples, unpaired Student's *t* test was performed. Error bars indicate means \pm SD. Sample sizes and statistical tests are indicated in the figures and figure legends, respectively. Selection of oocytes for different conditions was at random. No statistical analysis was used to determine sample size. Collection and analysis of the data were not performed blind to the conditions of the experiments, and no data from experiments were excluded from analysis. The number of independent replicates is indicated in the figure legends.

Online supplemental material

Videos 1 and 2 show chromosome movements in a representative control and a *Ccnb3*^{-/-} oocyte, respectively. Table S1 provides sequence information for the primers used in this study.

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Author contributions: M. Karasu generated the *Ccnb3*^{-/-} mouse strain and cloned plasmids used in this study, unless otherwise noted, and performed experiments in Figs. 1 (A–C), 3 C, and 6 A. Experiments in Figs. 4 (A and B), 6 (B and C), and 7 (A and B) were performed by N. Bouftas, and kinase assays in Figs. 3 D and 6 C by N. Bouftas and K. Wassmann. The remaining experiments and statistical analysis were done by N. Bouftas and M. Karasu. Overall supervision, funding acquisition, and project administration were done by K. Wassmann and S. Keeney. Figures were prepared by N. Bouftas, M. Karasu, and K. Wassmann, and the manuscript was written by K. Wassmann and S. Keeney with substantial input from other authors.

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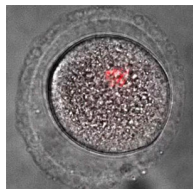
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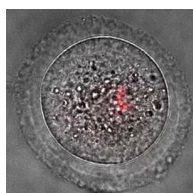
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Supplemental material

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Video 1. **Progression through meiosis I in control oocyte.** A *Ccnb3*^{+/-} oocyte incubated with SiR-DNA to visualize chromosomes. Acquisitions were started at GVBD + 6:30 (hours:minutes). 11 z-sections with 3- μ m steps were taken to follow chromosome movements, and shown are overlays of the stack. Acquisitions of the far-red and DIC channel were done every 20 min; 21 time points are shown. Related to Fig. 2 A. This is a representative video from 42 oocytes from six independent experiments.



Video 2. **Progression through meiosis I in *Ccnb3*^{-/-} oocyte.** A *Ccnb3*^{-/-} oocyte incubated with SiR-DNA to visualize chromosomes. Acquisitions were started at GVBD + 6:30 (hours:minutes). 11 z-sections with 3- μ m steps were taken to follow chromosome movements, and shown are overlays of the stack. Acquisitions of the far-red and DIC channel were done every 20 min; 21 time points are shown. Related to Fig. 2 A. This is a representative video from 63 oocytes from six independent experiments.

Table S1. Primers used for cloning expression plasmids

Primer ID	Primer (5'-3')	Primers used to generate the plasmid indicated
p20	ATCTGAATTCGGATCCGCCACCATGCCACCACCACTACTACCC	pRN3-eRFP-cyclin B3
p21	AGGAGGTCATGGATCCGTGGAATAATGCACAATTCAAAGATTCTC	
p30	CTTTACTGCGCCTTGAGTTGAAG	pRN3-eRFP-cyclin B3 Δ dbx
p31	GCATTCATAGTCAATGTGTCCAG	
p37	GAACTCACCAGTGATGCGGCAGCCATTGCTGTGGACTGGTTGGTG	pRN3-eRFP-cyclin B3 MRL
p38	CACCAACCAGTCCACAGCAATGGCTGCCGCATCACTGGTGAGTTC	
p92	CGCGGGCCCGGGATCCATGATGCCTTCTCTTCGTCATCTC	pRN3-eGFP- <i>X.laevis</i> cyclin B3
p93	TAGATCCGGTGGATCCTTAGCTCTGAAGGGCCTCTGTAAG	
p100	CGCGGGCCCGGGATCCATGCCGTTTTCAAAGGAAAAAGCCC	pRN3-eGFP- <i>D.rerio</i> cyclin B3
p101	TAGATCCGGTGGATCCTCACTTCAAAGCCCTTCAAGCTTCTC	
p104	CGCGGGCCCGGGATCCATGGCGCCCAAAAAGCAACAAC	pRN3-eGFP- <i>D.melanogaster</i> cyclin B3
p105	TAGATCCGGTGGATCCCTACGACAGATTGCTTTCGTTTCAGGTC	
p47	TACTTCCAATCCAATATTATGCCACCACCACTACTACCC	pFastBacI-MBP6His-cyclin B3 or cyclin B3 MRL
p48	TTATCCACTTCCAATATTTCAAGTGAATAATGCACAATTCAAAAG	

PART II: Cyclin B3 prevents a precocious CSF arrest in meiosis I oocytes

Our previous study has clearly shown the important role of cyclin B3 in meiosis for female mice. Oocytes depleted for cyclin B3 arrest at metaphase I. We also have previously shown that cyclin B3 from other species is able to fulfill the function of mouse cyclin B3 and rescue the metaphase I arrest in cyclin B3 KO oocytes. Strikingly, expression of cyclin B3 from *Xenopus* in mouse WT oocytes provoked a second round of segregation. After injection of *Xenopus* cyclin B3 in GV mouse oocytes and using live imaging with SiR-DNA staining chromosomes, meiotic maturation was observed. Control oocytes segregate chromosomes and arrest at metaphase II as expected. In oocytes injected with *Xenopus* cyclin B3, oocytes undergo meiosis I similarly to control oocytes. After polar body extrusion and chromosome segregation, oocytes enter meiosis II and seem to arrest in metaphase II for some time. This time interval ranges from 3h20 to 6h40 after chromosome segregation in anaphase I. Following this short arrest in metaphase II, oocytes undergo a second round of segregation, obviously being unable to properly arrest at metaphase II (**Fig. 1A**). This segregation is usually only provoked due to fertilization or artificial activation but expression of *Xenopus* cyclin B3 seems to be sufficient to induce anaphase II. The segregation of chromatids does not always occur in an orderly fashion. For certain injections, oocytes segregate chromatids almost immediately after anaphase I and polar body extrusion is not always clear or established in some of these oocytes. When there is no clear polar body extrusion or numerous polar body extrusions, the chromatids keep moving around the oocyte in a disorganized manner. To take a closer look, spreads of oocytes over-expressing *Xenopus* cyclin B3 were performed. Oocytes were spread at BD+16h and chromatids were counted for each condition. In control chromosome spreads, 100% of the spreads show metaphase II chromosomes where the two sister chromatids are still paired. However, spreads for oocytes injected with *Xenopus* cyclin B3 present 87,5% of oocytes with separated sister chromatids (**Fig.1B**). The second round of segregation and sister chromatid separation is also observed upon injection of *Drosophila* or zebrafish cyclin B3 (not shown). This signifies that the ectopic expression of cyclin B3 from these species induces precocious sister chromatid segregation and an apparent lift of the CSF arrest.

However, this phenotype is not observed when oocytes overexpress mouse cyclin B3 as seen from the chromosome spreads of cyclin B3 KO rescue from our previous results. A possible

reason could be the expression profile of exogenously expressed mouse cyclin B3 compared to *Xenopus* cyclin B3. Fluorescence from cyclin B3 KO oocytes injected with either mouse cyclin B3 or *Xenopus* cyclin B3 was quantified at two time points: meiosis I (BD+5h) and meiosis II (BD+12h). Figure 1C shows the quantification of the fluorescence of either mouse or *Xenopus* cyclin B3 during meiosis I and meiosis II. Meiosis I fluorescence is considered as 100% for both injections and the fluorescence observed in meiosis II was compared to it. The signal of mouse cyclin B3 does not re-accumulate in meiosis II, whereas *Xenopus* cyclin B3 is differently regulated and reaccumulates in metaphase II. The level is lower than meiosis I but still much higher than mouse cyclin B3. Therefore, cyclin B3 from these two organisms is regulated differently in mouse oocytes. Mouse cyclin B3 is not resynthesized or not stable during meiosis II, probably in order to only function during meiosis I, in accordance with its important role in meiosis I previously described. However, this regulation does not seem to affect *Xenopus* cyclin B3, and we hypothesize that other regulatory mechanisms are at work in frog oocytes to inhibit cyclin B3 in meiosis II.

In order to verify whether differences in expression levels in meiosis II are indeed the reason for the different phenotypes observed between *Xenopus* and mouse cyclin B3, we decided to exogenously express mouse cyclin B3 during meiosis II. Oocytes arrested in CSF arrest were injected with mouse WT cyclin B3. Oocytes injected with mouse WT cyclin B3 were observed for a couple of hours by staining with SiR-DNA to study chromosomes and using live imaging. Strikingly, 90,3% of oocytes show a second round of segregation rapidly after injection of cyclin B3. To confirm that this is due to cyclin B3-Cdk1 activity, a mutant previously described, cyclin B3 MRL mutant, was injected in metaphase II arrested oocytes. This mutant lacks associated Cdk activity. Injection of this mutant had no effect on the metaphase II arrested oocytes (**Fig.2**). Therefore, ectopic expression of cyclin B3 seems to be capable of inducing a second round of segregation pointing at a possible role in inhibiting CSF arrest and provoking anaphase II onset.

If the timing of expression of cyclin B3 determines if it can override CSF arrest, then it stands to reason that the non-degradable form of cyclin B3, Δ Dbox-cyclin B3, should also provide a similar effect. Live imaging was performed after injection of the Δ Dbox cyclin B3 in GV oocytes, which were stained again with SiR-DNA to analyze chromosome dynamics. The phenotype observed seems to be less organized than what is observed with expression of *Xenopus* cyclin B3. After regular polar body extrusion, different outcomes were observed. In

a small proportion of oocytes, chromosomes shortly arrest at metaphase II followed by the lift of the arrest with sister chromatid segregation. Most of the oocytes show a more chaotic progression with continuous segregation movements (**Fig. 3A**). If cell division occurred, it was not always asymmetrical. Chromosomes that move around sometimes keep floating around the oocyte. In order to have a clearer idea if these oocytes do indeed enter and exit meiosis II, securin or cyclin B1 injections and degradation patterns would be needed. Chromosome spreads were performed to confirm that sister chromatid segregation does indeed occur due to expression of stable cyclin B3 (**Fig. 3B**). Therefore, when cyclin B3 is stably expressed, it completely disorganizes the metaphase II arrest and induces chaotic anaphase II.

These findings demonstrate that cyclin B3 is capable of lifting CSF arrest when exogenously expressed in meiosis II. Therefore, a possible hypothesis explaining the arrest at metaphase I of cyclin B3 KO oocytes is that when cyclin B3 is lacking, a pseudo-CSF pathway is put in place blocking oocytes in meiosis I. If this is indeed the case, then inhibiting the CSF arrest should rescue the cyclin B3 KO phenotype. The CSF pathway is comprised of a series of kinases that are activated sequentially to finally activate the APC/C inhibitor Emi2 needed to establish and maintain metaphase II arrest. To inhibit this pathway, an inhibitor of MEK (U0126), the kinase needed for activation of MAPK and therefore Emi2, was used to treat cyclin B3 KO oocytes to assess whether this treatment rescues the metaphase I arrest. Cyclin B3 KO oocytes were treated at BD+4h and observed through live imaging. Inhibiting MEK led to partial rescue of cyclin B3 KO oocytes where we can observe polar body extrusion in 61,5% of oocytes (**Fig. 4A**). Chromosome spreads indicate that chromosome segregation indeed occurred in the oocytes (**Fig. 4B**). Hence, inhibition of one of the components of the CSF pathway during meiosis I in cyclin B3 KO oocytes allows exit from metaphase I arrest.

If cyclin B3 KO oocytes are arrested in metaphase I due to a pseudo CSF activity that is established precociously, then mimicking fertilization through activation may potentially release oocytes from this arrest. To activate oocytes, Ca^{2+} influx and waves must be induced in the cytoplasm. This Ca^{2+} influx leads to the activation of a kinase important to induce Emi2 degradation and therefore provokes APC/C activation. In order to mimic these waves, oocytes need to be treated with strontium (Sr^{2+}). To test the hypothesis that activation may rescue cyclin B3 KO oocytes, cyclinB3 KO oocytes were treated with strontium. These oocytes were treated at BD+7h30 assuming that by this time, CSF components would have

sufficiently accumulated, followed by chromosome spreads 20min after treatment to determine if chromosome segregation occurred. 63,6% of oocytes do indeed enter meiosis II and chromosomes have been able to segregate similarly to normal meiosis I exit in control oocytes (**Fig. 4C**). However, correct timing of this activation must be further tested. Therefore, it seems that mimicking activation is able to lift the metaphase I arrest in cyclin B3 KO oocytes.

In order to further explore if cyclin B3 has a role in inhibiting the CSF pathway present precociously in cyclin B3 KO oocytes, knockdown of one of the CSF components was carried out to determine if this would also lift the metaphase I arrest. Emi2 was the chosen target for knockdown (KD) by morpholino injection. This protein is the most downstream component of the CSF pathway that directly inhibits the APC/C. Therefore, depletion of Emi2 should demonstrate if this is indeed a sort of precocious metaphase II arrest put in place in meiosis I. Firstly, to determine if Emi2 KD is successful, the morpholino was injected in GV WT oocytes which were left arrested overnight before being released in meiosis I the next morning and studied through live imaging after staining with SiR-DNA. Previous studies have shown that Emi2 is important in establishing entry into meiosis II to allow re-accumulation of cyclin B1 needed to increase Cdk1 activity after meiosis I exit. Therefore, oocytes lacking Emi2 should fail at entering meiosis II shown by DNA decondensation. This was confirmed in our conditions in WT oocytes. These oocytes exit meiosis I normally but following polar body extrusion, chromosomes immediately decondense and oocytes fail to establish a metaphase II arrest (**Fig. 5A**). This KD was also performed on cyclin B3 KO oocytes. Injection of Emi2 morpholino in KO oocytes was shown to indeed rescue the metaphase I arrest. KO oocytes injected with Emi2 morpholino completed meiosis I with chromosome segregation and polar body extrusion. Like control oocytes, they then fail to enter meiosis II and in most cases their DNA decondenses (**Fig. 5B**). This indicates that cyclin b3 KO oocytes may indeed contain stabilized Emi2 in meiosis I that is able to inhibit the APC/C and keep oocytes arrested at metaphase I.

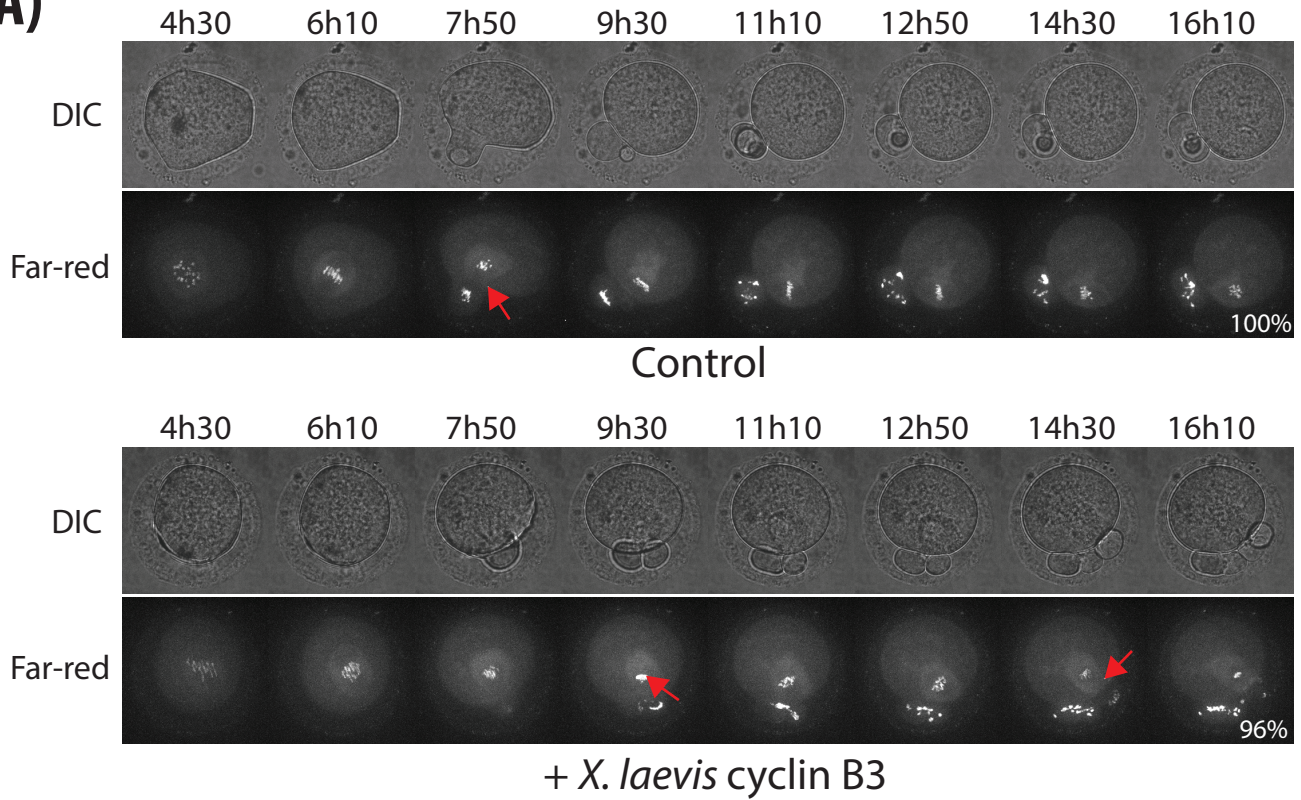
This data suggests that in a control condition cyclin B3 is able to inhibit Emi2 or some other protein responsible for its activation or stabilization during meiosis I in mouse oocytes. To verify if this is the case, we decided to use another model system, *Xenopus* oocytes. Since we observed in our previous study that *Xenopus* cyclin B3 is able to compensate functionally for mouse cyclin B3 and that *Xenopus* cyclin B3 can induce CSF arrest lift in mouse oocytes,

the choice of studying the molecular mechanism using *Xenopus* oocytes seems like a good option. It is simpler to perform western blots in *Xenopus* due to high protein content and conservation of the majority of pathways found in mouse oocytes. Our collaborators in Scott Keeney's lab have generated antibodies against *Xenopus* cyclin B3. First, I wanted to verify if cyclin B3 is present in *Xenopus* oocytes using this anti-guinea pig *Xenopus* cyclin B3 antibody. GV stage oocytes taken from ovaries were treated with progesterone to induce meiotic resumption. Oocytes were collected at different time points indicated in Figure 5C. *Xenopus* cyclin B3 is expressed in *Xenopus* oocytes during meiosis I. Staining is observed at GV, GVBD, and BD+1h where we can see a small decrease. No signal is observed in meiosis II, which in *Xenopus* oocytes is around BD+2-3h (**Fig. 5C**). This expression coincides with that observed with exogenous mouse cyclin B3 expression in mouse oocytes.

