Control of the cell cycle during meiotic maturation of mouse oocytes

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Declaration

The work presented in this thesis was conducted under the supervision of Professor John Carroll in the department of cell and developmental biology, University College London, and Professor Keith Jones in the department of reproductive physiology, University of Newcastle upon Tyne. I, Ibtissem Nabti confirm that the data presented in this thesis is my own and from original studies. Also, this dissertation has not previously been submitted, wholly or in part, to any other university for any degree or diploma.

Ibtissem Nabti

Abstract

The overall aim of the work presented in this thesis is to better understand the molecular mechanisms underlying the progression of the meiotic division in mammalian oocytes.

The first goal of this project was to investigate the relative contribution of securin and cyclin B1 to the control of the protease separase during the indeterminate period of metaphase II arrest. I found here that although there are conditions in which either securin or MPF can prevent chromosome disjunction, such as during meiosis I, securin is the predominant inhibitor of separase during metaphase II arrest. Thus, CDK1 pharmacological inhibition as well as antibody inhibition of CDK1/cyclin B1 binding to separase, both failed to induce sister chromatid disjunction. Instead, securin morpholino knockdown induced sister chromatid separation, which could be rescued by injection of securin cRNA.

I also examined the effect of CDK1 and MAPK activities on securin stability. Inhibition of both kinases, but not either one alone, using roscovitine and UO126, induced the premature destruction of securin during prometaphase of meiosis I. However, this effect is not dependent on securin phosphorylation, given that mutagenesis of CDK1 and MAPK phosphorylation sites on securin did not affect its stability.

Finally, I found that CDK1 and MAPK inhibition also causes the premature degradation of other APC/C substrates, such as cyclin B1 and geminin. Interestingly, only the D-box

containing substrates and not Ken-box substrates were subject to such effect. However, this prometaphase I instability of the D-box substrates was apparently not mediated by APC/C^{Cdc20} or APC/C^{Cdh1}, given that Cdc20 was also targeted for destruction and morpholino-knockdown of both Cdc20 and Cdh1 failed to rescue securin stability. Also, the inhibition of $SCF^{\beta\text{-}TrCP}$ using the dominant negative mutant of its F-box protein (β-TrCPΔ) could not restore securin levels, suggesting it is not $SCF^{\beta\text{-}TrCP}$-dependent. Future work will explore whether this effect could be attributed to activation of some meiosis-specific APC/C co-activators or the core APC/C, which was recently reported of being able to directly interact with the D-box containing substrates independently of Cdc20 and Cdh1 (Passmore et al., 2003; Yamano et al., 2004).

Publications containing work from this thesis

Nabti,I., Reis,A., Levasseur,M., Stemmann,O., and Jones,K.T. (2008). Securin and not CDK1/cyclin B1 regulates sister chromatid disjunction during meiosis II in mouse eggs. *Dev. Biol.* 321, 379-386.

Reis,A., Madgwick,S., Chang,H.Y., Nabti,I., Levasseur,M., and Jones,K.T. (2007). Prometaphase APC(cdh1) activity prevents non-disjunction in mammalian oocytes. *Nature Cell Biology* 9, 1192-1198.

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Abbreviations

AC	Adenylyl Cyclase
APC/C	Anaphase-Promoting Complex/Cyclosome
ATP	Adenosine Triphosphate
BP	Band Pass
BSA	Bovine Serum Albumin
Bub	Budding uninhibited by benzimidazole
BubR1	Bub1 Related
Ca^{2+}	Calcium
CaM	Calmodulin
CaMKII	Calmodulin-dependent protein kinase II
cAMP	Cyclic Adenosine 3', 5'-Monophosphate
CBD	Cyclin B1-Binding Determinants
Cdc20	Cell Division Cycle 20
Cdh1	Cdc20 Homologue 1
CDK	Cyclin-Dependent Kinase
CHX	Cycloheximide
cGMP	Cyclic Guanosine 3', 5'-Monophosphate
CSF	Cytostatic Factor
CUT	Cell Untimely Torn
DM	Dichroic Mirror
EDTA	Ethylenediaminetetracetic Acid
ECL	Enhanced Chemiluminescence
EGF	Epidermal Growth Factor
Emi	Early Mitotic Inhibitor
ER	Endoplasmic Reticulum
ERK	Extracellular-Regulated Kinase
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FSH	Follicle Stimulating Hormone

G1	Gap Phase 1
G2	Gap Phase 2
GFP	Green Fuorescent Protein
GPR	G Protein-coupled Receptor
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
H1	Histone 1
hCG	Human Chorionic Gonadotrophin
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HRP	Horseradish Peroxidase
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-triphosphate
IP_3R	IP_3 Receptor
LH	Luteinizing Hormone
LP	Long Pass
Mad	Mitotic Arrest Defective
MAPK	Mitogen-Activated Protein Kinase
MEM	Minimum Essential Medium
Met I	Metaphase I
Met II	Metaphase II
MF	Metafluor
MI	Meiosis I
MII	Meiosis II
MM	Metamorph
MOPS	Mopholinepropanesulphonic acid
MO	Morpholino Oligonucleotides
MPF	Maturation-Promoting Factor
NS	Not significant
OA	Okadaic Acid
$p90^{rsk}$	90-KD Ribosomal protein S6 Kinase

PB	Polar Body
PB1	First Polar Body
PB2	Second Polar Body
PBS	Phosphate Buffer Solution
PDE	Phosphodiesterase
PGC	Primordial Germ Cells
PIP_2	phophatidylinositol-4,5-bisphosphate
PKA	Protein Kinase A
PLC	Phospholipase-C
PLK1	Polo Like Kinase 1
PMSG	Pregnant Mares' Serum Gonadotrophin
PN	Pronuclei
PP2A	Protein Phosphatase 2A
PTTG	Pituitary Tumour-Transforming Gene
PVDF	Polyvinylidened Fluoride membrane
PVP	Polyvinylpyrrolidone
R	Roscovitine
RSK	Ribosome S6 Kinase
S phase	Synthesis Phase
SAC	Spindle Assembly Checkpoint
SCF	SKP1/Cullin/F-box protein
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
Sgo	Shugoshin
SMC	Structural Maintenance of Chromosome
Sr^{2+}	Strontium
TAE	Tris-Acetate-Ethylenediaminetetracetic
U	UO126
UTR	Untranslated Region
YFP	Yellow Fluorescent Protein
ZP	Zona Pellucida

Chapter 1: General introduction

Sexual reproduction is the creation of new offspring by the fusion of haploid gametes to form a diploid zygote. Gametes are generated from diploid *primordial germ cells* (PGC) by the process of *meiosis*. The female gamete is usually large and non-motile and is referred to as the egg (or *ovum*) (AUSTIN and Short, 1993). The male gamete, however, is small and motile and is referred to as the sperm (or *spermatozoon*) (AUSTIN and Short, 1993). Sexual reproduction has been favoured by evolution because it increases genetic diversity among offspring and generates individuals with unique assortments of genes inherited from both parents, therefore enhancing the reproductive success of parents and the survival of offspring (Alberts et al., 2000; Campbell et al., 1999).

The experiments presented in this thesis investigate part of the regulatory mechanisms underlying the progression of the meiotic division in mammalian oocytes. In order to introduce the research presented here, I will first provide an overview of meiosis, the process through which the haploid gametes are formed. Then, I will consider all the events that give rise to the fully-grown female gamete (the unfertilized egg) as well as the process of fertilization. Finally, I will provide a detailed description of the current understanding of how the meiotic cell divisions are controlled and how the oocyte segregates its chromatin in the first and second meiotic divisions.

1.1. Overview of meiosis

The finding that gametes are haploid in chromosome number and DNA content, a state that must therefore be achieved through cell divisions of an unusual kind, came from the discovery that the roundworm gametes contain only 2 chromosomes, whereas the zygote (the fertilized egg) contains 4 chromosomes (Van Beneden, 1883). This special type of cell division is called *meiosis*, from the Greek meaning diminution (Alberts et al., 2000; Van Beneden, 1883).

Meiosis is a unique process by which diploid germ cells (*oogonia* and *spermatogonia*) reduce their chromosome number by half, therefore allowing a diploid number of chromosomes to be reinstated at fertilization (AUSTIN and Short, 1993). It consists of a single phase of DNA replication followed by two consecutive rounds of division, called meiosis I (MI) and meiosis II (MII). During the first meiotic cell division, both the DNA content and chromosome number is halved, through the segregation of the two replicated homologs into separate daughter cells. In the second meiotic division, however, only the DNA content is halved via the separation of the sister chromatids (AUSTIN and Short, 1993).

Both meiotic divisions are divided into prophase, metaphase, anaphase and telophase, which are identical in purpose to their analogous mitotic sub-phases. Meiotic prophase I, however, lasts longer and is more complex than the prophase of the mitotic cell cycle (Alberts et al., 2000). It is characterised by the pairing of the two replicated homologs, a process called *synapsis*, and the crossing over of non-sister chromatids leading to

formation of *chiasmata*, which play a crucial role in holding homologous chromosomes together during metaphase of the first meiotic division (Met I), therefore ensuring their accurate segregation during anaphase I (Campbell et al., 1999). Chiasmata are also important in rearranging genes via DNA exchange between the homologous chromosomes. This is achieved during the process of homologous recombination, which is initiated by the genetically programmed formation of double-strand breaks, hence triggering the equivalent of DNA-damage repair using the homologous chromosome as a template (Campbell et al., 1999). During this repair process, DNA can be exchanged between the homologous chromosomal regions, leading to new combinations of DNA within the chromosomes (Campbell et al., 1999). In some cases, no exchange happens and the DNA is transferred from one helix, which remains unaltered, to the other helix, therefore leading to gene conversion, which plays an important role in gene mutagenesis and non-Mendelian inheritance (Campbell et al., 1999).

1.2. The mammalian ovum

The mammalian ovum, or egg, is a particularly fascinating cell, as it is the cell from which all the cells in a new organism are derived. As the sperm contributes virtually nothing to the new organism other than its chromosomes, the ovum has the difficult task of supplying most of the material for the body of the embryo and providing for its protection and maintenance during development (Campbell et al., 1999; Wilson, 1925). In order to prepare for this service, the ovum goes through an extensive period of growth during which it accumulates a large amount of cytoplasm, and in many cases becomes

surrounded by membranes or other protective envelopes (Campbell et al., 1999; Wilson, 1925).

1.2.1. The structure of the mammalian ovum

The mammalian egg is structurally specialised for fertilization. It consists of a spherical mass of cytoplasm, which is bounded by a plasma membrane and enclosed within a thick glycoprotein envelope called the *zona pellucida* (ZP) (Bleil and Wassarman, 1980; Dunbar et al., 1994). Beyond the zona pellucida, the egg is surrounded by a matrix of *granulosa* cells, called the *cumulus oophorus*. The oocyte and granulosa cells are interconnected via gap junctions, which are crucial in the maturation process of the oocyte through metabolites and regulatory elements transfer between the two cell types (Anderson and Albertini, 1976; Heller et al., 1981). Between the plasma membrane and the zona pellucida is a fluid-filled space, the *perivitelline* space. Lying immediately beneath the plasma membrane is a thin shell of actin-rich cytoplasm called the *cortex*. Within the cortex are the *cortical granules*, which contain proteolytic enzymes and mucopolysaccharides. These Golgi-derived structures play a crucial role in the egg's response to sperm penetration, especially preventing the entry of more than one spermatozoon (*polyspermy*) (Barros and YANAGIMA.R, 1971; Gwatkin et al., 1973).

1.2.2. Oogenesis: from primordial germ cells to eggs

The process of gametogenesis in the female is known as *oogenesis*, and a developing egg is called an *oocyte* (AUSTIN and Short, 1993). Unlike spermatogenesis, which is

initiated in puberty and continues throughout the male adult life, oogenesis begins during female embryonic life and ends months to years later in the sexually mature adult (AUSTIN and Short, 1993). The details of oogenesis (timing of events) are different between species, but the general stages are the same.

In mammals, oogenesis starts with the mitotic proliferation of the diploid primordial germ cells, as they migrate to the developing ovary to become *oogonia* (Bukovsky et al., 2005). Once they arrive to the gonads, the diploid oogonia begin to differentiate into *primary oocytes* (Bukovsky et al., 2005). At this stage, the oocytes enter meiosis, and become arrested in prophase of the first meiotic division for a period lasting from few months (mice) to many years (humans), until the female becomes sexually mature at puberty (Alberts et al., 2000; Bukovsky et al., 2005; Wolpert et al., 1998). After entry into meiosis, the oocytes never proliferate again, therefore, the number of oocytes at this embryonic stage is the total number of eggs the female mammal will ever have (Wolpert et al., 1998). In humans, most of these oocytes degenerate before puberty, leaving about 400,000 out of an original 6 million (Wolpert et al., 1998).

Following entry into prophase I arrest, the primary oocytes begin to grow. During this growth period, the oocytes undergo 300-fold increase in volume, accumulate a large amount of proteins, mRNA, ribosomes, cortical granules and become surrounded by a zona pellucida (Wassarman, 1988).

At puberty, under the influence of hormones, the fully-grown primary oocytes can resume the first meiotic division, a process known as *meiotic maturation* (Yanagimachi, 1994). At this stage of oocyte development, the nuclear (germinal vesicle - GV) envelope breaks down (GVBD), the chromatin recondenses, the replicated homologs separate and the oocyte divides asymmetrically to produce a large *secondary oocyte* (the precursor of the egg) and a small first *polar body* (PB1) (Yanagimachi, 1994). During oocyte maturation, meiosis proceeds directly to metaphase of the second meiotic division (Met II) without intervening S phase and then arrests again, with chromosomes aligned on the metaphase II spindle, until fertilization (Moore and Orr-Weaver, 1998). At ovulation, the secondary oocyte (Met II-arrested oocyte) is released from the ovary to the ampulla region of the oviduct (Runft et al., 2002). From this point the oocyte is fully mature and ready for fertilization; as such it is defined as an egg.

1.2.3. Folliculogenesis: follicular growth

Each developing oocyte is contained within a *follicle* and is surrounded by layers of *granulosa cells*. The ovarian follicle is the fundamental structure of the female reproductive biology and the process of its maturation is referred to as *folliculogenesis* (Gore-Langton and Armstrong, 1998; Yanagimachi, 1994). Folliculogenesis is a highly regulated developmental sequence by which the female germ cell develops within the somatic cells of the ovary and matures into a fertilizable egg (Knobil and Neil, 2006). The growing follicles are usually classified in terms of their morphology, oocyte size and granulosa cells number into primordial, primary, growing and pre-ovulatory follicles (or Graafian follicles), from where oocytes are ovulated (Knobil and Neil, 2006).

The process of follicular growth and oocyte maturation is mainly regulated by the gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are both peptide hormones secreted by the anterior pituitary (Bukovsky et al., 2005; Normann and Liteack, 1997; Yanagimachi, 1994). FSH stimulates granulosa cell proliferation, estrogens biosynthesis and LH receptor expression (Normann and Liteack, 1997). LH, however, induces thecal androgens production, which in turn stimulates estrogens production by the granulosa cells (Normann and Liteack, 1997). Follicular estrogens feedback on both the hypothalamus and pituitary to trigger a sudden and dramatic rise in the release of LH, the so-called "LH surge" (Normann and Liteack, 1997). The LH surge induces the pre-ovulatory maturation of the Graafian follicle, which includes meiotic maturation of the oocyte accompanied by changes in the structure of the Graafian follicle, to produce an ovulatory follicle consisting of a secondary oocyte (Met II-arrested oocyte), surrounded by an optimum number of highly differentiated granulosa cells and a large fluid-filled antrum (AUSTIN and Short, 1993). The antrum divides the granulosa cells into two separate compartments: the cumulus cells that directly surround the oocyte, whereas the outer layer consists of the mural granulosa cells (AUSTIN and Short, 1993).

1.2.4. Ovulation

The ovulatory process in mammals is a distinct biological phenomenon that begins when gonadotrophic hormones stimulate mature ovarian follicles and it ends when the follicles rupture and release eggs into the oviduct (Knobil and Neil, 2006). It is currently believed

that the ovarian follicle rupture at ovulation is caused by an acute inflammatory reaction that involves the granulosa and theca cells, and which is induced by the ovulatory surge in the gonadotrophins (Espey, 1980). This inflammatory response stimulates the biosynthesis of proteases, which degrade the wall of the follicle and the extracellular matrices within the ovarian connective tissue, resulting in rupture of the follicle and release of the egg, which remains in the ampulla region of the oviduct until fertilization (Espey, 1980).

1.2.5. Fertilization and egg activation

1.2.5.1. Fertilization

In mammals, the process of fertilization starts when the head of the sperm binds to the zona pellucida, the glycoprotein envelope that surrounds the egg. Fertilization takes place in the ampulla region of the oviduct, where the freshly ovulated metaphase II-arrested egg is located. At this stage, the egg is encased within a zona and is surrounded by a mass of cumulus cells, which are both physical barriers the sperm has to penetrate before it can contact and fuse with the egg plasma membrane. To become competent to overcome these vestments, the sperm must undergo two crucial events, known as *capacitation* and *acrosome reaction*.

It was observed in 1951 that freshly ejaculated mammalian sperm were not competent to fertilize eggs in *vitro*, unless they had spent a period of time in the female reproductive tract (AUSTIN, 1951; Chang, 1951). The series of modifications the sperm undergoes

while in the female uterus are referred to as capacitation (AUSTIN, 1952). During this process the sperm acquires the following functional capabilities: to penetrate the cumulus mass, to bind to the ZP and to undergo the acrosome reaction upon binding to the zona (AUSTIN, 1952; Visconti et al., 1998). These are achieved through widespread changes in the physiology and biochemistry of the sperm, including modifications of the plasma membrane potential and composition, changes in the intracellular pH, alteration of the metabolism and especially changes in the flagellar motility (Visconti et al., 1998).

Once capacitated, the next barrier the sperm encounters is the zona pellucida. As already mentioned, the zona is a glycoprotein envelope which consists of three main glycoproteins: ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980). ZP3 is a crucial protein, as ZP3-knockout mice are not fertile and their oocytes lack zona (Liu et al., 1996; Rankin et al., 1996). ZP3 serves as a receptor for species-specific binding of the sperm to the zona, leading to the acrosome reaction in which the hydrolytic content of the acrosomal vesicle is released by exocytosis (Florman and Wassarman, 1985; Wassarman, 1990; Wassarman, 2005). The enzymes released include β-*N*-acetylglucosaminidase and acrosin, which facilitate the sperm penetration of the ZP through enzymatic dissociation of its substances (Gilbert, 2006). In addition, the acrosomal reaction exposes proteins on the sperm plasma membrane that mediate the binding and fusion of sperm and egg membranes; a key player is the transmembrane protein *Izumo* (Inoue et al., 2005). Accordingly, it was demonstrated that Izumo-knockout male mice are sterile, and an antibody against human Izumo blocked the binding and fusion of human sperm with zona-free hamster eggs (Inoue et al., 2005).

1.2.5.2. Egg activation

Following sperm-egg membrane fusion, the egg initiates a series of processes that begin development. These series of events, called *egg activation*, happen in the cytoplasm of the egg upon fertilization and occur prior to the first cleavage (Knobil and Neil, 2006; Runft et al., 2002). The events of egg activation can be divided into early events, which occur within seconds of the sperm-egg fusion, and late events, which happen few hours after fertilization (Knobil and Neil, 2006; Runft et al., 2002).

The early events of egg activation are the block to polyspermy, which is necessary to prevent entrance of multiple sperms. In mammals, fertilization of the egg by only one sperm is ensured by the *cortical granule reaction* (Ducibella, 1996). Upon sperm entry, the cortical granules, which lie just beneath the egg plasma membrane, undergo exocytosis and release their contents into the perivitelline space (Ducibella, 1996). The released enzymes modify the ZP sperm receptors such that they are no longer recognizable and able to bind sperm (Ducibella, 1996). In mouse, for example, the cortical granules contain *N*-acetylglucosaminidase enzymes, which are capable of cleaving *N*- acetylglucosamine from ZP3 carbohydrate chains. It has been demonstrated that when *N*- acetylglucosamine residues are removed, ZP3 is no longer recognisable by sperm (Miller et al., 1993a; Miller et al., 1993b). Exocytosis also results in the hardening of the zona, making it more resistant to protease digestion during sperm penetration (Gilbert, 2006).

The late events of fertilization include resumption of the second meiotic division by the Met-II arrested egg, leading to extrusion of one half of the chromatin within the second polar body, leaving the egg in a haploid state (Runft et al., 2002). This is followed by decondensation of the egg and sperm chromatin, which become surrounded by their own nuclear envelopes, forming the female and male *pronuclei* (PN), respectively (Runft et al., 2002). Within the PN, the first mitotic S-phase takes place to generate two copies of each genome (Runft et al., 2002). Once the DNA has been replicated, the two pronuclei are brought together in a process known as *syngamy* (Evans and Florman, 2002). This is followed by the breakdown of the nuclear envelopes and recondensation of both genomes, which then orient themselves on the first mitotic spindle, just prior to undergoing the first embryonic cleavage (Evans and Florman, 2002).

Fertilization of the mammalian egg by sperm is associated with an explosive release in cytosolic calcium (Ca^{2+}), which spreads through the egg in waves (Stricker, 1999). These Ca^{2+} oscillations are crucial for stimulating cortical granule exocytosis and activating the egg to complete meiosis (Stricker, 1999). The cell cycle of the ovulated egg is maintained transiently in the metaphase of the second meiotic division by the high activity of the Maturation-Promoting Factor (MPF) (Jones, 2004; Madgwick and Jones, 2007; Mattioli et al., 1991). Loss of MPF is essential for the fertilized egg to resume its meiotic division and to begin development. This is achieved through the Ca^{2+} signals, which are transduced by calmodulin (CaM), which in turn associates with and activates calmodulin-dependent protein kinase II (CaMKII) (Lorca et al., 1991; Lorca et al., 1993). Activation of CaMKII results in the degradation of the regulatory subunit of MPF (cyclin

B1) through the Anaphase-Promoting Complex/Cyclosome (APC/C), leading to MPF inactivation (Lorca et al., 1991; Lorca et al., 1993). Having explained the structural and cellular events around oocyte development and maturation, the following section will focus on (i) the role of the two major kinases (MPF and MAPK) known to control oocyte maturation, and (ii) how the APC/C-mediated proteolysis of the various regulatory proteins co-ordinates cell cycle progression and chromosome segregation.

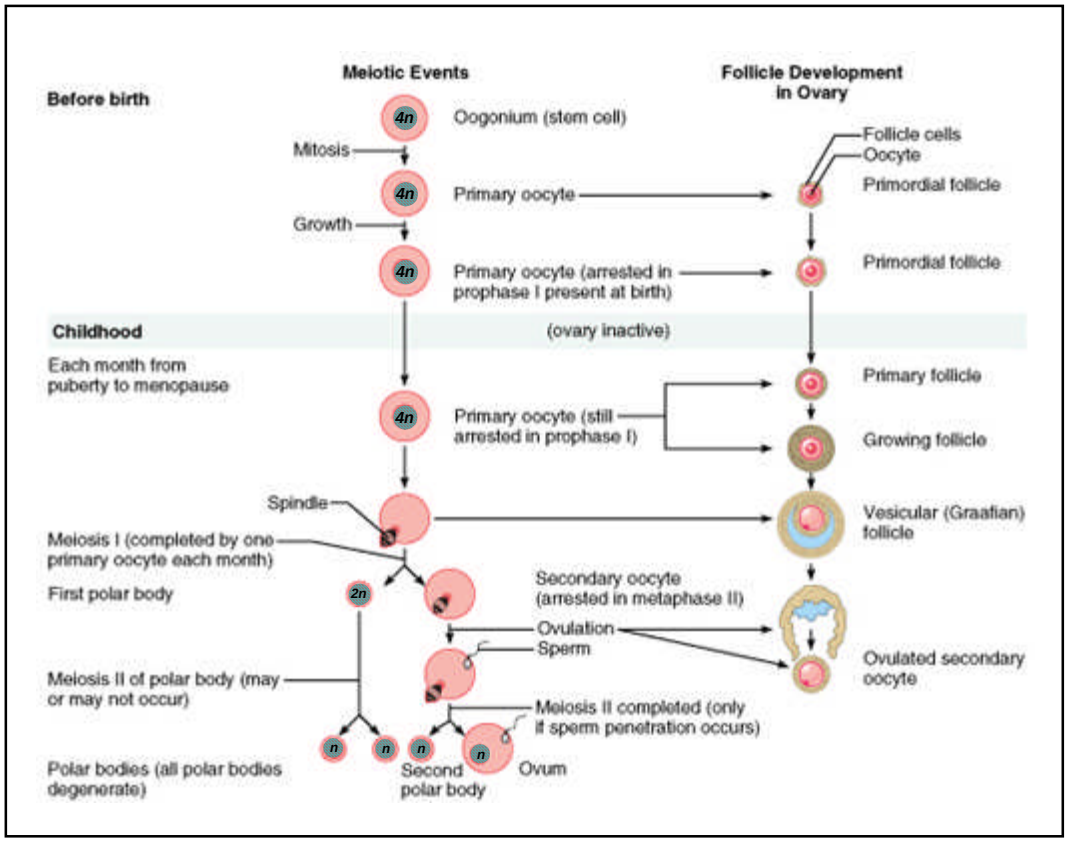

Figure 1.1. Overview of oogenesis and folliculogenesis.

Oogenesis (left) starts with the mitotic proliferation of the primordial germ cell as it migrates to the ovary to become oogonium. After a number of mitotic divisions, the oogonium enters the prophase of meiosis I and differentiates into a primary oocyte. The primary oocyte remains arrested in prophase I until the female becomes sexually mature. At this point, under the influence of hormones, the fully-grown primary oocyte resumes meiosis I to become a secondary oocyte, arrested at metaphase II. The egg is ovulated and remains in the metaphase II stage until fertilization, when it gets activated and resumes its second meiotic division. Each developing oocyte is contained within a follicle, whose process of maturation, folliculogenesis (right), occurs in parallel to oogenesis. The growing follicle is classified in terms of certain structural characteristics (morphology, oocyte size and number of follicle cells) into primordial, primary, growing and pre-ovulatory Graafian follicle, from where the secondary oocyte is ovulated (www.tarleton.edu/ ~anatomy/oogenesis.html).

1.3. Regulation of the meiotic cell cycle in oocytes

1.3.1. Maturation-Promoting Factor (MPF)

MPF is the universal M-phase kinase. It drives somatic cells into mitosis and eggs into meiosis (Doree and Hunt, 2002; Jones, 2004; Masui, 2001; Pines, 1999). MPF was first characterised by Masui and Markert in 1971 as an activity present in mature oocytes that was able to induce nuclear maturation when injected into immature prophase I-arrested frog oocytes (Doree and Hunt, 2002; Masui and Markert, 1971). It is a heterodimeric protein, composed of a catalytic CDK1 (Cyclin-Dependent Kinase 1) subunit and a regulatory cyclin B subunit (Doree and Hunt, 2002; Gautier et al., 1990; Lohka et al., 1988; Pines and Hunter, 1989).

Cyclins were initially purified in marine invertebrate embryos by their ability to fluctuate in a cyclical pattern along the cell division cycle (Evans et al., 1983). There are at least three types of cyclin B (B1, B2 and B3) in mammalian cells. Cyclin B1 appears to be the physiologically important regulator of MPF in oocytes, given that knocking out cyclin B1 in GV oocytes prevents their maturation (Ledan et al., 2001), and cyclin B1 knockout mice are non-viable (Brandeis et al., 1998). Cyclin B2 knockout mice, however, are viable and fertile (Brandeis et al., 1998). Whereas, cyclin B3 is absent from the ovarian tissue (Lozano et al., 2002).

MPF is the master controller of many aspects of the cell cycle due to its ability to phosphorylate a variety of substrates, such as: (i) histone H1, which has an important role

in chromatin condensation during cell division, (ii) nuclear laminins, leading to nuclear envelope breakdown and (iii) RNA polymerase II, explaining the inhibition of transcription at the final stages of oocyte maturation and during cell division (Bradbury et al., 1974; Cisek and Corden, 1989; Heikinheimo and Gibbons, 1998; Langan et al., 1989; Peter et al., 1990).

In mitosis, MPF activity is required in order for cells to enter M-phase and its inactivation is needed for mitotic exit. Meiotic division, however, is characterised by two peaks of high MPF activity occurring during meiotic maturation and metaphase II arrest. Between MI and II, MPF activity does not experience a mitotic like decrease, instead it undergoes a transient decrease to about 50% of the original activity, therefore allowing completion of the first meiotic division and a rapid reactivation of MPF that leads to direct progression to MII (Jones, 2004; Madgwick and Jones, 2007; Mattioli et al., 1991). The sustained high MPF activity during the metaphase II arrest is due to a cytoplasmic activity termed *cytostatic factor* (CSF), which was also discovered by Masui and Markert in 1971 (see section 1.3.6).