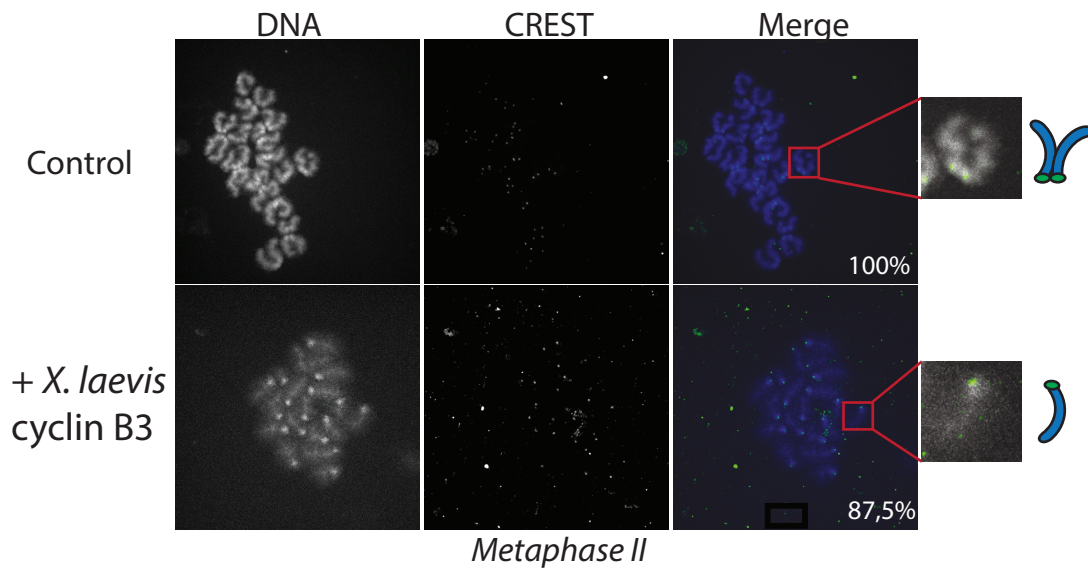
Next, *Xenopus* oocytes were collected and injected with *Xenopus* cyclin B3 RNA in GV stage similarly to what is done for mouse oocyte injections. Control and injected *Xenopus* oocytes were then released using progesterone to induce GVBD and meiotic maturation. Interestingly, overexpression of *Xenopus* cyclin B3 induces GVBD in both mouse and *Xenopus* oocytes. Oocytes were collected at different time points and western blots were performed to detect endogenous XErp1/Emi2. In control oocytes, Emi2 staining is observed at BD+2h, which in *Xenopus* meiosis is around end of meiosis I (Ohe et al 2007) beginning of meiosis II, and the signal accumulates until CSF. Oocytes overexpressing *Xenopus* cyclin B3 seem to have a different profile of XErp1/Emi2. Firstly, these oocytes have a perturbed progression through meiosis. Injected oocytes seem to be going through apoptosis between 4-6h following GVBD (not shown). Therefore, overexpression of *Xenopus* cyclin B3 clearly disrupts proper CSF arrest. XErp1/Emi2 western blot shows a lack of visible staining (**Fig. 5D**). This can be due to lack of accumulation of XErp1/Emi2, either because of destabilization or because of degradation of Xerp1/Emi2 due to overexpression of *Xenopus* cyclin B3. There could also be such a hyperphosphorylation making Xerp1/Emi2 migrate at such high molecular weight that we failed to detect it. This does need to be tested further. However, this result does fit with a possible role for cyclin B3 in inhibiting the CSF pathway leading to a degradation of Emi2.

Figure 1.

A)



B)



C)

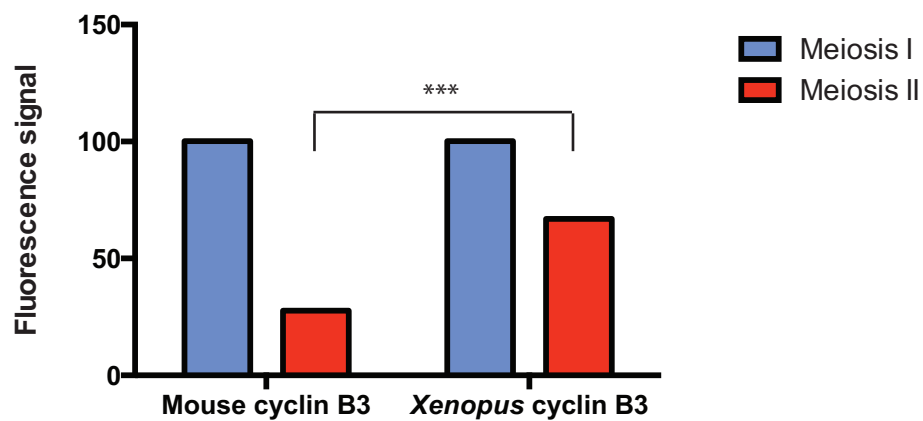
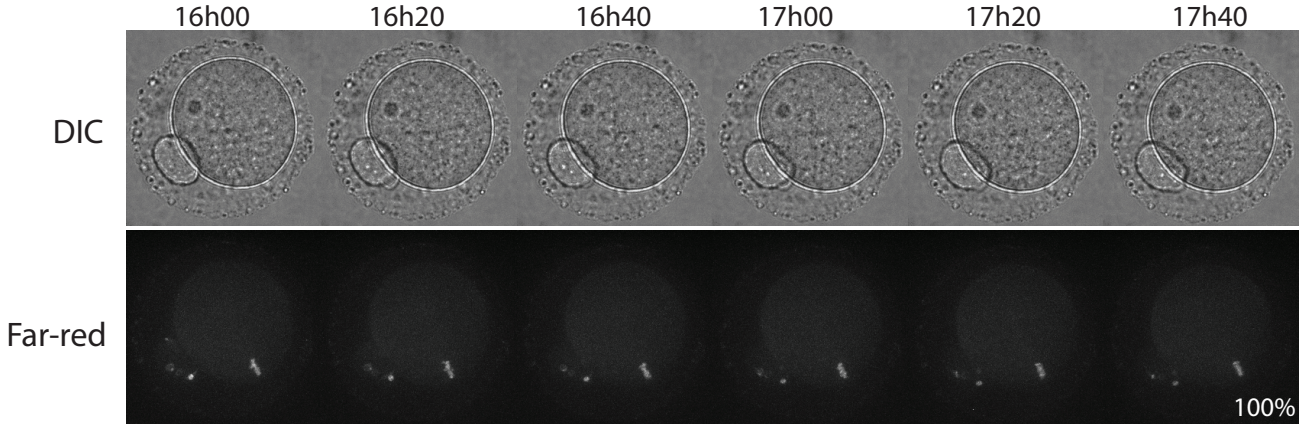
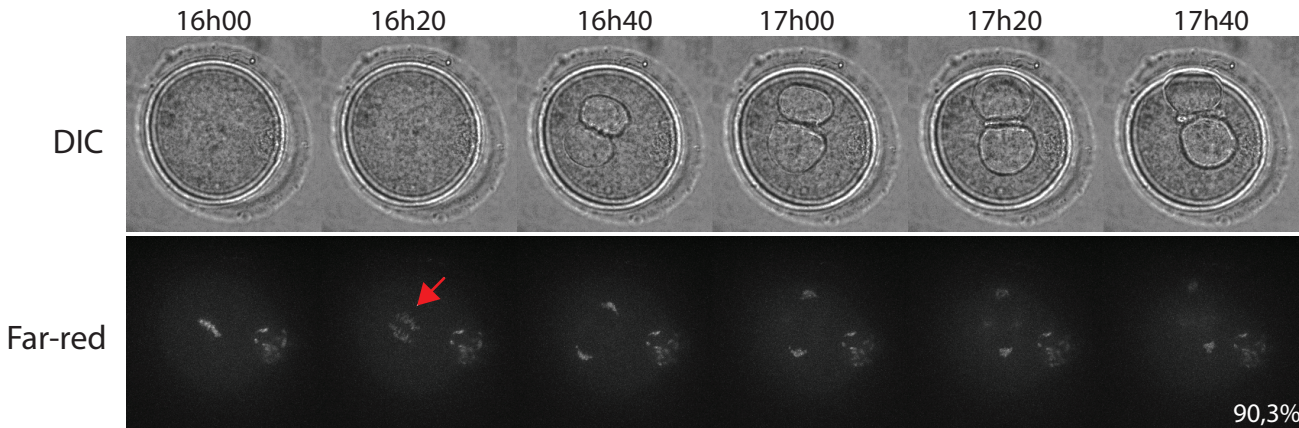


Figure 1: Expression of *Xenopus* cyclin B3 induces sister chromatid segregation. (A) *Xenopus* cyclin B3 prompts two rounds of DNA segregation. Selected time frames of control oocytes n=18 (top panel) or *Xenopus* cyclin B3 injection n=25 (bottom panel). Oocytes are treated with SiR-DNA observed with far-red before live-imaging and time points following GVBD are indicated on top. Red arrows indicate DNA segregation. **(B)** Injection of *Xenopus* cyclin B3 leads to precocious sister chromatid segregation. A representative image of chromosome spreads of control (n=22) and injected (n=16) done at metaphase II (BD+16h) stained with Hoechst (blue) and CREST (green). Insets show the usual chromosome or chromatid observed. Percentage shows the spreads that have the same phenotype. **(C)** Quantification of fluorescent expression of exogenous mouse cyclin B3 (n=10, mean= 27,7%) or *Xenopus* cyclin B3 (n=7, mean= 66,9%) in meiosis I (BD+5h) compared to meiosis II (BD+12h). For each oocyte, meiosis II fluorescent signal was compared to meiosis I (considered as 100% signal). $P=1,9924 \times 10^{-4}$ (from t test).

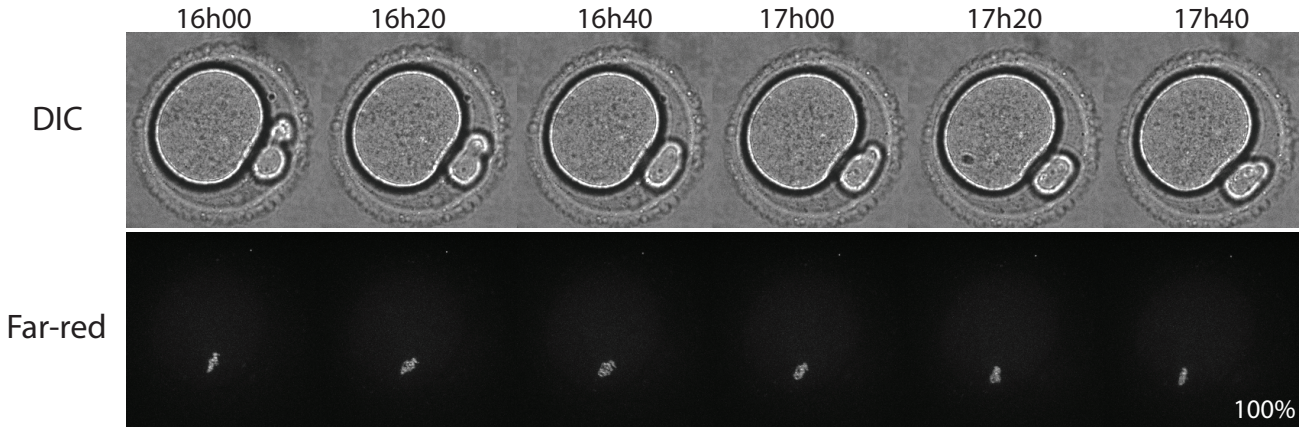
Figure 2.



Control



+ WT cyclin B3

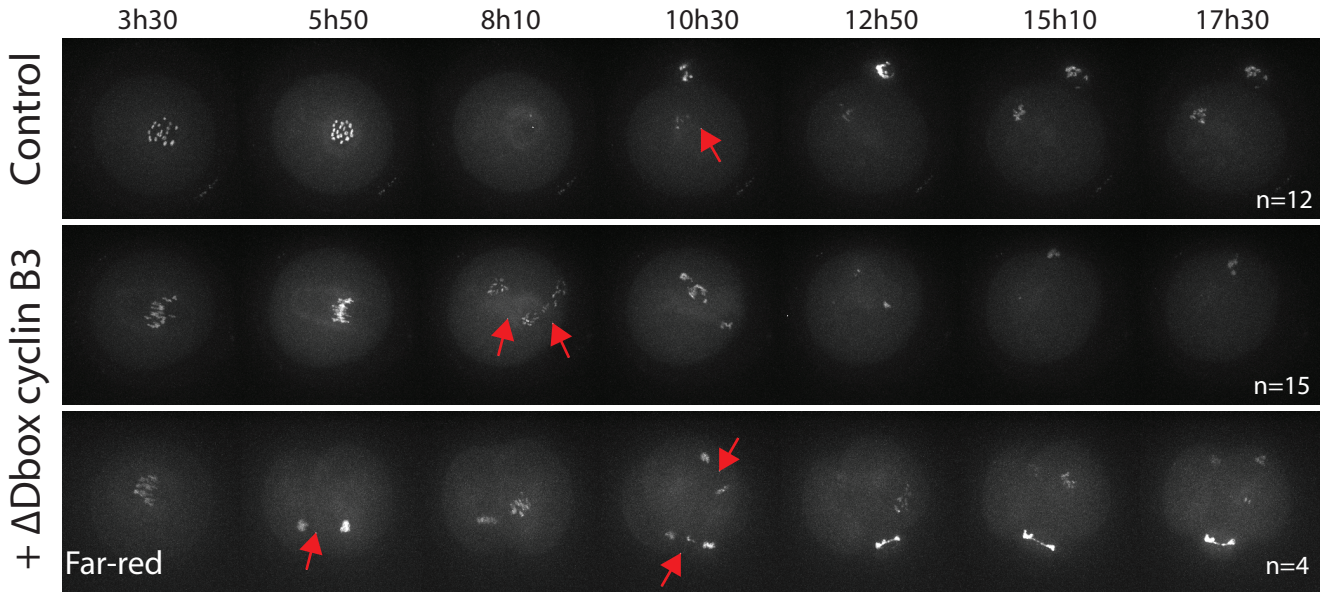


+ MRL mut. cyclin B3

Figure 2: Ectopic expression of cyclin B3 with its Cdk1 activity provokes sister chromatid segregation. Oocytes are treated with SiR-DNA before injection and observed in far-red panel. First two panels show a representative control MII oocyte (n=5) from spinning disk live imaging. The middle panels show an injected metaphase II oocyte with WT cyclin B3 (n=31). Frames of representative MII oocyte injected with MRL mutant cyclin B3 (n=9) are shown in the last two panels. Red arrow indicates sister chromatid segregation. Time points of selected frames indicated after GVBD.

Figure 3.

A)



B)

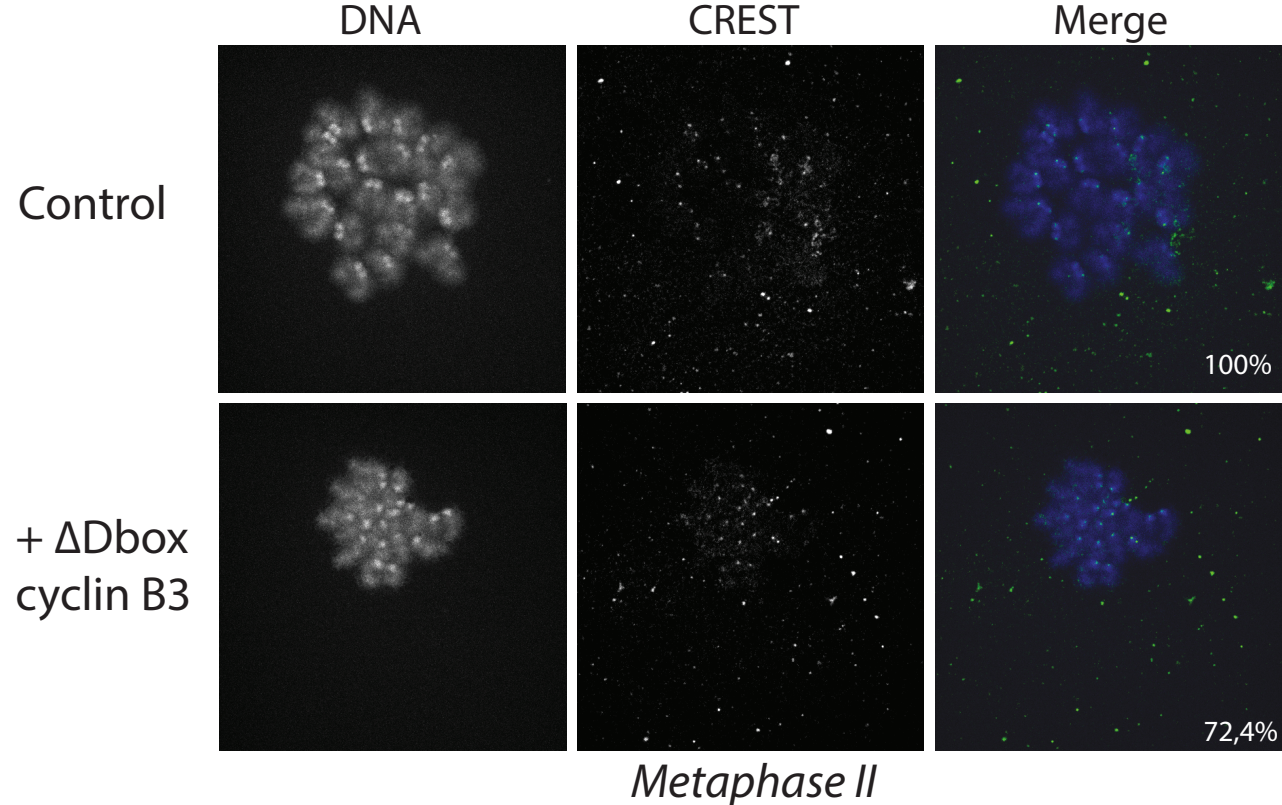
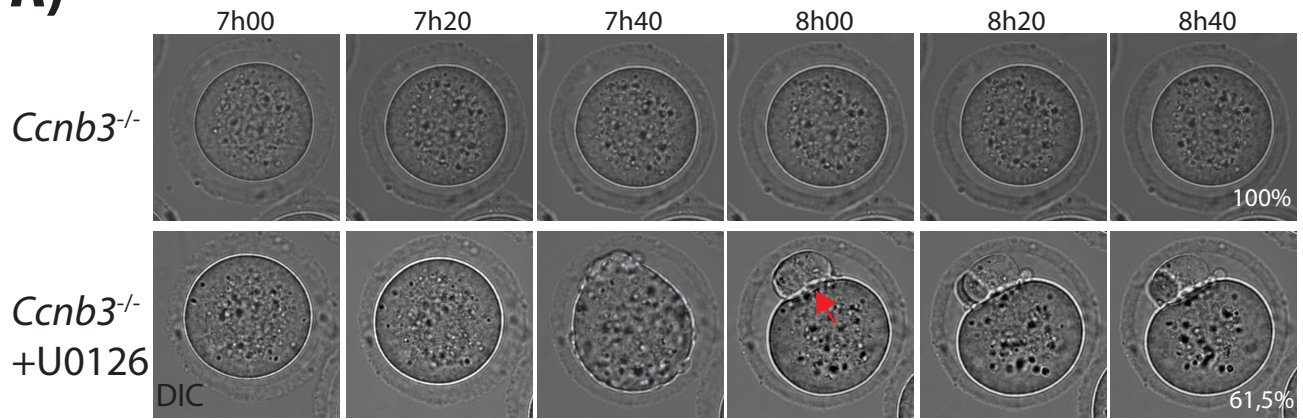


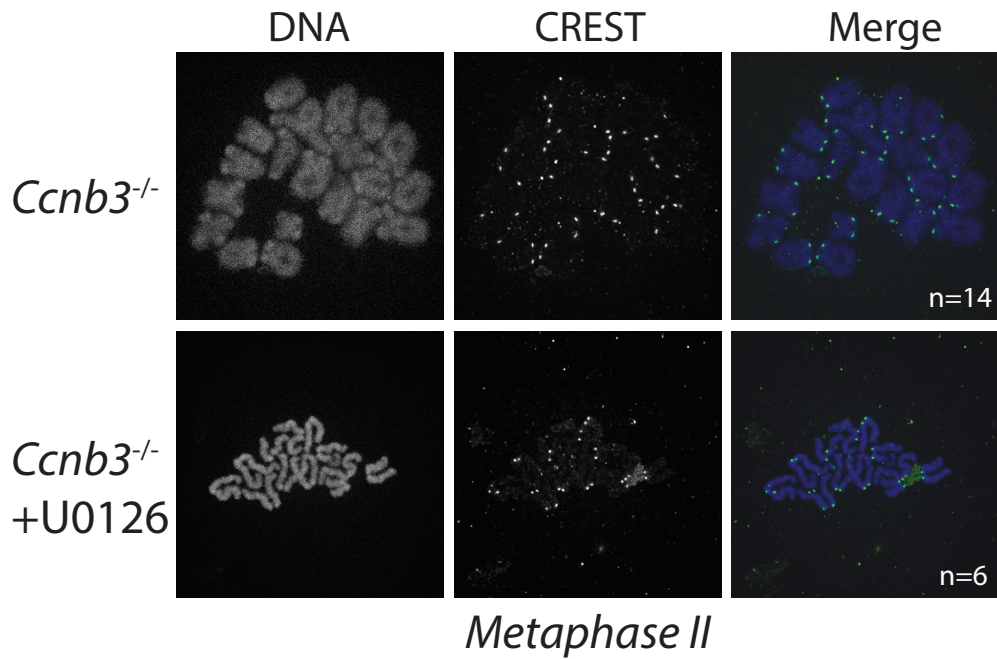
Figure 3: **Precocious sister chromatid segregation occurs due to stable expression of cyclin B3.** **(A)** \square Dbox cyclin B3 injection provokes disordered segregations. Oocytes are treated with SiR-DNA to analyze chromosome dynamics with far-red during live imaging. Top panel shows time frames of a control oocyte with one round of segregation. The two bottom panels show selected time frames from two representative oocytes showing different types of segregation, the top is more disordered while the bottom is more ordered and sequential. Red arrows point to segregations observed and timing after GVBD is indicated. **(B)** \square Dbox cyclin B3 injection leads to precocious sister chromatid segregation. Chromosome spreads were done at metaphase II. Representative images of control (n=20) and \square Dbox cyclin B3 injection (n=29) are shown. Hoechst (blue) was used to stain chromosomes and CREST (green) marks kinetochores. Percentages indicate the spreads with the same phenotype.

Figure 4.

A)



B)



C)

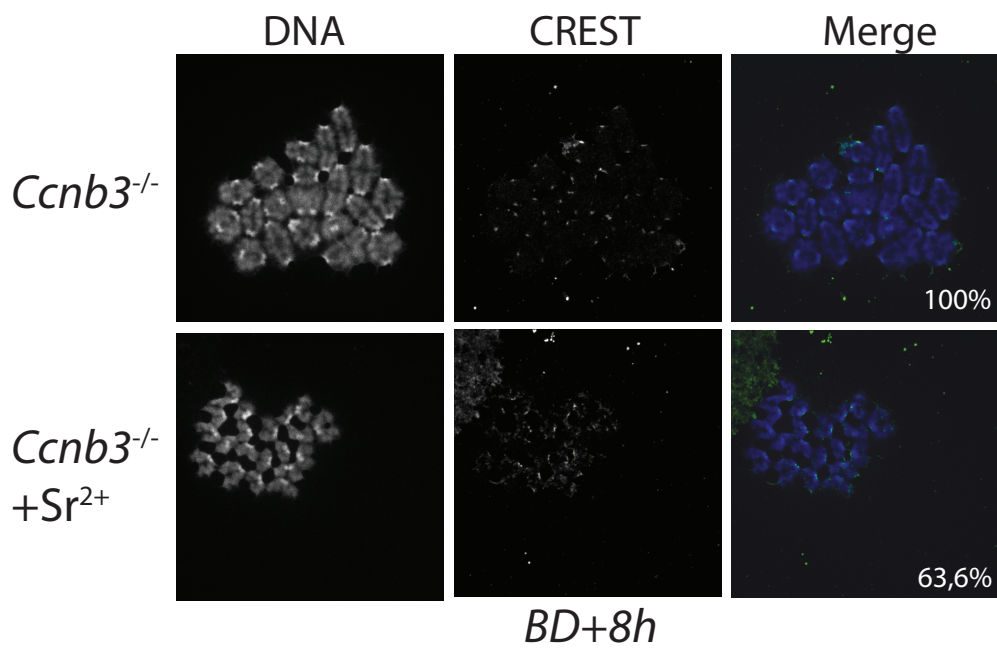
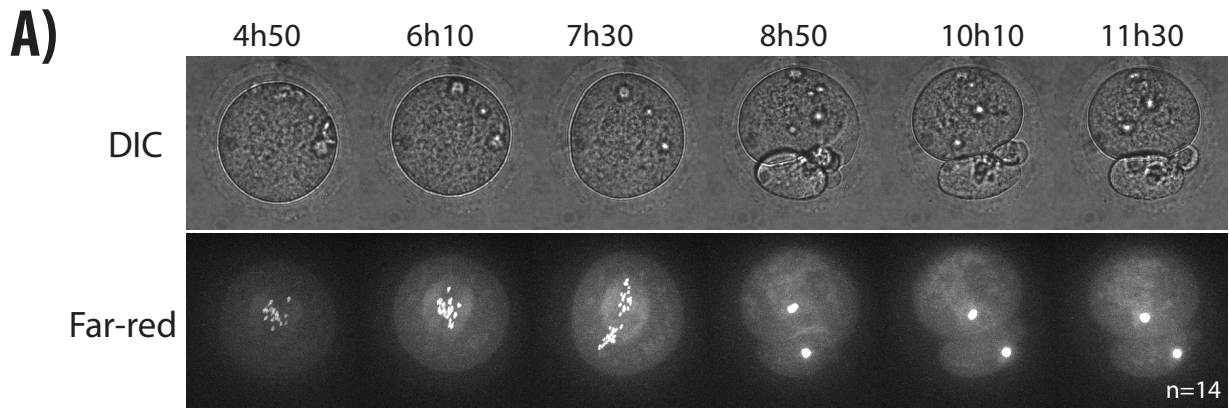
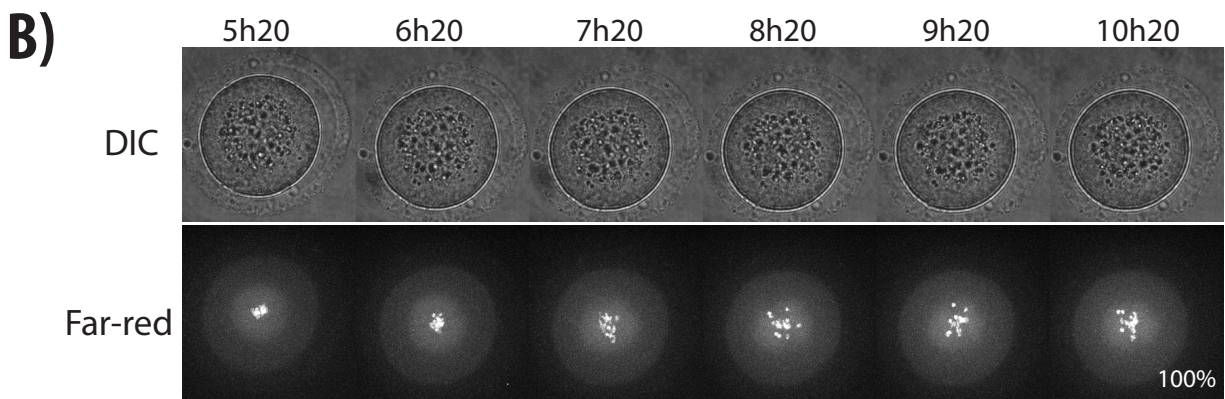


Figure 4: **Inhibition of CSF arrest components rescues metaphase I arrest of cyclin B3 KO oocytes.** **(A)** Inhibition of MEK partially rescues cyclin B3 KO phenotype. Cyclin B3 KO oocytes were either kept untreated (n=8) or treated with U0126 (n=13) at BD+4h. Frames from a representative video are shown with time following GVBD indicated. The red arrow shows polar body extrusion and percentage indicates the percent of oocytes with that phenotype. **(B)** Chromosome segregation is observed in cyclin B3 KO oocytes treated with a MEK inhibitor. Representative image of chromosome spreads of untreated and treated KO oocytes. Hoechst stains chromosomes (blue) and kinetochores are shown through use of CREST (green). **(C)** Activation of cyclin B3 KO oocytes can induce chromosome segregation. Representative images of chromosome spreads at BD+8h of untreated KO oocytes (n=6) or activated KO oocytes (n=11) for 20min using strontium (Sr^{2+}). CREST (green) shows kinetochores and Hoechst (blue) stains chromosomes. Percentages indicate the number of spreads with the same phenotype.

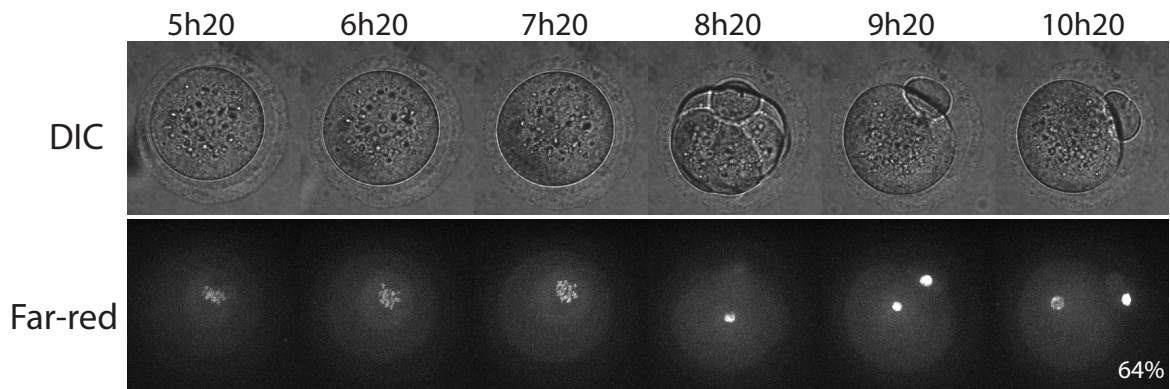
Figure 5.



Control + Emi2 MO



Ccnb3^{-/-}



Ccnb3^{-/-} + Emi2 MO

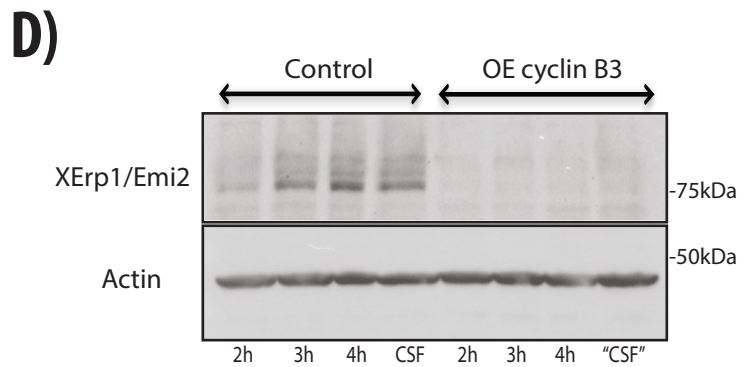
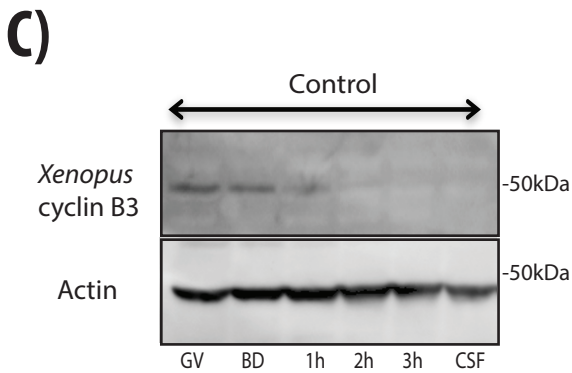


Figure 5: **Emi2 knockdown rescues cyclin B3 KO phenotype.** **(A)** Correct entry into meiosis II is abolished with morpholino Emi2 knockdown in WT oocytes. Representative images of spinning disk live imaging of an oocyte injected with Emi2 MO are shown. Oocytes are stained with SiR-DNA to observe chromosomes with far-red. Time after GVBD is indicated. **(B)** Knockdown of Emi2 rescues cyclin B3 KO metaphase I arrest. Selected time frames from representative images of cyclin B3 KO oocyte (n=9) and Emi2 MO injected KO oocytes (n=25) are shown. Chromosomes stained with SiR-DNA are shown (far-red) and timing after GVBD is indicated. **(C)** Cyclin B3 is expressed during *Xenopus* oocyte maturation. Western blot analysis of anti guinea pig *Xenopus* cyclin B3 in the top panel and loading control actin in the bottom panel. Timing of collection is indicated in the bottom and molecular weight is on the right. **(D)** Overexpression of *Xenopus* cyclin B3 in *Xenopus* oocytes leads to the degradation of Emi2. Western blot analysis of XErp1 (Emi2 in *Xenopus*) in the top panel and loading control actin in the bottom panel for control and injected oocytes. Timing after GVBD is indicated in the bottom and molecular weight on the right.

Discussion

Meiosis is composed of two sequential and distinct divisions that must be tightly regulated in order to obtain haploid gametes. Female mammalian meiosis is a long and complex process, because oocytes must complete two arrests in a timely manner in order to avoid errors in segregation of the genetic material. Oocytes are formed during embryonic development and mark a first arrest at prophase I. Once hormonal stimulation occurs, oocytes resume meiosis and must segregate chromosomes at anaphase I onset. Oocytes enter meiosis II importantly with no intervening S phase and mark a second arrest at metaphase II also called CSF arrest. It is only fertilization that triggers anaphase II onset with sister chromatid segregation. These arrest points are essential to undergo correct meiotic maturation and studies have suggested that due to these arrests and prolonged timing of maturation, female and not male mammalian meiosis requires additional regulation.

How does cyclin B3 affect APC/C activity in meiosis I?

During my PhD, I investigated the role of cyclin B3 in female meiosis. We showed that cyclin B3 is essential for female mouse fertility as cyclin B3 KO females are sterile. We found that cyclin B3 KO mouse oocytes are arrested at metaphase I and are unable to exit meiosis I. This arrest is not SAC dependent. The metaphase I arrest is due to inactive separase and high Cdk1 activity with lack of cyclin B1 degradation. Inhibition of Cdk1 activity using the Cdk inhibitor roscovitine is able to rescue the arrest. In addition, Cdk1 activity associated to cyclin B3 is necessary for its function. However, as seen in part I of my results section, there is a small proportion of oocytes, less than 10%, that were able to exit meiosis I. The reason for this is unclear. There may be a variation of APC/C activity in different oocytes. As seen with the degradation of exogenous APC/C substrates in cyclin B3 KO oocytes, APC/C is partially active in these oocytes. However, it is not able to efficiently degrade its endogenous substrates. It is possible that in the oocytes that escape the metaphase I arrest, the level of APC/C activity is sufficient for oocytes to complete meiosis I.