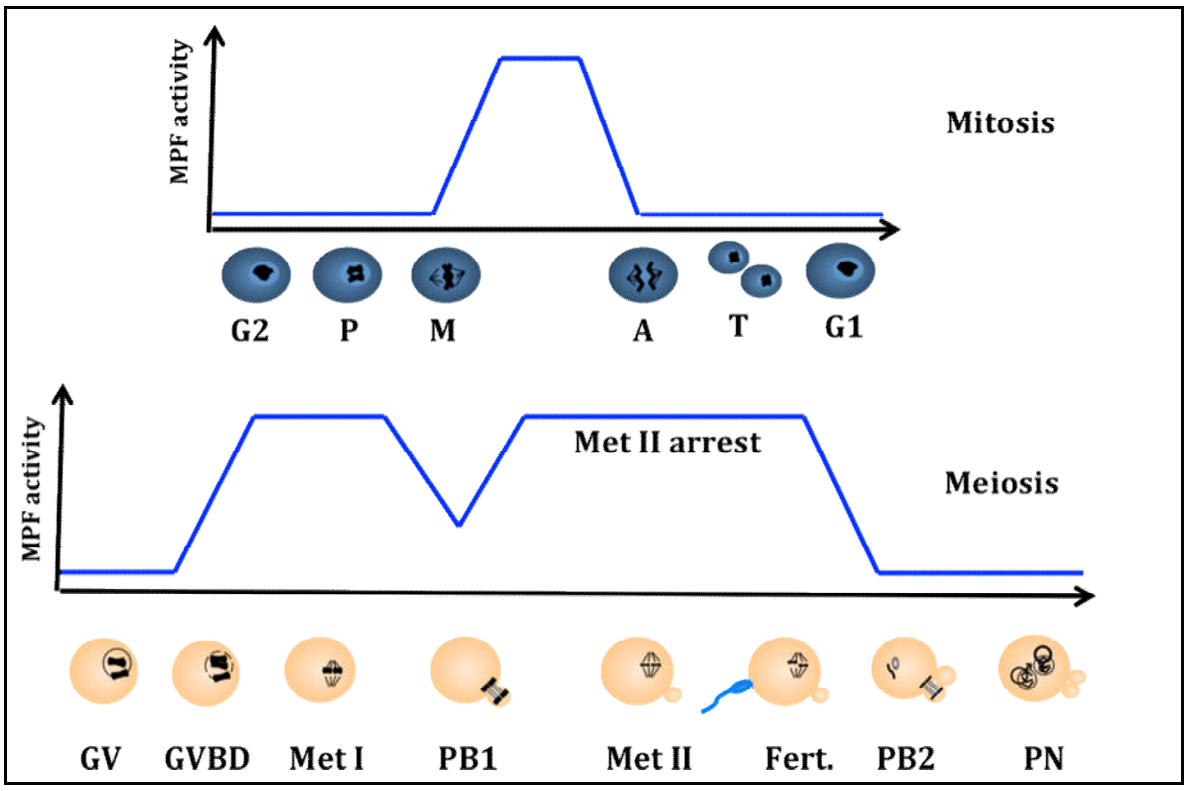

Figure 1.2. Profile of MPF activity during mitosis and meiosis.

In somatic cells (top), MPF activity is required for cells to enter M-phase and its inactivation is needed to exit mitosis. In contrast, meiosis (bottom) is characterised by two peaks of high MPF activity occurring during meiotic maturation and metaphase II arrest. Between MI and II, there is a transient decrease in MPF explaining the lack of interphase during this period.

The activity of MPF is highly regulated by mechanisms responding to cell cycle progression, which include both CDK1 phosphorylation and cyclin B1 degradation. The newly assembled MPF complex is usually maintained in an inactive state (termed pre-MPF) via phosphorylation of its catalytic subunit (CDK1) at Threonine-14 and Tyrosine-15 by the specific protein kinase, Wee1 (Gu et al., 1992; Lundgren et al., 1991; McGowan and Russell, 1993). These phosphorylation events can be reversed by the phosphatase cdc25, which causes an activating dephosphorylation of MPF at the same sites (Gautier et al., 1991; Millar et al., 1991). Therefore, the balance in cdc25 and Wee1 activities governs the status of CDK1 phosphorylation and MPF activation.

In addition, the activity of MPF depends on the presence or absence of its regulatory subunit (cyclin B1) during the cell cycle, explaining the periodic pattern of MPF activity (Evans et al., 1983; Murray et al., 1989). Cyclin B1 is usually synthesised prior to cell division and degraded at the metaphase-to-anaphase transition, allowing exit from M-phase (Clute and Pines, 1999; Evans et al., 1983; Murray et al., 1989). It was shown by Murray and Kirshner (1989) that cyclin B1 destruction was necessary for MPF inactivation and M-phase exit, given that metaphase-arrested *Xenopus* egg extracts that contain the non-degradable mutant of cyclin B1 (Δ90-cyclin B1) block at metaphase with high MPF activity following the addition of calcium (Murray et al., 1989). Cyclin B1 degradation at the onset of anaphase is triggered via ubiquitination by the APC/C, which is an E3 ubiquitin ligase (see section 1.3.3) (Glotzer et al., 1991).

1.3.2. MAPK signalling cascade during meiotic maturation

The c-Mos/MEK/MAPK/p90^{rsk} cascade is another regulatory system that is universally activated during meiotic maturation of vertebrate oocytes, and which functions in parallel to and interacts with MPF in driving meiosis (Sun et al., 1999). c-Mos is a 39 kDa germ cell-specific Ser/Thr protein kinase, which was first identified in cells transformed by Moloney murine leukemia virus (Papkoff et al., 1982). It is the product of the proto-oncogene *c-mos*, whose mRNA is stored as maternal information in the growing oocyte and is only translated upon exit from the prophase I arrest (Gebauer and Richter, 1997). c-Mos functions as a MAPK kinase kinase (MAPKKK), which directly phosphorylates the MAPK kinase, MEK1 (Gebauer and Richter, 1997). Activated MEK1, in turn, activates MAPK through dual phosphorylation on Threonine-183 and Tyrosine-185 (Crews and Erikson, 1992). MAPK, also known as Extracellular-Regulated Kinase (ERK), exists as two isoforms in mammalian oocytes, ERK1 and ERK2 (Boulton et al., 1991). The best-known downstream target of MAPK in oocytes is p90^{rsk}, a 90 kDa ribosome S6 kinase, which is activated by ERK via phosphorylation on Serine-369 and Threonine-577 (Dalby et al., 1998).

Unlike MPF, whose activation is crucial for resumption of oocyte meiotic maturation in all species studied so far, the timing and requirement for MAPK activation varies in different organisms. In *Xenopus* and other lower vertebrate oocytes, several lines of evidence suggest that MAPK activation is required for progesterone-induced MPF activation and meiotic resumption. It was shown that microinjection of constitutively activated thiophosphorylated MAPK or MEK into *Xenopus* oocytes leads to MPF

activation and GVBD in the absence of progesterone (Haccard et al., 1995; Huang et al., 1995). However, these events were inhibited upon microinjection of a MAPK phosphatase or anti-MEK antibody (Gotoh and Nishida, 1995; Kosako et al., 1994). Also, progesterone-induced GVBD is always accompanied by MAPK activation in vivo (Posada and Cooper, 1992). It is believed that progesterone causes MAPK activation through promoting c-Mos mRNA polyadenylation and translation, resulting in the firing of the MAPK signalling cascade, followed by MPF activation and GVBD (Sheets et al., 1995). Accordingly, microinjection of c-Mos triggers GVBD in a progesterone-independent manner (Sagata et al., 1989a). Conversely, progesterone-induced MPF activation and GVBD are prevented when the translation of c-Mos mRNA is inhibited by microinjection of antisense oligonucleotides (Sagata et al., 1988). Taken together these results confirm that progesterone-dependent stimulation of the c-Mos/MAPK cascade plays an important role in initial activation of MPF and meiotic resumption in lower vertebrate oocytes.

However, recent studies have challenged this idea, with the authors suggesting that progesterone-induced GVBD does not require MAPK, and other MAPK-independent pathways could trigger MPF activation. In agreement with this, it was reported that inhibition of MAPK activity using the MEK inhibitor UO126 or by over-expression of the MAPK phosphatase Pyst1, does not block MPF activation and GVBD upon progesterone stimulation (Fisher et al., 1999; Smythe and Stricker, 2005). Nevertheless, it is worth mentioning that in these cells although GVBD is not blocked, it is delayed and meiotic maturation happens less efficiently (Fisher et al., 1999).

In mammalian oocytes, the sequence of events differs with MAPK activation occurring after GVBD. This indicates that the MAPK phosphorylation cascade is not required for MPF activation and spontaneous meiotic resumption in mammals (Kalab et al., 1996; Verlhac et al., 1993). Indeed, inhibition of MAPK activation by the MEK inhibitor UO126 does not affect GVBD in mouse oocytes (Tong et al., 2003). Also, analysis of oocytes from c-*mos* knockout mouse has shown that GVBD occurs normally, while no activation of MAPK is detected in these cells (Hashimoto et al., 1994; Verlhac et al., 1996).

Although these studies show that MAPK activity is not necessary for GVBD in frog and mammalian oocytes, it is very important for the regulation of subsequent meiotic events. In addition to its vital role in keeping the CSF arrest (Colledge et al., 1994; Hashimoto et al., 1994), which I will discuss more later (see section 1.3.6), several lines of evidence suggest that the MAPK cascade is also required for the maintenance of microtubules and chromatin in the metaphase state during the MI to MII transition, when the levels of MPF activity are low. It was reported that addition of MAPK to *Xenopus* interphase extracts induces the shortening of the elongated microtubules (Gotoh et al., 1991), and microinjection of anti-Mos antibody into mouse oocytes prevents the assembly of a meiotic spindle (Zhao et al., 1991). Moreover, *c-mos*$^{-/-}$ mouse oocytes present abnormal spindles with interphase-like elongated microtubules, and fail to keep the chromosomes condensed at the MI to MII transition (Hashimoto et al., 1994; Verlhac et al., 1996).

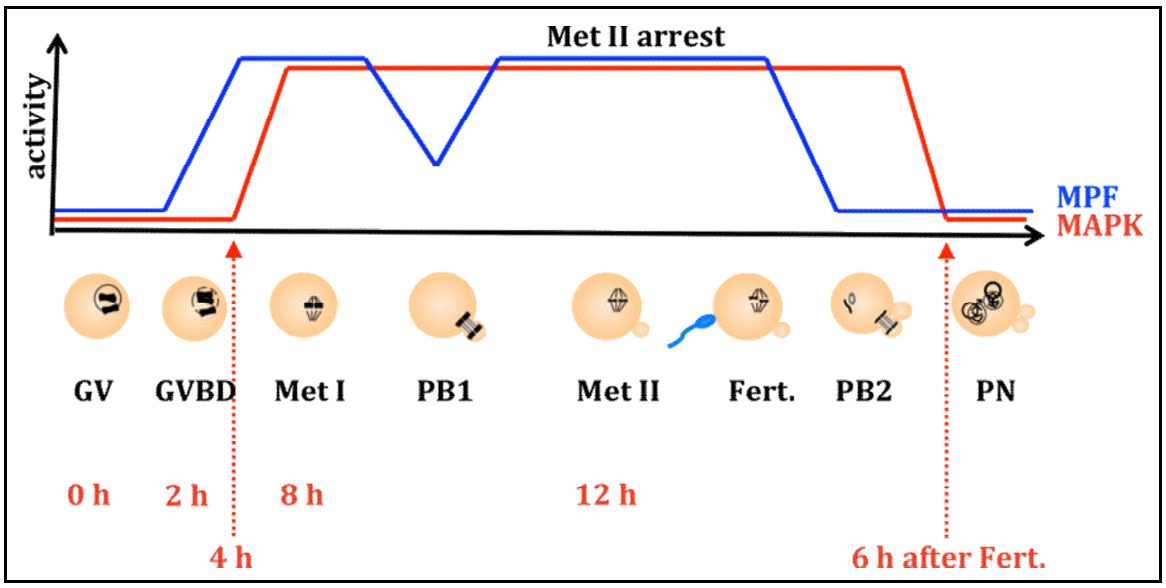

Figure 1.3. MAPK activity during meiosis in mammalian oocytes.

In spontaneous meiotic maturation of mammalian oocytes, MAPK activation occurs at approximately 2 hours after GVBD and is inactivated at 6 hours following egg activation, just prior to pronuclei formation. Between MI and II, unlike MPF whose activity drops slightly, MAPK activity remains high and this is important for the maintenance of microtubules and chromatin in the metaphase state.

Besides MAPK involvement in the organization and maintenance of the metaphase spindle, it is also important for the migration of the meiotic spindle to the cortex, leading to asymmetric divisions in oocytes and extrusion of small polar bodies. Accordingly, inhibition of MAPK leads to failure of spindle migration to the cortex, resulting in extrusion of abnormally large polar bodies (Choi et al., 1996; Hashimoto et al., 1994). Since, spindle migration to the cortex depends on actin microfilaments and not microtubules (Al et al., 2008; Azoury et al., 2008; Sun and Schatten, 2006), then the MAPK cascade might also control the actin-microfilament network.

Finally, there is also evidence associating MAPK with the spindle assembly checkpoint, suggesting that in the absence of MAPK activity, *Xenopus* and mouse oocytes fail to activate the SAC and to arrest the cell cycle at metaphase following microtubule depolymerisation (Takenaka et al., 1997; Verlhac et al., 1996). It has also been reported that MAPK is important for re-activating MPF after anaphase I (Gross et al., 2000; Taieb et al., 2001), and its inactivation seems to be crucial for pronuclei formation following fertilization (Moos et al., 1996).

1.3.3. The Anaphase Promoting Complex/Cyclosome (APC/C)

Cell proliferation in all eukaryotes depends on the E3 ubiquitin ligase activity of the anaphase promoting complex/cyclosome (APC/C), which is primarily required for mitotic/meiotic progression and exit (Peters, 2006). The APC/C is a 1.5 MDa protein complex that performs its various functions by assembling chains of at least four ubiquitin molecules on lysine residues in key regulatory proteins, such as cyclin B1 and

securin, thus targeting them for degradation by the 26S proteasome (Peters, 2006). The destruction of securin is important for activation of the protease separase, subsequently allowing segregation of chromosomes (Madgwick et al., 2004; Terret et al., 2003). The degradation of cyclin B1 is crucial for anaphase onset through MPF inactivation (Lorca et al., 1993).

The vertebrate APC/C is composed of at least a dozen different subunits, most of which were highly conserved throughout evolution and whose modification by genetic manipulation leads to early lethality in every species examined so far (Baker et al., 2007; Yoon et al., 2002; Zachariae et al., 1996; Zachariae et al., 1998b). APC/C-mediated ubiquitination of its substrates requires the help of 3 main cofactors, in addition to its E3 ubiquitin ligase activity it needs a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a co-activator protein (Peters, 2006).

The transfer of ubiquitin to a target substrate is performed in three steps. First, ubiquitin, which is a highly conserved 76-amino acids protein, is activated by E1 in a reaction that requires ATP in order to create a high-thioester bond between the cysteine residue at the enzyme's active site and the glycine residue of ubiquitin (Ciechanover et al., 1981; Haas et al., 1982; Hershko et al., 1981). The activated ubiquitin is then transferred to the active site of the ubiquitin-conjugating enzyme (E2) (Hershko et al., 1983). Finally, ubiquitin is transmitted to the side chain of a lysine residue on the target protein via an E3 ubiquitin ligase, such as the APC/C (Hershko and Ciechanover, 1998).

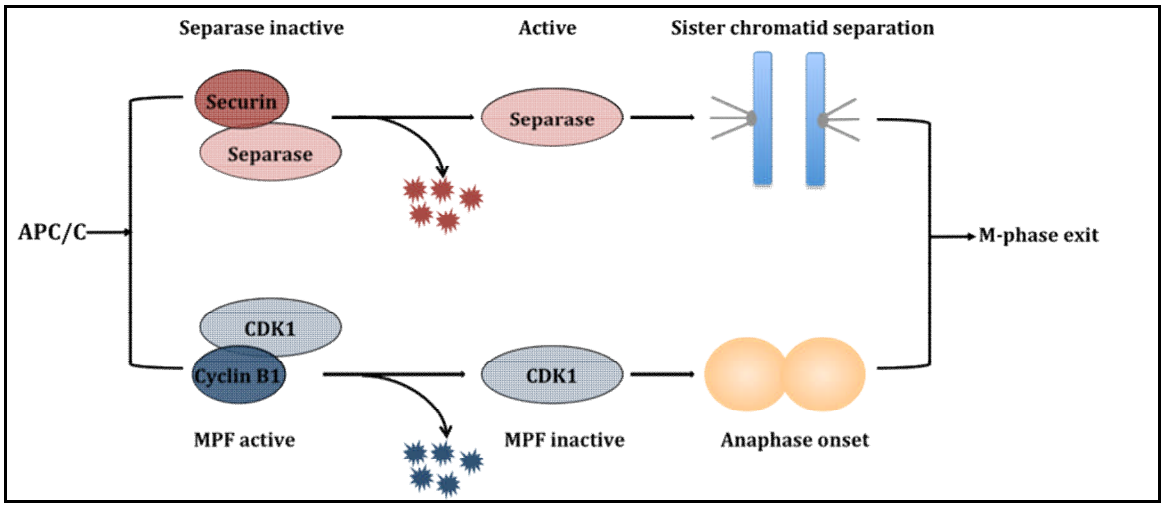

Figure 1.4. Separase activation and MPF inactivation are required for M-phase exit. At the metaphase-to-anaphase transition, APC/C targets cyclin B1 and securin for proteolysis. Cyclin B1 destruction is crucial for MPF inactivation and anaphase onset. However, Securin degradation is required for separase activation, and so sister chromatid separation.

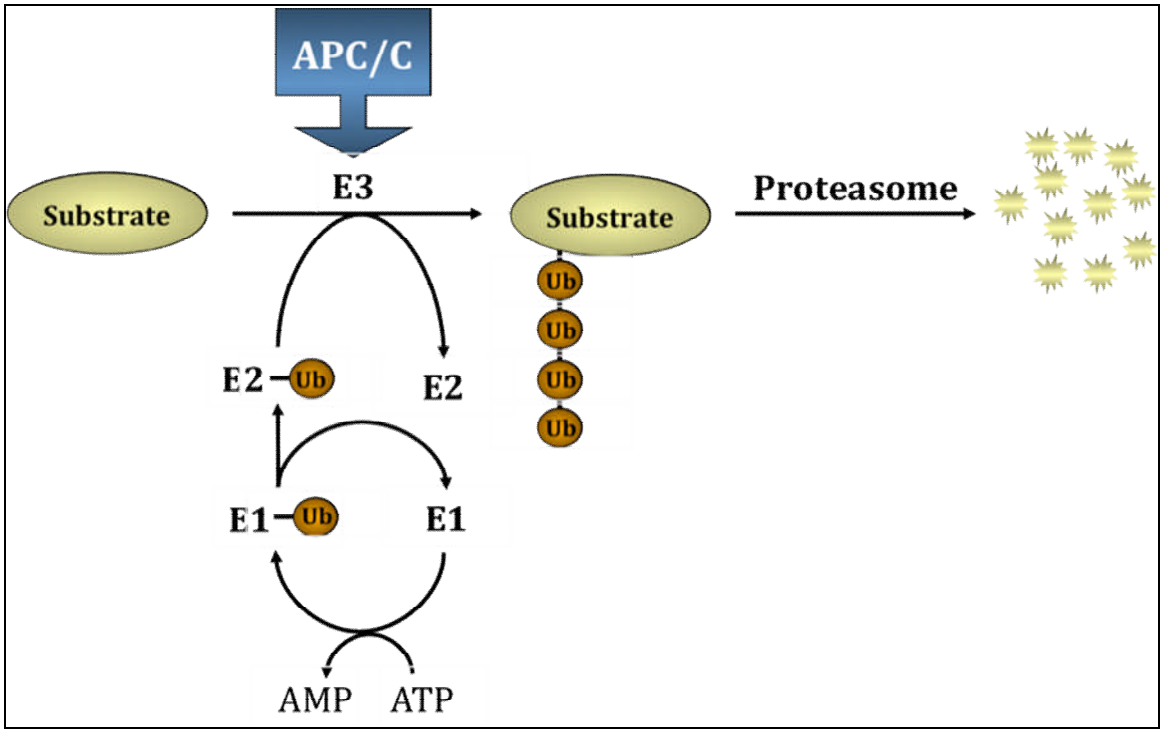

Figure 1.5. The ubiquitin pathway.

The transfer of ubiquitin to a target substrate is done in three steps. First, ubiquitin is activated by the ubiquitin-activating enzyme (E1) in a reaction that requires ATP. The activated ubiquitin is then transferred to the active site of the ubiquitin-conjugating enzyme (E2). Finally, an E3 ubiquitin ligase, such as the APC/C, transmits ubiquitin to the target protein, therefore triggering its recognition and destruction by the proteasome.

In addition to the core subunits, several co-activator proteins (non-core subunits) transiently associate with and activate the APC/C during specific periods of the cell cycle, and the best studied of these are Cdc20 (Cell division cycle 20) and Cdh1 (Cdc20 homologue 1) (Visintin et al., 1997). Cdc20 (also called *fizzy* in *Drosophila*) was initially identified in a mutant form of budding yeast that had similar arrest phenotypes as cells mutant for the APC/C subunits, and in *Drosophila* where the *fizzy* mutants failed to degrade the mitotic cyclins (Dawson et al., 1993; Hartwell et al., 1970; Hartwell et al., 1973; Sigrist et al., 1995). Similarly, Cdh1 (also known as *fizzy*-related in *Drosophila*) was found to be required for the activity of the APC/C during late mitosis and G1 in budding yeast, and for maintaining the low levels of cyclins during G1 in *Drosophila* (Schwab et al., 1997; Schwab et al., 2001; Sigrist and Lehner, 1997; Visintin et al., 1997).

These two co-activators are characterised by the presence of specific APC/C binding sequence elements, known as the IR-tails (Isoleucine-Arginine dipeptide motifs) (Visintin et al., 1997). They also contain WD40 domains (Tryptophan-Aspartate repeats) in their C terminus, which fold into seven-bladed propeller like structures that are believed to recognise the APC/C substrates by interacting with specific recognition elements, known as D-box, KEN-box and CRY-box (Glotzer et al., 1991; Pfleger and Kirschner, 2000; Reis et al., 2006b; Visintin et al., 1997). D-box (Destruction-box) recognition elements contain the consenus amino acid sequence RXXLXXXN (Glotzer et al., 1991), whereas the KEN-box is composed of a KEN amino acid sequence (Pfleger and Kirschner, 2000). CRY-box, however, consists of the consenus sequence CRYXPS (Reis et al., 2006b).

Different APC/C complexes associated with different co-activators tend to have different substrate specificities. APC/C^{Cdc20} recognises proteins that contain a D-box such as securin and cyclin B1, whereas APC/C^{Cdh1} is able to recognise proteins with either a D-, KEN- or CRY-box such as securin, cyclin B1 and Cdc20 (Hagting et al., 2002; Visintin et al., 1997).

1.3.3.1. Regulation of the APC/C during the mitotic cell cycle

In mitosis the APC/C is activated in prometaphase and remains active until late G1 but its activity is tightly controlled by various mechanisms to ensure proper timing of degradation of each of its substrates. Initial activation of the APC/C involves phosphorylation of its various subunits by the mitotic kinases Plk1 (Polo-like kinase 1) and CDK1, and the recruitment of Cdc20 (Hershko et al., 1994; Kraft et al., 2003). However, premature APC/C^{Cdc20} mediated ubiquitination of its substrates is prevented through the association of Cdc20 with the early mitotic inhibitor (Emi1) (Miller et al., 2006; Reimann et al., 2001). Also, via Cdc20 phosphorylation by CDK1 and MAPK, resulting in Cdc20 recognition and inhibition by the components of the spindle assembly checkpoint (SAC) (Chung and Chen, 2003).

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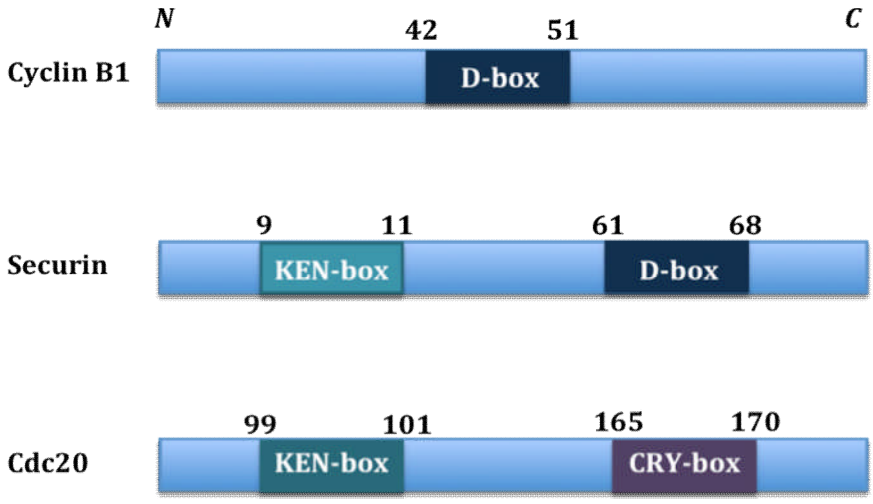

Figure 1.6. The APC/C recognition elements in cyclin B1, securin and cdc20

APC/C^{Cdc20} and APC/C^{Cdh1} recognise their substrates by interacting with different recognition elements, most commonly D-box, KEN-box and CRY-box. D-box containing proteins are targeted for destruction by either APC/C^{Cdc20} or APC/C^{Cdh1}, whereas, substrates with either KEN- or CRY-box are recognised by APC/C^{Cdh1}. The residues numbers indicate the location of the destruction motifs within each APC/C substrate in mouse.

In prophase, Emi1 becomes phosphorylated by Plk1 and is targeted for destruction through ubiquitination by SCF (Skp1/Cullin/F-box protein complex), which is another E3 ubiquitin ligase that is important in controlling the G1/S and G2/M transitions, whereas the APC/C is required for mitotic progression and exit (Hansen et al., 2004; Moshe et al., 2004). Upon Emi1 destruction, APC/C^{Cdc20} becomes active against the early mitotic substrates, such as Cyclin A and Nek2A (den Elzen and Pines, 2001; Geley et al., 2001; Hames et al., 2001), but it remains inhibited by the components of the spindle assembly checkpoint from prematurely targeting securin and cyclin B1 for degradation (Fang et al., 1998; Rieder and Maiato, 2004; Yu, 2002).

The spindle assembly checkpoint is a surveillance mechanism, which ensures the fidelity of chromosomes segregation through monitoring the state of their attachment to the spindle microtubules (Kops et al., 2005; Rieder et al., 1994). It consists of a network of proteins, and the key players include Bub1, Bub3, Mad1, Mad2, Mad3/BubR1, Mps1 and Aurora B. These proteins localize to the tensionless-unattached kinetochores, the structures that mediate the interactions between centromeric DNA and spindle microtubules, hence activating the SAC signalling pathway (Rieder et al., 1995). This in turn prevents anaphase onset and progression through the cell cycle via sequestration and inhibition of the APC/C co-activator, Cdc20 (Fang et al., 1998; Rieder et al., 1994; Rieder and Maiato, 2004; Yu, 2002). Therefore, the APC/C is incapable of ubiquitinating securin and cyclin B1, whose destruction is crucial for M-phase exit.

At anaphase onset, when all chromosomes achieve proper bipolar attachment to the spindle, the SAC is turned off and Cdc20 is released to trigger APC/C activation (Mao et al., 2003), which in turn targets securin and cyclin B1 for destruction by the proteasome, thereby allowing resumption of the cell cycle, sister chromatids separation and exit from mitosis.

Meanwhile, although Cdh1 levels are high, it is unable to associate with the APC/C due to its phosphorylation by CDK1 (Jaspersen et al., 1999). Following MPF inactivation via APC/C^{Cdc20}-induced degradation of cyclin B1, Cdh1 is dephosphorylated by the phosphatase cdc14, thus allowing its binding and activation of the APC/C (Jaspersen et al., 1999). APC/C^{Cdh1} induces the destruction of several regulatory proteins, known as the late mitotic proteins, such as Plk1 and the Aurora kinases, whose degradation allows exit from mitosis and entry into G1 (Castro et al., 2002; Lindon and Pines, 2004; Littlepage and Ruderman, 2002; Stewart and Fang, 2005). Accordingly, it was recently reported by Floyd *et al* (2008) that APC/C^{Cdh1} is required for the assembly of a spindle midzone at anaphase, and for normal timing of spindle elongation and cytokinesis through targeting the Aurora kinsases for destruction (Floyd et al., 2008).

The activity of APC/C^{Cdh1} continues through G1, where it targets Cdc20 and the mitotic cyclins for destruction, therefore preventing their premature accumulation, and so premature entry into S-phase (Prinz et al., 1998). Indeed, a study by Garcia-Higuera and colleagues (2008) demonstated that mouse MEFs lacking Cdh1 exhibit a slow exit from mitosis as well as a fast progression from G1 to S-phase (Garcia-Higuera et al., 2008). It

was also found by the same group that inactivation of mouse Cdh1 results in embryonic lethality due to placental insufeciency, which is believed to be arising from a failure of placental trophoblasts to re-duplicate, a phenotype that is consistent with the role of Cdh1 in controlling DNA replication (Garcia-Higuera et al., 2008).

In late G1 and during S phase, Cdh1 also becomes subject to ubiquitin-mediated degradation by the APC/C^{Cdh1} and SCF, respectively (Benmaamar and Pagano, 2005; Listovsky et al., 2004). It was demonstrated by Listovsky et al that addition of Cdh1 to *Xenopus* interphase extracts, which have an inactive APC/C, activates it to mediate its own destruction in a D-box dependent manner (Listovsky et al., 2004). Additionally, it was shown by Benaamar and Pagano that inhibition of SCF during S-phase leads to Cdh1 accumulation (Benmaamar and Pagano, 2005).

However, recent work on the nuclear transport factors Rae1 and Nup98 has challenged this view of APC/C^{Cdh1} being only active in late mitosis and during G1 (Jeganathan et al., 2005). It was found that mice with combined Rae1 and Nup98 haploinsufficiency exhibit premature separation of sister chromatids and severe aneuploidies, due to unscheduled APC/C^{Cdh1}-mediated destruction of securin during prometaphase (Jeganathan et al., 2005). This study demonstrates that securin associates with the APC/C^{Cdh1} during early mitosis, but its destruction is delayed by the nuclear transport factors Rae1 and Nup98 until the metaphase-to-anaphase transition (Jeganathan et al., 2005).

1.3.3.2. Regulation of the APC/C during the meiotic cell cycle

In meiosis, however, even less is known about the regulation of both APC/C^{Cdc20} and APC/C^{Cdh1} in higher organisms and most of our understanding comes from yeast, *Drosophila* and *Xenopus*. Cdc20, but not Cdh1, is believed to be the mediator of meiotic exit in eggs of species described thus far (Chang et al., 2004; Lorca et al., 1998). In *Xenopus* eggs, it was reported that Cdh1 is absent and is not expressed till the mid-blastula transition stage, and that Cdc20 inhibition results in prevention of exit from meiosis II upon egg activation (Chang et al., 2004; Lorca et al., 1998). Also in *Drosophila*, it was demonstrated that Cdh1 is absent during the early syncytial stages of embryogenesis, and it becomes functional only following cellularisation, where it targets cytoplasmic cyclin B1 for destruction (Chang et al., 2004; Raff et al., 2002; Sigrist and Lehner, 1997). Finally, Cdh1 knockdown using RNAi in *C.elegans* embryos does not affect their development (Fay et al., 2002), whereas depletion of Cdc20 leads to metaphase I arrest (Kitagawa et al., 2002).

In mice, on the other hand, Cdh1 is present during meiosis (Chang et al., 2004). However, unlike mitosis, the activity of APC/C^{Cdh1} precedes that of APC/C^{Cdc20}. Cdh1 appears to play an important role during prophase I and prometaphase I (Marangos et al., 2007; Reis et al., 2006a; Reis et al., 2007). It was shown that APC/C^{Cdh1} is required for maintaining the prophase I arrest by suppressing the rise in cyclin B1 levels (Marangos et al., 2007; Reis et al., 2006a). During this period, however, the activity of APC/C^{Cdh1} is restrained by Emi1, hence preventing complete depletion of cyclin B1 such that enough pre-MPF is available in readiness for meiotic resumption (Marangos et al., 2007). This

was reported by Marangos et al, who found that depletion of Emi1 through injection of morpholino antisense oligonucleotides (morpholinos) in GV mouse oocytes delays entry into meiosis I through inhibition of cyclin B1 accumulation (Marangos et al., 2007).

At GVBD, Emi1 is targeted for destruction through ubiquitination by SCF, resulting in an increase in APC/C^{Cdh1} activity (Marangos et al., 2007). Following germinal vesicle breakdown and during prometaphase I, APC/C^{Cdh1} targets Cdc20 for degradation in preference to cyclin B1 and securin, thereby delaying the onset of APC/C^{Cdc20} activity and anaphase I (Reis et al., 2007). It was reported by Reis et al that injection of mouse oocytes with Cdh1 morpholinos accelerates progression through meiosis I, resulting in premature anaphase onset and chromosomes segregation, but non-disjunction of homologs (Reis et al., 2007). Therefore, this delay in the onset of APC/C^{Cdc20} activity seems to be crucial in allowing more time for homologs to congress properly on the metaphase I plate (Reis et al., 2007).

The rising levels of MPF activity during meiosis I eventually lead to inactivation of APC/C^{Cdh1} (Jaspersen et al., 1999), which then allows the accumulation of Cdc20. APC/C^{Cdc20} activity, however, remains on hold by the components of the SAC until anaphase I onset, when it induces the degradation of securin and cyclin B1, thus promoting homolog segregation and exit from meiosis I (Brunet et al., 2003; Homer et al., 2005a; Homer et al., 2005b; Wassmann et al., 2003; Zhang et al., 2004).

Between MI and II, MPF activity does not experience a mitotic like decrease; instead it undergoes a transient decline to about 50% of its original activity, which is required for the MI-to-MII transition and the return to metaphase II (Hashimoto and Kishimoto, 1986; Hashimoto and Kishimoto, 1988; Madgwick et al., 2006; Madgwick and Jones, 2007; Mattioli et al., 1991). This is achieved by the Emi1 homologue, Emi2, which limits APC/C^{Cdc20} activity, thereby allowing cyclin B1 stability and a return of MPF activity (Madgwick et al., 2006; Ohe et al., 2007; Schmidt et al., 2004). In *Xenopus* and mouse oocytes, it was found that depletion of Emi2 reduces cyclin B1 re-accumulation after meiosis I, prevents entry into meiosis II and induces DNA replication (Madgwick et al., 2006; Ohe et al., 2007).

Following entry into the second meiotic division and metaphase II arrest, APC/C^{Cdc20} is kept inactive by the components of SAC and CSF (Madgwick and Jones, 2007; Tunquist and Maller, 2003). At fertilization, the Ca^{2+} oscillations that are transduced by calmodulin, activate calmodulin-dependent protein kinase II and the phosphatase calcineurin, leading to degradation of the components of CSF and activation of APC/C^{Cdc20} (Jones, 2005; Runft et al., 2002). This induces securin and cyclin B1 destruction, which in turn results in anaphase II onset and sister chromatid separation. Following PB2 extrusion, APC/C^{Cdh1} activity increases again and it remains high until pronuclei formation in the 1-cell embryo (Chang et al., 2004). Having provided an overview of the key players in the control of oocyte maturation, I will now focus on the molecular mechanisms underlying the cell cycle transitions in meiosis in mammalian oocytes.

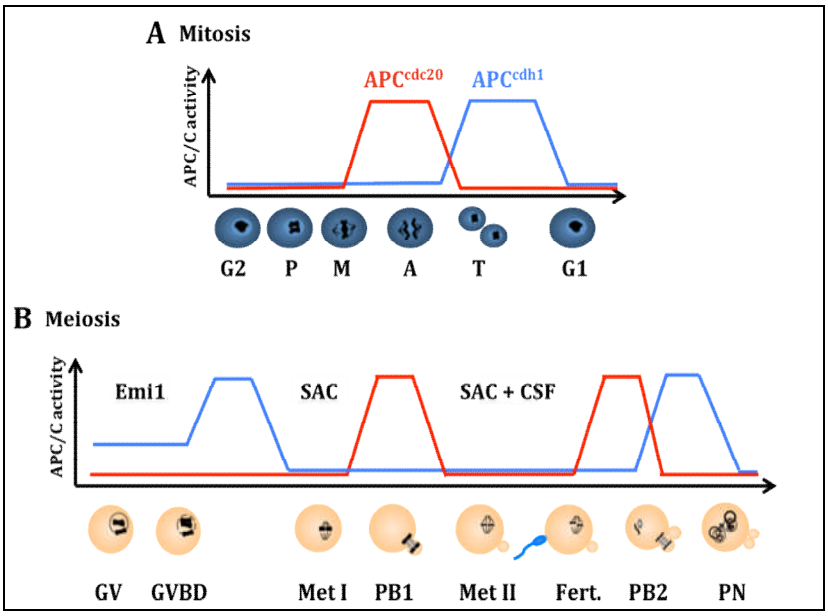

Figure 1.7. Profile of APC/C^{Cdc20} and APC/C^{Cdh1} activities during mitosis and meiosis. (**A**) In mitosis, at the metaphase-to-anaphase transition APC/C^{Cdc20} becomes fully active, targeting securin and cyclin B1 for degradation, thus allowing chromosomes separation. This is followed by APC/C^{Cdh1} activation at telophase, which continues until late G1, where it targets late mitotic proteins including Cdc20. (**B**) In meiosis, however, APC/C^{Cdh1} activity is present during prophase I arrest and prometaphase I. APC/C^{Cdh1} is first required for maintaining the GV arrest by suppressing cyclin B1 levels. However, during this period, APC/C^{Cdh1} is restrained by Emi1 to prevent complete depletion of cyclin B1 such that enough pre-MPF is available for meiotic resumption. After GVBD and during prometaphase I, APC/C^{Cdh1} is active again, and targets Cdc20 for degradation in preference to cyclin B1, therefore delaying the onset of APC/C^{Cdc20} activity and anaphase I. The rising levels of MPF activity eventually lead to inactivation of APC/C^{Cdh1} and Cdc20 accumulation. APC/C^{Cdc20} activity, however, remains on hold by SAC until anaphase I onset, when it induces securin and cyclin B1 destruction, thus allowing homlog segregation. Following entry into meiosis II, APC/C^{Cdc20} is kept inactive during metaphase II arrest by the components of SAC and CSF. At fertilization, APC/C^{Cdc20} is active again, therefore promoting sister chromatids separation. This is followed by APC/C^{Cdh1} activation after PB2 extrusion, which continues until pronuclei formation.

1.4. The molecular mechanisms governing cell cycle transitions in meiosis in mammalian oocytes

1.4.1. Prophase I arrest in oocytes

Following entry into meiosis, oocytes become arrested at prophase of the first meiotic division for a period lasting from few months (mice) to many years (humans), until puberty when the female becomes sexually mature. It is well established that this meiotic arrest is maintained by the high levels of intra-oocyte cyclic adenosine 3', 5'-monophosphate (cAMP) (Conti et al., 2002). It is also known that mammalian oocytes, in contrast to frog oocytes, when released from their follicles undergo spontaneous meiotic maturation (Pincus and Enzmann, 1935). This, however, can be prevented by addition of agents that raise intracellular cAMP, such as membrane-permeable cAMP analogs, cAMP phosphodiesterase (PDE) inhibitors or adenylyl cyclase (AC) activators, to the culture media (Cho et al., 1974; Conti et al., 2002; Dekel and Beers, 1978; Olsiewski and Beers, 1983).

The high cAMP level that is required for the maintenance of the prophase arrest is generated by the oocyte itself. This is achieved by way of a constitutively active G protein-coupled receptor (GPR3) in the oocyte plasma membrane that stimulates a heterotrimeric G protein of the G_s family, which in turn activates adenylyl cyclase to cause an elevation of cAMP. Consistent with this idea, it was reported that oocytes from GPR3 knockout mice fail to arrest at prophase I and undergo spontaneous meiotic maturation within intact follicles and in the absence of LH stimulation (Mehlmann et al.,

2004). Also, it was found that microinjection of a G_s blocking antibody into follicle-enclosed oocytes causes exit from prophase I arrest (Mehlmann et al., 2002). Moreover, AC3 deficient mice oocytes undergo GVBD within their follicles (Horner et al., 2003). Taken together, these findings demonstrate the essential role of the GPR3/G_s/AC pathway in the maintenance of the meiotic arrest.

In mammals, the somatic cells that surround the oocyte within the ovarian follicle are also required for maintaining the GV arrest, as removal of oocytes from their follicles causes spontaneous meiotic resumption due to the decrease in intra-oocyte cAMP levels (Pincus and Enzmann, 1935; Vivarelli et al., 1983). This suggests that the surrounding somatic cells also contribute to the maintenance of the elevated cAMP levels. Although the exact mechanism is still under investigation, it is believed that this is achieved through an inhibitory signal that diffuses from the follicular cells to the oocyte via gap junctions (Bornslaeger and Schultz, 1985; Larsen et al., 1986; Racowsky et al., 1989; Webb et al., 2002). Indeed, application of gap junction inhibitors to rat follicles causes a decrease in intra-oocyte cAMP and meiotic resumption (Sela-Abramovich et al., 2006). One possibility is that the signal that diffuses from the somatic cells into the oocyte is cAMP itself. However, it was found that the amount of cAMP generated by the follicular cells is insufficient to maintain the prophase arrest upon inhibition of cAMP generation in the oocyte (Mehlmann et al., 2002; Mehlmann, 2005b). Recently, it was reported that the inhibitory signal might well be cyclic guanosine 3', 5'-monophosphate (cGMP), which is produced by the follicular somatic cells and diffuses into the oocyte via gap junctions. Once inside the oocyte, it is believed that cGMP maintains the prophase arrest through

inhibiting cAMP hydrolysis by the phosphodiesterase type 3A (PDE3A) (Norris et al., 2009; Tornell et al., 1990). Consistent with this hypothesis, intra-oocyte cGMP decreases after removal of the oocyte from the follicle, and meiotic resumption is delayed upon injection of cGMP into the oocyte (Tornell et al., 1990). Also, decreasing cGMP in follicle-enclosed oocytes via injection of the cGMP-specific phosphodiesterase PDE9A, causes PDE3A-dependant decrease in cAMP levels and meiotic resumption (Norris et al., 2009).

The molecular mechanism underlying cAMP-mediated arrest in mammalian oocytes is not fully understood. However, it is generally accepted that the main downstream target of cAMP is Protein Kinase A (PKA), leading to its activation. Active PKA plays a crucial role in the maintenance of the GV arrest, given that injection of PKA inhibitors causes resumption of meiosis, and its over-activation blocks meiotic maturation (Bornslaeger et al., 1986; Duckworth et al., 2002; Maller and Krebs, 1977). PKA-mediated Prophase I arrest is believed to be achieved through prevention of pre-MPF activation, via direct regulation of the kinase Wee1 and the phosphatase cdc25 (Duckworth et al., 2002; Han et al., 2005; Pirino et al., 2009). As mentioned before, the newly assembled MPF is maintained in an inactive pre-MPF form through phosphorylation of CDK1 at Thr-14 and Tyr-15 by Wee1 (Fattaey and Booher, 1997; Mueller et al., 1995). These phosphorylation events can be reversed by cdc25 phosphatase, resulting in MPF activation and promotion of cell cycle progression (Lincoln et al., 2002). It has recently been reported that PKA directly phosphorylates both Wee1 and cdc25. In mouse oocytes, it was demonstrated that PKA-mediated

phosphorylation of Wee1 enhances its kinase activity, and therefore ability to prevent meiotic resumption through inhibition of MPF (Han et al., 2005). Phosphorylation of cdc25 by PKA, however, leads to its inactivation. In frog oocytes, it was reported that PKA-mediated phosphorylation of cdc25 results in its binding to 14-3-3 and sequestration away from pre-MPF, hence inhibiting pre-MPF activation by cdc25 and preventing exit from the prophase arrest (Duckworth et al., 2002).

1.4.2. Resumption of meiosis I

Mammalian oocytes remain arrested in prophase I until the preovulatory surge of LH from the pituitary gland (Mehlmann, 2005a). LH stimulates meiotic resumption indirectly, through acting on the mural granulosa cells in the outer region of the follicle rather than the oocyte itself, since the LH receptors are only found on the mural granulosa cells (Peng et al., 1991). The LH signal is then conveyed inward from the mural granulosa cells, through the cumulus cells to the oocyte, where it causes a decrease in cAMP levels. This consequently results in meiotic resumption through the inactivation of PKA, and the subsequent activation of pre-MPF that is brought about via the inhibition of CDK1 phosphorylation by Wee1 and the promotion of its dephosphorylation by cdc25.

Although, it is not completely understood how the action of LH on the somatic cells decreases oocyte cAMP, two hypotheses (pathways) have been put forth. In the first pathway, it is believed that LH causes the synthesis of EGF-like peptides by the follicular cells, resulting in activation of the EGF receptors and the MAPK cascade in the somatic cells (Norris et al., 2008). The active MAPK, in turn, triggers the closure of gap

junctions throughout the somatic compartment via phosphorylation of connexin 43 (CX43), a major component of the somatic cell junctions (Beyer et al., 1989; Norris et al., 2008; Okuma et al., 1996). The gap junction's closure reduces the flux of cGMP from the follicular cells to the oocyte, leading to a decrease in intra-oocyte cGMP levels (Norris et al., 2009; Norris et al., 2008). This loss of cGMP allows PDE3A to consume cAMP leading to meiotic resumption (Norris et al., 2009; Norris et al., 2008). Although this pathway is sufficient to cause exit from the prophase arrest, another pathway that does not involve the MAPK-dependent closure of gap junctions must exist, given that inhibition of MAPK activation using UO126 prevents gap junction closure but not meiotic resumption in response to LH (Norris et al., 2009). This suggests that the second pathway can cause a decrease in oocyte cAMP levels and meiotic progression without closure of gap junctions. Indeed, a recent work by Norris *et al* have demonstrated that LH also decreases the level of cGMP in the somatic cells of the follicle, leading to a decline in oocyte cGMP (Norris et al., 2009). This consequently results in PDE3A activation, a decrease in intra-oocyte cAMP levels and meiotic resumption (Norris et al., 2009).

Exit from prophase I arrest is also associated with a rapid increase in cyclin B1 synthesis, although most species contain enough pre-MPF to initiate GVBD. The timing and requirement for cyclin B1 synthesis varies between different species. In mouse, for example, cyclin B1 translation is only enhanced following GVBD (Fulka et al., 1986; Polanski et al., 1998). In *Xenopus* oocytes, however, cyclin B1 synthesis is a prerequisite to GVBD. This was demonstrated by Sagata and co-workers, who showed

that progesterone-dependent MPF activation and GVBD in *Xenopus* oocytes were suppressed upon the expression of a dominant negative mutant of CDK1, which was expected to bind cyclin B1 and to form inactive MPF complexes (Furuno et al., 1994). This result indicates that *de novo* synthesis of cyclin B1 is required for MPF activation and resumption of meiosis in *Xenopus* oocytes. This species-specific requirement for cyclin B1 synthesis might be due to the difference in the ratios of CDK1/cyclin B1 between species. The concentration of cyclin B1 in GV-arrested mouse ooctyes is about sevenfold higher than that of CDK1 (Kanatsu-Shinohara et al., 2000). In *Xenopus* oocytes, however, cyclin B1 levels are fiftyfold lower than those of CDK1 (Kobayashi et al., 1991). This suggests that in mouse oocytes most of CDK1 molecules are in complexes with cyclin B1, and therefore protein synthesis is dispensable for meiotic resumption. In *Xenopus* oocytes, on the other hand, cyclin B1 synthesis is necessary for CDK1 activation and the onset of maturation, given that most of CDK1 is free.

In addition to cyclin B1 synthesis, meiotic resumption is coupled with the translation of a pool of maternal mRNAs, which are stored in the GV oocyte with short poly (A) tails, generally 30-50 nucleotides, and therefore are translationally repressed (masked) (Richter, 1999). Upon release from the prophase arrest, those dormant mRNAs undergo polyadenylation, the process by which the poly (A) tails are extended to 80-250 residues, resulting in their unmasking and promotion of translation (Richter, 1999). One such mRNA that undergoes this type of modification is the one encoding c-MOS kinase. It is believed that PKA inactivation stimulates the polyadenylation of c-Mos mRNA leading to its translation, and activation of the MAPK phosphorylation cascade in the oocyte

(Dekel, 2005; Gebauer et al., 1994). As previously mentioned, although the c-Mos/MEK/MAPK/$p90^{rsk}$ pathway is not necessary for exit from prophase I arrest, several lines of evidence suggest that there is a link between MAPK cascade and pre-MPF activation. It has been reported that MAPK activation slows the rate of CDK1 inactivation in *Xenopus* oocytes (Abrieu et al., 1997). More importantly, it was found that $p90^{rsk}$ specifically associates with and phosphorylates Wee1 in vitro, decreasing its ability to phosphorylate CDK1 (Palmer et al., 1998). These results suggest that, at least in frog oocytes, the MAPK pathway is able to promote MPF activation through the downregulation of CDK1 inhibitory phosphorylation by Wee1.

An additional potential contribution of the c-Mos/ MAPK cascade to MPF activation at GVBD is through promoting the synthesis of its regulatory subunit, cyclin B1. It has been demonstrated that there is a strong temporal correlation between MAPK activity and cyclin B1 translation. It was found that *de novo* synthesis of cyclin B1 in c-Mos depleted mouse oocytes is suppressed (Okeefe et al., 1991). Also, cyclin B1 mRNA polyadenylation does not occur in response of progesterone if MAPK activity is inhibited in frog oocytes (deMoor and Richter, 1997). All these results suggest that MAPK activation is also required for the translation of cyclin B1 mRNA through promoting its polyadenylation.

Following GVBD, the chromatin recondenses and the replicated chromosomes align themselves on the metaphase plate of the first meiotic spindle, such that each pair of homologous chromosomes is attached to opposite poles of the spindle. Therefore, at the

onset of anaphase I, the homologs separate and half are lost from the oocyte during the asymmetric cell division, which gives rise to a large secondary oocyte and a small PB1. Without intervening S phase, the haploid oocyte proceeds directly to metaphase of the second meiotic division and then arrests again, with chromosomes aligned on the metaphase II spindle, until fertilization or parthenogenetic activation of the egg.

1.4.3. Metaphase II arrest in oocytes

To prevent parthenogenesis or completion of meiosis before fertilization, vertebrate eggs are arrested at metaphase of meiosis II with high MPF by a cytoplasmic activity termed cytostatic factor (CSF), which inhibits the APC/C from mediating cyclin B1 destruction (Harper et al., 2002; Madgwick and Jones, 2007; Masui and Markert, 1971; Peters, 2002). Unlike MPF, CSF identity has never been completely resolved, despite their simultaneous identification (Masui and Markert, 1971). CSF was first characterised by Masui and Markert in 1971 as an activity present in mature Met II-arrested eggs that was able to induce metaphase arrest when injected into dividing embryonic cells (Masui and Markert, 1971). It was found that CSF activity accumulates during oocyte maturation, is necessary and sufficient for metaphase arrest, and is inactivated during fertilization in a Ca^{2+}-dependent manner (Masui and Markert, 1971).

In frog and mammalian eggs, there is much evidence to suggest that the c-Mos/MEK/MAPK/$p90^{rsk}$ pathway is an important component of CSF. It was found that microinjection of c-Mos mRNA, active MAPK or active $p90^{rsk}$ in *Xenopus* embryos induces a metaphase arrest (Gross et al., 1999; Haccard et al., 1995; Sagata et al., 1989b).

Conversely, immunodepletion of c-Mos from Met II-arrested egg extracts causes a loss of the cleavage-arresting activity in the injected embryos (Sagata et al., 1989b). Also, the oocytes of *c-mos* knockout mouse, following "normal" progression through MI, they fail to arrest at MII and instead they spontaneously activate and extrude a second polar body (Colledge et al., 1994). Furthermore, treatment of Met II-arrested mouse eggs with the MEK inhibitor, UO126, induces their parthenogenetic activation (Tatemoto and Muto, 2001; Tong et al., 2003). All these results suggest that the MAPK pathway is necessary for the maintenance of the CSF arrest. However, it is unlikely to constitute the full CSF activity and the most compelling evidence is that although the MAPK cascade has already been activated before MI, it does not cause MI arrest (Fan et al., 2002). Also, MPF inactivation at fertilization precedes MAPK inactivation, showing that MAPK activity might be required for the establishment of the CSF arrest but its inactivation is not necessary for the release from the MII arrest (Fan et al., 2002).

Recently, the egg specific protein Emi2 has been identified as the molecule directly responsible for APC/C inhibition during CSF arrest in both *Xenopus* and mice (Schmidt et al., 2005; Shoji et al., 2006). It has been proven that Emi2 satisfies the three historical criteria for the molecular identification of CSF: **(i)** it accumulates during egg maturation (Schmidt et al., 2005), **(ii)** it is necessary and sufficient for metaphase arrest, since Emi2 deficient oocytes fail to enter MII after completion of MI (Madgwick et al., 2006; Ohe et al., 2007) and knocking down its activity in Met II-arrested eggs results in their parthenogenetic activation (Shoji et al., 2006), and finally **(iii)** it is rapidly degraded in response to Ca^{2+} signal (Liu and Maller, 2005; Madgwick et al., 2006; Rauh et al., 2005).

Emi2 blocks APC/C^{Cdc20} activity by directly binding to Cdc20, therefore allowing cyclin B1 re-accumulation following the MI to MII transition, and so MPF reactivation, until fertilization (Schmidt et al., 2005).

In frog eggs, the mechanism for establishment and maintenance of CSF arrest has just been reported, with the authors proposing the existence of a link between the long-standing CSF candidate c-Mos/MEK/MAPK/$p90^{rsk}$ pathway and Emi2 (Inoue et al., 2007; Nishiyama et al., 2007a; Tung et al., 2005). This study shows that in *Xenopus* eggs, Emi2 is directly phosphorylated by the kinase $p90^{rsk}$ resulting in recruitment of the protein phosphatase 2A (PP2A) (Nishiyama et al., 2007a; Wu et al., 2007). PP2A in turn dephosphorylates Emi2 at the MPF phosphorylation sites that would disrupt the interaction between Emi2 and APC/C^{Cdc20} (Wu et al., 2007). However, in contrast to frog, the link between $p90^{rsk}$ and Emi2 is unlikely to exist in mammalian eggs, given that constitutively active mutant forms of Rsk fail to induce the Met II arrest when injected in dividing mouse embryos or *mos*$^{-/-}$ oocytes (Dumont et al., 2005). Moreover, eggs from Rsk knockout mouse present normal CSF arrest (Dumont et al., 2005). Therefore, in mouse eggs, $p90^{rsk}$ might not be the downstream target of MAPK that is responsible for phosphorylating Emi2 during Met II arrest.

Upon fertilization of frog eggs, Emi2 is targeted for degradation. This is achieved through the Ca^{2+} signals that are transduced by CaMKII, which in turn phophorylates Emi2 and stimulates its association with the polo-like kinase, Plk1 (Hansen et al., 2006; Liu and Maller, 2005; Lorca et al., 1993; Schmidt et al., 2005). This is followed by Plk1-

mediated phosphorylation of Emi2 to generate a phosphodegron, which is then recognised by the SCF-E3 ubiquitin ligase, resulting in polyubiquitination of Emi2 and targeting for destruction by the proteasome (Hansen et al., 2006; Liu and Maller, 2005; Rauh et al., 2005; Schmidt et al., 2005). The resultant Emi2 degradation unleashes the APC/C^{Cdc20} to initiate anaphase II and complete the second meiotic division (Hansen et al., 2006; Liu and Maller, 2005; Rauh et al., 2005). This mechanism, however, has not yet been confirmed in mammalian eggs. Although, mouse Emi2 was also found to contain specific motifs for phosphorylation by Plk1 and CaMKII.

As well as CaMKII, the Ca^{2+}-dependent phosphatase calcineurin was proven to be involved in the exit from CSF arrest (Mochida and Hunt, 2007; Nishiyama et al., 2007b). Recently, it was reported that inhibition of calcineurin in the presence of Ca^{2+}, delays cyclin B1 destruction and prevents exit from meiosis II (Mochida and Hunt, 2007; Nishiyama et al., 2007b). Also, incubation of purified APC/C from CSF egg extracts with calcineurin causes dephosphorylation of the APC/C core subunit (Apc3) and its co-activator (Cdc20) (Mochida and Hunt, 2007). Therefore, these calcineurin-mediated dephosphorylation events might be crucial for APC/C^{Cdc20} activation and exit from CSF arrest.

1.4.4. Resumption of meiosis II

Fertilization of the mammalian egg by sperm is associated with an explosive increase in cytosolic Ca^{2+}, which is both necessary and sufficient to trigger resumption of meiosis II following the metaphase arrest (Stricker, 1999). The Ca^{2+} signal spreads through the egg

in a wave, starting at the point of sperm entry. In contrast to frog, whose eggs have a monotonic and short rise in $[Ca^{2+}]_i$ upon fertilization, mammalian eggs display a series of low frequency Ca^{2+} transients (oscillations) lasting for hours after sperm-egg fusion (Jones, 2004). Ca^{2+} oscillations were first demonstrated in fertilized mouse eggs by Cobbold and colleagues in the early 1980s (Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985). Using the Ca^{2+} specific photoprotein aequorin they found that, the fertilized egg generated a series of low frequency Ca^{2+} oscillations that lasted for several hours. Ca^{2+} is released from the endoplasmic reticulum (ER), and its release is regulated by ligand-gated ion channels. In other cells it is generally accepted that IP_3 (inositol 1,4,5-triphosphate) is the major effector in releasing Ca^{2+} via the IP_3 receptor (IP_3R) channel (Ayabe et al., 1995; Berridge et al., 2000; Miyazaki et al., 1993; Runft et al., 2002; Streb et al., 1983), and that IP_3 production is mediated by enzymes of the phospholipase-C (PLC) family through PIP_2 (phophatidylinositol-4,5-bisphosphate) hydrolysis (Jones and Nixon, 2000; Nuccitelli et al., 1993). However, in eggs, the mechanism of IP_3 production via PLC has been a matter of controversy. In brief, two hypotheses have been put forth. The first involves the binding of the sperm to a G protein-coupled receptor on the egg plasma membrane, which results in the activation of PLC, and so IP_3 synthesis (Halet et al., 2003; Jones, 2005; Nuccitelli et al., 1993). The most compelling evidence is that microinjecting mRNA of a G protein coupled-receptor into pig eggs induces their meiotic activation (Kim et al., 1998; Nuccitelli et al., 1993). The second hypothesis involves a soluble *sperm factor* that is introduced into the egg upon fertilization (Halet et al., 2003b; Jones, 2005; Nuccitelli et al., 1993; Swann, 1990a). The sperm factor theory was initially derived from experiments by Dale and

colleagues in 1985 showing that microinjection of soluble cytosolic sperm extracts into sea urchin eggs triggers the cortical reaction (Dale et al., 1985). This was further supported by a study done by Swann, with the author proposing that injection of sperm soluble factors into mammalian eggs results in fertilization-like Ca^{2+} oscillations (Swann, 1990).

Increasing evidence supports the latter of these scenarios, especially after the discovery of the sperm-specific PLC isoform (PLC zeta / PLCζ), which was initially cloned from mouse testes library (Saunders et al., 2002). It has been suggested that PLCζ is present at a sufficient concentration in the part of the sperm head that enters the egg first (Fujimoto et al., 2004; Saunders et al., 2002). It also has been reported that injection of PLCζ mRNA in mouse eggs generates Ca^{2+} oscillations very similar to those caused by sperm upon fertilization (Saunders et al., 2002), whereas other PLC isoforms were unable to stimulate such oscillations (Mehlmann et al., 2001). Moreover, depletion of PLCζ from sperm extracts removed their ability to induce Ca^{2+} rise (Knott et al., 2005). Finally, a recent study by Yoon et al also reported that human sperm lacking PLCζ is unable to initiate Ca^{2+} oscillations upon sperm-egg fusion, therefore leading to failure to activate the egg and sterility (Yoon et al., 2008). All these reports definitely support PLCζ as the most likely sperm factor candidate; however its physiological importance at fertilization and during early embryo development remains to be demonstrated.

Whatever the exact mechanism, Ca^{2+} signals are transduced by calmodulin, which in turn associates with and activates calmodulin-dependent protein kinase II. Activation of

CaMKII leads to degradation of cyclin B1 and securin through the APC/C. The resultant cyclin B1 and securin destruction allows MPF inactivation and unleashes the protease separase, to initiate anaphase and complete the second meiotic division (Lorca et al., 1991; Lorca et al., 1993). In what follows, I will focus on the molecular mechanisms underlying the regulation of separase activity, and on how cells ensure that cohesin cleavage occurs at the right time and place, making possible the complete and accurate distribution of their chromosomes.

1.5. Chromosome attachment and segregation

A fundamental aspect of cell division is the maintenance of genomic integrity through accurate transmission of the duplicated genome into daughter cells. Errors in chromosomal distribution lead to cells with supernumerary or missing chromosomes (Hassold et al., 2007; Hassold and Hunt, 2001). The resulting aneuploidy is usually associated with many cancers, and is also the leading cause of human birth defects (such as trisomies), malformations of the embryo and spontaneous foetal abortions (Hassold et al., 2007; Hassold and Hunt, 2001). In order to avoid chromosomal missegregation, the products of DNA replication, the sister chromatids, are held together by the chromosomal cohesin complex, which prevents them from drifting apart after synthesis (Nasmyth et al., 2000; van Heemst and Heyting, 2000). This cohesion is also important in facilitating DNA repair by homologous recombination throughout G2, and allowing chromosomes to be aligned in the middle of the metaphase spindle and to be separated simultaneously at anaphase onset (van Heemst and Heyting, 2000).