It is interesting that in cyclin B3 KO oocytes, the APC/C is able to discriminate between endogenous and exogenous substrates. As shown by Levasseur *et al*, binding to their partners sometimes protects substrates from APC/C ubiquitination. They show that cyclin B1 during meiosis I is protected from degradation due to its binding to Cdk1 while free cyclin B1 is degraded earlier. The binding of cyclin B1 to Cdk1 masks a possible APC/C motif found in

cyclin B1 protecting it from APC/C targeting. They also show that a portion of Cdc20 is able to escape SAC dependent inhibition given that the APC/C is active in prometaphase targeting free cyclin B1 for degradation while the SAC is on. Therefore, it is possible that in cyclin B3 KO oocytes, the exogenously expressed substrates, cyclin B1 and securin, are at a disadvantage as they bind less to Cdk1 or separase respectively and are not protected from the APC/C. Some endogenous securin is nevertheless degraded in cyclin B3 KO oocytes, this degradation however is not sufficient for anaphase I onset. Therefore, it is tempting to speculate that cyclin B3 is able to fine-tune APC/C activity in some way regulating its specificity towards some substrates over others.

As mentioned in part II of my results, we use *Xenopus* oocytes as an alternative model. The role of cyclin B3 seems to be conserved in *Xenopus* as seen by the rescue experiments in part I. Injection of *Xenopus* cyclin B3 in cyclin B3 KO mouse oocytes allows exit from meiosis I and chromosome segregation. Therefore, we have started a collaboration with the Thomas U. Mayer group from the University of Konstanz. In our collaboration, we are planning to address whether cyclin B3 binds to or phosphorylates sub-units of the APC/C. It would also be interesting to analyze if cyclin B3 influences APC/C activity by performing APC/C ubiquitination assays. Nonetheless, that cyclin B3 is acting only on the APC/C seems less likely due to my more recent results, found in part II that I will discuss below. Moreover, even if certain oocytes were able to escape the metaphase I arrest, cyclin B3 KO female mice were always found to be sterile. We can not exclude the possibility that cyclin B3 may play a role at later stages during meiotic maturation like at exit from meiosis II, or embryonic development.

Special characteristics of cyclin B3 during meiotic maturation.

Interestingly, we observed in our study that oocytes lacking cyclin B3 have a slight increase in endogenous cyclin B1. It is therefore worth mentioning that cyclin B3 could play a role in regulating translation of certain proteins such as cyclin B1, similarly to cyclin B2. It has been suggested that cyclin B2 could play a role in regulating cyclin B1 translation as oocytes lacking cyclin B2 show a decreased level of cyclin B1 (Daldello et al. 2019). In addition, Cdk1 was shown to have a role in regulating translation of cyclin B1 through the phosphorylation of the RNA binding protein CPEB1 (Han et al. 2017). However, Cdk1 phosphorylation is usually up-regulating translation, and it seems unlikely that in association with cyclin B3,

Cdk1 would do the opposite and inhibit translation of cyclin B1 to allow anaphase onset. Nevertheless, this hypothesis has to be tested in the future.

Another interesting characteristic of mouse cyclin B3, like in other placental mammals, is its size. Compared to cyclin B3 from other species, mouse and human cyclin B3 is a much larger protein. This is because of the extension of exon 8 creating a 3-fold increase of the protein (J. C. Lozano et al. 2012). This was thought to hold significance and mediate a difference in function or additional protein-to-protein interactions. Our data suggests that this increase in size is not essential for the function of cyclin B3 in meiosis I. Smaller sized cyclin B3 from other species lacking the exon 8 extension, *Xenopus*, zebrafish and *Drosophila*, were able to rescue the metaphase I arrest of cyclin B3 KO mouse oocytes. Therefore this 3-fold increase isn't needed for the meiosis I function of cyclin B3 but could be required for additional unknown roles or regulation of where and when it is expressed.

It was quite surprising to see that male meiosis was not perturbed in mice lacking cyclin B3 since it is readily expressed in testis (Karasu and Keeney 2019; J.-C. Lozano et al. 2002). Cyclin B3 could either play a non-essential role during male meiosis or be redundant with another cyclin. In addition, our data shows that expression of exogenous cyclin B3 in mouse oocytes doesn't increase Cdk1 activity significantly although this could also be due to the role of cyclin B3 inducing lower overall Cdk1 activity for anaphase onset. Moreover, in the study of Hochegger *et al*, cyclin B3 was thought to not have any role in *Xenopus* oocyte maturation due to undetectable levels of expression, using an antibody produced in their lab. However, we have shown in part II of the results that we are able to detect cyclin B3 in *Xenopus* oocytes during meiosis I with a new polyclonal antibody generated in the lab of our collaborators. Whether cyclin B3 is indeed essential for meiotic maturation in *Xenopus* is currently unknown, but rescue of mouse cyclin B3 KO oocytes through expression of *Xenopus* cyclin B3 suggests that it could have a similar role.

Cyclin B3-Cdk1 activity down regulates overall Cdk1 activity in mouse oocytes

Cyclin B3 has a specific role during meiosis that can not be compensated for by other cyclins, such as cyclin B1. This is not the case for example for cyclin B1 and B2. In oocytes lacking cyclin B1, oocytes are able to enter and complete meiosis I due to an up-regulation of cyclin B2. These oocytes can also enter meiosis II correctly if cyclin B2 is expressed at that time (J. Li et al. 2018). Therefore, cyclin B1 and B2 can compensate for each other indicating that,

with their Cdk1 associated activity, both cyclins can phosphorylate the same substrates. Cyclin B3-Cdk1 seems to play on different substrates. Injection of cyclin B1 or Cyclin A2 (my unpublished data) does not induce chromosome segregation in cyclin B3 KO oocytes. This signifies that cyclin B3 with associated Cdk1 activity has a distinct role from cyclin B1-Cdk1 indicating that they may act on different substrates. This further shows that during mammalian female meiosis, cyclins do confer specificities to Cdk1 activity impinging on different substrates and pathways during maturation. Interestingly, cyclin B3-Cdk1 kinase seems to be able to have a negative effect on overall Cdk1 activity as overexpression of stable cyclin B3 in WT oocytes does not significantly increase overall kinase activity during metaphase. On the contrary, kinase activity seems to slightly decrease with the expression of stable cyclin B3. This could be due to a decrease in cyclin B1-Cdk1 activity provoked by cyclin B3-Cdk1 activity. In addition, cyclin B3-Cdk1 activity is needed to promote anaphase I onset inducing a decrease of overall Cdk1 activity. Hence, cyclin B3-Cdk1 could play a role in regulating cyclin B1-Cdk1 activity directly or indirectly.

Precocious CSF arrest in cyclin B3 KO mouse oocytes.

During the second part of my PhD, and in part II of the results section, we have shown that cyclin B3 KO oocytes seem to have a precocious CSF arrest put in place in meiosis I. This is unexpected as high Cdk1 activity in meiosis I usually suggests destabilization of Emi2 (Isoda et al. 2011; Tang et al. 2008). Inhibition of the CSF kinase MEK, and artificial activation creating calcium waves similar to fertilization in these KO oocytes led to chromosome segregation. This data suggests that there is indeed a CSF arrest put in place in cyclin B3 KO oocytes that maintains the metaphase I arrest observed and that cyclin B3 is able to inhibit the CSF pathway somehow. At what level it acts negatively on the pathway establishing CSF arrest is still unknown and needs to be investigated.

Correct timing of activation still needs to be determined as not all oocytes were rescued. Furthermore, even in WT metaphase II arrested oocytes, activation does not have a 100% success rate. It would be interesting to try using IVF (*in vitro* fertilization) to rescue metaphase I arrested oocytes. This could further indicate that the metaphase I arrest observed in cyclin B3 KO oocytes is indeed a physiological, precocious CSF arrest similar to the metaphase II arrest in WT oocytes. In addition, this technique might be more effective. It would also be of interest to see if after activation, cyclin B3 KO oocytes enter meiosis II

correctly with re-accumulation of cyclin B1 and if another CSF arrest is put in place. Possible alignment of chromosomes after activation could be verified through live imaging. If chromosomes do not decondense then it would suggest that these oocytes are able to enter meiosis II.

Conserved role of cyclin B3 in mouse and *Xenopus* oocyte maturation.

It is interesting to note that overexpression of *Xenopus* cyclin B3 in *Xenopus* oocytes leads to abnormal meiotic progression. *Xenopus* oocytes overexpressing cyclin B3 present aberrant meiotic maturation since oocytes seem to go through apoptosis after 4-5 hours following GVBD. This indicates that something in these oocytes is perturbed. If the role of cyclin B3 is conserved and *Xenopus* cyclin B3 overexpression is able to lift CSF arrest, then it is possible that these oocytes have exited meiosis II and go through apoptosis due to lack of fertilization. In addition, and interestingly, overexpression of *Xenopus* cyclin B3 induces accelerated GVBD, around 1h30 earlier than control *Xenopus* oocytes (data not shown). This is also observed when *Xenopus* cyclin B3 is expressed in mouse oocytes. This signifies that *Xenopus* cyclin B3 seems to have an additional role of being capable of inducing GVBD unlike mouse cyclin B3.

To observe if cyclin B3 is needed for meiotic maturation in *Xenopus* oocytes, morpholino knockdown is currently being performed. In these oocytes, GVBD seems to be slightly affected as it is delayed by 1-2h compared to control oocytes. Analysis of Cdk1 activity or cyclin B1/2 levels should indicate if *Xenopus* oocytes do arrest in meiosis I similarly to mouse oocytes. In addition, levels of XErp1/Emi2 protein expression in morpholino knockdown oocytes compared to control oocytes could show us if there is accumulation of Emi2 earlier in knockdown oocytes.

In the lab, we are currently trying to answer this question in mouse oocytes. It is essential to verify if Emi2 is expressed earlier (already in meiosis I) and at higher levels in cyclin B3 KO oocytes compared to control. First, we are checking Emi2 expression in WT oocytes on western blots at different time points during meiosis. This should give us an idea of the expression profile of endogenous Emi2 in mouse oocytes. Furthermore, oocytes injected with WT or stable cyclin B3 in meiosis II will be analyzed. If it acts similarly to *Xenopus* oocytes, we should see a degradation of Emi2. This would act as a negative control and also show if -like in *Xenopus* CSF extracts, as shown by our collaborators in Konstanz- expression

of cyclin B3 during CSF arrest induces degradation of Emi2 in the mouse. The next step would be to analyze Emi2 expression in cyclin B3 KO mouse oocytes. These oocytes could have an earlier expression or accumulation of Emi2 leading to the metaphase I arrest. We could also analyze the expression profile of other CSF components, such as Mos. However, differences could be more difficult to detect as Mos-MAPK is expressed during meiosis I in control oocytes. Nevertheless, first preliminary data indicate that there is indeed increased in vitro MAPK activity in meiosis I KO oocytes.

How does cyclin B3 inhibit CSF arrest?

Emi2 degradation:

As described in part II of the results section, expression of *Xenopus* cyclin B3 in mouse oocytes induces precocious sister chromatid segregation. In addition, ectopic expression of mouse cyclin B3 in metaphase II arrested oocytes also induces a lift of the CSF arrest with chromatid segregation. Moreover, morpholino knockdown of Emi2 rescues cyclin B3 KO metaphase I arrest inducing anaphase I onset. In addition, overexpression of *Xenopus* cyclin B3 in *Xenopus* oocytes seems to induce degradation of Emi2 as XErp1/Emi2 staining was not observed on the western blot from oocytes overexpressing *Xenopus* cyclin B3. This was indeed confirmed by our collaborators in the Thomas U. Mayer lab. They collected CSF extracts from *Xenopus* oocytes expressing *Xenopus* cyclin B3. XErp1/Emi2 degradation was observed to occur rapidly in less than 30min after overexpression of *Xenopus* cyclin B3. The degradation of Emi2 due to cyclin B3 overexpression suggests that it might not only play on APC/C activity directly since Emi2 degradation does not depend on APC/C activity. This result indicates that cyclin B3 could be a potent inhibitor of CSF arrest inducing the degradation of Emi2. Cyclin B3 could also act by destabilizing Emi2 mRNA and expression. On which component of the pathway required for CSF arrest cyclin B3 acts is still unclear.

Emi2 was shown to be important for entry into meiosis II after anaphase I onset (Madgwick et al. 2006; Shoji et al. 2006). In *Xenopus* oocytes, it was shown that XErp1/Emi2 is present in meiosis I but is degraded due to high Cdk1 activity. Cdk1 can phosphorylate XErp1/Emi2 on residues on its N-terminus to destabilize it. Once the APC/C is active and Cdk1 activity decreases at anaphase I onset, Xerp1/Emi2 can accumulate and inhibit APC/C activity allowing cyclin B1 re-synthesis. This provides correct MII entry. Because Cdk1 activity is lower in meiosis II than in meiosis I, Emi2 can act as an APC/C inhibitor (Isoda et al. 2011;

Tang et al. 2008). However, it is tempting to speculate that the ability of Emi2 to act as an APC/C inhibitor is determined by absence (meiosis II) or presence (meiosis I) of cyclin B3.

Phosphorylation of Emi2 not only controls its stability, but also its activity. As seen in *Xenopus* oocytes, Cdk1 phosphorylates Emi2 on specific sites on the N-terminus to destabilize it and in the C-terminus to inactivate it. These phosphorylation sites have been identified in *Xenopus* oocytes (Isoda et al. 2011). It would be interesting to see if Cdk1 phosphorylates these sites when it forms a complex with cyclin B3. Phosphorylation status of Emi2 could be analyzed in *Xenopus* cyclin B3 knockdown or overexpression *Xenopus* oocytes. Furthermore, injecting Emi2 phospho-mutants could inform us on if cyclin B3 acts on Emi2 by phosphorylating it. For example, we could mutate sites usually phosphorylated by Cdk1 into non-phosphorylatable sites. These constructs can then be injected into metaphase II arrested mouse oocytes, where WT cyclin B3 was injected. If chromatid segregation does not occur, it would suggest that these sites need to be phosphorylated for cyclin B3-Cdk1 function. This can also be verified in *Xenopus* oocyte CSF extracts by adding different peptides of mutant Emi2 together with *Xenopus* cyclin B3 to analyze Emi2 degradation or lack thereof.

Additionally, pull-down of *Xenopus* cyclin B3 could be performed to verify if it interacts with different components of the CSF pathway. This could inform us of different possible interactions. Several proteins, at least in *Xenopus*, affect Xerp1/Emi2 stability and activity. Phosphorylation on certain sites on the N-terminus of Xerp1/Emi2 recruits Plk1 to Xerp1/Emi2 leading to its degradation. Cyclin B3 could regulate Plk1 activity or/and cyclin B1-Cdk1. Cyclin B1-Cdk1 and Plk1 were found in pulldowns with specific phosphopeptides of Emi2 in the N-terminus (Isoda et al. 2011). Therefore, similar pulldown experiments could allow us to see where and if *Xenopus* cyclin B3 binds to XErp1/Emi2 or other CSF components.

In addition, if cyclin B3 acts by destabilizing Emi2 through phosphorylation, then PP2A activity would be needed to stabilize Emi2 by dephosphorylating those sites. Therefore, PP2A inhibition could rescue the KO phenotype. Use of a PP2A inhibitor such as okadaic acid could be used in first assays. Our preliminary data suggests that indeed inhibiting PP2A allows chromosome segregation in some cyclin B3 KO oocytes (data not shown). However, no polar body extrusion was observed. Further experiments must be completed to have a clearer result. We could also use a more specific PP2A inhibitor by using a PP2A-B56

inhibitor peptide (the PP2A isoform responsible in part for Emi2 stabilization). Live imaging and chromosome spreads can be used to verify if this inhibition successfully rescues cyclin B3 KO oocytes.

Upstream of Emi2: the Mos-MAPK pathway

Cyclin B3 may act on one of the components of the Mos-MAPK pathway directly decreasing the stabilizing phosphorylation of Emi2. If this were the case, less PP2A would be recruited to dephosphorylate Emi2 to stabilize it. Consequently, Cdk1 would successfully phosphorylate Emi2 on its N-terminus leading to destabilization of Emi2 and its degradation. To test this, we could verify if stabilizing phosphorylation by Mos-MAPK is present on XErp1/Emi2 in *Xenopus* oocytes overexpressing *Xenopus* cyclin B3 or in meiosis I in *Xenopus* cyclin B3 knockdown. Furthermore, pull-down experiments of cyclin B3 in *Xenopus* oocytes could inform us on if it binds to some component of the Mos-MAPK pathway. In addition, to verify if cyclin B3 acts on the components of the CSF pathway upstream of Emi2, MAPK activity could be analyzed in mouse oocytes. However, analyzing MAPK activity is more difficult than looking at different phosphorylations of Emi2, as MAPK is needed for other processes in oocytes and is present and somewhat active throughout meiotic maturation. In addition, its inactivation takes a longer time than Emi2 degradation beginning only 2 hours following Sr^{2+} activation (Gonzalez-Garcia et al. 2014). Emi2 on the other hand can be degraded within 30min and reappears only 6h later coinciding with pronuclear formation (Madgwick et al. 2006). This hints at a possible additional role of Emi2 later in development. Nevertheless, we could utilize a reporter similar to the one published by Karl Swann's group that is able to show MAPK declining activity in mouse oocytes. This luciferase reporter does not emit any luminescence when MAPK (Erk1) is phosphorylated. When it is not phosphorylated, corresponding to its inactive state, the construct changes conformation, emitting luminescence. This indicates when MAPK is not active (Gonzalez-Garcia et al. 2014). This tool could be used to see if cyclin B3 affects MAPK activity and not Emi2 directly. If ectopic expression of cyclin B3 in metaphase II arrested oocytes affects MAPK activity, then an increase in luminescence should be observed during live imaging. On the other hand, if cyclin B3 acts directly on Emi2, then MAPK activity may be less, differently, or not altered. In addition, as previously mentioned, western blots of mouse oocytes are being performed in the lab to analyze MAPK activity in cyclin B3 KO oocytes.

Whether cyclin B3 has a role later in development in female mice is not known. The phenotype observed in meiosis I does not exclude that cyclin B3 could have a role following meiosis I. All data found with the use of *Xenopus* oocytes will be tested in mouse oocytes to test if the role of cyclin B3 is conserved and dissect its mechanism. Cyclin B3 in mice is required for female meiotic maturation but is dispensable for male meiosis. Hence, it makes sense that it plays a role affecting a pathway that is necessary for correct oocyte maturation and not during spermatogenesis. By inhibiting in some way the CSF pathway, cyclin B3-Cdk1 activity allows correct exit from meiosis I and anaphase onset. Therefore, through the use of different systems and techniques, we will be able to define the role of cyclin B3 during female meiosis. In conclusion, during my PhD I was able to uncover a novel role of cyclin B3 needed for correct female mammalian meiotic maturation, a function that seems to be evolutionarily conserved.

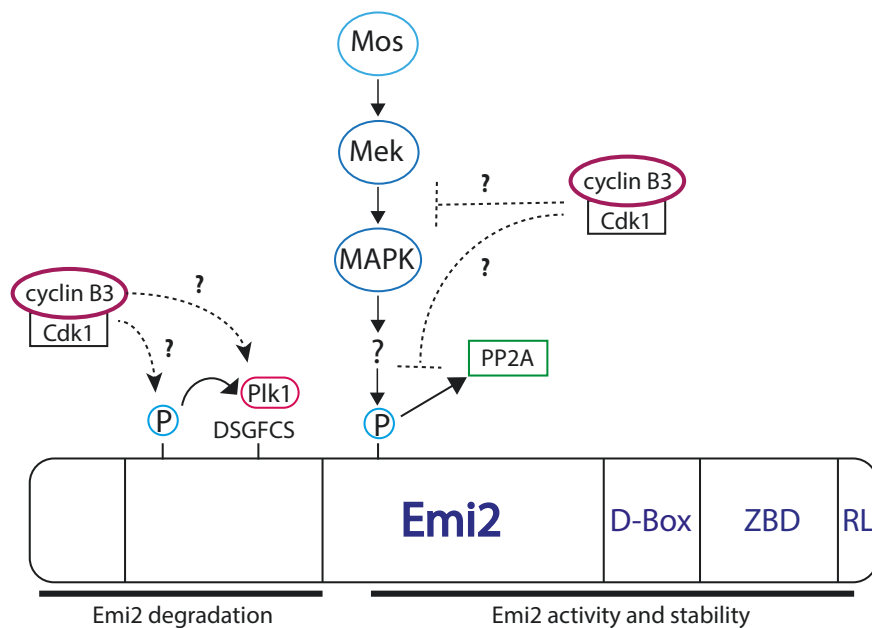


Figure 19: Cyclin B3 inhibits the CSF pathway. Cyclin B3-Cdk1 activity is able to inhibit the CSF pathway and avoid precocious arrest in meiosis I. It could do so by acting on different regulators of the CSF arrest. Cyclin B3-Cdk1 could phosphorylate the N-term of Emi2 leading to its degradation. It could also play on the Plk1 activity needed for Emi2 degradation. In addition, cyclin B3-Cdk1 could inhibit the stabilization of Emi2 by inhibiting in some way Mos-MAPK pathway or PP2A activity. One or more of these actions would induce Emi2 degradation and anaphase onset in meiosis I.

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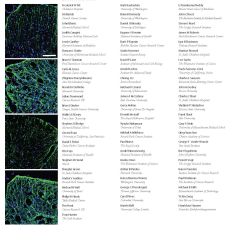
Résumé

Tout au long de ma thèse, je me suis focalisée sur l'étude de la régulation du cycle cellulaire méiotique chez les femelles et plus particulièrement sur les deux divisions méiotiques et la ségrégation chromosomique dans les ovocytes. La méiose est un processus étroitement réglementé qui doit être effectué de manière ordonnée pour obtenir des gamètes avec le bon nombre de chromosomes. Les deux divisions qui se produisent pendant la méiose (appelées méiose I et II) ont deux schémas de ségrégation différents. A noter que la phase S doit être inhibée entre les deux divisions. Cela conduit à la création de gamètes haploïdes. Toutes les étapes de la division des cellules méiotiques sont régulées par des cyclines associées à leurs partenaires catalytiques, les Cdks. La méiose chez les femelles mammifères présente de nombreux défis. Il a été démontré qu'il s'agit d'un processus sujet aux erreurs, où des erreurs de ségrégation se produisent, créant des gamètes aneuploïdes. L'aneuploïdie est plus fréquente pendant la méiose chez la femme que chez l'homme. De plus, l'incidence d'aneuploïdie augmente en corrélation avec l'âge. Comprendre les mécanismes impliqués dans la régulation de la méiose chez les femelles mammifères est donc essentiel.

La progression dans les divisions méiotiques dans les ovocytes de mammifères (également appelée maturation méiotique) est caractérisée par deux arrêts. Les ovocytes formés pendant le développement embryonnaire sont arrêtés en prophase I, jusqu'à ce que la stimulation hormonale induise la reprise de la méiose. Le deuxième arrêt est marqué à la métaphase II, également appelée arrêt CSF. Cet arrêt n'est levé que lors de la fécondation. Au cours de ma thèse, j'ai étudié le rôle d'une cycline, la cycline B3, grâce à l'utilisation de souris cycline B3 KO. J'ai trouvé qu'un manque d'activité de la cycline B3-Cdk1 induit un arrêt en métaphase I dans les ovocytes de souris. Cela est dû aux niveaux élevés de taux de cycline B1 et d'activité de Cdk1 ainsi que la séparase inactive. L'activité de l'APC/C a également été affectée, car les substrats endogènes de l'APC/C n'étaient pas dégradés efficacement. En outre, le rôle de la cycline B3 s'est révélé être conservé, car la cycline B3 d'autres espèces a pu sauver le phénotype des ovocytes cycline B3 KO.

En explorant d'avantage les rôles possibles de la cycline B3 et en utilisant des ovocytes de *Xénope*, j'ai pu montrer que la cycline B3 était capable d'inhiber l'arrêt CSF. Le mécanisme de cette inhibition en cours d'étude, mais mes dernières données suggèrent que la cycline B3 est capable d'induire la dégradation de l'inhibiteur de l'APC/C, Emi2. De plus, les ovocytes dépourvus de cycline B3 ont mis en place un arrêt CSF précoce conduisant à l'arrêt en métaphase I observée. Imiter la fécondation ou l'inhibition de l'arrêt CSF sauve cet arrêt en métaphase I. Par conséquent, mon travail de thèse a montré que la cycline B3 est essentielle dans la méiose chez les femelles afin d'activer correctement l'APC/C, prévenant ainsi un arrêt CSF précoce dans la méiose I au lieu de la méiose II.

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REVIEW



Cycling through mammalian meiosis: B-type cyclins in oocytes

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ABSTRACT

B-type cyclins in association with Cdk1 mediate key steps of mitosis and meiosis, by phosphorylating a plethora of substrates. Progression through the meiotic cell cycle requires the execution of two cell divisions named meiosis I and II without intervening S-phase, to obtain haploid gametes. These two divisions are highly asymmetric in the large oocyte. Chromosome segregation in meiosis I and sister chromatid segregation in meiosis II requires the sharp, switch-like inactivation of Cdk1 activity, which is brought about by degradation of B-type cyclins and counteracting phosphatases. Importantly and contrary to mitosis, inactivation of Cdk1 must not allow S-phase to take place at exit from meiosis I. Here, we describe recent studies on the regulation of translation and degradation of B-type cyclins in mouse oocytes, and how far their roles are redundant or specific, with a special focus on the recently discovered oocyte-specific role of cyclin B3.

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Introduction

The generation of healthy, haploid gametes depends on the correct execution of a specialized form of cell division named meiosis. Unlike mitosis, meiosis contains two steps of DNA division with only one round of DNA replication. These two divisions are called meiosis I and meiosis II. In oocytes, these two rounds of chromosome segregation are regulated temporally, are highly asymmetric and depending on the species, contain cell cycle arrest points that are crucial for female gametogenesis. In mammalian meiosis, after premeiotic S-phase and meiotic recombination, oocytes are arrested at prophase I from birth of the female onwards. Upon hormonal stimulation, this arrest is lifted and meiosis resumes with entry into the first meiotic division. Oocytes undergo the first metaphase-to-anaphase transition where chromosomes segregate and half the DNA is discarded in the smaller cell, the polar body. Oocytes enter meiosis II immediately following meiosis I exit. Oocytes must then arrest at metaphase II awaiting fertilization which triggers the second division with segregation of sister chromatids (Figure 1). Once the oocyte is fertilized, the embryo can begin its development [1, 2, 3].

It is primordial to understand the mechanisms that regulate the correct execution of female mammalian meiosis. Aneuploid embryos harbouring incorrect numbers of chromosomes, are mainly caused by maternal errors during female meiosis. 10–30% of all human oocytes are found to be aneuploid while only 1–2% of spermatozoa contain the wrong number of chromosomes [4–6]. This difference is increased in correlation with maternal age [5,7]. By age 42, the incidence of aneuploidy may increase up to 80%, according to [8]. Not all segregation errors are due to aberrant meiotic divisions though, and additionally, a high rate of segregation errors is observed in the following mitotic divisions at later stages of embryogenesis, up to the blastocyst stage [9,10]. Today, with more commonly used medically assisted reproduction and the increase of maternal age at first pregnancies, it is indispensable to gain a better comprehension of the regulatory mechanisms put in place to segregate the genetic material in meiosis and early mitotic divisions in the embryo. Insights into cell cycle regulation during meiotic maturation will help us understand what goes wrong so often in oocytes, which consequently leads to the generation of aneuploidies and hence, failure to generate healthy offspring.

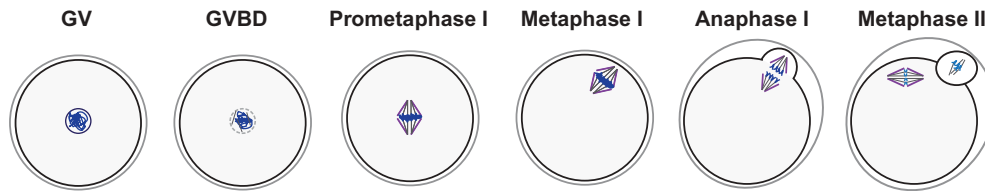


Figure 1. Mammalian oocyte maturation. Oocytes are arrested at prophase I, also known as germinal vesicle (GV) stage. Upon hormonal stimulation, fully-grown oocytes resume meiosis with germinal vesicle breakdown (GVBD). Chromosomes (in blue) condense, the bipolar spindle forms and microtubules attach to the chromosomes throughout prometaphase. In metaphase I chromosomes that are properly attached to the spindle and aligned at the metaphase plate migrate to the cortex. Chromosomes segregate during anaphase I with the extrusion of a first polar body. Oocytes immediately enter meiosis II and arrest at metaphase II awaiting fertilization.

Mammalian meiosis

Although meiotic divisions are composed of the same steps as mitosis, the fact that two divisions without intervening S-phase are executed with entirely different segregation patterns requires additional regulatory mechanisms. Detailed reviews on different aspects of chromosome and sister chromatid segregation in meiosis I and II, respectively, have been published and are beyond the scope of this review [2, 11, 12]. Here, we will focus on very recent results concerning the role of B-type cyclins for cell cycle progression in mammalian oocytes.

In oocytes, the size of the cell, as well as the time frame of meiotic maturation to enter meiosis I and ensure fertilization takes place at the right moment of cell division, demands distinct cell cycle regulation. Below we introduce progression through the meiotic divisions (meiotic maturation) from a cell cycle point of view, before describing the roles of the three existing mammalian B-type cyclins B1, B2 and B3 [13–15], all three of which have now been studied by knock-out approaches in mouse oocytes [16–21].

Mammalian oocytes are formed during embryonic development and following DNA replication and recombination, oocytes arrest at prophase I. This is known as GV stage due to the presence of a germinal vesicle. This signifies that oocytes must stay arrested for extended periods of time (months for mice, decades for humans) before the meiotic divisions. Before resuming meiosis, selected oocytes undergo a short growth phase in the adult female. Upon hormonal stimulation, the GV breaks down (GVBD) followed by chromosome condensation and spindle formation [1, 2, 3]. In mammals,

a prolonged prometaphase in meiosis I allows the spindle to fully form and microtubules (MT) to attach correctly to kinetochores (KT), hence chromosomes can align at the metaphase plate. Similarly to mitosis, the Spindle Assembly Checkpoint (SAC) is present and active during meiotic cell division. It imposes a delay in anaphase I onset by inhibiting the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase essential for this cell cycle transition, to allow time for establishing correct KT-MT attachments [7,22,23]. In order to allow metaphase-to-anaphase I transition to take place, the APC/C must be activated in order to ubiquitinate proteins that block separase activity, namely securin and cyclin B1. Once ubiquitinated, these proteins are degraded and separase becomes active leading to chromosome segregation [24–26]. Oocytes then enter meiosis II immediately, and crucially, enter meiosis II without carrying out a second S-phase. Chromosomes then align and arrest at metaphase II. APC/C activation is inhibited to maintain this second meiotic arrest (also called Cystostatic factor (CSF-) arrest) until anaphase II is triggered by fertilization [11,27,28].

M-phase promoting factor (MPF)

Upon hormonal stimulation, the protracted GV arrest is lifted and this depends on the increase in MPF (M-phase promoting factor) kinase activity, the main driver of mitosis and meiosis. MPF phosphorylates a large number of substrates, and is required for entry into and progression through mitosis and meiosis, chromosome condensation, dissolution of the nuclear membrane, spindle formation, and chromosome alignment. It has to be

inactivated for anaphase onset and exit from cell division. What makes up this factor has been extensively debated, but in both mitosis and meiosis MPF is considered as corresponding to the complex cyclin B-Cdk1 [29]. However, MPF activity is not only due to cyclin B-Cdk1 complexes but also the kinase Greatwall (Mastl in mammals) that is essential to protect the phosphorylated substrates of cyclin B-Cdk1 from the counteracting phosphatase PP2A, allowing appropriate entry and progression through mitosis and meiosis [29–31].

During the prolonged GV arrest in oocytes, Cdk1 is kept inactive through phosphorylation established by the Wee1 and Myt1 kinases, and APC/C dependent degradation of cyclin B. Although the molecular mechanism is not completely known in mouse oocytes, depending on hormonal stimulation and release from surrounding follicle cells, activation of Cdk1 is triggered by the phosphatase Cdc25 that removes the inhibitory phosphorylation on Cdk1. To be active, Cdk1 needs to be associated with cyclins, in this case cyclin B1 or B2, to induce GVBD. Once Cdk1 is dephosphorylated and sufficient MPF activity is generated, GVBD occurs with resumption of meiosis [32,33].

Overview of M-phase B type cyclins

Most of the components needed for meiosis are also present during mitosis. This is the case for the main regulators of the cell cycle, cyclins. Several cyclins are expressed depending on the species and cell cycle phase. In order to fulfill their purpose, cyclins bind to their catalytic partners, Cdks (cyclin-dependent kinases). Progression through mitotic cell division is thought to require just one B-type cyclin together with Cdk1, as in both budding and fission yeast, Clb2 or Cdc13 respectively, is the only cyclin required to be paired up with Cdk1 to drive cells through mitosis. In *S. pombe*, it was even shown that Cdc13-Cdk2 (which corresponds to Cdk1) can -in principle- drive progression through all stages of the cell cycle, indicating that cyclin specificity is not determinant for ordering cell cycle events [34–36]. This has led to a model proposing that threshold levels of Cdk1-associated activity determine substrate specificities, and not individual cyclins. Importantly though, this

model does not seem to apply to mitosis and meiosis in other organisms. At least in mammalian oocytes, specific B-type cyclins harbour distinct roles. Individual knock-outs of all three B-type cyclins have been described in the mouse and provide useful insights to the specificities of each cyclin during the meiotic divisions. In this review, we will discuss the redundant and distinct roles that B-type cyclins occupy in mouse oocyte meiosis, highlighting the importance of each cyclin in the correct timing of cell cycle events during this specialized cell division.

Cyclin B1

In mammals, cyclin B1 is the main cyclin driving entry into and progression through mitosis and meiosis. Apart from phosphorylations impinging on Cdk1, protein levels of cyclin B1 directly correlate with Cdk1-associated kinase activity, regulating key steps of mitotic and meiotic cell cycle progression. In mouse oocytes, cyclin B1-Cdk1 activity gradually increases for the first hours after GVBD, as oocytes progress through prometaphase, to reach its highest levels in metaphase I. This is due to inactivation of the APC/C activator Cdh1, and concomitant SAC activation, preventing activation of the APC/C in a Cdc20-dependent manner [25,37–40]. Slow progression through prometaphase allows the establishment of KT-MT attachments and correct alignment of chromosomes at the metaphase plate. For anaphase I onset, cyclin B1-Cdk1 activity must decrease drastically for a switch-like transition from metaphase to anaphase, and this is brought about by inactivation of the SAC and cyclin B1 degradation [7,11,22]. Cyclin B1-Cdk1 activity has to rise again quickly to inhibit S-phase onset, and instead promote entry into meiosis II with metaphase II or CSF-arrest. Upon fertilization, cyclin B1-Cdk1 activity must drop again for anaphase II onset with sister chromatid segregation [11,28,41].