1.5.1. Chromosome attachment: the cohesin complex

Newly replicated sister chromatids tend to remain linked by the cohesin complex from S-phase until the metaphase-to-anaphase transition, when cohesion is dissolved (Nasmyth et al., 2000; van Heemst and Heyting, 2000). This molecular glue, which is believed to embrace both sisters in a ring-like structure, consists of five core subunits: two SMC subunits that belong to the family of Structural Maintenance of Chromosome (Smc1 and Smc3) and three non-SMC subunits (Scc1, Scc3 and Pds5) (Darwiche et al., 1999; Guacci et al., 1997; Hartman et al., 2000; Losada et al., 1998; Michaelis et al., 1997; Panizza et al., 2000; Sumara et al., 2000; Toth et al., 1999). The components of the cohesin complex were first identified by genetic studies in yeast, in which they isolated five genes whose mutagenesis caused premature sister chromatid separation and high rates of chromosome loss (Michaelis et al., 1997; Panizza et al., 2000; Toth et al., 1999).

At anaphase onset, the resolution of sister chromatid cohesion is catalysed by the cysteine protease, separase, which cleaves the Scc1 subunit (Uhlmann et al., 1999; Uhlmann et al., 2000). Separase also triggers chromosomes separation during meiosis by cleaving the Scc1 meiotic homolog (Rec8), which is believed to confer some specialized meiotic purposes, such as formation of the synaptonemal complex, reciprocal recombination and protection of centromeric cohesion (Deveaux and Smith, 1994; Klein et al., 1999; Lorenz et al., 2004; Parisi et al., 1999). In plants and fission yeast, it has been reported that loss of Rec8 results in disruption of the monopolar orientation of sister kinetochores, leading to equational rather than reductional division during meiosis I, which cannot be rescued by expression of Scc1 (Chelysheva et al., 2005; Watanabe and Nurse, 1999; Yokobayashi

et al., 2003). This implies that the meiotic cohesin complex also plays a crucial role in establishing the mono-orientation of the MI kinetochores.

1.5.2. Chromosome segregation: dissolving cohesion

In somatic cells and during mitosis, centromeric cohesin is lost at anaphase onset by the protease separase (Uhlmann et al., 1999; Uhlmann et al., 2000; Waizenegger et al., 2000). Cohesin bound to chromosome arms, however, is removed during early stages of mitosis via the "prophase pathway" (Waizenegger et al., 2000). This mechanism does not depend on separase-mediated Scc1 cleavage, but it does require the two mitotic kinases Plk and Aurora B (Losada et al., 2002; Sumara et al., 2002). Accordingly, it was demonstrated that Plk directly phosphorylates Scc1 and Scc3 in *vitro* (Sumara et al., 2002). Also, phosphorylated cohesin when incubated with sperm chromatin associate less efficiently to DNA than the non-phosphorylated one (Sumara et al., 2002). Therefore, the release of the cohesin complexes during the prophase pathway might be triggered by the phosphorylation of its two subunits (Scc1 and Scc3) by Plk. However, the role of Aurora B in this process is less clear and requires further investigation.

In contrast to mitosis, during meiosis cohesins are lost in a stepwise manner and the whole process is separase-dependent (Kudo et al., 2006; Lee et al., 2006). Removal of cohesion from chromosome arms does not take place until anaphase I onset, hence allowing homologs segregation during meiosis I (Lee et al., 2006). Whereas, centromeric cohesin is only cleaved during anaphase II, leading to sister chromatid separation (Lee et al., 2006).

During meiosis I and mitotic prometaphase, protection of centromeric cohesin is achieved by a specific cohesin protector, called shugoshin (Sgo), which means “guardian spirit” in Japanese (Kitajima et al., 2004). Mammals have two shugoshin homologs, Sgo1 and Sgo2, which are expressed in both somatic and germ cells. Nonetheless, Sgo1 is the key protector of centromeric cohesin during mitosis, whereas the predominant role of Sgo2 is during meiosis. Indeed, depletion of human Sgo1 causes removal of the whole bulk of cohesin including the centromeric one during prometaphase, and knockdown of Sgo2 in mouse oocytes results in loss of centromeric cohesion and separation of sister chromatids by the time the oocytes enter metaphase II (Kitajima et al., 2004; Kitajima et al., 2005; Lee et al., 2008; Llano et al., 2008; McGuinness et al., 2005; Tang et al., 2004).

Recruitment of shugoshin to the centromeric region of chromosomes is catalysed by the SAC protein Bub1, which also promotes shugoshin association with the protein phosphatase PP2A (Kitajima et al., 2005; Kitajima et al., 2006; Tang et al., 2004). The Sgo/PP2A complex, in turn, induces the dephosphorylation of centromeric cohesin, thus preventing its cleavage and dissociation from the chromatin (Kitajima et al., 2006; Riedel et al., 2006). Accordingly, it was reported that purified Sgo/PP2A complexes are able to dephosphorylate the cohesin subunits Scc1 and Scc3 that have been phosphorylated by Plk in *vitro* (Kitajima et al., 2006). Also, inactivation of PP2A in yeast and mouse oocytes causes premature sister chromatid separation and higher rate of aneuploidy (Lee et al., 2008; Mailhes et al., 2003; Riedel et al., 2006). At anaphase onset, the Sgo/PP2A complex is displaced away from the centromeres as a result of the microtubule pulling

tension generated across the sister kinetochores, therefore allowing phosphorylation of the centromeric cohesin by Plk, which seems to be crucial for cohesin cleavage by separase (Gomez et al., 2007).

1.5.3. Separase

1.5.3.1. Structure and function of separase

Separase was initially identified in a mutant form of fission yeast, in which cell division occurs in the absence of nuclear division, causing the cut (cell untimely torn) phenotype (Hirano et al., 1986). Separase homologs exist in most if not all eukaryotes examined so far, and its name varies from one organism to another: CUT1 in fission yeast, ESP1 in budding yeast and separin/separase in human (Hirano et al., 1986; Mcgrew et al., 1992; Nagase et al., 1996).

Separases are generally large proteins of 150-230 kDa, and only a C-terminal domain seems to be conserved amongst them. This conserved domain, the *separin* domain, contains the signature motif for the cysteine endopeptidases of the CD-clan subclass, a superfamily of proteases that also includes the caspases, which are involved in programmed cell death (Aravind and Koonin, 2002; Uhlmann et al., 2000). The N-terminal region of separase, however, does not show obvious conservation between species (Hornig et al., 2002). This large region is believed to be the site of interaction with separase's binding partner, securin (Hornig et al., 2002).

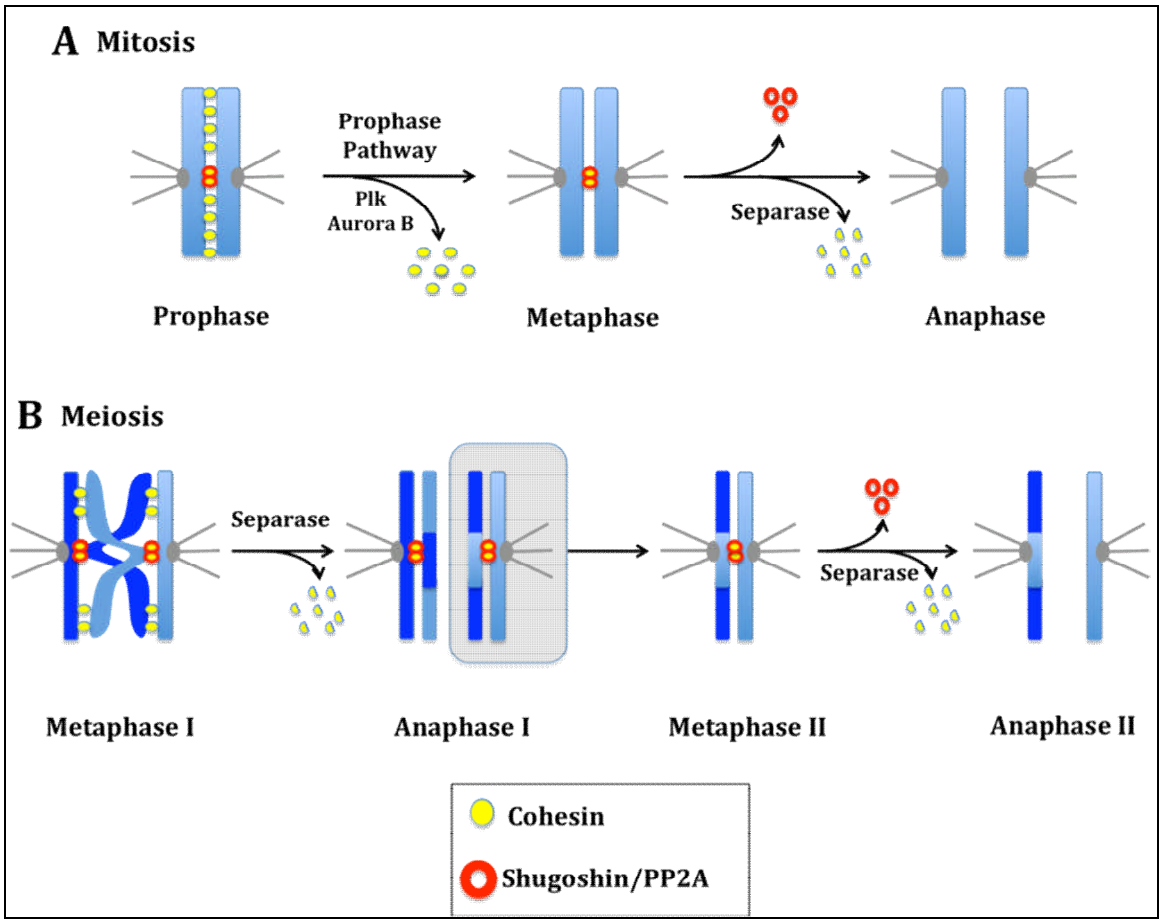

Figure 1.8. Cohesin cleavage during mitotic and meiotic cell divisions.

(A) In somatic cells and during mitosis, cohesin complexes bound to chromosomes arms are removed during prometaphase via the prophase pathway, which involves Plk and Aurora B-mediated phosphorylation of cohesin. Centromeric cohesin, however, is protected from prophase dissociation by the Shugoshin/PP2A complex. At anaphase onset and as a result of the displacement of the Shugoshin/PP2A complex, centromeric cohesin is destroyed by separase, leading to sister chromatid separation. **(B)** In contrast to mitosis, during meiosis cohesin is lost in a stepwise manner and the whole process is separase-dependent. During meiois I, only cohesion from chromosomes arms is lost, while centromeric cohesin is protected by Shugoshin/PP2A, therefore only allowing homologs separation at anaphase I. In meiosis II, the Shugoshin/PP2A complex is moved away from the centromeres vicinity, thus resulting in centromeric cohesin cleavage by separase and sisters' segregation during anaphase II.

In yeast, it was reported that esp1 mutants fail to dissociate Scc1 from their chromosomes and are completely defective in sister chromatid separation, whereas in the wild type cells Scc1 suddenly disappears from the chromosomes at anaphase onset (Ciosk et al., 1998; Michaelis et al., 1997). Also, it was demonstrated that budding yeast and human separases are capable of cleaving Scc1 in *vitro*, and the Scc1-derived peptide inhibitor binds the active site of yeast separase (Uhlmann et al., 2000; Waizenegger et al., 2000). These observations confirm the hypothesis that separase triggers chromosomes segregation through removal of cohesin, which solely relies on Scc1 cleavage. Indeed, separase recognises and cleaves the Plk-phosphorylated Scc1 at two distinct sites, each with an arginine residue (Uhlmann et al., 1999). Mutation of either site does not abolish the cleavage of Scc1, whereas simultaneous mutation of both arginines does, leading to lethality (Uhlmann et al., 1999).

The universal role of separase in triggering anaphase and chromosomes segregation through cohesin cleavage is an irreversible step, once executed it cannot be undone. This suggests that separase must be highly regulated by surveillance mechanisms that allow its activation and progression of the cell cycle into anaphase only if the cells are sure that their DNA is damage-free and chromosomes are properly attached to the spindle microtubules.

1.5.3.2. Separase regulation

The best-known regulator of separase is securin, which is a functionally conserved protein, although there is little conservation of its primary amino acid sequence except for

the presence of D-box and KEN-box motifs (Ciosk et al., 1998; CohenFix et al., 1996). It was originally identified in budding yeast as Pds1, whose depletion results in precocious separation of sister chromatids and increased rates of chromosome loss (Ciosk et al., 1998; Yamamoto et al., 1996). Securin, also known as the product of the pituitary tumour-transforming gene (PTTG) in vertebrates, CUT2 in fission yeast and PIM in *Drosophila*, binds to and inhibits separase for most of the cell cycle, but at metaphase onset it is targeted for degradation via ubiquitination by the APC/C, thus unleashing separase (Funabiki et al., 1996a; Funabiki et al., 1996b; Leismann et al., 2000; Pei and Melmed, 1997; Stratmann and Lehner, 1996).

In fission yeast and *Drosophila*, securin is essential for chromosomes disjunction and its absence leads to impairment in separase activity and failure in separating sister chromatids, suggesting that securin must be more than a simple inhibitor of separase (Funabiki et al., 1996b; Jensen et al., 2001; Leismann et al., 2000). Indeed, it was recently reported that securin inhibits the proteolytic activity of separase in a 2-fold manner (Funabiki et al., 1996a; Hornig et al., 2002). While inhibiting separase, securin is able to promote the spindle localization of the protease, and securin destruction then promotes the full activation of separase at anaphase (Hornig et al., 2002; Kumada et al., 1998). The specific architecture of the separase-securin complex is believed to be at the origin of this dual mechanism of regulation. Securin binds to both the N and C terminus of separase leading to their separation and therefore preventing them from inducing an activating conformational change at the protease active site (Hornig et al., 2002). Upon securin degradation at anaphase onset, the N and C terminus are free to interact and

therefore to induce the conformational change that is necessary for the activation of separase (Hornig et al., 2002).

Many studies have demonstrated the importance of securin degradation for normal anaphase. In frog eggs, it was demonstrated that over-expression of non-degradable securin results in failure to separate chromosomes (Zou et al., 1999). A similar phenotype was observed in human somatic cells upon the expression of stable securin (Hagting et al., 2002). However, despite its crucial role as an inhibitor of separase, it is not essential for viability of budding yeast, and for maintaining cohesion in mammals (Waizenegger et al., 2002; Yamamoto et al., 1996). It also has been reported that securin knockout mice are viable and fertile, and human cultured cells lacking securin execute mitosis normally (Huang et al., 2005; Waizenegger et al., 2002). This suggests that other securin-independent mechanisms must contribute to the control of separase. Regulation of phosphorylation of the Scc1 subunit of cohesin (Rec8, in meiosis) could be one such mechanism (Holland and Taylor, 2006; Waizenegger et al., 2002). Shugoshin-dependent protection of centromeric cohesin could be another mechanism (Kitajima et al., 2004). Also, recruitment of cyclin B1 by phosphorylated separase is an additional mechanism. It was reported that CDK1-mediated phosphorylation of separase is required for the stable binding of CDK1 to separase via its regulatory subunit cyclin B1, leading to inhibition of both protease and kinase (Gorr et al., 2005), which seems to be crucial for PB1 extrusion and exit from meiosis I in mouse oocytes (Gorr et al., 2006). In *vitro* and in *vivo* studies showed that non-degradable cyclin B1 blocks anaphase and chromosomes separation, which could be rescued by mutating the CDK1 phosphorylation sites in

separase (Chang et al., 2003; Herbert et al., 2003; Stemmann et al., 2001). Also, incubation of separase with CDK1 leads to separase inactivation despite the absence of securin (Gorr et al., 2005). CDK1 and securin are unable to bind separase simultaneously; therefore the loss of one inhibitory mechanism is expected to be compensated by the other. Indeed, a recent study demonstrated that ES cells lacking either the securin- or CDK1-separase inhibition mechanism maintained sister chromatid cohesion (Huang et al., 2005).

Finally, vertebrate separase itself undergoes auto-cleavage at anaphase (Papi et al., 2005). The consequences of this cleavage in mitosis have just been described, with the authors suggesting that the cleaved separase acts positively to induce entry into the next mitotic cell cycle (Papi et al., 2005). This is believed to be achieved through promoting the destruction of the kinase Wee1, therefore leading to pre-MPF activation during late G2 (Papi et al., 2005). This hypothesis, however, remains to be tested during the meiotic cell cycle.

Chapter 2: Materials & Methods

2.1. Materials

Female MF1 mice were obtained from Harlan (UK). Mouse oocyte collection and culture equipment was from VWR (UK), unless stated otherwise. All chemicals were purchased from Sigma-Aldrich (UK), unless noted otherwise. Antibodies were obtained from AbCam (UK). All antisense morpholinos (MO) were designed by Genetools (USA).

All chemicals used were tissue culture or embryo-tested grade where appropriate.

2.2. Methods

2.2.1. Mouse oocyte collection and culture

2.2.1.1 Collection of germinal vesicle oocytes

Germinal vesicle (GV) oocytes were collected from 4-6 weeks old MF1 mice (Harlan). The females were superovulated by intraperitoneal injection of 7.5IU of pregnant mares' serum gonadotrophin (PMSG) (Calbiochem), which mimics endogenous FSH. Mice were killed by cervical dislocation at 46-48 h after PMSG injection. The ovaries were removed and immediately transferred to dissection medium, consisting of M2 medium (**Table 1**) supplemented with 1μM milrinone or 200μM IBMX, in order to keep the oocytes arrested at the GV stage. The cumulus-enclosed oocytes were isolated by mechanical perforation of the ovaries with a 27-gauge needle. The cumulus masses were removed by repeated

mouth-pipetting, using narrow-bore glass Pasteur pipettes. Following few rinses in drops of dissection medium, the oocytes were ready for further use.

2.2.1.2 Collection of metaphase II-arrested eggs

For collection of Met II eggs, 4-6 weeks old MF1 mice were superovulated by intraperitoneal injection of 7.5IU of PMSG, followed 46-48 h later by 5IU of human chorionic gonadotrophin (hCG) (Calbiochem), which has strong homology with LH. Mice were killed by cervical dislocation at 13-15 h after hCG injection. The oviducts were removed and immediately transferred to M2 medium. The oviducts were then placed in M2 medium containing 300IU/ml hyaluronidase. The cumulus-enclosed Met II eggs were released using fine forceps to tear the oviducts. Following a brief exposure to hyaluronidase (maximum 5 min), the cumulus cells were removed. The denuded Met II eggs were then washed in M2 medium and ready for further manipulations.

For bench handling, microinjections and imaging experiments, GV and Met II oocytes were cultured in M2 medium. For longer-term incubation, the oocytes were cultured in minimum essential medium (MEM) (Gibco) supplemented with 20% of foetal bovine serum (FBS) (Invitrogen), 60µg/ml penicillin and 50µg/ml streptomycin in a 5% CO_2 humidified incubator at 37ºC (Lab Impex Research). To keep the GV oocytes arrested, the culture media (M2 or MEM) was supplemented with 1µM milrinone or 200µM IBMX. For parthenogenetic activation of eggs, the Met II-arrested oocytes were incubated in Sr^{2+} medium (**Table 2**).

Table 1: Composition of M2 culture medium

M2 was used for oocytes collection and short term culture. This is a modified Kreb's Ringer solution with some of the bicarbonate substituted with Hepes buffer.

Component	Concentration (mM)
NaCl	94.66
KCl	4.78
$CaCl_2.2H_20$	1.71
$KH_2.PO_4$	1.19
$MgSO_4.7H_20$	1.19
$NaHCO_3$	4.15
Hepes	20.85
Na Lactate	23.28
Na Pyruvate	0.33
Glucose	5.56
BSA	4.00g/l
Penicillin G. K salt	0.06g/l
Streptomycin sulphate	0.05g/l
Phenol Red	0.01g/l

Table 2: Composition of Sr^{2+} medium

Sr^{2+} medium was a used for parthenogenetic activation of eggs. This is a Ca^{2+}-free M2 medium with $CaCl_2.2H_20$ replaced by $SrCl_2$. BSA was added right before usage.

Component	Concentration (mM)
NaCl	94.66
KCl	4.78
$SrCl_2$	10
$KH_2.PO_4$	1.19
$MgSO_4.7H_20$	1.19
$NaHCO_3$	4.15
Hepes	20.85
Na Lactate	23.28
Na Pyruvate	0.33
Glucose	5.56
BSA	4.00g/l
Penicillin G. K salt	0.06g/l
Streptomycin sulphate	0.05g/l
Phenol Red	0.01g/l

2.2.2. Microinjection and imaging

All microinjections of GV and Met II oocytes were done in M2 medium (± milrinone or IBMX) on the heated stage of an inverted microscope (Leica DM IRB, Leica, Wetzlar, Germany). Borosilicate glass capillaries (outer diameter 1.5mm and inner diameter 0.86mm) with an inner filament (Harvard Apparatus Ltd) were used to make microinjection pipettes using a micropipette puller (Model P-97, Sutter Instrument, USA). The micropipettes were then backfilled with approximately 1µl of injection solution, and inserted into oocytes using the negative capacitance overcompensation facility on an electrophysiological amplifier (World Precision Instruments, UK); while cells were immobilised with a holding pipette (Hunter Scientific). This procedure ensures a high rate (>90%) of oocytes survival and a low rate (<1%) of Met II oocytes parthenogenetic activation. A precise injection volume (2-5% of the total oocyte volume) was achieved by using a Pneumatic PicoPump (World Precision Instruments). The volume of the oocyte is approximately 250pl.

Brightfield and epi-fluorescence images were recorded using a (20 X 0.75 NA) objective and a Princeton Instruments MicroMax interline cooled CCD camera (Roper Scientific, Buckinghamshire, UK). Filters sets were used as appropriate for the fluophore being studied. Hoechst staining, for example, was examined using a UV filter set at BP 330-380 nm for excitation, DM 400 nm and LP 450 nm for emission. Whereas, GFP and YFP tagged proteins were imaged using a fluorescein isothiocyanate (FITC) filter set at BP 450-490 nm for excitation, DM 510 nm and BP 520 nm for emission. Metamorph (MM) and metafluor (MF) software 6.1 (Universal Imaging Corp, PA, USA) were used for

image capture (control of the filter and shutter settings) and data analysis. Eggs were imaged every 10-20 minutes to minimise photobleaching and photodamage. Also, to accurately measure multiple oocytes within a single image, it was important to correct the illumination variation across the field of view. This was done using the Metamorph's "Reference Image" command, which allows the acquisition of a background image with the same experimental conditions as the sample image (wavelength and exposure time) but with a closed-shutter. The background was then subtracted from the experimental image, therefore improving quantitative accuracy and overall throughput.

2.2.3. Western blotting

Oocytes were washed in phosphate-buffered saline (PBS) with 1% polyvinylpyrrolidone (PVP) solution and frozen in 5X sample buffer (0.06M Tris/HCL at pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.025% bromophenol blue and 5% 2-mercaptoethanol). The samples were then boiled at 95ºC for 5 min using a heat block (QBT2, Grant) and briefly centrifuged at 8,000Xg (MSE, Micro Centaur, Sanyo, Japan).

Proteins were fractionated at 200mV for 50 min on an X Cell II™ blot Module (Invitrogen) and power pack (Power Pac 300, BioRad); using 10% NuPage Bis-Tris pre-cast gels (Invitrogen) with mopholinepropanesulphonic acid (MOPS) running buffer (Invitrogen). Coloured standard markers (Kaleidoscope, BioRad) were also loaded on the gel. Following electrophoresis, proteins were blotted onto polyvinylidened fluoride membranes (PVDF) for 1h30min at 100mV. The PVDF membranes used were pre-rinsed in 100% methanol, washed in water and then placed in blotting buffer (25mM Tris

buffer, 192mM glycine, 20% methanol). Following blotting, the membranes were saturated with 5% non-fat dry milk in blocking buffer (10mM Tris, 100mM NaCl and 0.1% Tween-20) at room temperature for 1h and incubated with primary antibody (**Table 3**) overnight at 4°C. After the overnight incubation, membranes were washed in blocking solution without milk for 1 h with buffer changed every 15 min, then incubated with anti-rabbit/mouse horseradish peroxidise (HRP)-linked secondary antibody (**Table 4**) for 1h at room temperature. Finally, membranes were washed again in blocking buffer for 1h with continuous change of buffer.

Standard enhanced chemiluminescence (ECL) techniques (Amersham Biosciences) were used to detect the protein-bound HRP-labelled secondary antibodies according to the manufacturer's instructions. The membranes were incubated in ECL detection solution mixture (40:1), wrapped in Clingfilm and exposed to Hyperfilm (Amersham Biosciences). The exposure time depended on the strength of the signal.

Table 3: Primary antibodies used in this study.

Primary antibody	Species	Concentration	Manufacturer & catalogue number
Anti-securin	Mouse	1:1000	AbCam (ab3305)
Anti-cyclin B1	Mouse	1:400	AbCam (ab72)
Anti-Cdh1	Mouse	1:200	AbCam (ab3242)
Anti-Cdc20	Rabbit	1:200	Santa Cruz (sc-8358)
Anti-PLK1	Rabbit	1:500	AbCam (ab14209)
Anti-actin	Mouse	1:2500	Millipore (MAB1501R)

Table 4: Secondary antibodies used in this study.

Secondary antibody	Concentration	Manufacturer & catalogue number
Goat anti-mouse	1:5000	BioRad (170-6516)
Goat anti-rabbit	1:5000	BioRad (172-1019)

2.2.4. Chromosome spreads

Chromosome spreads were prepared by the air-drying technique, which involves swelling eggs, fixing them on slides and staining them with Giemsa. Eggs' swelling was done through incubation in a dish filled with 1% trisodium citrate for 7-10 min. The eggs were then placed onto clean microscope slides and immediately fixed by dropping onto them

one drop of freshly prepared fixative, 3:1 (Methanol:glacial acetic acid), from a height of at least 2 cm to avoid premature fixation by vapour. Following fixation, the chromosomes were spread by gentle mouth blowing, and left to dry overnight. The slides were then incubated for 5 min in 5M HCL, washed with running distilled water and stained with 9:1 (Gurr buffer:Giemsa). Finally, the slides were rinsed in Gurr buffer (pH 6.8 - BDH) and left to dry.

C-spreads, however, were prepared by treating the fixed oocytes with 0.2M HCL for 1 h, and then incubating the slides in a freshly prepared aqueous solution of 4% barium hydroxide at 37°C for 30 seconds. This was followed by a subsequent incubation in 2 x standard saline citrate (SSC; 0.3M sodium chloride/0.03M trisodium citrate) at 60°C for 1 h, and Giemsa staining for 15 min. Slides were rinsed in distilled water following each step.

To visualise the chromatin, the slides were applied to a microscope (TE300, Nikon). (20 X 0.75 NA) objective was used to find the chromatin and (100 X 0.75 NA) oil immersion lens was used to count the chromosomes and to record brightfield images of the spreads, as previously described.

2.2.5. H1 kinase assay

MPF (CDK1-cyclin B) activity can be measured in vitro by its ability to phosphorylate histone H1. Five oocytes collected in 2µl of PBS were transferred in 3µl of storing solution (10µg/ml leupeptin, 10µg/ml aprotinin, 10mM p-nitrophenyl phosphate, 20mM

β-glycerophosphate, 0.1mM sodium orthovanadate and 5mM EGTA), and immediately frozen on dry ice. After three thaw-freeze cycles, 2 x concentrated kinase buffer [60µg/ml leupeptin, 60µg/ml aprotinin, 24mM p-nitrophenyl phosphate, 90mM β-glycerophosphate, 4.6mM sodium orthovanadate, 24mM EGTA, 24mM $MgCl_2$, 0.2mM EDTA, 4mM MOPS, 0.6mM ATP, 2mg/ml histone H1 (HIII-S from calf thymus, Sigma) and 0.25mCi/ml [^{32}P] ATP] was added to the samples. This was followed by 30 min incubation at 30°C. The reaction was then stopped by addition of 2 x SDS-sample buffer (0.125M Tris-HCL, 4% SDS, 20% glycerol, 10% mercaptoethanol, 0.002% bromophenol blue). The samples were then boiled at 95°C for 3-5 min, analysed by 10% SDS-PAGE gel at 7V for 18h (**Table 5**), which was followed by autoradiography. Autoradiographs were imaged using Fuji Bas-1000 phosphorimager system and analysed with TINA 2.0 software.

Table 5: 10% SDS-PAGE gel

Separating gel 10%	
40% acrylamide	24ml
2% Bis-acrylamide	13ml
1.5M Tris-HCl pH8.8	25ml
10% SDS	1ml
10% APS (1g/ml)	500µl
TEMED	50µl
Water	↑ 100ml

Stacking gel 4%	
40% acrylamide	3ml
2% Bis-acrylamide	1.5ml
0.5M Tris-HCl pH8.8	7.5ml
10% SDS	300µl
10% APS (1g/ml)	150µl
TEMED	30µl
Water	↑ 30ml

The stacking gel was applied to the separating gel following solidification

2.2.6. Protein knockdown using morpholino antisense oligos

To better understand the role of securin, cdc20 and cdh1 in the control of meiosis, I have decided to deplete them using morpholino oligonucleotides (MO) (**Table 6**). Morpholinos prevent protein translation by binding to their complementary sequences in the 5' untranslated region of target mRNA, therefore blocking access of the translation initiation complex. Unlike other antisense oligos (e.g. siRNA and sDNA), morpholinos are completely stable to nucleases, non-toxic, water soluble and have very long-term activity. Those criteria are due to key structural features, with morpholine rings replacing the ribose sugars in the DNA bases (A, T, G and C) and non-ionic inter-subunit linkages replacing the anionic phosphates (www.gene-tools.com). To achieve very high sequence specificity, morpholinos tend to be 20-25 bases long, which assures strong and specific binding to their complementary target sites. Like all gene knockdown reagents, morpholinos were actively delivered into GV oocytes through microinjection. The oocytes were then cultured for 24 h in MEM supplemented with milrinone or IBMX, in order to keep them arrested at the GV stage. To resume maturation, the oocytes were incubated in MEM following release.

Table 6: Morpholinos used and their sequences.

Morpholino	Sequence (5' → 3')	Concentration
Securin MO	GATAAGAGTAGCCATTCTGGATTAC	1.5mM
5MM-securin MO	GATAACACTACCGATTCTCGATTAC	1.5mM
Cdh1 MO	CCTTCGCTCATAGTCCTGGTCCATG	3.0mM
Cdc20 MO	CGGTCTCAAACACCAAGTGCGGCAT	3.0mM

2.2.7. Preparation of RNA for microinjection

2.2.7.1. Bacteria transformation and plasmid DNA purification

To propagate a plasmid carrying the gene of interest, 10pg-100ng of plasmid DNA was transformed into 50µl of TOP10 chemically competent *E.coli* cells. The cells were then incubated on ice for 30 min, followed by a brief heat-shock for 30 seconds at 42°C before being placed back on ice for 2 min. 250µl of pre-warmed S.O.C. medium (GIBCO) was then added to the cells, which were further incubated for 1 h at 37°C. The transformation reaction was then spread on agar plates containing 100µg/ml ampicillin and incubated overnight at 37°C. After the incubation, single colonies were picked and grown overnight at 37°C in LB medium (Sigma) containing 100µg/ml ampicillin. The cells from the overnight culture were then collected by centrifugation for 5 min at 10,000Xg and the plasmid DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Finally, the presence of the plasmid DNA within the collected sample was confirmed by agarose gel electrophoresis

and its concentration was measured using NanoDrop spectrophotometer (ND 1000, Thermo Scientific).

2.2.7.2. Synthesis of RNA

Plasmid DNA used in this thesis contained T3 RNA polymerase promoter site and therefore mMESSAGE mMACHINE T3 Ultra kit (Ambion) was used for RNA synthesis. The template DNA was first linearised with the restriction enzyme *sfi*I, analysed on agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. After linearization, the plasmid DNA was incubated with T3 RNA polymerase enzyme mixture, reaction buffer and ribonucleotides for 3 h at 37°C. This was followed by addition of 1µl of TURBO DNase, which removes the template DNA, and further incubation at 37°C for 15 min. Once the reaction was completed, RNA poly(A) tailing was done by adding the tailing reagents (*E*-PAP enzyme, *E*-PAP buffer, $MnCl_2$ and ATP solution) to the mMESSAGE mMACHINE T3 Ultra reaction and incubating it at 37°C for 90 min. Finally the RNA was purified and cleaned up from the transcription reaction using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The recovered RNA was then run on agarose gel and quantified using NanoDrop spectrophotometer (ND 1000, Thermo Scientific), before being aliquoted into 1 µl aliquots and stored at -80°C until further use.

2.2.7.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used to check the size and quantity of purified DNA or RNA. 0.8% agarose gel was prepared by mixing 0.4g of agarose powder with 50ml of 1 x Tris-acetate-ethylenediaminetetracetic acid (EDTA) (TAE buffer) (Promega). The mixture was boiled in a microwave oven until the agarose powder has completely dissolved. 10mg/ml of ethidium bromide was then added to the agarose-TAE buffer, and poured into a gel casting apparatus (BioRad). Once the gel has set, it was placed in a BioRad mini-sub filled with 1 x TAE buffer. The DNA and RNA samples mixed with loading buffers (DNA blue loading dye and RNA formaldehyde loading buffer) were loaded onto the gel alongside a 1kb DNA ladder (Promega) as a molecular weight indicator. The gel was run for 20-30 min at 100V using a BioRad Power Pac 300, and finally visualised on a UV transilluminator (TFX-20M, Vilber Lourmat).

2.2.8. Site-directed mutagenesis

Site-specific mutation and amino acid substitution in a plasmid carrying the gene of interest was done using the QuikChange II site-directed mutagenesis kit (Stratagene). This system utilises a double-stranded DNA vector with an insert, two mutagenic oligonucleotide primers and a high fidelity *PfuUltra* DNA polymerase. The oligonucleotide primers were carefully designed using Stratagene's web-based QuikChange primer design program (www.startagene.com/qcprimerdesign); such that each primer was complementary to the opposite strand of the vector and carried in the middle the desired mutation, which was flanked by 10-15 bases of correct sequence on

both sides (**Table 7**). The mutagenesis was done in three steps, according to the manufacturer's instructions. Briefly, the first step was to synthesise the mutant strand, which required oligonucletoide primers extension by *PfuUltra* DNA polymerase during temperature cycling (**Table 8**). This was followed by *Dpn* I endonuclease treatment for 1h at 37°C to digest the methylated parental DNA template and to select for mutation containing synthesised DNA. Finally, the plasmid DNA carrying the desired mutation was transformed into XL1-Blue supercompetent cells and purified using Miniprep DNA purification system (section 2.7.1). To confirm the presence of the mutation, 50ng/µl of plasmid DNA together with sequencing primer (**Table 9**) was sent to UCL scientific support services for sequencing. The mutant plasmid DNA was then used as template for RNA synthesis (section 2.2.7.2).

Table 7: Mutagenic primers

Primer	Orientation	Sequence (5' → 3')
Securin T43A	Forward	GCCTTAGATGGGAGATCTGAAGTTGCAGCACCACGTTTTGG
	Reverse	CCAAAACGTGGTGCTGCAACTTGAGATCTCCCATCTAAGGC
Securin S165A	Forward	GCTGGGCCCCCCTGCACCTGTGAAGATGCCC
	Reverse	GGGCATCTTCACAGGTGCAGGGGGGCCCAGC
Securin S171A	Forward	CCTGTGAAGATGCCCGCTCCACCATGGGAATCC
	Reverse	GGATTCCCATGGTGGAGCGGGCATCTTCACAGG
$Securin^{DM}$ RXXL→AXXL	Forward	GCCTTACCTAAAGCTACTGCAAAGGCTTTGGGAACTGTC
	Reverse	GACAGTTCCCAAAGCCTTTGCAGTAGCTTTAGGTAAGGC
$Securin^{DM}$ AXXL→AXXA	Forward	GCTACTGCAAAGGCTGCGGGAACTGTCAACAGAGC
	Reverse	GCTCTGTTGACAGTTCCCGCAGCCTTTGCAGTAGC

Table 8: Cycling parameters for site directed mutagenesis

Segment		Temperature	Time	Cycles
1	Initial denaturation	95°C	30 seconds	1
2	Denaturation	95°C	30 seconds	16
	Annealing	55°C	1 minute	
	Extension	68°C	5 minutes (1 min/kb of plasmid length)*	
3	Hold	37°C	--	1

* Plasmid used in this study was 4.6 kb long; therefore it required 5 minutes at 68°C per cycle.

Table 9: Sequencing primer

Primer	Sequence (5’ → 3’)
Sequencing primer	GGAAAATGGAGAACCAGGCACCC

2.2.9. Statistical analysis

All t-tests are two-tailed and based on two samples (unpaired) with similar variance. All error bars represent standard deviations (SD).

Table 10: P values and their statistical significance.

P value	Significance	Symbol
>0.05	Not significant	NS
0.01-0.05	Significant	*
0.001-0.01	Very significant	**
<0.001	Extremely significant	***

Table 11: Reagents used in this study.

Reagent	Concentration	Function
Milrinone	1 μM	Inhibition of GVBD
IBMX	200 μM	Inhibition of GVBD
$SrCl_2$	10 mM	Parthenogenetic activation
Hoechst	10ng/ml	Staining of the Chromatin
Roscovitine	50 μM	CDK1 inhibitor
UO126	50 μM	MEK1 inhibitor
MG132	50 μM	Proteasome inhibitor
Cycloheximide	10 μM	Protein synthesis inhibitor

Chapter 3: Securin and not MPF regulates sister chromatid disjunction during meiosis II in mouse eggs.

3.1. Introduction

On completion of the first meiotic division, the mammalian oocyte enters the second meiotic division and becomes arrested at the Met II stage until fertilization. This arrest is due to the cytoplasmic activity, CSF, which stabilizes cyclin B1 by inhibiting the APC/C (Tunquist and Maller, 2003). At fertilization, the sperm-triggered Ca^{2+} oscillations induce CSF inactivation, which in turn results in the stimulation of the APC/C, followed by cyclin B1 and securin targeted destruction by the proteasome (Runft et al., 2002). The degradation of cyclin B1 is necessary for MPF inactivation, hence release from the metaphase II arrest and completion of meiosis II. Securin destruction, on the other hand, is required for separase activation and sister chromatid separation at anaphase II.

During metaphase II arrest, which might last several hours, the egg has the difficult task of ensuring the continued attachment and bi-orientation of the sister chromatids. This is very important, because any premature loss of sister chromatid cohesion might lead to aneuploidy, which is the leading cause of human birth defects (such as trisomies), malformations of the embryo and spontaneous foetal abortions (Hassold and Hunt, 2001). The maintenance of the pairing of sister chromatids during metaphase II arrest is achieved through inhibition of separase, the protease that is responsible of cleaving the cohesin complex, which holds chromatids together (Petronczki et al., 2003).

It is well established that separase is regulated by two mechanisms, securin and CDK1/cyclin B1 (Ciosk et al., 1998; Petronczki et al., 2003; Zou et al., 1999). They both bind to and inhibit separase for most of the cell cycle until anaphase onset, when securin and cyclin B1 are targeted for destruction via ubiquitination by the APC/C. Securin inhibits separase by binding to both separase's N and C terminus, therefore preventing them from inducing an activating conformational change at the protease active site (Hornig et al., 2002). MPF, on the other hand, only binds to CDK1-phosphorylated separase via its regulatory subunit, cyclin B1, leading to the reciprocal inhibition of both protease and kinase (Gorr et al., 2005; Holland and Taylor, 2006; Stemmann et al., 2001).

The two inhibitory mechanisms are mutually exclusive at the molecular level, given that separase only binds one inhibitor at a time (Gorr et al., 2005). This implies that the loss of one inhibitory mechanism could be compensated for by the other, and activation of separase would require the loss of both mechanisms. Indeed, during the first meiotic division of mouse oocytes separase activity is regulated by both securin and cyclin B1, and proteolysis of both proteins is required for separase activation and homologue disjunction (Herbert et al., 2003). This was demonstrated by over-expression of either non-degradable D-box-truncated form of cyclin B1 (Δ90-cyclin B1) or non-degradable D-box mutated variant of securin, which were shown to inhibit both homologue disjunction and polar body formation (Herbert et al., 2003). However, the relative contribution of securin and cyclin B1 to separase inhibition and sister chromatid attachment during the metapahse II arrest is not fully understood. Although, it was reported that following fertilization sister chromatid separation was blocked upon over-

expression of either Δ90-cyclin B1 or D-box mutant securin (Madgwick et al., 2004). These observations, however, fail to give any context to the importance of securin and cyclin B1 in the inhibition of separase, especially since sister chromatid separation eventually occurs at later stages in the Δ90-cyclin B1 injected eggs (Madgwick et al., 2004).

This study is set out to examine what controls separase during the metaphase II arrest of mouse oocytes. I used the CDK1 inhibitor roscovitine and an antibody designed to block the interaction of MPF with separase (Gorr et al., 2006), to demonstrate that separase is not regulated by MPF during the metaphase II arrest. Instead, securin knockdown using an antisense morpholino induces sister chromatid separation, which could be rescued by injection of securin cRNA. Therefore, securin and not CDK1/cyclin B1 regulates separase activity during metaphase II arrest.

3.2. Results

3.2.1 Exogenous cyclin B1 and securin show equal stability during Met II arrest

To determine the relative contribution of cyclin B1 and securin to separase inhibition during the Met II arrest, I performed timelapse fluorescence measurements of oocytes injected with cRNA constructs for yellow fluorescent protein (YFP) tagged cyclin B1 (cyclin B1-YFP) or securin (securin-YFP) during maturation, from GV to Met II arrest. The measurements demonstrated that both proteins gradually accumulated during meiosis I, declined at the time of PB1 extrusion, then increased again following entry into meiosis

II **(Fig. 3.1A, B)**. To establish whether cyclin B1 and securin are equally stable during the Met II arrest, I calculated the mean rate of their re-expression in eggs two hours after PB1 extrusion **(Fig. 3.1C)**. There was no statistical difference between cyclin B1 and securin fluorescence rises following entry into meiosis II. These results suggest that exogenous cyclin B1 and securin are equally stable during metaphase II arrest.

3.2.2 Endogenous cyclin B1 and securin expression varies after PB1 extrusion

Having shown the equal stability of exogenous cyclin B1 and securin during Met II arrest, I went on to examine the changes in the endogenous proteins using Western blotting. An equal number of oocytes at different maturation stages and following arrest at Met II, were blotted for cyclin B1 and securin **(Fig. 3.2A)**. Similar to their YFP-labeled constructs, cyclin B1 and securin levels increased during maturation, reaching their highest point at metaphase I. This was followed by a period of intense degradation, ending with PB1 extrusion. However, following polar body extrusion very marked differences in the re-establishment of endogenous cyclin B1 and securin were observed. Cyclin B1 levels rose significantly, and went on to reach similar levels to those observed during metaphase I (6 h post release). In contrast, securin levels failed to recover and remained at levels near those at PB1 extrusion **(Fig. 3.2A, B)**. The securin band in the Met II lane was not visible in the first blot (n=20), however it was observed when the number of eggs was doubled (n=40) **(Fig. 3.2C)**. Also, endogenous securin was lost upon activation of eggs using Sr^{2+}-containing medium, which mimics the sperm-triggered Ca^{2+} release **(Fig. 3.2C)**.

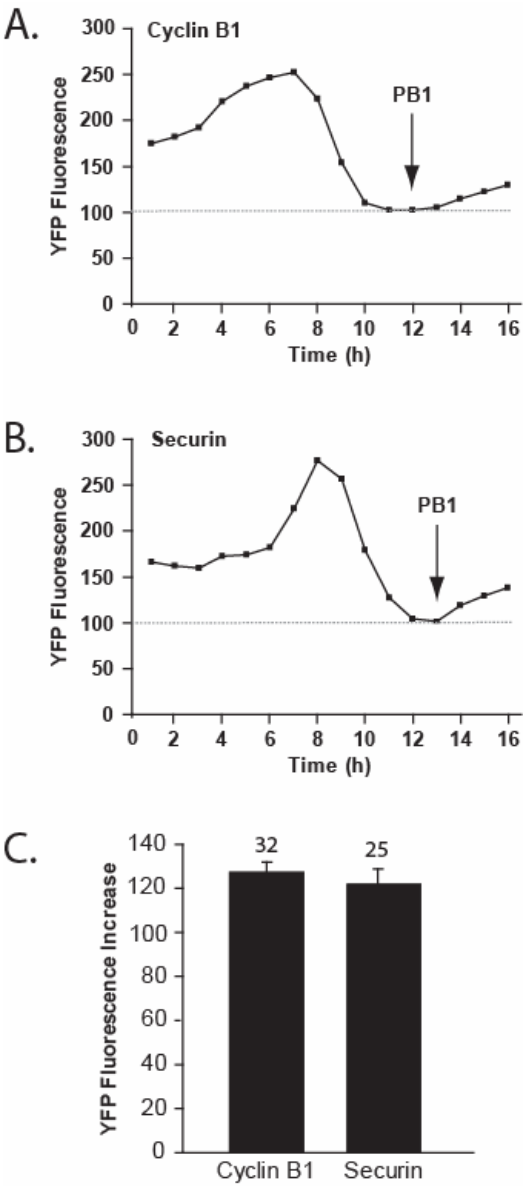

Fig. 3.1. Exogenous cyclin B1 and securin synthesis rates during meiotic maturation. Cyclin B1-YFP **(A)** or securin-YFP **(B)** were microinjected into GV oocytes, which were then matured in culture. YFP fluorescence readings were recorded at the times shown during oocyte maturation and following extrusion of the first polar body (PB1). Brightfield images were also captured at the same times to determine when PB1 extrusion occurred (arrow). **(C)** Comparison of cyclin B1-YFP and securin-YFP fluorescence levels in eggs two hours after PB1 extrusion. There was no statistical difference between fluorescence levels (*t*-test). To allow comparison between eggs of YFP fluorescence levels after PB1, fluorescence at time *t* (F*t*) were all normalized with respect to the minimum fluorescence obtained, which invariably corresponded to when the PB1 was extruded (F_{min}, plotted as a dashed horizontal line). YFP fluorescence was plotted as $F_t/F_{min} \times 100$.

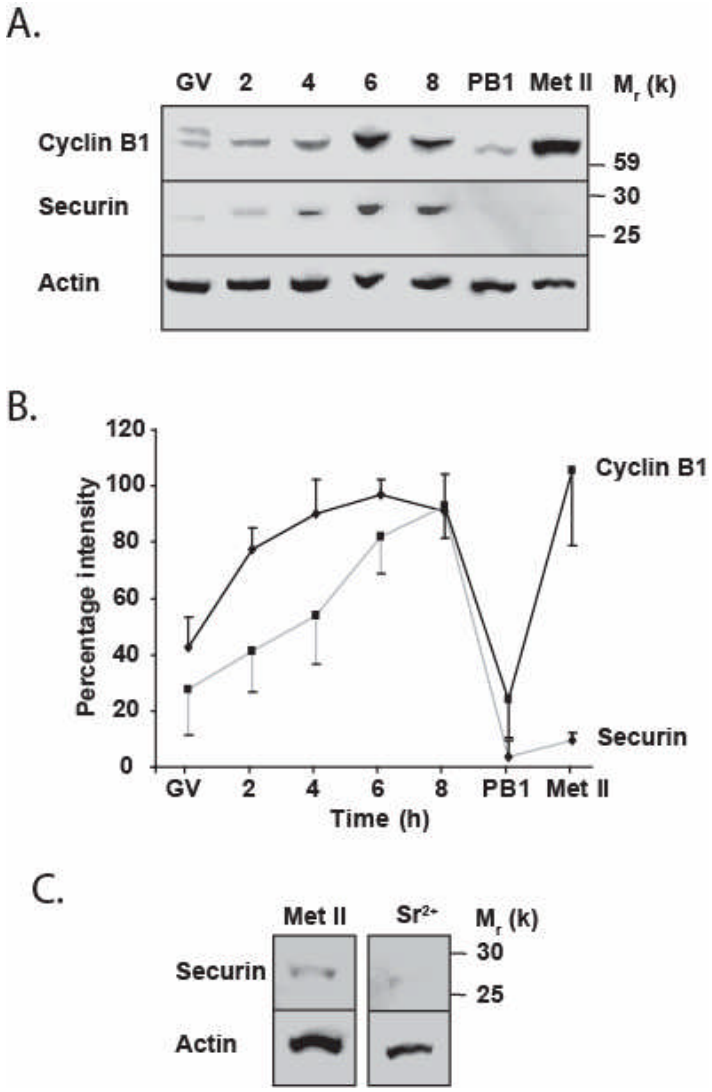

Fig. 3.2. Endogenous cyclin B1 and securin expression varies after polar body extrusion. (A) Western blot of oocytes (*n = 20* per lane) for cyclin B1 and securin during GV arrest, at the times indicated in hours during maturation relative to milrinone wash out, immediately following PB1 extrusion or during Met II arrest at 14 h. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. Cyclin B1 levels rise appreciably following PB1 extrusion, however there is little change in securin. **(C)** Western blot of eggs for securin (*n* = 40) with or without incubation in Sr^{2+} containing medium for 3 h. In panels A and C actin was used as a loading control.

3.2.3. CDK1 inhibition does not induce sister chromatid separation

During the Met II arrest, low securin and high cyclin B1 levels suggest that at this stage separase could be either inhibited by CDK1/cyclin B1, or by the low levels of securin. To further investigate this, I decided to disturb CDK1 activity in the Met II-arrested eggs using the CDK1 pharmacological inhibitor, roscovitine. This inhibitor was chosen because it was previously reported to be able to induce egg activation and PB2 extrusion (Gorr et al., 2006; Phillips et al., 2002). In agreement with this, within half an hour of exposure to roscovitine, the eggs started to demonstrate signs of parthenogenetic activation through PB2 extrusion, although this happened at lower rates and in some eggs over a longer timeframe than Sr^{2+}-treated eggs **(Fig. 3.3A, B)**. Many of the roscovitine-treated eggs attempted to extrude PB2, however the extrusion was transitory and the polar body ended up being reabsorbed **(Fig. 3.3C)**. Unexpectedly, in addition to the transitory polar bodies, when the chromatin of the roscovitine-treated eggs was labelled with Hoechst dye, I found that sister disjunction was aberrant. In some eggs all the chromatin mis-segregated into the PB2 **(Fig. 3.3D)**, in others it ended up stretched between the egg and PB2 **(Fig. 3.3E)**. The Hoechst real time observations were further confirmed by chromosome spreads, which showed that sister chromatids remained attached in the roscovitine-activated eggs **(Fig. 3.3F)**. On the other hand, in the control Sr^{2+}-activated eggs, anaphase was readily observed and it was always coupled with equal segregation of the chromatin between the egg and PB2 **(Fig. 3.3G)**.

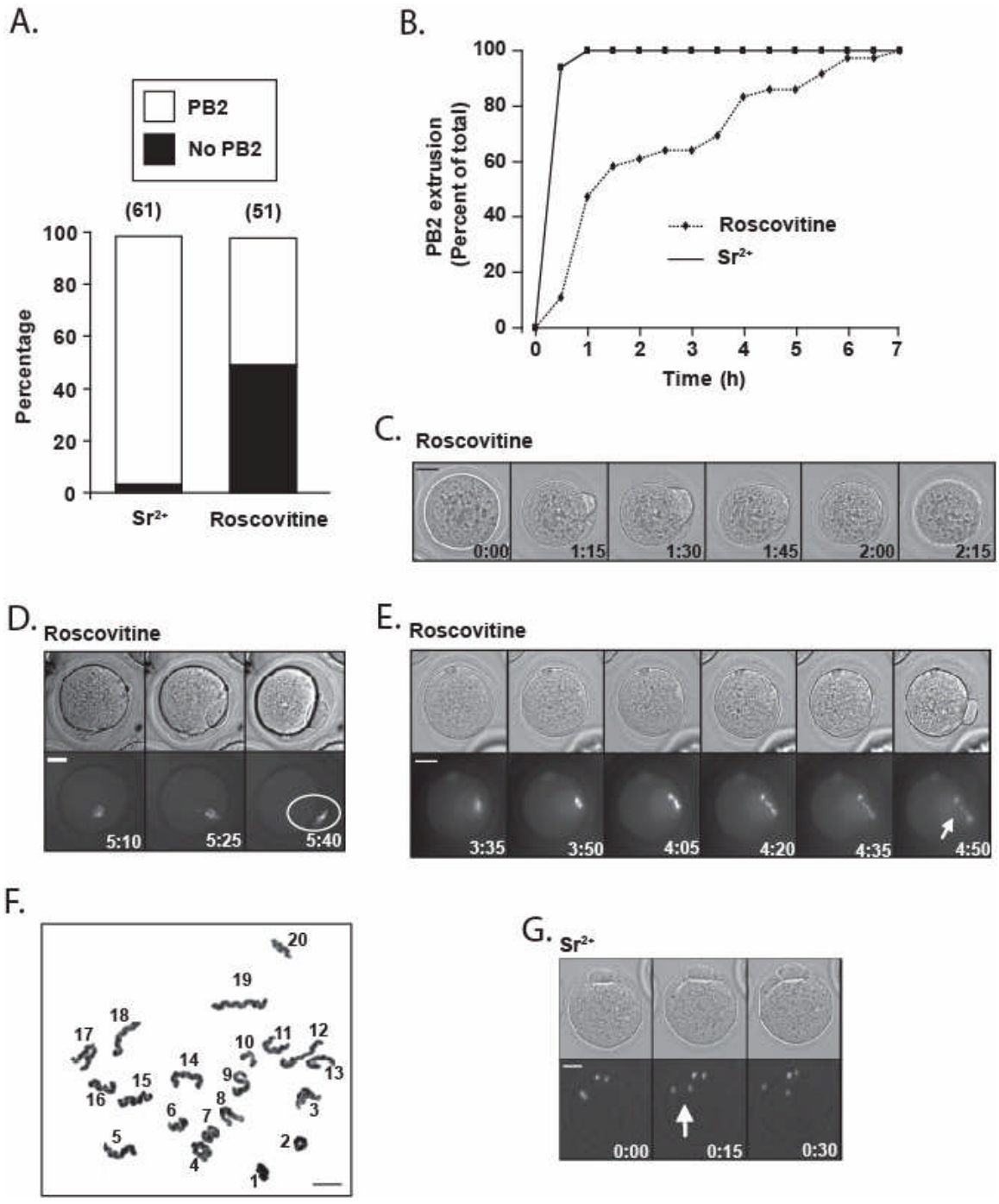

Fig. 3.3. CDK1 inhibition does not induce sister chromatid separation. (A) Rates of PB2 extrusion in Met II eggs following incubation in Sr^{2+} containing medium, or addition of roscovitine; assessed at 7 h. **(B)** Timing of PB2 extrusion following addition of roscovitine or Sr^{2+}, plotted as a cumulative percent of the total number of eggs extruding

a PB2. Some eggs were slower in extruding a PB2 following roscovitine addition than Sr^{2+}. **(C-E)** Brightfield and Hoechst images of eggs following addition of roscovitine at the times indicated. Note the abnormal events associated with roscovitine addition: a transitory extrusion of a polar body (C), an egg in which all the chromatin enters the polar body (D, circle); a cut phenotype in which chromatin is stretched between the egg and PB2 (E, arrow). **(F)** Chromosome spread of a roscovitine treated egg showing attached sister chromatids ($n = 25$). **(G)** Brightfield and Hoechst images of eggs following Sr^{2+} addition at the times indicated. Note that anaphase appears normal with chromatin visible in both the PB2 and the egg (arrow). Scale bars 20 μm (C-E, G); 10 μm (F).

3.2.4 Inhibition of separase-CDK1 binding fails to induce sister chromatid separation

Inhibition of CDK1 led to parthenogenetic stimulation of eggs but it could not induce sister chromatid separation, suggesting that during Met II arrest, MPF activity is not responsible for inhibiting separase. To further confirm this, I decided to use a more specific approach, an antibody that blocks the interaction of MPF with separase (Gorr et al., 2006; Phillips et al., 2002). This antibody, called anti-CBD antibody, was raised against the two known cyclin B1-biniding determinants of mouse separase (amino acids 1120-1134 and 1340-1354). Injection of the anti-CBD antibody into eggs did not disrupt the Met II arrest, as assessed by Hoechst staining, and failed to induce sister chromatid separation, as judged by chromosome spreads **(Fig. 3.4A, B)**. However, it remained possible that the anti-CBD antibody is only effective at preventing the interaction between MPF and separase prior to their mutual binding. To verify that this antibody is indded capable of dissolving the separase/MPF complex, I decided to inhibit homologue disjunction by injecting the cRNA for the non-degradable Δ90 cyclin B1 into GV oocytes, which were then allowed to mature in culture. These oocytes went as far as Met I and then arrested again with paired homologues, as reported previously (Herbert et al., 2003). Interestingly, injection of the anti-CBD antibody, but not control IgG, induced homologue disjunction, as assessed by chromosome spreads **(Fig. 3.4C, D)**. However, no PB1 was extruded, and this is attributed to the high MPF activity, which is unaffected by the antibody (Gorr et al., 2006). These results are consistent with the ability of the anti-CBD antibody to dissolve preformed MPF-separase complexes.

Taken together, both the roscovitine and the anti-CBD antibody data demonstrate that MPF is not responsible for inhibiting separase activity during Met II arrest.

3.2.5 Securin Knockdown induces sister chromatid separation

Having shown that MPF is not the inhibitor responsible for regulating separase during the Met II arrest in mouse eggs, I went on to investigate whether it is the low levels of securin that inhibit separase activity. Therefore, I performed knockdown of securin expression in oocytes using a securin antisense morpholino (securin^{MO}). Microinjection of securin^{MO} in GV oocytes, which were then kept arrested for 24 h, dramatically reduced securin levels. The control 5 base mismatch securin morpholino (5MM-securin^{MO}), however, had no effect **(Fig. 3.5A)**. GV oocytes that were depleted of securin by this protocol, alongside control morpholino-injected oocytes, were cultured for 14 h and then assessed for their abilities to extrude PB1 and to align chromatin on Met II spindle. There was no difference between both groups in the ability to extrude PB1 **(Fig. 3.5B)**. However, when the chromatin of the securin^{MO}-injected eggs was examined using Hoechst staining, I noticed that it was dispersed throughout the cytoplasm. In contrast, the chromatin was perfectly aligned on the Met II plate in the 5MM-securin^{MO} injected oocytes **(Fig. 3.5B, C)**. To determine if the status of chromatin observed in the securin-knocked down oocytes was due to a lack in sister chromatid pairing, chromosome spreads were performed. In the control morpholino-injected oocytes, the sister chromatids were perfectly attached, as assessed by Giemsa staining and C-banding **(Fig. 3.5D)**.

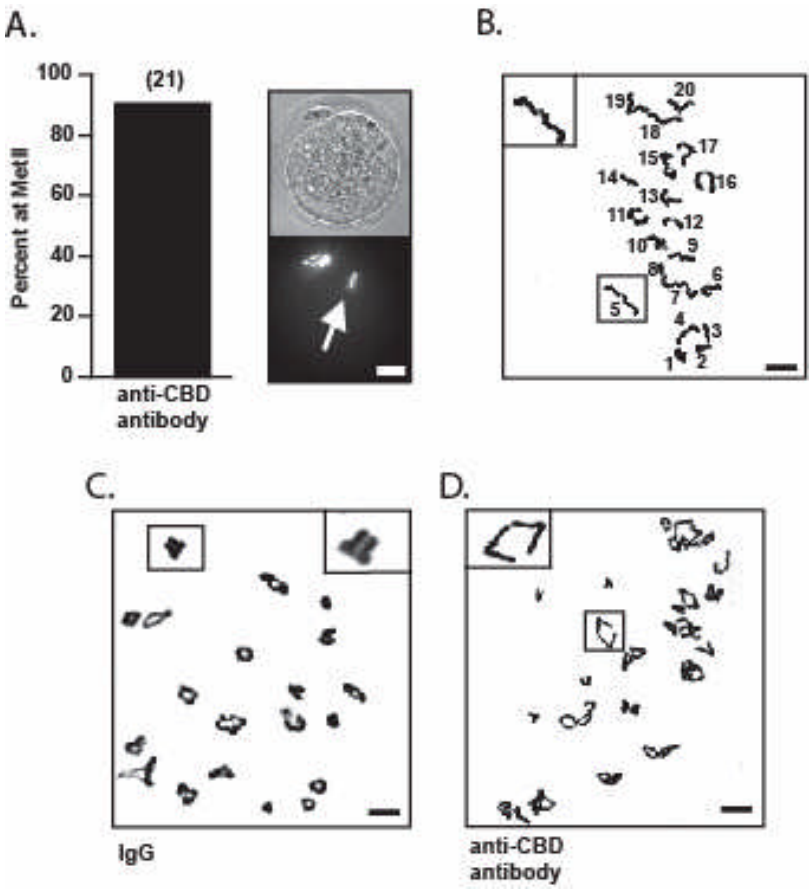

Fig. 3.4. Inhibition of separase–CDK1 binding fails to induce sister chromatid separation. (A) Assessment of Met II status in eggs injected with anti-CBD antibody, as judged by alignment of chromatin on a Met II plate (arrow). The antibody had little effect on chromatin. **(B)** Chromosome spread of an anti-CBD antibody injected egg (n = 6) at 3 h after injection showing attached sister chromatids (one chromatid is magnified in inset). **(C, D)** Chromosome spread of an oocyte arrested at Met I by injection of Δ90-cyclin B1, which is then further injected with either control IgG (C, n = 7) or anti-CBD antibody (D, n = 8) and incubated for 3 h. IgG injection had no effect on homologues, which remain attached; however the anti-CBD antibody caused separation of homologues. (Insets show a magnified bivalent or dyad). Scale bar 20 μm (A), or 10 μm (B–D).