Although protein synthesis is not necessary for GVBD, Cyclin B synthesis is essential for correct progression through meiosis in mouse oocytes [42, 43]. Following GVBD, the increase of cyclin B1 synthesis is responsible for the rise in Cdk1 activity, inducing chromosome condensation and congression as well as alignment during metaphase I. Another specificity of meiosis in mouse oocytes is that -unlike in cultured

human cells – cyclin B1 is expressed in excess compared to its partner Cdk1 [44,45], indicating that there is a distinct regulation of MPF activity in meiosis compared to mitosis. The large size of the cell and the extended time needed for prometaphase and metaphase might be the reason for this difference. Similarly to mitotic securin degradation which occurs preferentially when securin is not found in association with separase [46, 47], Levasseur et al demonstrated in a very recent study that there are different pools of cyclin B1; free cyclin B1 and Cdk1-bound cyclin B1, and that association with Cdk1 determines temporal control of cyclin B1 degradation. Free cyclin B1 is degraded before all chromosomes are correctly attached and the SAC is turned off, in an APC/C-dependent manner. It continues to be degraded afterwards, through only its destruction box (Dbox), which is a motif recognized by the APC/C in association with Cdc20. The first degradation wave of free cyclin B1 depends on a newly identified motif within the N-terminal helix of cyclin B1. However, when cyclin B1 is bound to Cdk1, it is somewhat protected from the first wave of degradation that depends on this new motif. The authors show that this newly identified degron-like motif in cyclin B1 is recognized by the APC/C leading to prometaphase degradation in mouse oocytes, and that the sequence is masked through binding of Cdk1. This would allow the cell to eliminate progressively excess cyclin B1 in prometaphase I in a SAC-independent manner, to allow the

rapid inactivation of cyclin B1-Cdk1 needed for the anaphase switch to occur on time [44]. At the same time, this would provide a stock of cyclin B1 available for association with Cdk1, to make sure that cyclin B1 does not become a limiting factor for Cdk1 activity in oocytes (Figure 2). Indeed, reducing the amount of free cyclin B1 in oocytes leads to defects in chromosome alignment at the metaphase I plate and hence, missegregation events [44]. The authors propose that excess cyclin B1 compensates for weak SAC control in oocytes to ensure correct chromosome segregation [44]. It is interesting to note in this context that endogenous cyclin A2 degradation occurs also in two waves in oocytes, with most of cyclin A2 being degraded in prometaphase I, and a small fraction of cyclin A2 localized to kinetochores escaping degradation until anaphase I onset [48]. Future studies will show whether a similar motif as the one identified in cyclin B1 can be identified in the N-terminal helix of cyclin A2, or other B-type cyclins.

Additionally, the location of cyclin B1 and binding to separase may also play a role in regulating its ubiquitination by the APC/C. *In vitro* cultured mitotic cells seem to harbour a pool of cyclin B1 that preferentially inhibits separase near chromosomes, compared to cytoplasmic cyclin B1. The degradation of this pool of cyclin B1 inhibiting separase is slower compared to the cytoplasmic pool [49], indicating that APC/C dependent degradation is determined by the partners cyclin B1 is

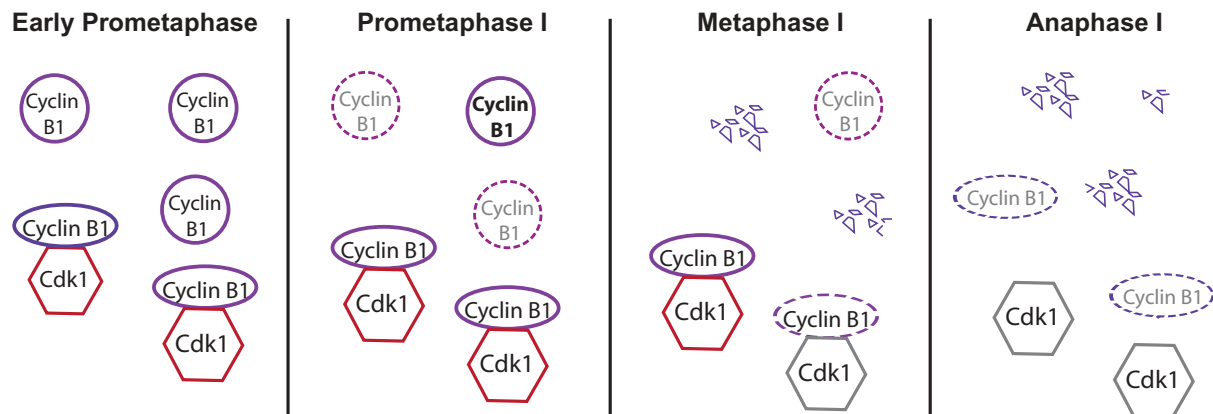


Figure 2. Regulation of cyclin B1 degradation during meiosis I. At early prometaphase, different pools of cyclin B1 are present in the oocyte: free cyclin B1 and cyclin B1 bound to Cdk1. During prometaphase, free cyclin B1 starts to be degraded while the SAC is on, whereas Cdk1-bound cyclin B1 is protected from APC/C-dependent ubiquitination and degradation through its binding. Most of the free cyclin B1 is degraded once oocytes progress into metaphase I, while bound cyclin B1 begins to be degraded only once the SAC is satisfied and Cdc20-APC/C under SAC control becomes active. Once the APC/C is fully active, cyclin B1 bound to Cdk1 is degraded leading to a sharp decrease of Cdk1 activity and anaphase I onset.

associated with. Whether location of cyclin B1 and binding to separase regulate cyclin B1 ubiquitination also in oocytes is currently unknown.

Altogether, these studies indicate that additional regulation of cyclin B1 in meiosis and mitosis is accomplished through differential APC/C affinity for the separate pools present in the cell in prometaphase. When the SAC is satisfied, APC/C with its co-activator Cdc20, targets cyclin B1 for degradation through ubiquitination. This brings about a sharp decrease of Cdk1 activity and exit from meiosis I. Once vertebrate oocytes exit meiosis I, they continue into meiosis II and arrest at metaphase II awaiting fertilization. In order for this arrest to be maintained, Cdk1 activity must be kept at high enough levels. Cyclin B1 is necessary for this very dynamic arrest, with an equilibrium of cyclin B1 degradation and synthesis to keep Cdk1 activity at an appropriate threshold [50,51]. In *Xenopus* oocytes, it was found that cyclin B1 and B2 synthesis continues during CSF arrest while Cdk1 activity level seems to be constant. It is thought that once a high amount of B-type cyclins is synthesized this leads to an increase in Cdk1 activity past a specific threshold. The APC/C with its co-activator Cdc20 becomes active inducing degradation of B-type cyclins. As a consequence, Cdk1 activity drops until another regulatory pathway, the Mos-MAPK pathway, counteracts APC/C activation allowing once again synthesis of cyclin B1 and B2. Therefore, CSF-arrest is dynamic, brought about by an equilibrium between cyclin B1 and B2 synthesis and APC/C activation [52]. Additionally, Cdc25 phosphatase is required during the arrest to remove inhibitory phosphorylation on Cdk1. Upon fertilization, the Mos-MAPK pathway is inhibited, allowing full APC/C activation and cyclin B1 and B2 degradation, while Cdk1 is additionally downregulated by the kinase Wee1B, which phosphorylates Cdk1 for further inhibition [53, 54, 55, 56]. Oocytes exit meiosis II and form a female pronucleus, which fuses with the male pronucleus provided by the sperm, to re-establish the diploid chromosome content in the zygote [57].

Cyclin B1 vs cyclin B2

Overall, cyclin B1 expression seemed to be necessary for development and fertility in mice while cyclin B2 is dispensable [20]. Cyclin B1 null mice are not viable

while cyclin B2 null mice are viable and fertile, although litters are smaller in size and numbers [20]. Lack of cyclin B1 in embryos induces an arrest after only two divisions at the 4-cell stage, underlining its significance in embryonic development [58]. Both cyclins are expressed during mitosis although they are localized differently, next to microtubules (cyclin B1) and the Golgi region (cyclin B2), respectively, and Cyclin B1 is translocated to the nucleus during prophase unlike cyclin B2 [59]. In mouse oocytes, the distribution of exogenous cyclin B1 evolves before GVBD with a cytoplasmic localization at first followed by its import into the nucleus [60]. Nuclear targeting of exogenous cyclin B1 significantly increased its capacity to induce entry into meiosis I [61], indicating that localization is an important factor regulating cyclin B1's capacity to induce GVBD. But as the importance of cyclin B1 and B2 subcellular localization for its role during meiotic maturation has not been further studied in mouse oocytes, it will not be subject of further discussion here.

Translational regulation is specific to each cyclin in mouse oocytes. The translation of cyclin B1 is tightly regulated in mouse oocytes since at different time points, cyclin B1 has to be rapidly synthesized. mRNA granule formation plays a role in inhibiting translation while their dispersion allows quick synthesis of cyclin B1 in mouse oocyte meiosis I [62]. Additionally, timing of cyclin B1 translation is regulated through polyadenylation mediated by specific lengths of the 3'UTR. Long and intermediate 3'UTR length of cyclin B1 mRNA leads to repression of translation in GV arrested oocytes, and to their translation after GVBD while cyclin B1 mRNA with short 3'UTR is constitutively translated already in GV oocytes [63]. Following GVBD, the synthesis and amount of cyclin B1 increases while cyclin B2 remains at stable levels (Figure 3). Unlike cyclin B2, cyclin B1 transcripts are regulated through ribosome loading, dependent on the RNA-binding protein CPEB1. This loading of ribosomes on cyclin B1 increases after GVBD while ribosome loading on cyclin B2 does not change after resumption of meiosis. Intriguingly, Cdk1-associated activity was found to play a role in the phosphorylation of CPEB1 and therefore, to regulate cyclin B1 translation [64]. It is attractive to

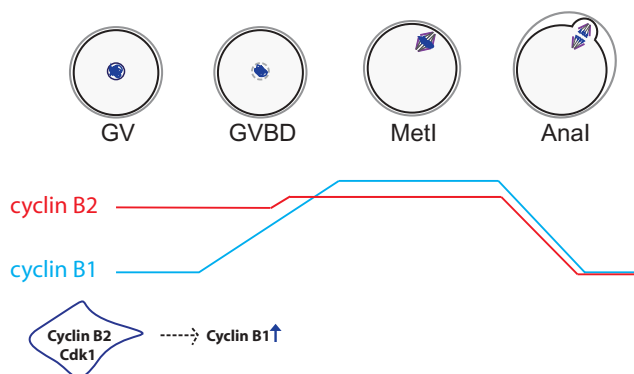


Figure 3. Protein levels of cyclin B1 vs. cyclin B2 in meiosis I. According to [64], preceding resumption of meiosis, cyclin B2 (in red) is translated at higher levels than cyclin B1 (in blue) while their degradation rates are similar. Therefore, at GV stage, cyclin B2 protein is more abundant than cyclin B1. After entry into meiosis I, protein levels of cyclin B2 remain similar with only a slight increase in translation. However, cyclin B1 is actively translated leading to an increase of protein levels following GVBD reaching its maximum at metaphase I. Cyclin B1 translation depends on cyclin B2 protein at entry into meiosis I. Hence, protein levels of both cyclins are regulated differentially and temporally in mouse oocytes.

speculate that cyclin A2, a strong inducer of GVBD when expressed exogenously [48], may act as a trigger for entry into meiosis I by mediating phosphorylation of CPEB1, but this has not been addressed yet.

Complete loss of either cyclin B1 or B2 in mouse oocytes

To elucidate the contributions of cyclin B1 and B2 for meiotic progression in oocytes, two recent studies examined meiotic maturation in mice harbouring oocytes devoid of either cyclin with knock-out approaches targeting the gene encoding cyclin B1 (*Ccnb1*) or cyclin B2 (*Ccnb2*). In mitotic tissues, only cyclin B1 seems to be essential, and a conditional knock-out approach using a Gdf9-dependent deletion strategy was necessary to determine its role in oocytes. Surprisingly though, oocytes without cyclin B1 are still able to enter meiosis I and extrude a polar body. No abnormalities as far as chromosome alignment and spindle formation is concerned, were observed in oocytes devoid of cyclin B1. However, once these oocytes exit meiosis I, they fail to enter meiosis II and to establish a CSF arrest. At entry into meiosis I, cyclin B2 is upregulated and promotes GVBD in

these *Ccnb1*^{-/-} oocytes, but after meiosis I, except when over-expressed, cyclin B2 cannot substitute for cyclin B1. Because oocytes fail to re-activate Cdk1 after meiosis I, *Ccnb1*^{-/-} oocytes decondense chromosomes and seem to enter an interphase-like state. Importantly, injection of mRNA coding for cyclin B2 can rescue meiosis II in *Ccnb1*^{-/-} oocytes, indicating that the failure of cyclin B1 oocytes to progress beyond meiosis I may be due to a failure to re-accumulate sufficient levels of cyclin B2 to compensate for loss of cyclin B1 such as in meiosis I, and not due to a specific role of cyclin B1 in meiosis II. Collectively these data indicate that cyclin B2 can substitute for cyclin B1 in oocyte meiosis [16,20].

Ccnb2 knock-out mice are viable, allowing the analysis of meiosis in oocytes devoid of cyclin B2. Without cyclin B2, entry into meiosis I is significantly delayed [16,21,65], because oocytes fail to activate Cdk1 efficiently for GVBD. Unlike cyclin B2 in *Ccnb1*^{-/-} oocytes, no upregulation of cyclin B1 to compensate for loss of cyclin B2 was observed. Quite the opposite, translation of mRNA coding for cyclin B1 was affected in the absence of cyclin B2 in a proportion of oocytes, indicating that this cyclin has a specific role in translational control of cyclin B1 and other, oocyte-specific mRNAs such as coding for *Mos* [21]. How cyclin B2 brings about translational control of meiotic transcripts is currently unknown, but may involve phosphorylation of CPEB1, which occurs in a Cdk1-dependent manner [64]. If cyclin B2 together with Cdk1 is able to phosphorylate CPEB1, it will be important to determine whether this is due to specific substrate specificity of cyclin B2, or due to the fact that cyclin B2 translation occurs independently of the CPEB1. Because of missing cyclin B2 protein in GV oocytes and failure to accumulate sufficiently high levels of cyclin B1, overall MPF activity is lower in *Ccnb2*^{-/-} oocytes, resulting in defects in spindle formation, delayed and inefficient APC/C activation, delay in anaphase I onset and reduced number of oocytes that succeed to extrude a polar body and exit meiosis I. These defects were rescued by overexpression of cyclin B1 [21]. The authors suggest that the metaphase I arrest observed in a significant proportion of *Ccnb2*^{-/-} oocytes is due to SAC activation because inhibition of the essential SAC kinase *Mps1* can rescue polar body extrusion. *Ccnb2*^{-/-} oocytes that fail to extrude a polar body

show kinetochore recruitment of the SAC component Mad2 indicating prolonged SAC activation, even though under normal conditions the SAC is activated only very transiently in meiosis I [21]. A small caveat of this assay is the fact that without a functional SAC, oocytes undergo metaphase-to-anaphase transition precociously and with much lower Cdk1 activity [66,67]. Therefore, oocytes devoid of cyclin B2 may harbour just enough cyclin B1-Cdk1 activity to undergo metaphase-to-anaphase transition in the absence of a functional SAC, explaining the rescue. Overall, the data obtained from complete loss of either cyclin indicates that cyclin B1 and B2 harbour specific roles during meiotic maturation mainly because of their expression profiles brought about in part by a specific role of cyclin B2 for translational control of cyclin B1. They can substitute for each other when expressed at the required times during meiotic maturation [16,21], indicating that they can phosphorylate the same substrates throughout meiosis I and II.

Cyclin B3

Evidence from the nucleotide sequence, cyclin box conservation, expression pattern and Cdk binding site pointed to cyclin B3 being a distinct and unique cyclin. Cyclin B3 was first described in chicken and found to exhibit 33% identity with cyclin B2 and 30% identity with A-type cyclins showing that it resembles both types of cyclins. Cyclin B3 was found to be located in the nucleus in interphase, similarly to A-type cyclins, while cyclin B1 is located in the cytoplasm [68, 69, 70]. In *C. elegans*, cyclin B3 was found to be close to chicken cyclin B3 with 50% identity in the cyclin box. Cyclin B3 is mainly expressed in maternal germ cells and conserved in other worms [71]. In humans, cyclin B3 mRNA was detected by RT-PCR in testis and 8 other tissues, but only in testis by northern blot analysis, suggesting low abundance in tissues other than germ cells. Protein levels and mRNA expression was found at highest levels in prepachytene spermatocytes and in oocytes during embryonic development [70]. This particular expression hinted at a role in early meiosis I (prophase) in both male and female meiosis.

Concerning Cyclin B3's sequence encoded by *Ccnb3* in mice, an important extension of exon 8 is observed in placental mammals and not in other species, suggesting that the resulting 3-fold increase in the size of the protein occurred during evolution of placental mammals. The extension and increase in size makes placental mammals' cyclin B3 of substantial size compared to other species; human cyclin B3 is 1395 aa long while chicken and *Xenopus* cyclin B3 are 403 aa and 416 aa long, respectively. This extended sequence could mediate new protein-protein interactions that may be important for its function specifically in these species [72]. Contrary to human and mouse cyclin B3, *Drosophila* and *C.elegans* cyclin B3 is expressed in both meiotic and mitotic cells during embryogenesis [71,73–77]. Although it is expressed in mitotic *Drosophila* cells, cyclin B3 was found to be dispensable for mitosis but essential for female meiosis [74]. This differs from *C. elegans* cyclin B3 which is essential for embryonic development. In humans, aberrant expression of cyclin B3 during mitosis has been observed in a specific type of cancer, Ewing-like sarcoma, where a fusion of cyclin B3 and a ubiquitously expressed protein has been found [78–80], indicating that untimely expression of this cyclin can have serious consequences on cell fate.

The importance and role of cyclin B3 has been established in several species although the molecular mechanism is not fully understood. In *Drosophila* and *C. elegans* oocytes and embryos, cyclin B3 was found to be a partner of Cdk1 although a possible interaction with Cdk2 was not examined [74, 81]. In chicken mitotic cells, cyclin B3 was able to bind to both Cdk1 and Cdk2 [68]. Surprisingly, human cyclin B3 did not co-immunoprecipitate with Cdk1 but was found to interact with Cdk2 resulting in very low *in vitro* kinase activity [82]. Mouse cyclin B3 purified from insect cells is able to be partnered up with Cdk1 and associated *in vitro* kinase activity was detected [18].