Unexpectedly, spreads performed on the securin^{MO}-injected oocytes showed that although homologues and sister chromatids were attached before PB1 **(Fig. 3.5E)**, following PB1 extrusion sister chromatids were separated, which was further confirmed by C-banding **(Fig. 3.5F)**.

To certify that the lack in sisters pairing, which occurs as a result of securin knockdown, was not due to an off-target effect, I decided to rescue the phenotype by injecting securin cRNA into securin^{MO}-injected oocytes, just before their release. This rescue is made possible by the fact that exogenous cRNA lacks the 5' UTR that is targeted by the morpholino. Spreads performed on these oocytes showed that securin cRNA rescued the loss of sister attachment in securin^{MO}-injected oocytes, and this was further confirmed by C-banding **(Fig. 3.5G)**.

3.2.6 Securin knockdown during Met II arrest induces sister chromatid separation

The above data show that securin knockdown induces sister chromatid separation in oocytes that had extruded PB1, but not in those yet to extrude PB1. To ensure that sister chromatid disjunction happens during meiosis II, I performed knockdown of securin expression in eggs already arrested at Met II. Therefore, freshly ovulated Met II eggs were collected at 12 h post hCG, microinjected with either 5MM-securin^{MO} or securin^{MO} and examined 12h later for attachment of sister chromatids. Western blots confirmed that 12 h of culture following securin^{MO} injection was enough to cause a dramatic decrease in securin levels, whereas the 5MM- securin^{MO} had no effect **(Fig. 3.6A)**.

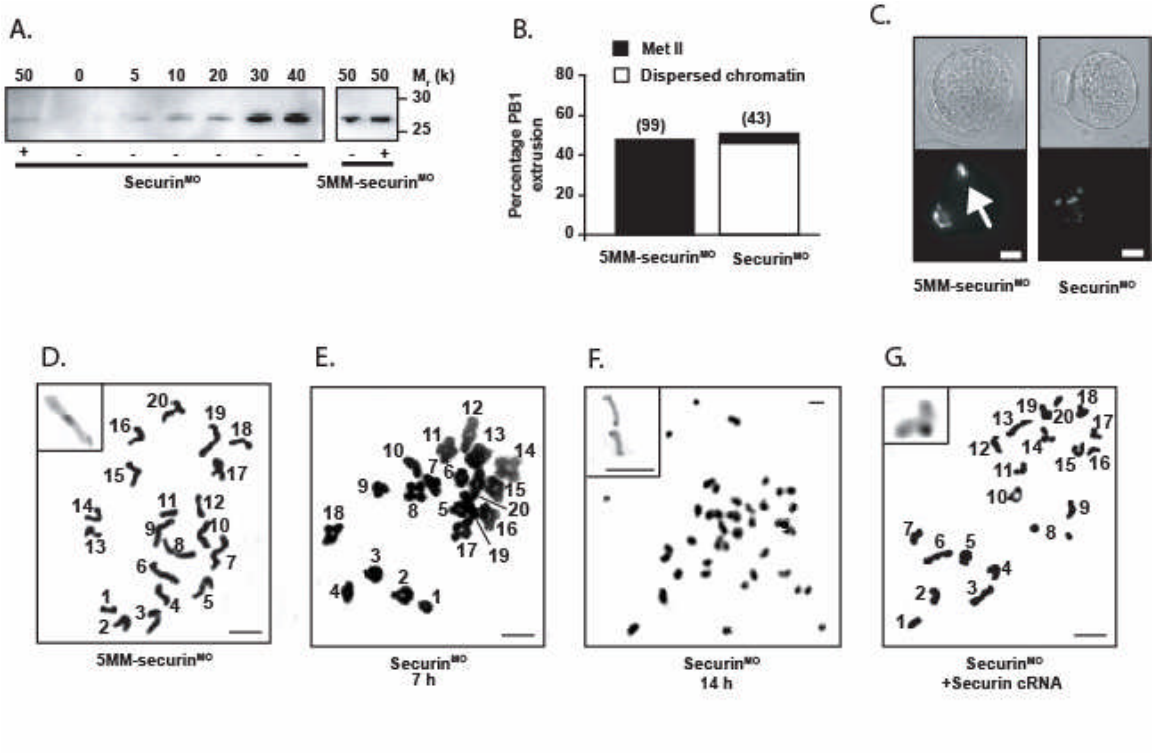

Fig. 3.5. Securin knockdown induces separation of sister chromatids. **(A)** Western blot for securin in GV arrested oocytes, n = 0–50 per lane, cultured for 24 h following injection of securin^{MO} or 5MM-securin^{MO} as indicated (n = 3). Securin^{MO} but not its 5 base mismatch control morpholino caused a large knockdown in securin expression (estimated 80%). **(B)** Percentage PB1 extrusion rates in oocytes injected with either securin^{MO} or 5MM-securin^{MO} as in panel A, assessed at 14 h following milrinone washout. All eggs extruding a PB1 were stained with Hoechst and categorised as having an intact Met II spindle (solid bar) or chromatin that was dispersed in the cytoplasm (open bar). **(C)** An example of a brightfield and Hoechst image in an egg that had been injected with the morpholinos indicated and matured as in panel B. The arrow shows a normal Met II plate which was prevalent in the 5MM-securin^{MO} injected oocytes, while chromatin is dispersed in the oocytes injected with securin^{MO}. **(D–G)** Chromosome spreads and associated C-banding (D, F, and G, inset) in oocytes at either 7 h (E) or 14 h (D, F and G) following microinjection of either 5MM-securin^{MO} (D) or securin^{MO} (E-G). In panel G securin cRNA was further microinjected into oocytes immediately before maturation was induced. Scale bar 20 μm (C) and 10 μm (D–G).

Chromosome spreads performed on these eggs revealed that most (86%) of the control morpholino injected eggs kept their sister chromatids attached **(Fig. 3.6B, C)**, whereas the majority (71%) of securin^{MO}-treated eggs showed a mixture of attached and unattached sisters, with complete disjunction evident in some eggs **(Fig. 3.6B, D)**.

Finally, to confirm that securin knockdown using morpholino does not affect MPF activity and that sister chromatid disjunction is only caused by the loss of securin, I performed H1 kinase assay on securin^{MO}-injected eggs, along with control morpholino-injected, non-injected and roscovitine or Sr^{2+}-treated eggs. Both roscovitine and Sr^{2+} induced a big decrease in the H1 kinase activity of the treated eggs. However, there was no difference between the non-injected and the morpholinos-injected eggs in their H1 kinase activity, suggesting that securin^{MO} does not affect MPF activity **(Fig. 3.6E)**. Taken together, these results demonstrate that only securin and not MPF is required for continued attachment of sister chromatids during Met II arrest in mouse eggs.

3.3 Discussion

In vertebrates, it is well established that separase is controlled by two mechanisms, securin and MPF (Ciosk et al., 1998; Petronczki et al., 2003; Zou et al., 1999). These two inhibitory systems are mutually exclusive at the molecular level, such that if one goes missing the other one can compensate for it (Gorr et al., 2005; Herbert et al., 2003).

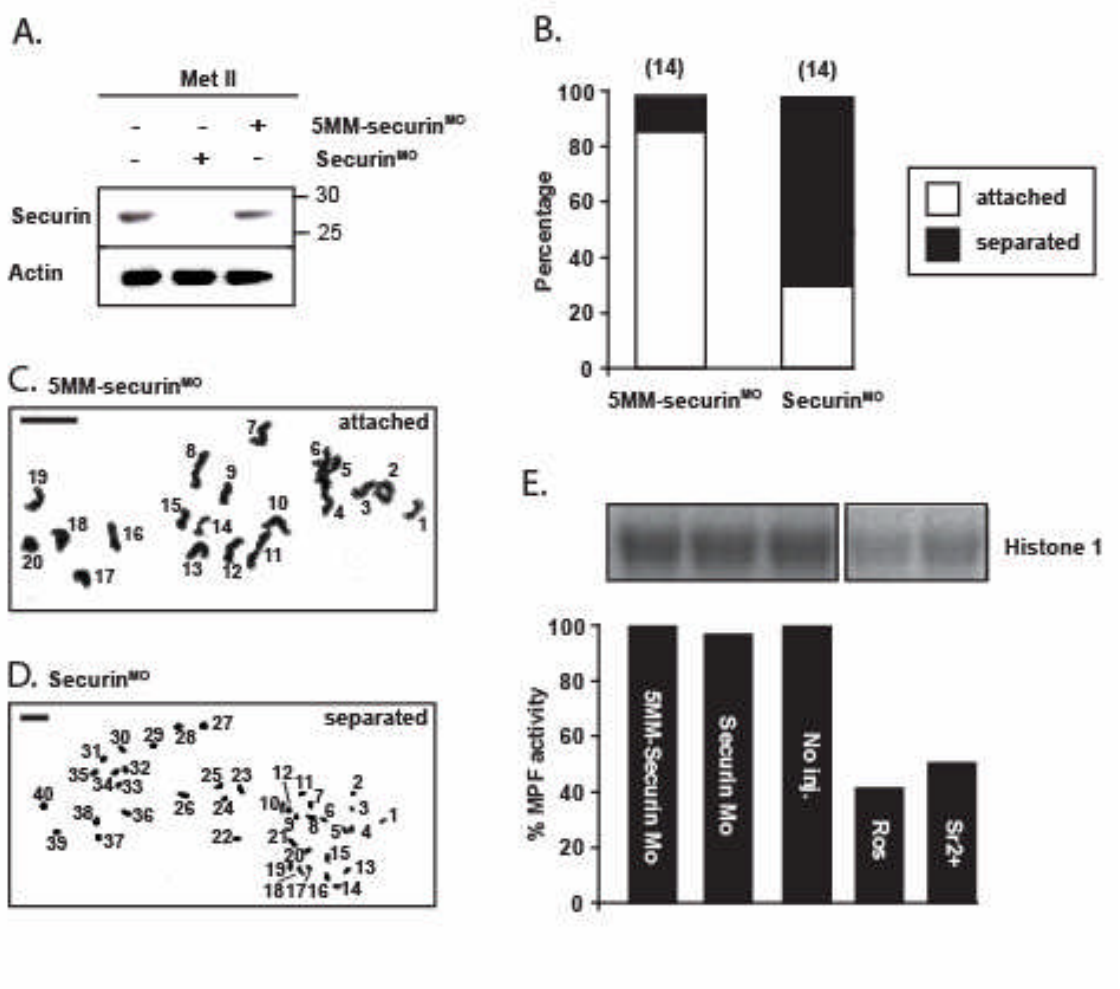

Fig. 3.6. Securin morpholino induces sister chromatid separation in Met II eggs. **(A)** Western blot for securin in Met II eggs, *n=40* per lane, cultured for 12 h following collection or injection with either securin^{MO} or 5MM-securin^{MO}. Securin^{MO} but not its control morpholino caused a large knockdown in securin expression. Actin was used as a loading control. **(B)** Percentage of eggs having separated (black bars) or attached (white bars) sister chromatids following microinjection of either 5MM-securin^{MO} or securin^{MO} and culture for 12 h. **(C)** Chromosome spread of a 5MM-securin^{MO} injected egg, demonstrating attached sisters**. (D)** Chromosome spread of a securin^{MO} injected egg in which all of the sister chromatids had undergone disjunction. **(E)** MPF activity in 5 eggs per lane, following injection with 5MM-securin^{MO}, securin^{MO}, no injections (no inj.) and incubation with either roscovitine or Sr^{2+}. MPF activity was assessed after 12 h of culture, and normalized with respect to the non-injected eggs. Scale bar 10 μm (C, D).

This mutuality has been proven in many systems including eggs, where we already know, by over expression of non-degradable constructs for either cyclin B1 or securin, either mechanism has the capacity to prevent sister chromatid separation upon egg activation (Madgwick et al., 2004). These observations, although important in implicating separase in sister chromatid disjunction, fail to give any context to the relative contribution of securin and cyclin B1 to the inhibition of separase during Met II arrest in eggs, especially that sister chromatid separation eventually occurs at later stages in the non-degradable cyclin B1-injected eggs (Madgwick et al., 2004).

The experiments described in this study show that even though endogenous and exogenous securin and cyclin B1 display similar profiles of expression during meiosis I, very marked differences were observed following PB1 extrusion. While the exogenous constructs show equal re-accumulation and stability during Met II arrest, the endogenous securin failed to recover and remained at levels near those at PB1 extrusion. This failure in securin recovery can only be attributed to the limited rates of the protein re-synthesis rather than degradation, given that Emi2 inhibits the activity of the APC/C at this time (Liu and Maller, 2005; Rauh et al., 2005; Shoji et al., 2006; Tung et al., 2005).

The lack of appreciable securin re-accumulation after PB1 extrusion suggests that CDK1/cyclin B1 could be the predominant inhibitor of separase during Met II arrest. Interestingly, preventing the interaction of CDK1/cyclin B1 with separase,

pharmacologically using the CDK1 inhibitor roscovitine or directly by injection of an anti-CBD antibody, failed to induce separase activation and sister chromatid separation.

Roscovitine was specifically chosen because of its reported ability to induce PB2 extrusion (Phillips et al., 2002). Indeed, addition of roscovitine induced egg activation and PB2 extrusion, although the metaphase-to-anaphase transition was always abnormal. In many of the roscovitine-treated eggs PB2 extrusion was transitory, indicating that roscovitine is able to induce higher rates of PB2 extrusion than those reported in Fig. 3.3A, but they were missed because of the transitory effect. The same phenotype was reported during meiosis I in mouse oocytes expressing non-degradable securin (Herbert et al., 2003), and it is believed that the inability of those oocytes to completely extrude polar bodies is due to failure in formation of a central spindle structure, which is necessary for proper cytokinesis (Mollinari et al., 2005). Although the decrease in CDK1 activity was clearly inducing the initial events of cytokinesis, it failed to induce sister chromatid separation. Instead, I observed a variety of defects all associated with lack of sister disjunction. In some eggs all the chromatin mis-segregated to the PB2. In others it ended up stretched between the egg and PB2, which is reminiscent of the cut phenotype that happens when cells carrying a mutant form of separase or expressing non-degradable securin fail to induce separation of sister chromatids even though they undergo cytokinesis (Funabiki et al., 1996). Failure of roscovitine in inducing sisters disjunction could be attributed to the fact that this pharmacological inhibitor, while it inhibits CDK1 activity, it is unable to dissolve the pre-formed CDK1/cyclin B1-separase complexes. However, given that CDK1-mediated phosphorylation of separase is required prior to

CDK1 binding, and separase dephosphorylation is readily observed during passage through mitosis (Herbert et al., 2003; Stemmann et al., 2001), I assume that roscovitine should be able to reverse the association of CDK1 with separase, at least in the presence of the cellular phosphatases.

The lack of efficacy for CDK1 in inhibiting separase during Met II arrest was further confirmed using a more specific approach, the anti-CBD antibody, which prevents the interaction of CDK1/cyclin B1 with separase (Gorr et al., 2006). Injection of the antibody into eggs had no effect on neither metaphase II arrest nor sister chromatid attachment. In contrast, the anti-CBD antibody induced homologue disjunction in Met I-arrested oocytes that were pre-injected with the non-degradable Δ90 cyclin B1, confirming its ability to dissolve the pre-formed MPF-separase complexes. Interestingly, even though the antibody was able to cause homologue segregation in these oocytes, it was unable to induce cytokinesis and PB1 extrusion. This can only be attributed to the sustained MPF activity, which is unaffected or even increased in the presence of the antibody, given that MPF binding to separase was proven to induce inhibition of both kinase and protease (Gorr et al., 2005). Taken together all these data confirm that MPF is not required for the continued attachment of sister chromatids during Met II arrest.

In contrast, a securin antisense morpholino injected either into GV oocytes, which were then allowed to mature, or into Met II-arrested eggs, induced sister chromatid separation. This effect was specific since it was not observed with the control morpholino, and could be rescued by injection of securin cRNA into the securin^{MO}-treated ooytes. Interestingly,

securin knockdown only induced chromosomes separation following PB1 extrusion and not during meiosis I. This suggests that securin is the predominant inhibitor of separase during Met II arrest, whereas during meiosis I a second mechanism that is securin-independent is involved in regulating separase activity. Indeed, it was reported that during the first meiotic division separase is regulated by both securin and MPF, and loss of both systems is required for separase activation and homologue disjunction (Herbert et al., 2003).

Also, a recent study by Marangos and Carroll (2008) reported the existence of competitivity between the APC/C substrates, such that excess in securin levels is important for the stabilization of cyclin B1 and hence progress through MI (Marangos and Carroll, 2008). So, it remained possible that sister chromatid disjunction, which has occured upon securin knockdown, was also attributed to a drop in MPF activity as a result of cyclin B1 targeted destruction by the APC/C in the absence of securin. However, this was unlikely since there was no difference in MPF activity of either securin^{MO} or 5MM-securin^{MO} injected eggs, which further proves that only securin is required for separase inhibition and continued attachment of sister chromatids during Met II arrest in mouse eggs.

In conclusion, this study demonstrates that although there are conditions in which either securin or MPF can prevent chromosome disjunction, such as during meiosis I, securin is the predominant inhibitor of separase during the indeterminate period of Met II arrest. The low levels of securin during meiosis II were proven to be sufficient for regulating

separase activity, therefore it would be interesting to investigate what other functions securin might have during meiosis I, given that its levels at this stage were much higher than those observed during Met II arrest. It will also be worth to examine if the maintenance of securin levels present problems for ageing eggs in keeping sister chromatids attachment. Moreover, it would be interesting to investigate the mechanisms behind separase regulation in eggs from securin knockout mice, given that securin inactivation was only found to cause sub-fertility in those mice (Wang et al., 2001). It would also be crucial to further confirm the results obtained in this chapter using additional securin morpholinos and CDK1 inhibitors.

Chapter 4: MPF and MAPK are required for inhibiting premature destruction of securin during prometaphase of meiosis I.

4.1 Introduction

As already mentioned in previous chapters, securin is the best-known regulator of the protease separase. It is a functionally conserved protein, although there is little conservation of its primary amino acid sequence, except for the presence of D-box and KEN-box motifs. Hence, allowing its targeted destruction by the APC/C at the metaphase-to-anaphase transition, which in turn triggers chromosomes separation through activation of separase (Ciosk et al., 1998; CohenFix et al., 1996; Funabiki et al., 1996; Uhlmann, 2001).

Securin was originally identified in budding yeast as Pds1, which is important but not essential for sister chromatid disjunction (Ciosk et al., 1998; CohenFix et al., 1996). Its depletion does not cause lethality but results in precocious separation of sister chromatids and increased rates of chromosome loss (Yamamoto et al., 1996). In contrast, securin of fission yeast (Cut2) and *Drosophila* (PIM) are essential (Funabiki et al., 1996a; Funabiki et al., 1996b; Leismann et al., 2000; Stratmann and Lehner, 1996). Their absence leads to impairment in separase activity and failure in separating sister chromatids, suggesting that Cut2 and PIM must be more than simple inhibitors of separase (Funabiki et al., 1996b; Jensen et al., 2001; Leismann et al., 2000). Indeed, fission yeast securin, for example, was proven to be required in promoting spindle localization of separase (Kumada et al., 1998). In *Drosophila* and fission yeast, non-degradable PIM and Cut2

cause the cut phenotype (Funabiki et al., 1996b; Leismann et al., 2000), whereas budding yeast with non-degradable Pds1 arrests in M phase (Clarke et al., 1999; Gardner et al., 1999).

Vertebrate securin was first described as the product of the pituitary tumour-transforming gene (PTTG), which is over-expressed in several neoplasms, including the pituitary ones (Pei and Melmed, 1997). It has subsequently been identified as securin due to its ability to bind and regulate separase (Leismann et al., 2000; Zou et al., 1999). The oncogenic nature of PTTG over-expression is believed to be caused by defective sister chromatid separation, resulting in aneuploidy, which is one of the main criteria in tumour development. Accordingly, it was demonstrated that human cell lines expressing non-degradable PTTG exhibit the cut phenotype (Zur and Brandeis, 2001), whereas those lacking PTTG show high chromosome loss and abnormal anaphases (Jallepalli et al., 2001). Also, PTTG knockout mice, although they are viable, the females are sub-fertile and their embryonic fibroblasts display aberrant chromosome morphology, aneuploidy and prolonged G2 and M phases (Mei et al., 2001; Wang et al., 2001).

The crucial role of securin in preventing premature activation of separase and maintaining chromosome attachment until anaphase onset, suggests that it must be highly regulated by mechanisms responding to cell cycle progression. Indeed, in addition to being expressed in a cell-cycle-dependent manner, peaking in M phase and getting destroyed at anaphase onset (Nabti et al., 2008; Ramos-Morales et al., 2000), during the course of this study it has been reported that securin is subject to another layer of

regulation, phosphorylation. This was demonstrated by Holt et al (2008), who found that MPF-dependent phosphorylation of securin near its D-box motif reduces its rate of ubiquitination by the APC/C in budding yeast (Holt et al., 2008). Securin phosphorylation, however, can be reversed by the phosphatase Cdc14, whose activation requires separase activity (Holt et al., 2008). Therefore, at anaphase onset and due to accumulation of APC/C activity, some securin is destroyed, leading to activation of separase. This in turn results in Cdc14 activation, more securin being targeted for degradation and more separase getting active (Holt et al., 2008). Accordingly, these findings suggest the existence of a positive feedback loop that makes separase activation more switch-like, therefore increasing the abruptness of anaphase and sister chromatid separation (Holt et al., 2008). It also has been suggested in other studies that securin interacts with and is a substrate of MAPK (Pei, 2000), although the significance of such interaction on securin stability and function as a separase regulator is still unknown.

I found here that inhibition of both MPF and MAPK, but not either one alone, induces premature destruction of securin during prometaphase of meiosis I. Also, securin stability could be rescued through inhibition of the proteasome or cellular phosphatases. However, mutations of the three candidate phosphorylation sites of MPF and MAPK had no effect on securin stability. Therefore, MPF and MAPK-mediated stability of securin during prometaphase I could not be brought about through its phosphorylation and may be mediated via an indirect mechanism.

4.2. Results

4.2.1. Securin undergoes a gel-mobility shift at the GV to MI transition.

In somatic cells, it was reported that securin is phosphorylated upon entry into the M-phase (Ramos-Morales et al., 2000). At the exit from prophase arrest and upon entry into meiosis I, mouse securin also undergoes a gel-mobility shift, which is suggestive of phosphorylation **(Fig. 4.1)**. This shift was noted in numerous earlier studies but has not yet been investigated (Marangos and Carroll, 2008; Nabti et al., 2008).

4.2.2. Inhibition of both MPF and MAPK induces premature destruction of endogenous securin.

The transition from prophase arrest to MI is driven by an increase in CDK1/cyclin B1 activity. After GVBD, there is also an increase in MAPK activity. Therefore, to determine whether the observed mobility shift of securin at the GV to MI transition was indeed caused by phosphorylation and if this is catalysed by CDK1 and MAPK, the activity of both kinases was inhibited using the pharmacological inhibitors, roscovitine and UO126, respectively. The oocytes were exposed to the drugs between 3-5 h after release from GV arrest. Western blots performed on these oocytes demonstrated that inhibition of both CDK1 and MAPK results in a loss of the band shift **(Fig. 4.2.A),** as well as a decrease in the levels of securin **(Fig. 4.2A, B, C)**. However, inhibition of either kinase alone did not affect securin levels, which remained as high as those observed in the non-treated MI oocytes **(Fig. 4.2.B, C)**.

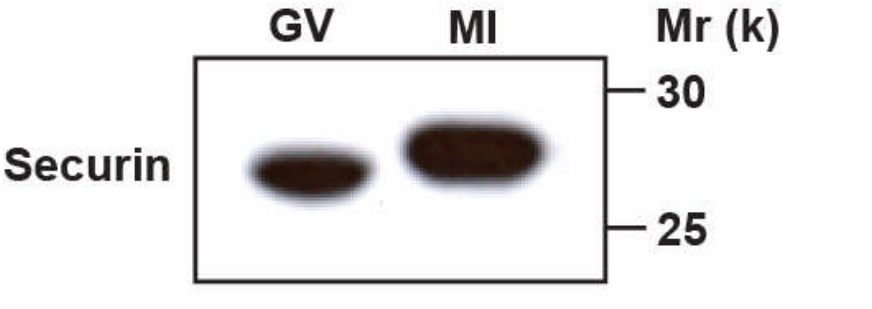

Fig. 4.1. Securin undergoes a gel-mobility shift at the GV to MI transition. Western blot of oocytes (*n=20* per lane) for securin during GV arrest and MI (at 5 h post release from IBMX). This blot is representative of three independent experiments.

These results indicate that the band shift observed at the GV to MI transition could be due to securin phosphorylation, and suggest that this might be catalysed by CDK1 and MAPK. Furthermore, CDK1 and MAPK activities seem to be required for accumulation of securin during prometaphase I.

4.2.3. Inhibition of both MPF and MAPK induces premature destruction of exogenous securin.

Simultaneous inhibition of CDK1 and MAPK induced a decrease in the levels of endogenous securin. To further investigate this, I performed timelapse fluorescence measurements of oocytes injected with the cRNA construct for green fluorescent protein (GFP) tagged securin (securin-GFP) during maturation in the presence or absence of roscovitine and UO126. The measurements demonstrated that in the inhibitors-treated oocytes, securin destruction occurs prematurely (at around 3 h post GVBD) **(Fig. 4.3.A, C)**, whereas in the control non-treated oocytes, securin degradation starts much later (at 7 h post GVBD) **(Fig. 4.3.B, C)**. Taken together, these results further confirm that CDK1 and MAPK activities are required for securin stability during prometaphase of meiosis I.

4.2.4. Proteasome and phosphatase inhibitors restore securin levels.

Next, it was important to examine whether the observed decrease in securin levels required the 26S proteasome. Therefore, the oocytes were further incubated with the proteasome inhibitor (MG132).

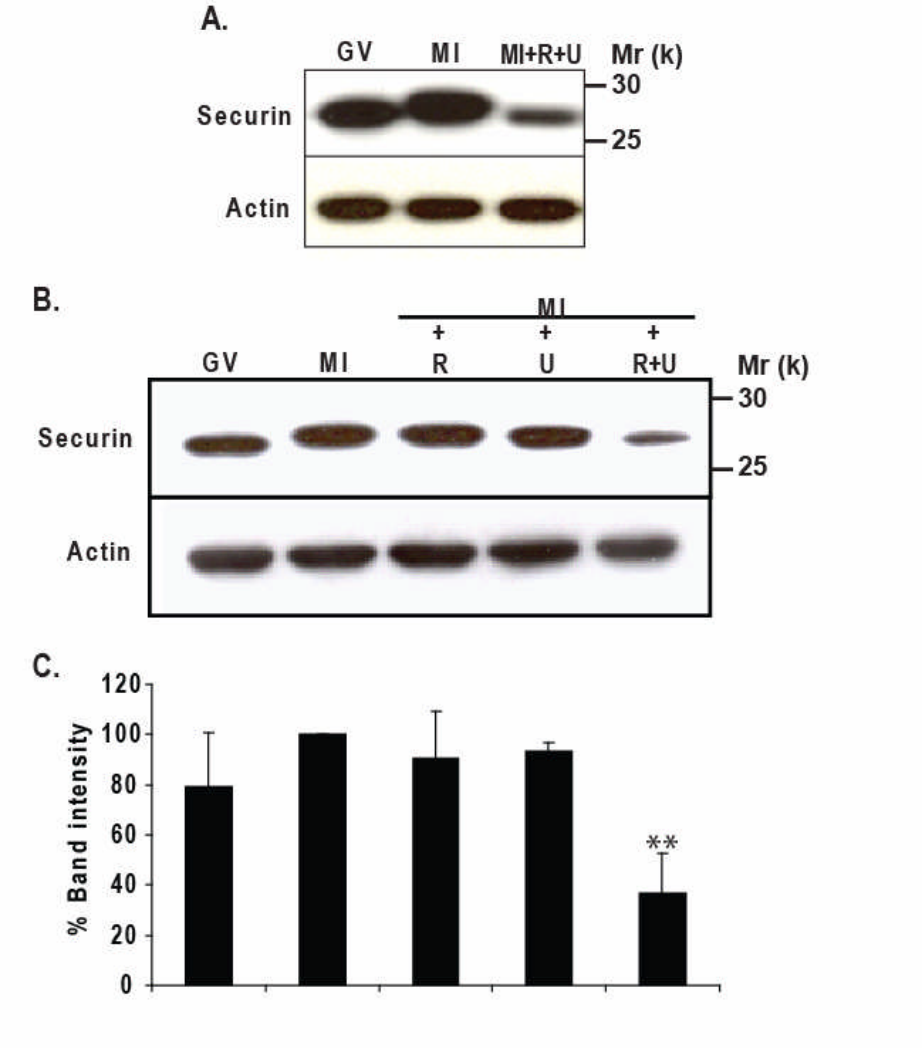

Fig. 4.2. Inhibition of MPF and MAPK induces premature destruction of endogenous securin. (A) Western blot of oocytes (*n=20* per lane) for securin during GV arrest, MI, and MI following incubation with roscovitine and UO126 (MI+R+U). **(B)** Western blot of oocytes (*n=20* per lane) for securin during GV arrest, MI, and MI following treatment with either roscovitine (R), UO126 (U) or both roscovitine and UO126 (R+U). **(C)** Densitometric analysis of the Western blot in panel B. The results are mean ± s. d. from three independent experiments. MI is set at 100%. The asterisks represent a value significantly different from control (the MI value) at $^{**}P$ (0.001-0.01). In panels A and B, all MI samples were collected at 5 h post release from IBMX, and drug treatment was performed between 3 and 5h. Actin was used as a loading control.