In *C. elegans*, cyclin B3 knockdown is lethal and detrimental to development. Cyclin B3 deficient embryos show several phenotypic defects: in meiosis, cyclin B3 depleted oocytes are not able to segregate sister chromatids during anaphase II although other cell cycle steps occur. Once both male and female

pronuclei are formed, their migration is slower in mutant embryos. During the first mitosis, metaphase was found to be delayed and chromosomes do not segregate. This phenotype is partially rescued by simultaneously knocking down SAC components indicating that cyclin B3's role in *C. elegans* is SAC dependent and essential for embryonic development [77]. As previously discussed, cyclin B3 in *Drosophila* is dispensable for viability and male meiosis but essential for female meiosis. Cyclin B3 deficient *Drosophila* females are sterile [74]. In *Drosophila* oocytes, cyclin B3 shares some functions with other cyclins, cyclin A and cyclin B, but also harbours a unique role in promoting anaphase onset in both oocytes and embryos, contrary to cyclin B1 that inhibits anaphase onset [69,73–75]. Furthermore, together with cyclin A, cyclin B3 inhibits entry into S phase between meiosis I and II [75]. Although cells progress slower through the cell cycle, cyclin B1 is dispensable for mitosis in *Drosophila*. Only double knockdown of cyclin B3 and B1 leads to embryonic lethality once maternal reserves are depleted [74,75]. Surprisingly, a different role of cyclin B3 was uncovered in the ascidian *Ciona intestinalis*. *Ciona intestinalis* cyclin B3 was found to be essential for maternal to zygotic transition, where zygotic genome activation (ZGA) occurs. The authors described a correlation between the decrease of cyclin B3 mRNA and start of ZGA. Knockdown of cyclin B3 led to advanced initiation of ZGA [83] demonstrating yet another role for this cyclin.

Through the analysis of *Ccnb3* knock-out male mice it was found that although expressed in testis in mice, its role is dispensable during spermatogenesis [17,18]. Furthermore, its protein and mRNA levels are low in adult ovaries; nevertheless, its role is essential for correct meiotic maturation, as oocytes cannot progress beyond metaphase I without cyclin B3 [18,19,84]. Complete loss of cyclin B3 leads to a SAC-independent metaphase I arrest, with high Cdk1 activity and a failure to correctly degrade endogenous APC/C substrates [18,19], even though exogenously expressed substrates are degraded [18]. This may indicate that cyclin B3 is required for targeting specific pools of cyclin B1 and securin for APC/C dependent ubiquitination, such as cyclin B1 associated with Cdk1, and securin in association with separase [44,46,49]. Alternatively, cyclin B3 may be

involved in regulating substrate specificity of the APC/C by phosphorylating one of its subunits, or by activating phosphatases that determine substrate specificity through some yet unknown mechanism (Figure 4). Lastly, cyclin B3 may be required for translational regulation of cyclin B1 and securin mRNAs, and in its absence, translation is upregulated leading to the observed failure to degrade endogenous substrates and metaphase I arrest. A cyclin B3 mutant that does not yield *in vitro* kinase activity with Cdk1 was also not able to rescue oocytes devoid of cyclin B3 [18], strongly suggesting that Cdk1 activity associated with cyclin B3 is essential for its role in female meiosis. Most importantly, cyclin B1 cannot substitute for cyclin B3, showing that cyclin B3 has a non-redundant and specific role in oocytes, indicating that it confers distinct substrate specificity to Cdk1 than cyclin A2, B1, or B2 [18]. As mentioned above, cyclin B3 in placental mammals is three times bigger than from other vertebrates [72]. It came therefore as a surprise that the function of cyclin B3 seems to be conserved between species, because expression of cyclin B3 derived from *Drosophila*, zebrafish and *Xenopus* was able to rescue the meiotic defects of *Ccnb3* knock-out mouse oocytes [18]. The extended exon 8 in placental mammals seems therefore not absolutely required for cyclin B3 function in meiosis.

Conclusion

Recent studies using knock-out mouse models and sophisticated imaging approaches have provided important insights on the roles of B-type cyclins during female mammalian meiosis. Cyclin B2 occupies a specific role in oocytes, regulating translation of meiotic mRNAs, and this role of cyclin B2 is essential for accumulating sufficient levels of MPF activity for meiotic cell cycle progression. Cyclin B1 and B2 seem to be largely redundant as far as MPF substrate phosphorylation for progression through meiosis I and meiosis II are concerned, and their individual roles in oocyte meiosis are most likely due to differences in expression and localization. Cyclin B3 clearly occupies a distinct role in meiotic cell cycle progression, specific to oocytes. Cyclin B3 is required for metaphase-to-anaphase transition in meiosis I, whereas cyclin B1 and B2 inhibit anaphase I onset. Recent data indicate

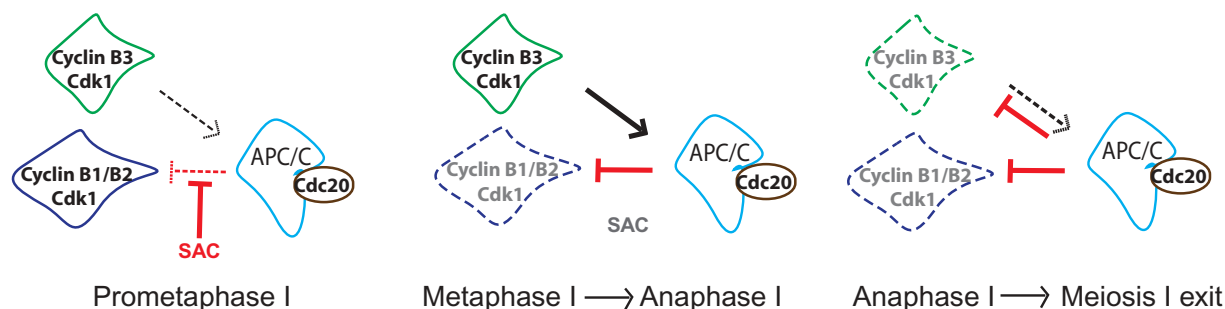


Figure 4. Model of how cyclin B3 may promote anaphase I onset in mouse oocytes. Cyclin B1/B2-Cdk1 (in blue) activity increases as oocytes progress through prometaphase into metaphase, because the APC/C (light blue) in association with its activator Cdc20 (brown) is kept in check by the SAC. At the metaphase-to-anaphase transition, the SAC is satisfied and inactivated, allowing full APC/C activity, ubiquitination and hence degradation of cyclin B1 and B2 in association with Cdk1. In oocytes, full APC/C activity requires the function of cyclin B3-Cdk1. Cyclin B3-Cdk1 (in green) promotes APC/C activity leading to chromosome segregation and exit from meiosis I. Therefore cyclin B3, a late substrate of the APC/C, shows an opposing role to cyclin B1 and B2 during mouse oocyte meiosis I.

that cyclin B3-Cdk1 substrates are not shared with other A- and B-type cyclins, because none of them can substitute for cyclin B3, putting this cyclin apart. Future work will aim at identifying the targets of cyclin B3 to elucidate why this cyclin is required specifically in oocytes. It is attractive to speculate that the size of the oocyte requires the additional contribution of cyclin B3 for ensuring switch-like transitions in meiosis.

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Detection of Separase Activity Using a Cleavage Sensor in Live Mouse Oocytes

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Abstract

Separase proteolytically removes cohesin complexes from sister chromatid arms in meiosis I, which is essential for chromosome segregation. Regulation of separase activity is essential for proper cell cycle progression and correct chromosome segregation. Onset of endogenous separase activity has not yet been observed in live oocytes.

We describe here a method for detecting separase activity in mouse oocytes *in vivo*. This method utilizes a previously described cleavage sensor made up of H2B-mCherry fused with Scc1(107–268 aa)-YFP. The cleavage sensor is loaded on the chromosomes through its H2B-tag, and the signal from both mCherry and YFP is visible. Upon separase activation the Scc1 fragment is cleaved and YFP dissociates from the chromosomes. The change in the ratio between mCherry and YFP fluorescence intensity is a readout of separase activity.

Key words Separase, Cohesin, Cleavage sensor, Oocyte, Chromosome segregation, Anaphase, Female meiosis, Scc1, Live imaging, Time lapse

1 Introduction

During meiosis I, chromosomes are organised as bivalents, where homologous chromosomes are held together via chiasmata and sister chromatids are locked together by a ring complex named cohesin [1, 2]. At the metaphase-to-anaphase I transition, separase proteolytically cleaves cohesin at the chromosome arms allowing chiasmata resolution and chromosome segregation [3, 4]. In meiosis II, sister chromatids are held together by the remaining cohesin localized at the centromeres [3]. During the second meiotic division, separase cleaves centromeric cohesin leading to sister chromatid segregation.

Regulation of separase activity is essential for proper mitotic and meiotic chromosome segregation [3, 4]. Upon translation, separase is folded with the help of its chaperon and inhibitor securin [5]. Separase remains in a stable complex with securin, until securin is

targeted for degradation through APC (Anaphase Promoting Complex)-dependent ubiquitination [6–8]. Separase is additionally kept inactive by inhibitory phosphorylation mediated by Cyclin B1-Cdk1 kinase [9, 10]. In oocytes, Cyclin B1 is ubiquitinated in an APC-dependent manner at the same time as securin [11]. Dephosphorylation of separase and degradation of securin result in activation of separase [10]. Separase then cleaves its substrates, the kleisin subunits of cohesin (Scc1 in mitosis or Rec8 in meiosis) [12].

Observing and measuring separase activity during meiosis in mouse oocytes presents a challenge. Since cohesin complexes containing Rec8 are loaded during embryonic development in low quantities with no turnover [13, 14], it is impossible to introduce exogenously expressed fluorescently tagged cohesin subunits to follow their cleavage without creating a transgenic mouse. In addition, introducing fluorescent tags may inhibit the subunit's ability to integrate into a complex. A different approach for the detection of separase activity *in vivo* has been developed in mitotic cells [15], which we have adapted in oocyte meiosis.

We describe a method of detecting separase activity in mouse oocytes *in vivo*. This method utilizes a cleavage sensor, which is a protein construct made up of H2B-mCherry fused with Scc1(107–268 aa)-YFP. The H2B-tag serves to bind the sensor to the chromosomes, the Scc1 fragment is a very efficient separase substrate, and the ratio of YFP and mCherry fluorescence levels on the chromosomes allows the detection of the cleavage event.

mRNA encoding the cleavage sensor is injected in competent prophase arrested oocytes and cleavage sensor protein expression begins. After allowing sufficient time for expression, the prophase arrest is lifted, chromosomes condense and the cleavage sensor binds to the chromosomes. Its fluorescence can be followed through live imaging.

When separase is activated at the metaphase-to-anaphase transition of meiosis I, it cleaves Scc1-YFP with high efficiency leading to YFP dissociation from the chromosomes, visible as a sharp decrease of YFP compared to mCherry fluorescence. This can be clearly observed by live imaging of oocytes (Fig. 1).

The cleavage sensor is not included into the endogenous cohesin complexes and its expression does not impede cleavage of the endogenous Rec8 and chromosome separation. This sensor may be used in different genetic backgrounds. Moreover, variants of this sensor can be created depending on the aim of the experiments. It allows the observation of separase activity through time lapse imaging, detection of activation and inactivation of separase activity as well as its efficiency under different conditions. The cleavage sensor is a powerful and elegant tool useful for observation and measuring separase activity in mouse oocytes *in vivo*.

In this chapter we describe collection of competent prophase arrested mouse oocytes, microinjection of the cleavage sensor mRNA, time lapse imaging of oocytes during metaphase-to-anaphase I transition and observation and measurement of separase activity.

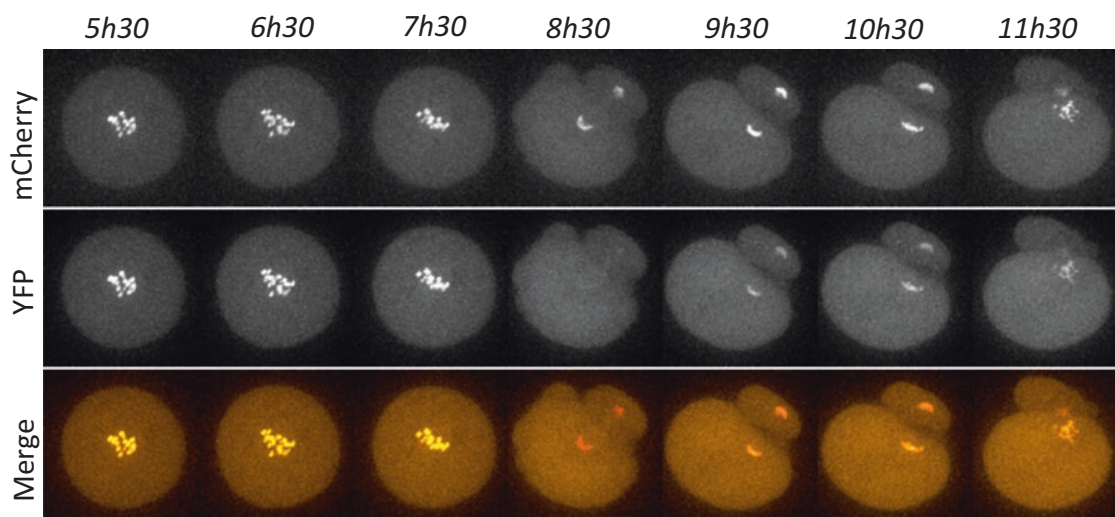


Fig. 1 Live imaging of separase activity in mouse oocyte. At 5 h30 mark (metaphase I), the intact cleavage sensor (H2B-mCherry-Scc1-YFP) is observed at the chromosomes. Once separase is activated at anaphase I onset (8 h30 mark), it cleaves Scc1. This leads to a sharp decrease of YFP, leaving only the signal from mCherry on the chromosomes

2 Materials

2.1 Collection of Oocytes

1. 9- to 14-week-old mice, strain CD1 Swiss (Janvier labs, France).
2. Sterile-filtered, suitable for mouse embryo culture M2 medium (Sigma) supplemented with penicillin (final concentration 1.678 mM) and streptomycin (final concentration 0.689 mM), and where indicated supplemented with 100 mg/mL dbcAMP (dibutyryl cyclic AMP).
3. Mineral oil suitable for mouse embryo cell culture (Sigma).
4. 60 mm easy grip polystyrene tissue culture dishes (Falcon).
5. Mouth pipette: plastic tubing connected to a mouthpiece. Attach the drawn out glass Pasteur pipette to the tubing and use it for collection and cleaning of oocytes.
6. 25 G 16 mm BD Microlance hypodermic needle mounted on a 1 mL syringe.
7. Dissection scissors and forceps.
8. Incubator set to 38 °C.
9. Binocular dissection microscope combined with a heating plate.

2.2 Microinjection of Oocytes

1. Glass capillaries: outer diameter 1 mm, inner diameter 0.75 mm, length 100 mm (Harvard apparatus).
2. Magnetic puller, for example Narishige PN-30.

3. Microinjection microscope, we use a Nikon Eclipse Ti with Eppendorf TransferMan NK2 micromanipulators and TOKAI HIT Thermoplate.
4. Eppendorf FemtoJet Microinjector connected to the microinjection needle holder.
5. Eppendorf CellTram Oil (or similar) manual microinjector connected to the holding pipette holder.
6. Depression glass slide.
7. Holding pipette: VacuTip microcapillaries, inner diameter 15 μm , outer diameter 100 μm , angle 35°.
8. Microloader tips 20 μL (Eppendorf).
9. Linearized and purified plasmid encoding for the cleavage sensor under T7, T3, or SP6 promoter and allowing synthesis of a poly(A)-tail (*see Note 1*).
10. T7/T3/SP6 mMessage mMachine transcription kit (Invitrogen).
11. Microcentrifuge.
12. RNase-free water.

2.3 Set-Up for Imaging

1. We use an inverted microscope Zeiss Axiovert 200M, combined with an MS-2000 automated stage (Applied Scientific Instrumentation), a Yokogawa CSU-X1 spinning disc and a nanopositioner MCL Nano-Drive. The spinning disc unit is supplemented with a Quad-Band Dichroic filter 13 \times 15 \times 0.5 DI-T405/488/568/647 for CSU X1. The light sources are 50 mW 488 nm and 561 nm lasers (Roper Scientific). The images are taken with an EMCCD camera, pixel size 16 \times 16 μm (Photometrics).
2. Objective: Plan-APOCHROMAT 40 \times /1.4 Oil DIC (UV) VIS-IR (Zeiss).
3. The microscope is operated with MetaMorph software.
4. The body of the microscope is encased in an incubation chamber heated by a heating unit (PeCon).
5. Heating insert: incubator cover, incubator main body, lens warmer, operated by a touch screen three-channel temperature controller (Chamlide).
6. Imaging chamber: Chamlide CMB 35 mm 1-well magnetic chamber for round coverslips.
7. Round cover glasses, diameter 25 mm.
8. Immersion oil with refractive index of 1.518 (e.g., Cargille Type 37 immersion oil).

3 Methods

3.1 Collection of Oocytes

1. Prepare a 60 mm polystyrene dish with 6–7 evenly spaced drops of around 50 μL prewarmed M2 medium supplemented with 100 mg/mL dbcAMP. Cover the drops entirely with mineral oil. Store this dish in a 38 °C heated environment (an incubator or a heated surface).
2. Place another 60 mm polystyrene dish on a 38 °C heated stage of a binocular microscope and put a 500 μL drop of prewarmed 38 °C M2 medium supplemented with dbcAMP in the center. Do not cover it with mineral oil.
3. Sacrifice a nonprimed 8- to 12-week-old OF1 mouse (*see Note 2*) by cervical dislocation. Open the peritoneal cavity with a pair of scissors and remove both ovaries.
4. Transfer the ovaries into the prepared 500 μL drop of M2 medium supplemented with dbcAMP. From this step on, all procedures should take place on a 38 °C heated surface.
5. Release the mature GV oocytes from the ovaries by piercing the follicles with a syringe-mounted needle. During this procedure, the ovaries can be held down with another needle, or a pair of forceps.
6. Carefully collect the biggest (mature/competent) oocytes. Transfer of the oocytes is done by mouth-assisted aspiration with a glass pipette (*see Note 3*).
7. Transfer all collected oocytes into a drop in the prepared multidrop dish. Some oocytes have clusters of small follicle cells stuck to them. Separate the oocytes from the follicle cells by a series of aspirations into a glass pipette with a diameter of the opening similar in size to the diameter of the oocyte. Cleaning the oocytes can be performed in other drops of M2 medium in the same dish.
8. Take only the mature GV oocytes and transfer them into an unused drop of M2 medium. Mature GV oocytes are the largest, with a smooth thick zona pellucida and with a germinal vesicle in the center of the oocyte.