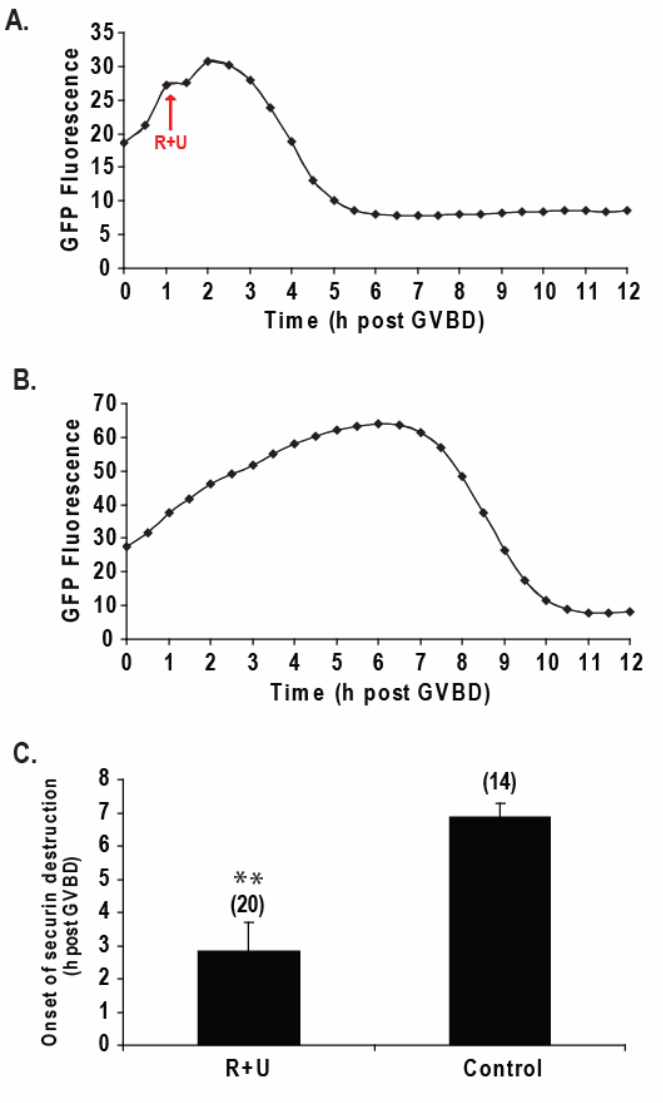

Fig. 4.3. Inhibition of MPF and MAPK induces premature destruction of securin-GFP. Representative traces of oocytes injected with securin-GFP. The oocytes were injected at the GV stage and then matured in culture in the presence **(A)** or absence **(B)** of roscovitine and UO126 (R+U)**.** The fluorescence readings were recorded at the times shown during oocyte maturation. In panel A, the drugs were added at 1 h post GVBD (red arrow). **(C)** Time of onset of securin-GFP destruction in the inhibitors-treated oocytes (R+U) and non-treated oocytes (control). The peak of the graph was taken as a marker for the start of destruction. The results are mean ± s. d. from three independent experiments. The asterisks represent a value significantly different from control at $^{**}P$ (0.001-0.01).

Western blot performed on these oocytes showed that MG132 restores securin levels to the MI levels, suggesting that the decrease in securin levels upon CDK1 and MAPK inhibition is due to 26S proteasome-mediated destruction **(Fig. 4.4.A, B)**. Moreover, the protein phosphatase 1 and 2A inhibitor (okadaic acid) also rescued securin levels **(Fig. 4.4.A, B)**, and oocytes incubated with the phosphatase inhibitor were less efficient at destroying securin-GFP at the metaphase-to-anaphase transition when compared to oocytes in control conditions **(Fig. 4.4.C)**, which suggest that securin stability during meiosis I may depend on its phosphorylation status.

4.2.5. Prometaphase I securin stability does not depend on the phosphorylation status of the three MPF and MAPK sites.

Having shown in the previous experiment that okadaic acid treatment prevents the premature destruction of securin when CDK1 and MAPK are inhibited, I went on to investigate whether the possible phosphorylation of securin by those two kinases increases its stability. Examination of the amino acid sequence for mouse securin using the Scansite program (http://scansite.mit.edu) showed six consenus motifs for MPF phosphorylation and five for MAPK phosphorylation. However, since securin stability requires the activity of either MPF or MAPK, only the sites that can be phosphorylated by either kinase were chosen for mutagenesis **(Fig. 4.5.A)**. Therefore, I created a Phos^{-} mutant of securin in which the three-shared sites (T43, S165 and S171) were substituted for alanine residues, thus preventing them from becoming phosphorylated.

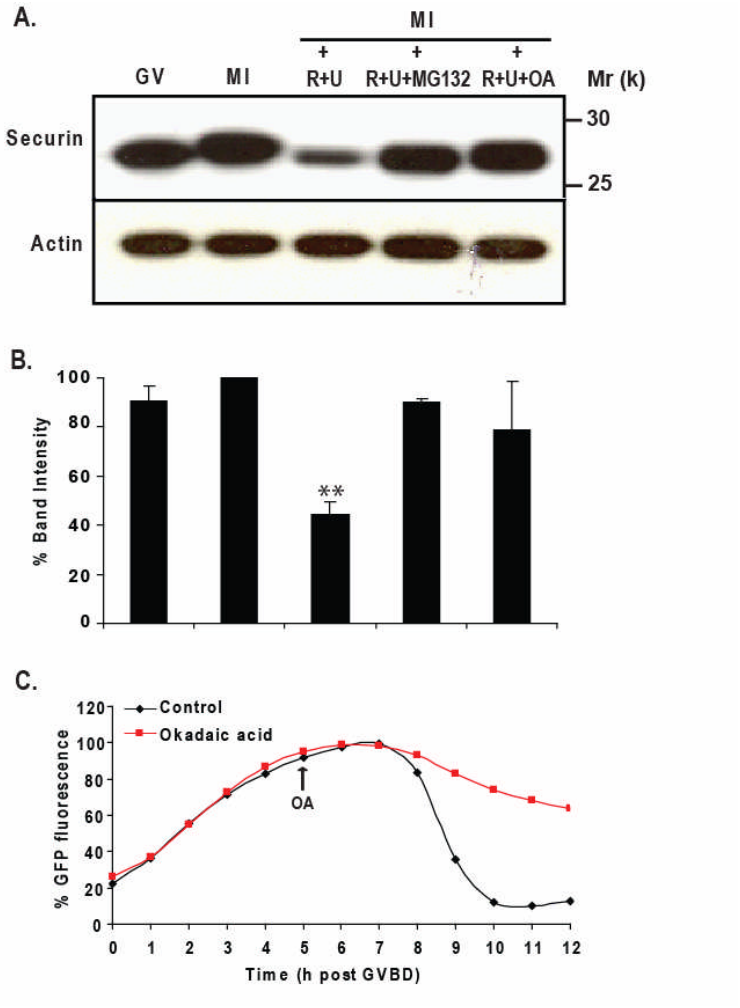

Fig. 4.4. Proteasome and phosphatase inhibitors restore securin levels. (A) Western blot of oocytes (*n=20* per lane) for securin during GV arrest, MI, MI following incubation with roscovitine and UO126 (R+U), and MI after treatment with either MG132 or okadaic acid (OA) in addition to roscovitine and UO126. All MI oocytes were collected at 5 h post release from IBMX, and drug treatment was performed between 3 and 5 h. Actin was used as a loading control. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. MI is set at 100%. The asterisks represent a value significantly different from control (the MI value) at $^{**}P$ (0.001-0.01). **(C)** Representative traces of oocytes (from two independent experiments) injected with securin-GFP at the times shown during maturation, in the presence (*n=10*, red) or absence (*n=10*, black) of okadaic acid. The arrow indicates time of okadaic acid addition.

Timelapse fluorescence records of oocytes injected with cRNA constructs for GFP tagged Phos$^-$ securin (Phos$^-$ securin-GFP) or wild type securin (securin-GFP) demonstrated similar expression profiles and stability during oocyte maturation **(Fig. 4.5.B)**. However, since these measurements represent the balance between protein synthesis and destruction, it remained possible that the changes in destruction are masked by the rates of synthesis. To exclusively monitor protein destruction, the oocytes were incubated in the presence of cycloheximide to inhibit protein synthesis while monitoring the levels of securin-GFP. This also failed to reveal any difference in the behaviour of the non-phosphorylable mutant and wild type securin during prometaphase I **(Fig. 4.5.C)**. These data suggest that in mouse oocytes, the phosphorylation of securin at the three MPF and MAPK sites may not underlie the changes in its stability when MPF and MAPK are inhibited.

4.2.6. Phos$^-$ mutations do not affect securin function.

Next, I wanted to investigate whether the Phos$^-$ mutations had any effects on securin function. In the previous chapter, I have shown that securin is the main inhibitor of separase during Met II arrest, and that securin morpholino knockdown during this period resulted in chromatin misalignment, due to sister chromatid separation. However, this phenotype could be rescued by injection of securin cRNA in the morpholino-treated eggs. Therefore, to test whether the non-phosphorylable securin mutant was also capable of rescuing this phenotype, Met II-arrested eggs were co-injected with securin^{MO} and the cRNA for either Phos$^-$ or wild type securin. The eggs were then assessed for their chromatin status by Hoechst staining 12 h following the microinjections.

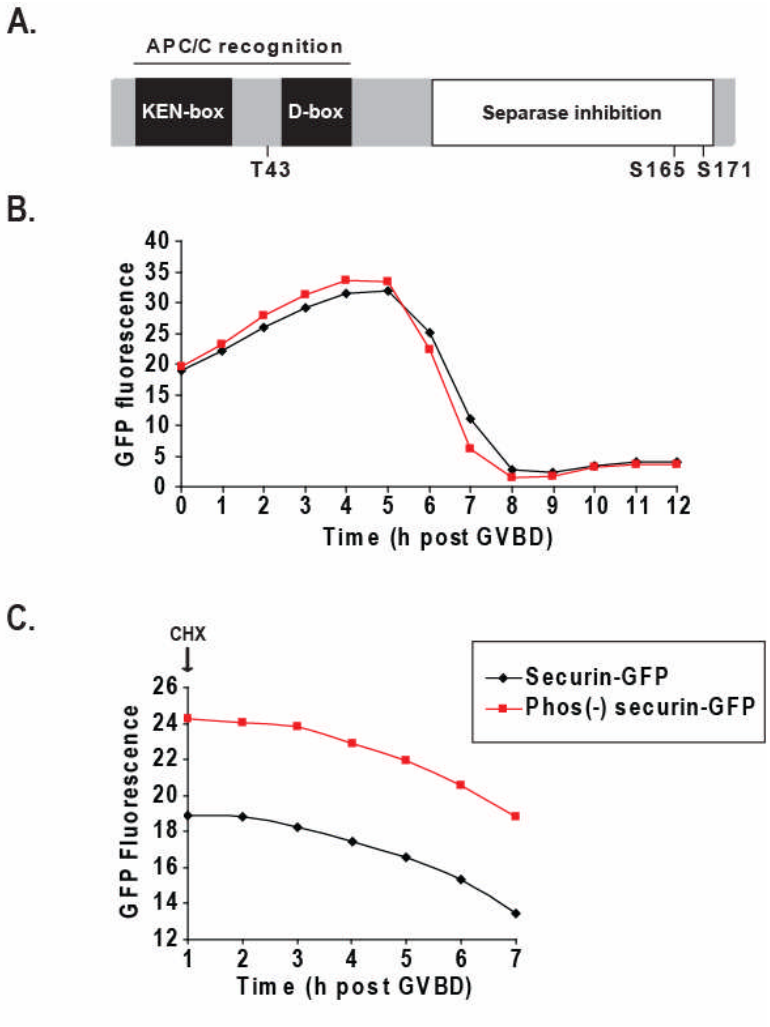

Fig. 4.5. The phosphorylation status of securin at the three MPF and MAPK sites has no affect on its stability during prometaphase I. **(A)** A schematic diagram of mouse securin showing the relative position of CDK1 and MAPK phosphorylation sites to the APC recognition motifs and the separase inhibition domain. **(B)** Representative traces of oocytes (from two independent experiments) injected with either Phos$^-$ securin-GFP (*n=15*) or wild type securin-GFP (*n=13*) at the times shown during maturation. **(C)** Representative traces of oocytes (from two independent experiments) injected with either Phos$^-$ securin-GFP (*n=12*) or wild type securin-GFP (*n=12*) at the times shown during maturation, in the presence of cycloheximide (CHX).

As reported previously, the chromatin in the morpholino-treated eggs was misaligned and this phenotype could be rescued by injection of wild type securin **(Fig. 4.6.A, B)**. More importantly, injection of the Phos^{-} securin mutant in the securin^{MO}-treated eggs also rescued sister chromatid attachment, as judged by the proper alignment of the chromatin **(Fig. 4.6.A, B)**. Taken together, these results suggest that securin phosphorylation at the three MPF and MAPK sites was not critical for its function as a separase inhibitor.

4.3. Discussion

It is well established that securin plays a crucial role in ensuring the faithful segregation of chromosomes through regulating the activity of the protease separase. For that reason, securin is highly regulated by mechanisms responding to cell cycle progression. In mitosis, as well as meiosis, this is mainly achieved through the control of its expression, which is done in a cell-cycle-dependent manner, with securin peaking in M-phase and getting destroyed at anaphase onset (Nabti et al., 2008; Ramos-Morales et al., 2000), therefore allowing separase activation and chromosomes separation (Ciosk et al., 1998; CohenFix et al., 1996; Funabiki et al., 1996b; Uhlmann, 2001). Recently, however, and during the course of this investigation it was reported by Holt et al (2008) that phosphorylation could provide an additional layer of regulation. According to this study, MPF-dependent phosphorylation of securin near its D-box motif reduces its rate of ubiquitination by the APC/C in budding yeast (Holt et al., 2008). More importantly, this additional regulatory mechanism seems to be conserved, as it was also observed in human PTTG and *Drosophila* PIM (Holt et al., 2008).

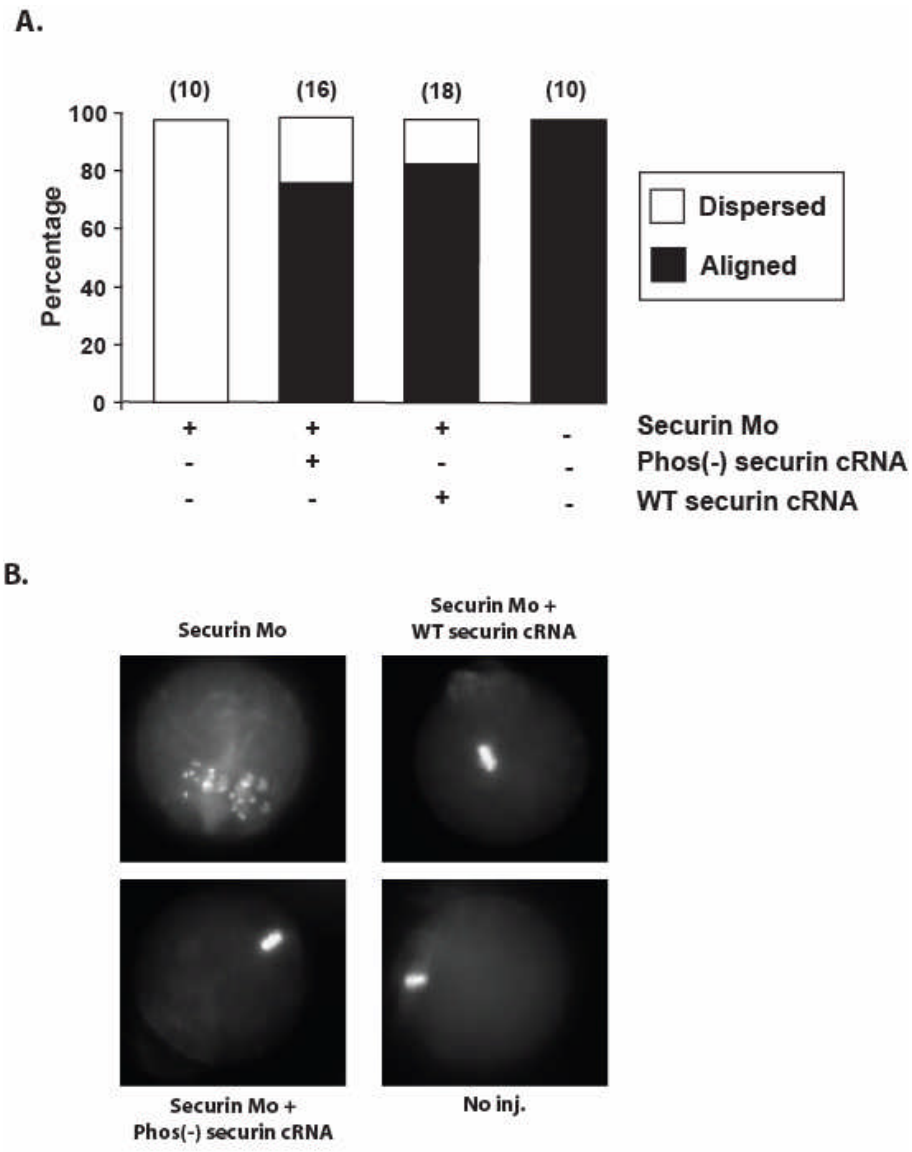

Fig. 4.6. Phos$^-$ mutations do not affect securin function. (A) Percentage of eggs with dispersed (white bars) or perfectly aligned chromatin (black bars) following microinjection of securin^{MO} alone, securin^{MO} and the cRNA for either Phos(-) or WT securin, or no injections (no inj.). The eggs were assessed for their chromatin status by Hoechst staining at 12 h following microinjections. **(B)** Representative images of the eggs described in panel A.

In previous experiments, it was noted that upon entry into meiosis I, mouse securin undergoes a gel-mobility shift, which is suggestive of phosphorylation (Marangos and Carroll, 2008; Nabti et al., 2008). To test this hypothesis, the two major M-phase kinases, CDK1 and MAPK were inhibited using the pharmacological inhibitors, roscovitine and UO126, respectively. The inhibition of both kinases induced a loss of the band shift, which suggests that the mobility-shift observed at the GV to MI transition could be due to securin phosphorylation, and suggests that this might be catalysed by CDK1 and MAPK. Interestingly, inhibition of both kinases, but not either one alone, also induced a decrease in the levels of endogenous securin and premature destruction of exogenous securin during prometaphase I, indicating that the activity of either CDK1 or MAPK might be required for the accumulation of securin during meiosis I.

The decrease in securin levels upon inhibition of both kinases was proven to be caused by its destruction rather than inhibition of synthesis, given that the proteasome inhibitor MG132 restored the levels of securin. More importantly, the phosphatase inhibitor, okadaic acid, also prevented the premature degradation of endogenous securin during prometaphase I, and stabilised the levels of exogenous securin at the metaphase-to-anaphase transition. These findings imply that okadaic acid protects securin when CDK1 and MAPK are inhibited. There are a number of possible explanations for such effect. First, securin destruction may be dependent on the phosphorylation of one of the components of the degradation machinery. Alternatively, it may be that the phosphorylation of securin results in increased stability, as reported by Holt et al 2008.

To further investigate the latter hypothesis, the three candidate sites for MPF and MAPK phosphorylation were substituted for alanine residues, thus preventing them from becoming phosphorylated. Two of the sites (S165 and S171) are situated within the separase inhibition domain, whereas the third site (T43) is near the D-box motif. Securin mutants carrying single alanine substitutions at the three sites individually were as stable as the wild type securin (results not shown). Also, the triple alanine securin mutant failed to reveal any difference in its behaviour when compared to the wild type securin. These results are not consistent with the study by Holt et al, who found that securin phosphorylation near its D-box motif reduces its rate of ubiquitination by the APC/C, hence promoting its stability (Holt et al., 2008). Therefore, in contrast to budding yeast, human and *Drosophila*, in mouse oocytes securin phosphorylation and in particular at the three MPF and MAPK sites may not underlie the changes in its stability.

Having shown that the Phos$^-$ mutations had no effect on securin stability, I went on investigating whether the phosphorylation of securin upon entry into M-phase was important for its function as a separase inhibitor. In budding yeast, it was reported that securin phosphorylation by MPF is required for its binding to separase and promoting the nuclear localization of the protease (Agarwal and Cohen-Fix, 2002). Moreover, it is well established that securin interacts with separase through its carboxy (C) terminal domain, and mutations within this domain would modulate securin ability to bind separase (Agarwal and Cohen-Fix, 2002; Hornig et al., 2002). Therefore, it was important to determine whether the triple alanine securin mutant, which carries two mutations in the separase-binding domain, maintained its function as a separase inhibitor. Indeed, the

mutant was able to rescue the chromatin misalignment in securin^{MO}-injected eggs, suggesting that the phosphorylation status of securin in general and of residues S165 and S171 in particular does not affect its function. These results, although they are not consistent with earlier observations, they agree with a study by Boelaert et al (2004), which demonstrated that mutagenesis of the conserved residue S165 does not influence human PTTG stability and function, even though it affects its transforming ability (Boelaert et al., 2004).

In summary, the experiments reported here demonstrate that although the inhibition of MPF and MAPK induces the premature destruction of securin during prometaphase of meiosis I, the stability of securin during this period is not dependent on the three phosphorylation sites for MPF and MAPK, as proven by the mutagenesis experiments. Therefore, it would be interesting to determine what causes such premature destruction of securin in the absence of MPF and MAPK. It will be worth examining whether this effect is due to activation of the APC/C, whose activity is well known to be modulated by its phosphorylation status (Hershko et al., 1994; Kotani et al., 1998; Kraft et al., 2003). It will also be important to confirm these results using additional inhibitors of MPF and MAPK. Finally, it will be interesting to investigate the reasons behind securin phosphorylation upon entry into meiosis I, other than regulating its stability and function as a separase inhibitor.

Chapter 5: MPF and MAPK are required for inhibiting premature destruction of the APC/C D-box substrates during prometaphase of meiosis I.

5.1. Introduction

The initiation of anaphase and exit from M phase in both mitotic and meiotic divisions depend on the E3 ubiquitin ligase activity of the APC/C, which ubiquitinates key cell cycle regulators, such as securin and cyclin B1. Therefore, targeting them for destruction by the proteasome at the metaphase-to-anaphase transition (Clute and Pines, 1999; Herbert et al., 2003; Zur and Brandeis, 2001). As a key driver of the cell cycle, the activity of the APC/C is subject to many regulatory mechanisms, mainly through direct binding to co-activators, which confer substrate specificity during specific periods of the cell cycle. The best-studied co-activator proteins are Cdc20 and Cdh1 (Visintin et al., 1997a).

In meiosis, it is well established that Cdc20, but not Cdh1, is the mediator of the meiotic exit in eggs of species described thus far (Herbert et al., 2003; Salah and Nasmyth, 2000). In mammals, however, Cdh1 has been shown to play a more predominant role during prophase I (Marangos et al., 2007; Reis et al., 2006a), prometapahse I (Reis et al., 2007) and following PB2 extrusion (Chang et al., 2004). In mouse oocytes, APC/C^{Cdh1} is required for preventing unscheduled entry into M-phase by suppressing the levels of cyclin B1 (Marangos et al., 2007; Reis et al., 2006a). During prometaphase I, however, APC/C^{Cdh1} targets Cdc20 for destruction thereby delaying the onset of APC/C^{Cdc20}

activity and the onset of anaphase, until correct assembly of the MI spindle (Reis et al., 2007).

Because of their essential role for the activity and specificity of the APC/C, the regulation of Cdc20 and Cdh1 is also important. During mitosis, Cdc20 is mainly regulated through the control of its expression, which is done in a cell-cycle-dependent manner, with Cdc20 peaking in M-phase and getting destroyed in G1 (Fang et al., 1998; Prinz et al., 1998). Another level of regulation is through association with different inhibitors, such as Emi1 and SAC proteins (Fang et al., 1998; Miller et al., 2006; Musacchio and Salmon, 2007; Reimann et al., 2001a; Reimann et al., 2001b; Yu, 2002). Cdh1, however, while subject to similar levels of control as APC/C^{Cdc20}, is also inhibited through phosphorylation by MPF (Jaspersen et al., 1999; Zachariae et al., 1998a), as well as binding to numerous inhibitors, including Emi1 and the nuclear transport factors Rae1 and Nup98 (Jeganathan et al., 2006; Miller et al., 2006; Reimann et al., 2001b).

The core-subunits of the APC/C are also subject to regulatory phosphorylation during mitosis. However, unlike Cdh1, APC/C phosphorylation by CDK1 and Plk1 is required for its activation and association with Cdc20 (Hershko et al., 1994; Kotani et al., 1998; Kraft et al., 2003). Accordingly, it was demonstrated that phosphatase treatment of mitotic APC/C in *vitro* prevents its activity, whereas addition of CDK1 stimulates it (Lahavbaratz et al., 1995). Moreover, Cdc20 also gets phosyphorylated upon entry into mitosis. However, unlike the APC/C core-subunits, this CDK1 and MAPK-dependent phosphorylation of Cdc20 is not required for the activity of the ligase, although it is necessary for the recognition and inhibition of the co-activator by the components of the

SAC. This was demonstrated by Chung and Chen (2003), who found that phosphorylation deficient mutants of *Xenopus* Cdc20 are insensitive to the spindle checkpoint signal and unable to associate with its components (Chung and Chen, 2003).

In meiosis, unlike mitosis, the mechanisms of APC/C regulation are as yet largely unexplored, especially during the prolonged period of prometaphase I. In this study, I show that inhibition of MPF and MAPK induces premature destruction of the APC/C D-box substrates but not Ken-box substrates. This prometaphase I instability of the D-box-containing substrates was apparently not mediated by APC/C^{Cdc20} or APC/C^{Cdh1}, given that Cdc20 is also targeted for destruction and morpholino-knockdown of both Cdc20 and Cdh1 failed to rescue securin stability. Also, the inhibition of $SCF^{\beta\text{-}TrCP}$ using the dominant negative mutant of its F-box protein (β-TrCPΔ) could not restore securin levels, suggesting it could not be $SCF^{\beta\text{-}TrCP}$-dependent. Therefore, this effect may be attributed to activation of another APC/C co-activator or the core APC/C, which was recently reported of being able to directly interact with the D-box containing substrates independently of Cdc20 and Cdh1 (Passmore et al., 2003; Yamano et al., 2004).

5.2. Results

5.2.1. Inhibition of MPF and MAPK induces premature destruction of the APC/C D-box substrates.

In the previous chapter, I have demonstrated that inhibition of both CDK1 and MAPK induces the premature destruction of securin during prometaphase I. To determine whether this phenotype was specific to securin, the stability of other APC/C substrates,

such as cyclin B1 and geminin, was investigated. Cyclin B1 and geminin were chosen because, similar to securin, they both accumulate during the cell cycle and are targeted for destruction at the metaphase-to-anaphase transition by the APC/C^{Cdc20} in a D-box-dependent manner. Cyclin B1 stability was examined using Western blotting, whereas geminin was tested using timelapse fluorescence measurements, due to the lack of the anti-geminin antibody in the lab. Interestingly, Western blots performed on MI oocytes that were treated with roscovitine and UO126 demonstrated that cyclin B1 is also subject to the premature degradation upon CDK1 and MAPK inhibition **(Fig. 5.1.A, B)**. Moreover, timelapse fluorescence measurements of oocytes injected with the cRNA construct for GFP tagged geminin (geminin-GFP) during maturation showed that in the control oocytes, geminin destruction starts at 6 h post GVBD; whereas in the inhibitor-treated oocytes it starts significantly earlier, at around 3 h post GVBD **(Fig. 5.1.C, D, E)**. Taken together, these results suggest that in addition to the premature degradation of securin, inhibition of CDK1 and MAPK also induces the premature destruction of other D-box substrates of the APC/C, including cyclin B1 and geminin.

5.2.2. Inhibition of MPF and MAPK does not induce premature destruction of Ken-box substrates.

Having shown that the APC/C D-box containing substrates (securin, cyclin B1 and geminin) are prematurely targeted for degradation in the absence of CDK1 and MAPK activities, it was important to address whether this was the case for PLK1 and the D-box mutated variant of securin (securin^{DM}), two substrates that rely on the KEN-box for destruction.