3.2 Microinjection of Oocytes

1. Prepare mRNA coding for the cleavage sensor for microinjection using a mMessage mMachine transcription kit well ahead and freeze down small aliquots for single use. Spin the mRNA solution for at least 30 min at 13,000 rpm ($15,682 \times g$) at 4 °C prior to microinjection. Keep the mRNA solution on ice at all times.
2. Prepare an injection needle from a glass capillary using a magnetic puller (*see Note 4*). Avoid touching both ends of the capillary or the resulting needle.

3. Set up a microinjection microscope: turn on the microinjector, the thermoplate and the light source. Set up the microinjector to Function 0 (Change capillary).
4. Fill the microinjection needle with 1–1.6 μL of mRNA solution (*see Note 5*). Attach the microinjection needle to the needle holder. Attach the holding pipette to the holding pipette holder.
5. Form a drop of 3–5 μL of prewarmed M2 medium supplemented with dbcAMP on a depression glass slide and cover it with a small amount of mineral oil (about 200 μL).
6. Transfer the oocytes into the drop on the prepared depression glass slide. Keep the oocytes grouped together.
7. Put the glass slide with the oocytes on the thermoplate of the microinjection microscope, lower the holding pipette and the microinjection needle into the drop so that their tips appear at the same level as the oocytes (*see Note 6*). Change the microinjection pump setting to Function 4 (Continuous flow) and press “Inject” (*see Note 7*).
8. Bring the tip of the holding pipette close to an un-injected oocyte and apply light suction with the holding pipette pump. The suction force should be strong enough to attach the oocyte to the holding pipette, but light enough so that the oocyte does not change shape or enter into the holding pipette.
9. Bring the tip of the microinjection needle close to the oocyte and adjust the positions of the holding pipette and the microinjection needle (*see Note 8*).
10. Keeping the holding pipette steady bring the tip of the microinjection needle into the oocyte until you see a burst-like change in the oocyte cytoplasm (*see Note 9*). Avoid piercing the nucleus.
11. Quickly withdraw the needle at the same angle (*see Note 10*).
12. Move the holding pipette with the injected oocyte aside and release the suction force (apply negative force) in the holding pipette pump. Keep injected oocytes in a separate group from uninjected oocytes.
13. Repeat **steps 8–12** until all oocytes have been injected.
14. Set the microinjector back to function 0. Lift the microinjection needle and the holding pipette from the drop. Transfer the glass slide with the injected oocytes on a heated stage of a binocular microscope. Transfer the oocytes to a clean drop of M2 medium supplemented with dbcAMP. Separate and remove dead oocytes (*see Note 11*).
15. Incubate the injected oocytes at 38 °C from 20 min to 4 h (*see Note 12*).

16. To release oocytes from the GV block, prepare a 60 mm polystyrene dish containing 6–7 evenly spaced drops of M2 medium (without dbcAMP). Cover the drops with mineral oil. Prepare another 60 mm polystyrene dish containing 4–5 drops of M2 medium (without dbcAMP) and place on the heated stage of a binocular microscope. Do not cover with mineral oil.
17. Transfer oocytes consecutively through four or five drops (on the dish without the oil), each time mixing the oocytes in the medium and then allowing them to settle. Change the medium in the pipette completely each time. Transfer all the oocytes into a clean drop of M2 medium in the prepared dish, covered with mineral oil. Incubate from 45 min to an hour at 38 °C.
18. Check for the germinal vesicle breakdown (GVBD) event—a sign of meiotic resumption and entry into prometaphase I. When this happens, the germinal vesicle in the oocyte disappears. Collect these oocytes and transfer them to an unused drop of M2 medium in the same dish. If not all oocytes had gone through GVBD, check again in 10–15 min (*see Note 13*).
19. Incubate for 4–6 h at 38 °C (*see Note 14*). GVBD event serves as a reference point to all subsequent events in the first meiotic division. Anaphase I usually takes place between 7 and 9 h after GVBD, depending on the mouse strain and culture conditions. OF1 mouse oocytes undergo metaphase–anaphase I transition at GVBD+7–8 h.

3.3 Setting Up for Imaging

1. At least 1 h before starting the imaging, prewarm the imaging platform. This includes switching on a heating chamber or a heating plate and an objective warmer in case of an immersion objective (*see Note 15*). Right before the imaging turn on the whole microscope system.
2. Assemble the imaging chamber with a clean cover glass. Place on a 38 °C heated surface or incubate in the 38 °C incubator until warm.
3. Form small 2 μ L M2 medium (without dbcAMP) drops in the center of the cover glass in the imaging chamber and cover with mineral oil. The number of drops depends on the number of the oocytes and the number of experimental conditions (*see Note 16*).
4. Transfer the injected oocytes into the drops in the imaging chamber, up to 15 per drop, evenly spaced.
5. Apply preheated immersion oil without air bubbles on the objective, place the imaging chamber with the oocytes on the heating plate above the objective. Adjust the position of the objective so that the immersion oil is in contact with the bottom of the imaging chamber.

3.4 Time-Lapse Imaging

1. Using MetaMorph interface, set up the acquisitions as follows.

2. Create a folder where the files from the specific time lapse experiment will be stored.
3. Find and save the positions of all the oocytes in the imaging chamber.
4. Use these channels: 491 nm for YFP and 561 nm for RFP fluorescence. Adjust the laser power and the exposure time of 491 nm and 561 nm channels to as low as possible (*see Note 17*).
5. Set time lapse: acquisitions 10–20 min for 8–10 h depending on when the first metaphase–anaphase transition is expected.
6. Z stacks: 11 slices with a 3 μm interval. Adjust the position of the saved point so that Z stacks would cover the whole volume of the chromosome mass.
7. Start the acquisition (*see Note 18*).

3.5 Quantification and Analysis with ImageJ

1. Transfer the files to the analysis station. Using ImageJ assemble raw images into Z-projection stacks by individual oocyte and wavelength. You can do this manually or by using a macro MetaMorph Raw into Stacks (available upon request).
2. Open the stacks for 491 and 561 nm channels for the first oocyte.
3. Select a square region of interest around the chromosome mass in one of the images at the first time point and measure the fluorescence intensity within this region with the ImageJ function.
4. Select the window with the image of the same oocyte taken on another channel and restore the selected region of interest through “Image – Selection – Restore Selection” function of ImageJ. Measure the fluorescence intensity of this region, too (*see Note 19*).
5. Return to the image taken with the first channel, switch to the next time point, adjust the region of interest so that it covers the moved chromosome mass and repeat the measurement on both channels.
6. Repeat the measurement steps until the chromosomes segregate, then measure only the chromosome mass that stays in the oocyte until the end of the time lapse.
7. Measure the intensities of oocyte cytoplasm fluorescence in both channels (background fluorescence) in a similar manner.
8. Subtract background fluorescence from the chromosome fluorescence in both channels respectively for each time point.
9. Normalize the fluorescence intensities to the first time point for both channels individually. On a line chart the fluorescence value will drop in both channels upon chromosome segregation (Fig. 2a, b left panels), indicative of chromosome segregation.

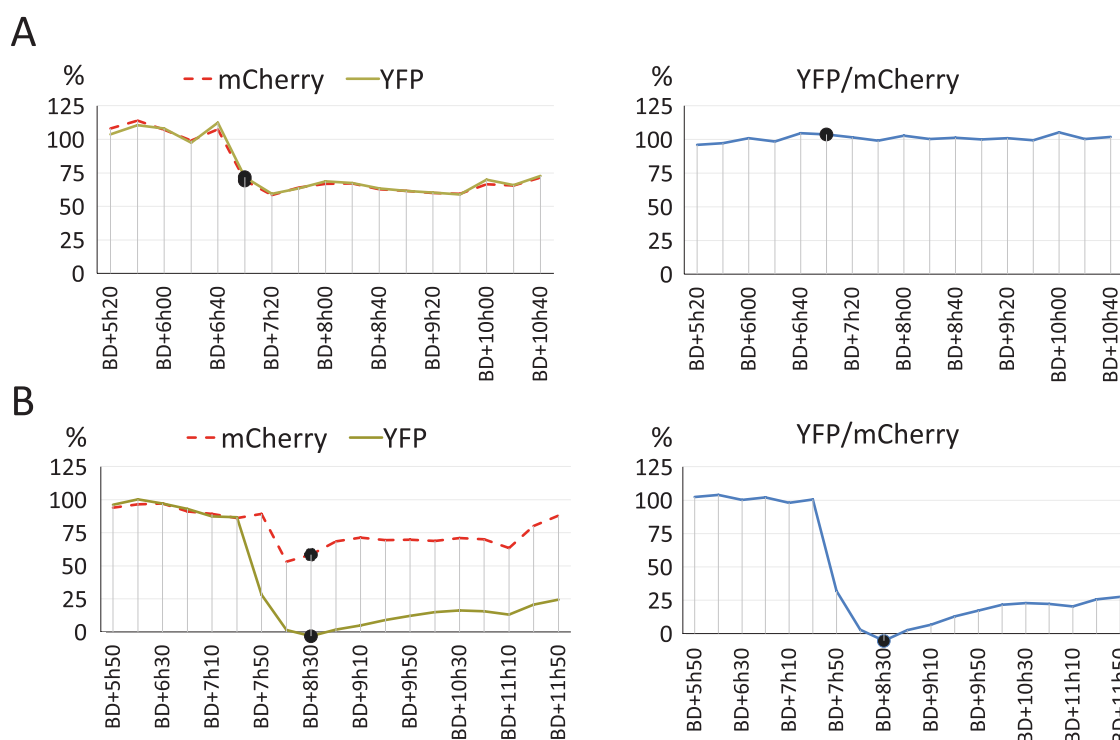


Fig. 2 Quantification of YFP and mCherry fluorescence. **(a)** Quantification of YFP and mCherry fluorescence intensity of the negative control (H2B-mCherry-YFP) measured on the chromosomes during live imaging of a mouse oocyte at metaphase-to-anaphase I transition (left) and the ratio of normalized YFP–mCherry fluorescence intensity (right). Black circle: chromosome segregation time point. **(b)** Quantification of YFP and mCherry fluorescence intensity of the cleavage sensor (H2B-mCherry-Scc1(107–268 aa)-YFP), measured on the chromosomes during live imaging of a mouse oocyte at metaphase-to-anaphase I transition (left) and the ratio of normalized YFP–mCherry fluorescence intensity (right). Black circle: chromosome segregation time point

10. Calculate the ratio between YFP and RFP fluorescence intensities for each time point.
11. Build a line chart of the ratio changes over time. Mark the time point when the chromosomes segregate, i.e., when they are first seen as two masses. The dip in the YFP–mCherry fluorescence ratio represents the time of separase activation (Fig. 2b, right panel) (*see Note 20*).
12. Repeat **steps 2–11** for all oocytes.

4 Notes

1. Any plasmid suitable for in vitro transcription would serve. It has to contain a transcription start (usually T7, T3, or SP6 promoter), a Kozak sequence, the sensor cDNA (H2B, mCherry, Scc1 107–268 aa, YFP as a single reading frame) and a polyadenylation signal sequence (can be replaced by a string of around 30 adenosines in the coding strand). Include a unique restriction enzyme cut site after the poly(A) signal for plasmid linearization.

2. One 8- to 10-week-old mouse of this strain usually harbors around 50 competent GV oocytes.
3. The collection pipette can be produced from a glass Pasteur pipette. Heat up a thin section of the pipette over a flame until the glass becomes malleable and rapidly pull on both ends of the pipette. Break off the extended thin portion at 5–7 cm and check the diameter of the opening. The diameter of the pipette opening should not be smaller than the diameter of the oocyte. It is better to prepare pipettes of two sizes, one around 2–3 diameters of the oocyte for oocyte collection, and another with the diameter very close to the diameter of the oocyte for cleaning.
4. To determine the puller settings, follow the manufacturer's instructions. The settings and the resulting needle will have to be adjusted for individual conditions in the lab.
5. Use a P-10 automatic pipette with Eppendorf Microloader tips to reach inside the microinjection needle from the wide end. In case air bubbles appear after loading the needle with mRNA solution, tap lightly on the side of the needle. mRNA can also be loaded with a regular 10 μ L tip by placing the drop of the mRNA solution on top of the wider end of the microinjection needle. The solution will travel to the working end of the needle by capillary forces within 5–10 min.
6. After the tip of the holding pipette is lowered into the medium, use the holding pipette pump to take in some medium in the holding pipette and equilibrate the suction so that it does not take any medium in or out. Also, sometimes the tip of the microinjection needle can be sealed during pulling. Test this by pressing "Clean" button on the microinjector for a second. If the tip of the needle is not sealed, an air bubble should exit. If the tip is sealed, it can be broken off by tapping the tip of the needle against the holding pipette.
7. The force of the flow required for a successful injection depends on the diameter of the needle. At the beginning of the injections set the force to 150 hPa and adjust accordingly to the injection outcome.
8. In absence of an antivibration table, if the holding pipette tremors slightly adjust it so that it touches lightly the glass slide.

The microscope should be focused on the middle of the opening of the holding pipette, which is also the centre of the oocyte attached to the pipette. The microinjection needle should be adjusted so that only the very end of the needle is in focus.
9. Sometimes the tip of the needle is not well positioned against the centre of the oocyte and upon injection, it can slide between the zona pellucida and the oocyte membrane. If this happens, a bubble appears, created by the constant flow of mRNA from

the needle. Withdraw the needle, readjust its position and try again.

10. It is very easy to rupture the membrane of the oocyte. Always withdraw the needle at the same angle as the injection path. Do not inject too much: the area of the cytoplasmic burst upon injection should not exceed the diameter of the nucleus. Make sure that the tip of the injection needle is thin enough and is clean. Sometimes the material from the cytoplasm can stick to the tip of the needle. This greatly increases the probability of the membrane rupture of the oocytes injected after that. Clean the needle by rubbing its sides against the sides of the holding pipette. Exchange the needle if needed.

Sometimes mRNA solution can contain a small amount of precipitates, which can clog the injection needle. It is visible in the injection microscope as a mass inside the needle. Sometimes it can be cleared by applying extra pressure from the microinjector (press “Clean” several times). If possible (depending on the length of the injection needle and its diameter), try to break off the end of the injection pipette by bumping it softly onto the holding pipette. If the clog does not exit the needle, exchange the needle.

The concentration of the mRNA solution can also influence the clogging. If the injection is very difficult, try diluting the mRNA solution with RNase-free water and repeat the microcentrifugation step.

11. Dead oocytes have a more granulated cytoplasm and are slightly less transparent than intact oocytes. If the membrane is ruptured, a slow movement of cytoplasm can be visible. Sometimes the membrane remains intact but the oocytes die anyway. Do not be disappointed if most oocytes die in the beginning!
12. The incubation time depends on the expression of the target protein. Longer incubation tends to increase the expression levels.
13. The time of meiosis resumption after release from dbcAMP depends on the mouse strain, source of the medium, and the time of incubation in the arrested state (in dbcAMP-supplemented M2). Variations between 45 and 80 min are normal. To keep the oocyte culture synchronised do not collect any oocytes that went through GVBD 15–20 min after the first batch.
14. Due to oocyte sensitivity to phototoxicity, it is best not to expose oocytes to the laser light before GVBD+4 h in this assay.
15. Setting up the right temperature conditions is the key to successful imaging. Oocyte culture is very sensitive to temperature changes and even 1 °C variation interferes with meiotic

progression. The best way to adjust the temperature is by experimental testing. The temperature inside of the drop in the imaging chamber should be around 38 °C. This can be measured initially using a thermometer with a wire temperature probe. Then try incubating the oocytes overnight in the heated microscope in the imaging chamber. If the conditions are optimal, the oocytes should be alive and have extruded polar bodies (PB) at GVBD+7 h. If the oocytes are still in metaphase I, but visually unaffected, the temperature is probably too low. If the oocytes are in metaphase I, but look unhealthy, i.e., with very granulated cytoplasm, uneven surface, full of bubbles, or even dead, the temperature may be too high. Another reason for failures in meiotic progression can be the composition of the imaging chamber (certain materials are toxic for the oocytes) or the source of the medium and mineral oil.

To keep the microscope temperature stable, we keep the heating chamber around the microscope at a constant temperature at all times.

16. We place 15 oocytes per drop, evenly spaced. Time-lapse imaging with the current set up (described later) is optimal for up to 30 oocytes. If there is no difference in oocyte treatment conditions, two drops of medium is enough. If the conditions are different (i.e., drug treatments, injection of different constructs), use separate drops of medium to place oocytes from different conditions. In case of drug treatments, it is essential to make sure that the drug is not lipophilic as it may affect the untreated oocytes through diffusion in the mineral oil. If the drug is lipophilic, also add it to the oil in the matching final concentration so there is an equilibrium maintaining the correct concentration in the medium.
17. Oocytes are very sensitive to phototoxicity. Try using a very low laser power and exposure times, where chromosomes are still visible. If there are still effects of phototoxicity (chromosome missegregations, lagging chromosomes), reduce the number of the oocytes per time-lapse experiment.
18. As acquisitions take place for 8–10 h, it is advisable to schedule the time lapse imaging overnight.
19. It is important that the region of interest has the exact same location in images from both channels at the same time point. To simplify the measuring routine you can use the Sensor Measurement Plugin (available upon request).
20. The rapid decrease of the YFP–mCherry ratio upon chromosome segregation represents the cleavage of the sensor by separase (Fig. 2b). Note that despite the drop in both mCherry and YFP fluorescence intensity in the negative control H2B–mCherry–YFP (Fig. 2a, left panel), the YFP–mCherry ratio

remains unchanged during the chromosome segregation event (Fig. 2a, right panel). As *Scc1* is a very good separase substrate, it is cleaved almost completely (Fig. 2b). Often, the area of the chromosomes after sensor cleavage has lower fluorescence intensity than the rest of the cytoplasm; this is why the curve dips below zero. After the cleavage and a rapid decrease in YFP–mCherry ratio, it slowly grows back up. We believe this behavior represents replacement of the cleaved sensor molecules with the intact ones still found in the cytoplasm (or newly synthesized molecules of the sensor). We believe that the time between the start of sensor cleavage (the dip of the ratio curve) and the reaccumulation of uncleaved sensor (end of the dip) represents the time window of separase activity. The efficiency of the cleavage can be estimated by the speed of the cleavage and whether or not most of the sensor molecules were cleaved during the time where separase was active.

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