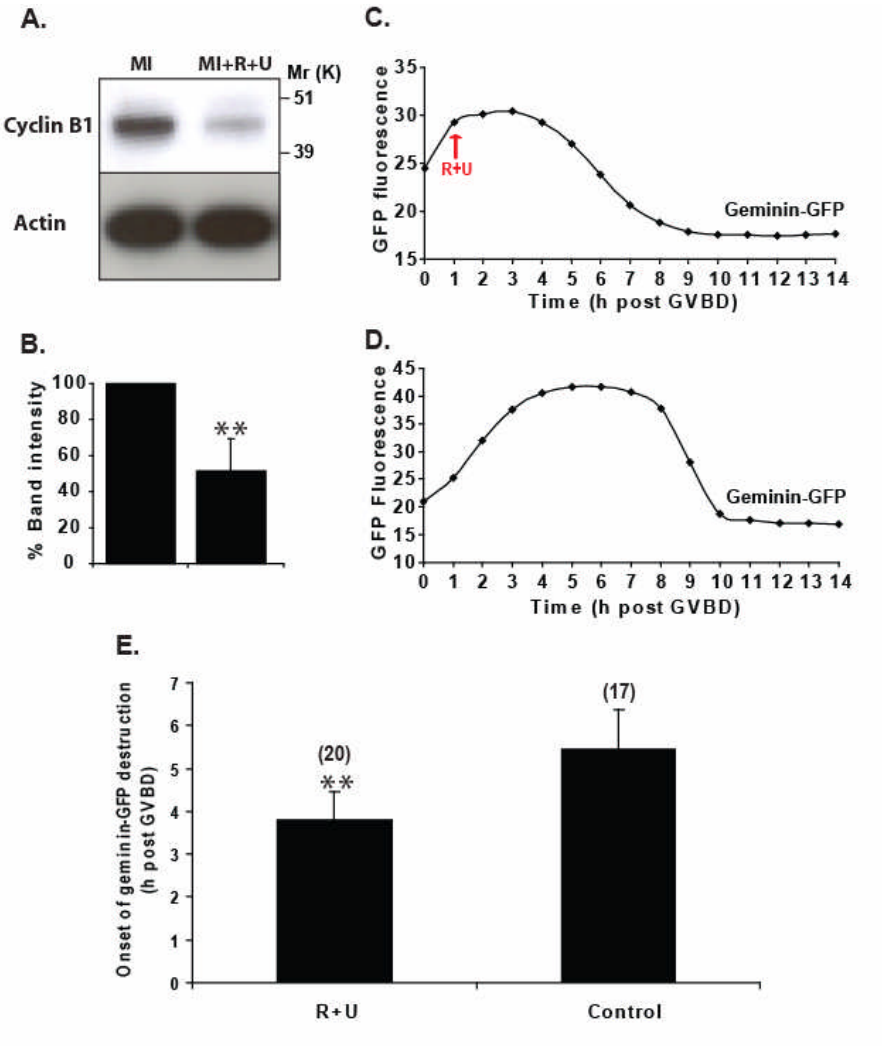

Fig. 5.1. Inhibition of MPF and MAPK induces premature destruction of cyclin B1 and geminin. (A) Western blot of oocytes (*n=30* per lane) for cyclin B1 at MI in the presence or absence of roscovitine and UO126 (R+U). The oocytes were collected at 5 h post release from IBMX, and drugs treatment was performed between 3 and 5 h. Actin was used as a loading control. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. MI is set at 100%. **(C)** Representative traces of oocytes (*n=20*, from three independent experiments) injected with geminin-GFP at the times shown during maturation in the presence of roscovitine and UO126. The drugs were added at 1 h post GVBD (red arrow). **(D)** Representative traces of oocytes (*n=17*, from two independent experiments) injected with geminin-GFP at the times shown during maturation, in the absence of the inhibitors. **(E)** Time of onset of geminin-GFP destruction in the inhibitors-treated oocytes (R+U) and non-treated oocytes (control). The peak of the graph was taken as a marker for the start of destruction. The results are mean ± s. d. The asterisks represent values different from controls at $^{**}P$ (0.001-0.01).

Surprisingly, Western blots performed on the inhibitor-treated and non-treated MI oocytes showed no significant difference in the levels of PLK1 between both groups **(Fig. 5.2.A, B)**. Next, I performed timelapse fluorescence measurements of oocytes injected with cRNA constructs for GFP tagged wild type securin (WT securin-GFP) or securin^{DM} (securin^{DM}-GFP) during maturation, in the presence or absence of roscovitine and UO126. The recordings demonstrated that wild type securin was destroyed in both the inhibitor-treated and non-treated oocytes **(Fig. 5.2.C, D, E)**. Although, as reported in the previous chapter, the onset of securin degradation in the treated oocytes occurs prematurely (at 3 h post GVBD), whereas in the non-treated oocytes, securin destruction starts 2 h later (at 5 h post GVBD) **(Fig. 5.2.C, D)**. Interestingly, securin^{DM} was stable throughout oocytes maturation and failed to be destroyed in the presence or absence of the drugs **(Fig. 5.2.C, D, E).** To ensure that securin^{DM} stability during MI is real, the destruction rates of both mutant and wild type securin were monitored in GV-arrested oocytes, which were incubated in the presence of cycloheximide to inhibit protein synthesis. Securin^{DM} contains a functioning Ken-box and therefore can only be targeted for destruction by APC/C^{Cdh1}. Indeed, the readings demonstrated that during the GV-arrest, when APC/C^{Cdh1} is active, securin^{DM} is destroyed, although its destruction was much slower than the wild type securin **(Fig. 5.2.F)**, presumably due to the presence of a single KEN-box rather than a KEN- and D-box. Taken together, these results further confirm the necessity of the D-box for the APC/C^{Cdc20}-mediated degradation of securin at the metaphase-to-anaphase transition, and suggest its requirement for the efficient destruction of securin by APC/C^{Cdh1}. More importantly, the D-box seems to be indispensable for the premature destruction of securin upon MPF and MAPK inhibition.

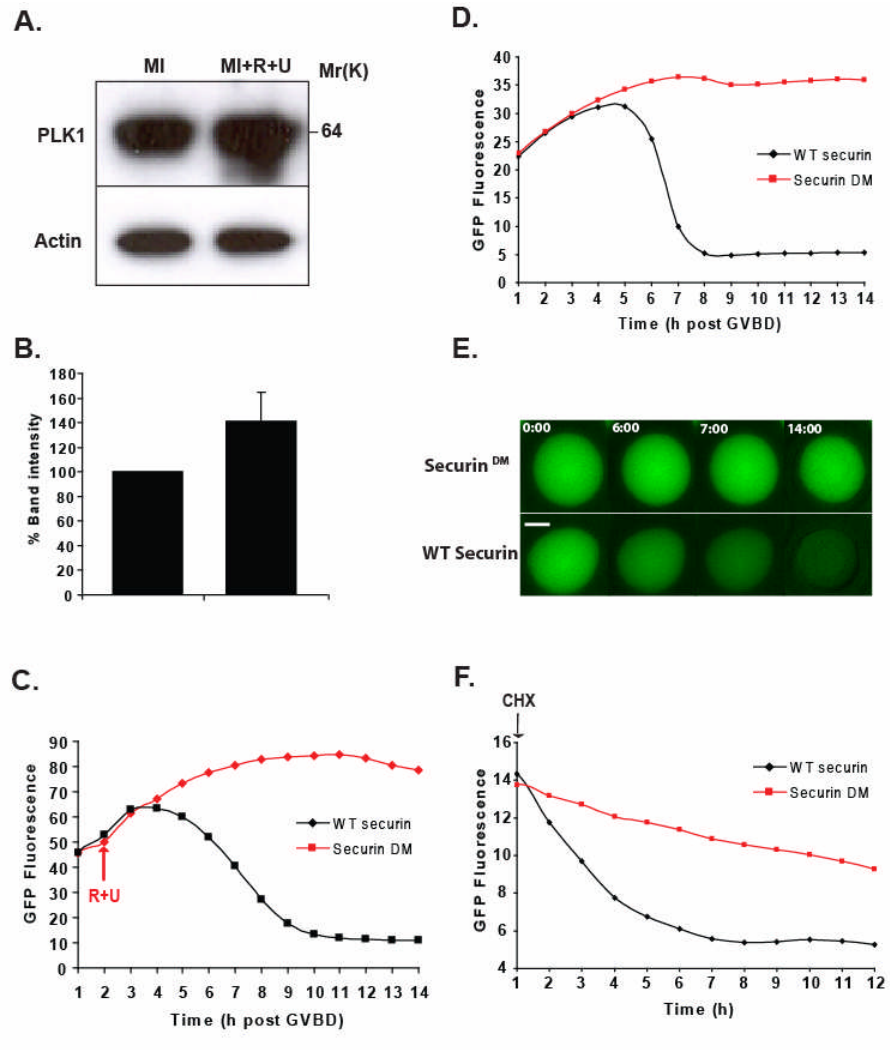

Fig. 5.2. Inhibition of MPF and MAPK does not induce premature destruction of PLK1 and securin^{DM}. (A) Western blot of oocytes (*n=20* per lane) for PLK1 at MI in the presence or absence of roscovitine and UO126 (R+U). The oocytes were collected at 5 h post release from IBMX, and drugs treatment was performed between 3 and 5 h. Actin was used as a loading control. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. MI is set at 100%. **(C)** Representative traces of oocytes (from three independent experiments) injected with either WT securin-GFP (*n=11*) or securin^{DM}-GFP (*n=11*) at the times shown during maturation, in the presence of roscovitine and UO126. The drugs were added at 2 h post GVBD (red arrow). **(D)** Representative traces of oocytes (from two independent experiments) injected with either WT securin-GFP (*n=11*) or securin^{DM}-GFP (*n=11*) at the times shown during maturation, in the absence of the inhibitors. **(E)** Representative images of the oocytes analysed in panel D at the times shown during maturation (h post GVBD). Scale bar 20 µm. **(F)** Representative traces of oocytes injected with either WT securin-GFP (*n=12*) or securin^{DM}-GFP (*n=12*) at the times shown during GV-arrest, in the presence of cycloheximide (CHX). These results are representative of two independent experiments.

5.2.3. Premature destruction of securin is not APC/C^{Cdc20}-dependent.

Inhibition of MPF and MAPK induced premature destruction of the D-box containing substrates only and not Ken-box substrates, suggesting that APC/C^{Cdc20} is likely to be the mediator of this effect. To test this hypothesis, the levels of Cdc20 in the roscovitine and UO126 treated MI oocytes were examined by Western blotting. Interestingly, inhibition of both kinases also induced the premature degradation of Cdc20 **(Fig. 5.3.A)**, which suggests that the instability of the D-box substrates upon MPF and MAPK inhibition could not be brought about via the activation of APC/C^{Cdc20}. To further investigate this, Cdc20 morpholino (Cdc20^{MO}) was utilised to deplete oocytes of Cdc20. The morpholino was injected in GV-stage oocytes, which were kept arrested for 24 h. These oocytes were then released, cultured for 5 h in the presence of roscovitine and UO126 and assessed for their securin levels using Western blotting. There was no significant difference in the levels of securin between Cdc20^{MO}-injected and non-injected oocytes in the presence of the inhibitors **(Fig. 5.3.A, B)**, which provides further evidence to suggest that securin instability upon MPF and MAPK inhibition may not be APC/C^{Cdc20}-dependent. Next, it was important to to confirm the effeciency of the Cdc20 morpholino at inhibiting the activity of APC/C^{Cdc20}, given that the Cdc20 blot was hard to replicate due to problems with the antibody. This was done through testing the abilitiy of the morpholino to block oocytes at MI, since it is well established that the APC/C^{Cdc20} is the mediator of the MI exit. Therefore, the morpholino was injected into GV-stage oocytes, which were kept arrested for 24h, released and then assesessed for PB1 extrusion at 14 h post realease. Indeed, all the Cdc20 morpholino-injected oocytes blocked at MI and failed to extrude PB1, whereas 54% of the non-injected control oocytes extruded PB1 **(Fig. 5.3.C)**, which

suggests that the Cdc20 morpholino is efficient at inhibiting APC/C^{Cdc20} activity and further confirms the previous findings.

5.2.4. Premature destruction of securin is not APC/C^{Cdh1}-dependent.

Having shown that the premature degradation of the D-box containing substrates could not be mediated by APC/C^{Cdc20}, the role of APC/C^{Cdh1} was investigated. Firstly, the levels of Cdh1 in roscovitine and UO126 treated MI oocytes were examined by Western blotting. Unexpectedly, inhibition of both kinases induced a significant increase in Cdh1 levels, which were approximately twofold higher than those observed in the control MI oocytes **(Fig. 5.4.A, B)**. Next, it was important to determine whether the stabilisation of Cdh1 levels upon MPF and MAPK inhibition was responsible for APC/C^{Cdh1} activation and hence the premature destruction of securin. Therefore, oocytes were depleted of Cdh1 using Cdh1 morpholino ($Cdh1^{MO}$). The morpholino was injected into GV-stage oocytes, which were kept arrested for 24 h. These oocytes, alongside non-injected oocytes, were then released, cultured for 5 h in the presence of the inhibitors and assessed for their Cdh1 and securin levels using Western blotting. The morpholino successfully decreased Cdh1 levels to about 25% of those observed in the inhibitor-treated but non-injected oocytes. Nevertheless, the low level of securin in the treated oocytes was not affected by the depletion of Cdh1 **(Fig. 5.4.C, D)**.

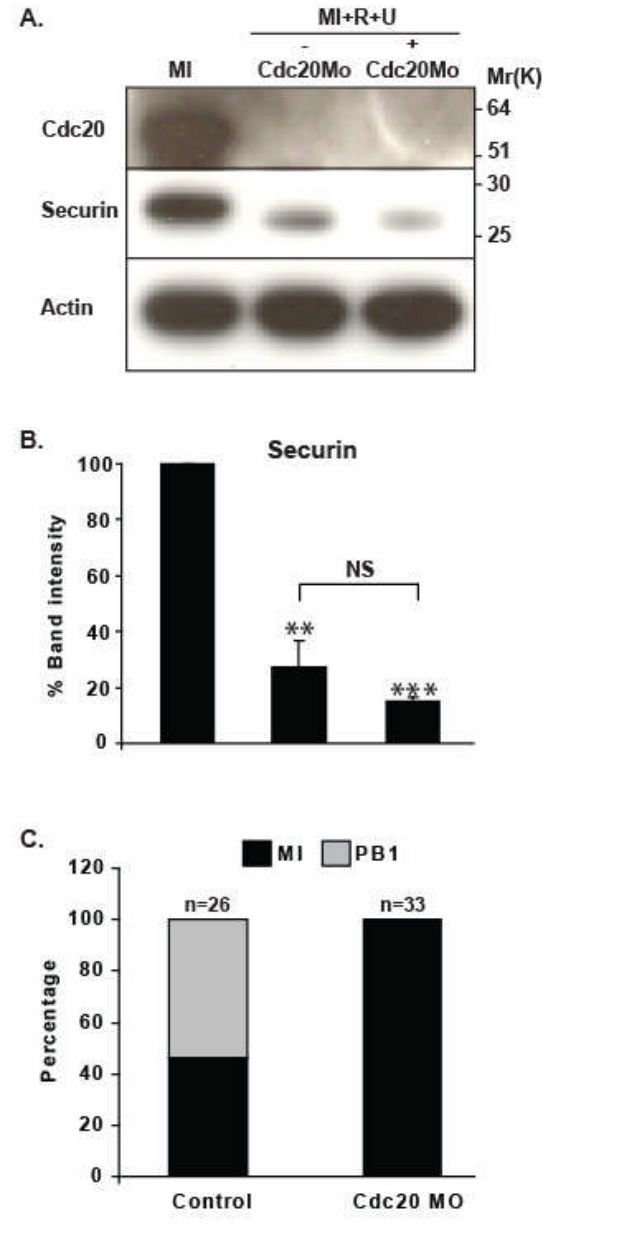

Fig. 5.3. Premature destruction of securin is not APC/C^{Cdc20}-dependent. (A) Western blot of oocytes (*n=20* per lane) for Cdc20 and securin at MI, and MI following incubation with roscovitine and UO126 (R+U) in the presence or absence of Cdc20 morpholino (Cdc20^{MO}). Oocytes were collected at 5 h post release from IBMX, and drugs treatment was performed between 3 and 5h. Actin was used as a loading control. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. MI is set at 100%. Asterisks indicate values significantly different from control (the MI value) at $^{**}P$ (0.001-0.01) and $^{***}P < 0.001$, whereas NS represents values not significantly different at $^{NS}P > 0.05$. **(C)** Rates of MI arrest and PB1 extrusion in Cdc20^{MO}-injected (*n=33*) and non-injected oocytes (*n=26*).

To further confirm the efficiency of the Cdh1 morpholino at inhibiting the activity of APC/C^{Cdh1}, the morpholino was injected into GV-stage oocytes, when APC/C^{Cdh1} is known to be active. These oocytes, alongside non-injected oocytes, were kept arrested for 24 h, and then assessed for their securin levels using Western blotting. The levels of securin in the morpholino treated oocytes were twofold higher than those observed in the control GV oocytes, suggesting that the Cdh1 morpholino was efficient at inhibiting APC/C^{Cdh1} activity **(Fig. 5.4.E, F)**. Taken together, these results suggest that securin instability upon CDK1 and MAPK inhibition could not be mediated by the APC/C^{Cdh1}.

5.2.5. Combined morpholino-knockdown of Cdc20 and Cdh1 induces more destruction of securin.

In the previous sections I have demonstrated that depletion of either Cdc20 or Cdh1 failed to rescue the premature destruction of securin upon CDK1 and MAPK inhibition. Next, I decided to deplete the oocytes of both co-activators. Therefore, Cdc20 and Cdh1 morpholinos were simultaneously injected into GV-stage oocytes, which were kept arrested for 24 h. These oocytes, alongside non-injected oocytes, were then released, cultured for 5 h in the presence or absence of roscovitine and UO126 and assessed for their securin levels using Western blotting. Unexpectedly, simultaneous knockdown of Cdc20 and Cdh1 induced an increased destruction of securin, whose levels were significantly lower than those observed in the non-injected oocytes, in the presence or absence of the inhibitors **(Fig. 5.5.A, B)**. These data raise the possibility that neither APC/C^{Cdc20} nor APC/C^{Cdh1} mediates the premature destruction of securin upon MPF and MAPK inhibition, and that additional factors may be acting.

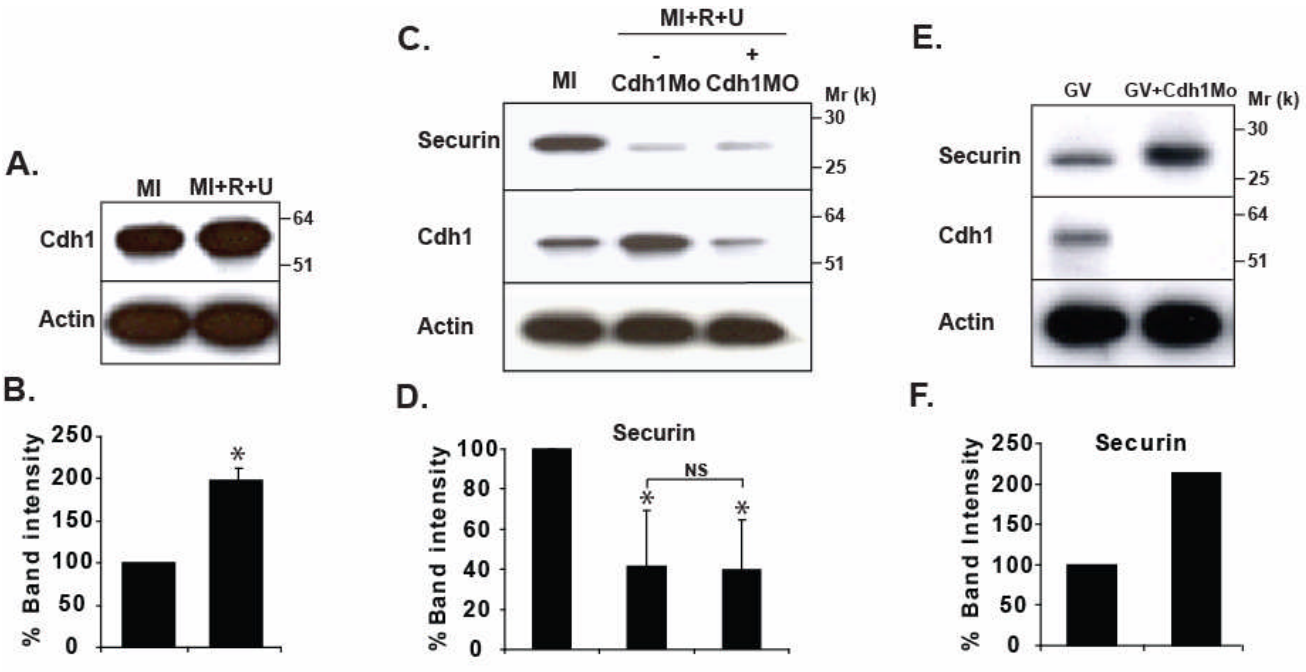

Fig. 5.4. Premature destruction of securin is not APC/C^{Cdh1}-dependent. (A) Western blot of oocytes (*n=20* per lane) for Cdh1 at MI in the presence or absence of roscovitine and UO126 (R+U). **(B)** Densitometric analysis of the Western blot in panel A. The results are mean ± s. d. from three independent experiments. **(C)** Western blot of oocytes (*n=30* per lane) for securin and Cdh1 at MI, and MI following incubation with roscovitine and UO126 (R+U), in the presence or absence of Cdh1 morpholino (Cdh1 MO). **(D)** Densitometric analysis of the Western blot in panel C. The results are mean ± s. d. from three independent experiments. In panels A and C, all MI samples were collected at 5 h post release from IBMX, and drugs treatment was performed between 3 and 5h. In panels B and D, MI is set at 100% and the asterisks indicate values significantly different from controls (MI values) at $^{*}P$ (0.01-0.05), whereas NS represents values not significantly different at $^{NS}P > 0.05$. **(E)** Western blot of oocytes (*n=20* per lane) for securin and Cdh1 at the GV stage, in the presence or absence of Cdh1 morpholino. **(F)** Densitometric analysis of the Western blot in panel E. GV is set at 100%. Actin was used as a loading control.

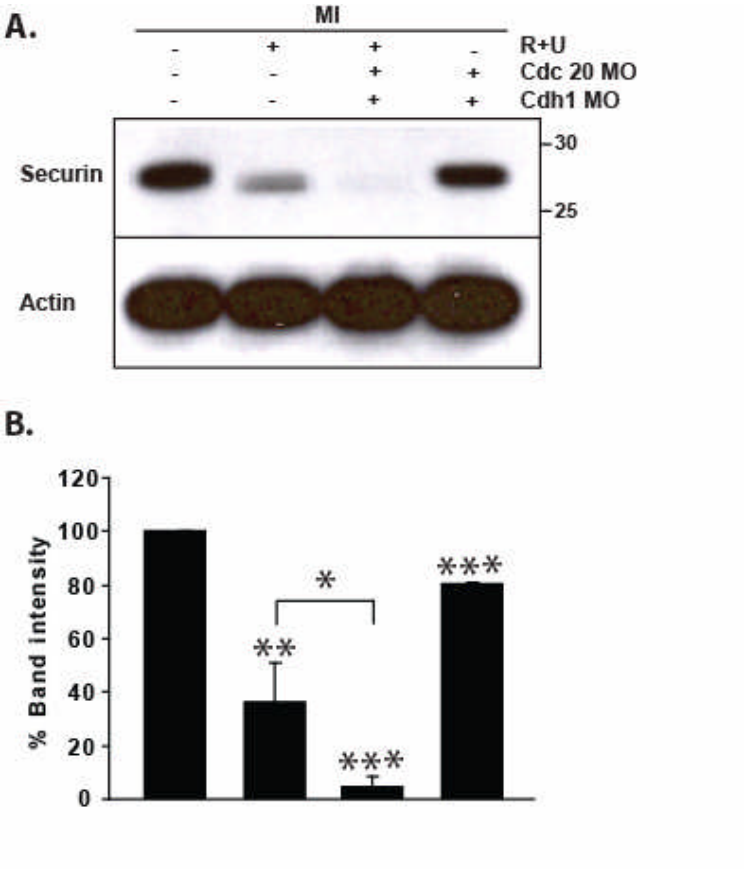

Fig. 5.5. Combined knockdown of Cdc20 and Cdh1 induces more destruction of securin. (A) Western blot of oocytes (*n=20* per lane) for securin at MI in the presence or absence of roscovitine and UO126 (R+U), and MI following microinjection with Cdc20 and Cdh1 morpholinos in the presence or absence of the inhibitors. MI oocytes were collected at 5 h post release from IBMX, and drugs treatment was performed between 3 and 5h. Actin was used as a loading control. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. MI is set at 100%. Double and triple asterisks indicate values significantly different from control (the MI value) at $^{**}P$ (0.001-0.01) and $^{***}P < 0.001$. Single asterisk represents two significantly different values at $^{*}P$ (0.01-0.05).

5.2.6. Premature destruction of securin is not $SCF^{\beta\text{-}TrCP}$-dependent.

Having shown that the premature destruction of securin in the absence of CDK1 and MAPK activities could not be caused by APC/C^{Cdc20} or APC/C^{Cdh1}, I went on to investigate whether this effect was due to activation of the second major E3 ubiquitin ligase, SCF (Skp1/Cullin/F-box protein). The possible involvement of SCF in the premature destruction of securin was tested through inhibition of its activity, using the dominant negative mutant of its F-box protein (β-TrCP) (Guardavaccaro et al., 2003; Marangos et al., 2007). The cRNA construct for the mutant β-TrCP (β-TrCPΔ) was microinjected in oocytes at GVBD, in order to avoid any interference with meiotic resumption. These oocytes, alongside non-injected oocytes, were cultured for 5 h in the presence or absence of roscovitine and UO126, and then assessed for their securin levels using Western blotting. There was no significant difference in the levels of securin between β-TrCPΔ-injected and non-injected MI oocytes **(Fig. 5.6.A, B)**. More importantly, the mutant in the inhibitor-treated oocytes failed to rescue securin levels, which remained as low as those observed in the non-injected oocytes that were also treated with roscovitine and UO126 **(Fig. 5.6.A, B)**. These results suggest that the targeted destruction of securin upon CDK1 and MAPK inhibition may not be $SCF^{\beta\text{-}TrCP}$-dependent.

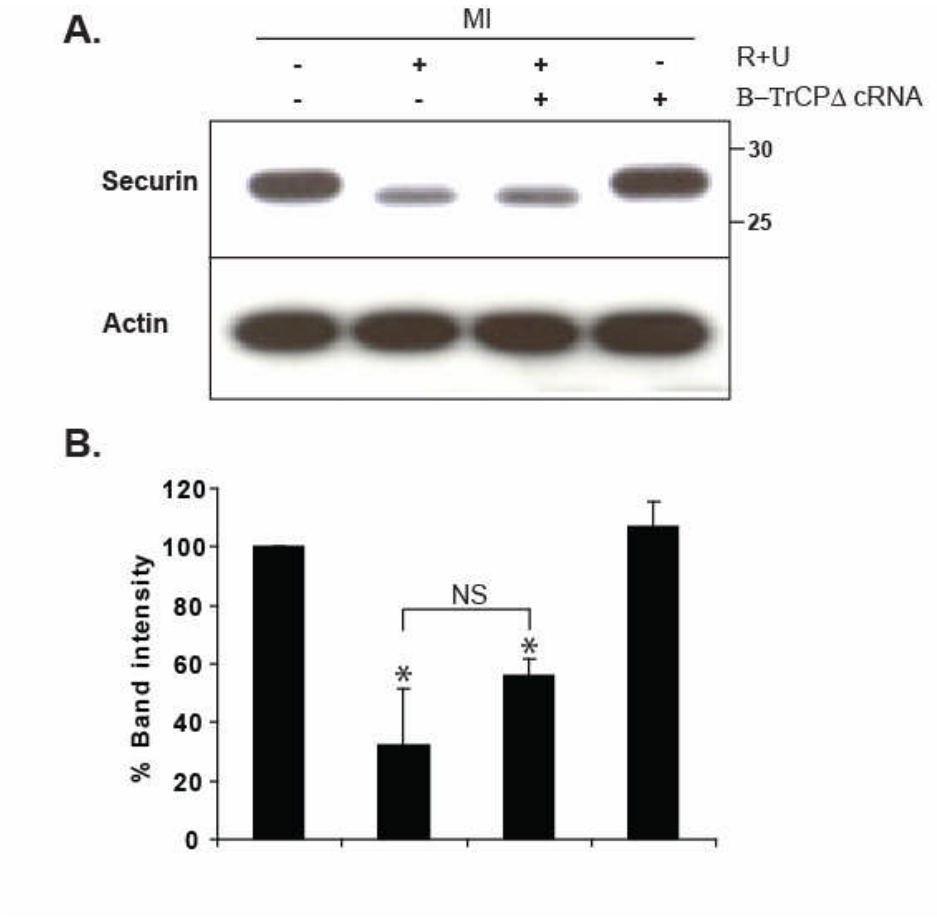

Fig. 5.6. Premature destruction of securin is not SCF$^{\beta\text{-TrCP}}$-dependent. (A) Western blot of oocytes (*n=20* per lane) for securin at MI, MI after treatment with roscovitine and UO126 (R+U) and MI following microinjection with the cRNA for β-TrCPΔ, in the presence or absence of the inhibitors. All MI samples were collected at 5 h post release from IBMX, and drugs treatment was performed between 3 and 5 h. Actin was used as a loading control. **(B)** Densitometric analysis of Western blots from three independent experiments. The results are mean ± s. d. MI is set at 100%. Asterisks indicate values significantly different from control (MI value) at $^{*}P$ (0.01-0.05), whereas NS represents values not significantly different at $^{NS}P > 0.05$.

5.3. Discussion

In meiosis, APC/C^{Cdc20} ubiquitinates cyclin B1 and securin to induce anaphase onset and chromosomes separation. More recently, it was shown that APC/C^{Cdh1} is required for maintaining the GV arrest by suppressing the levels of cyclin B1 (Marangos et al., 2007; Reis et al., 2006a). Also, during prometaphase I, APC/C^{Cdh1} targets Cdc20 for destruction thereby delaying the onset of APC/C^{Cdc20} activity and the onset of anaphase (Reis et al., 2007). In contrast to mitosis, the meiotic mechanisms of APC/C regulation are as yet unexplored, especially during the prolonged prometaphase I.

This study was set out to examine what causes the premature destruction of securin during prometaphase I in the absence of MPF and MAPK activities. In the previous chapter, I have demonstrated that securin stability is unlikely to be dependent on its phosphorylation state, as determined by the mutagenesis experiments. Here, I found that inhibition of both kinases also induces the premature degradation of other APC/C substrates, suggesting that this effect might be due to activation of the APC/C. Interestingly, only the D-box containing substrates, such as cyclin B1, geminin and wild type securin were subject to destruction when MPF and MAPK were inhibited. The Ken-box substrates, however, were not prematurely targeted for degradation. For example, $securin^{DM}$, which only contains a functioning Ken-box, was stable throughout the cell cycle and failed to be destroyed not only at the metaphase-to-anaphase transition, but also during prometapahse I in the presence of MPF and MAPK inhibitors. These findings further confirm the requirement of the D-box for the APC/C^{Cdc20}-mediated degradation of

securin at the metaphase-to-anaphase transition, and also suggest its necessity for the premature destruction of the APC/C substrates upon MPF and MAPK inhibition.

The targeted destruction of the D-box containing substrates and not Ken-box substrates implies that APC/C^{Cdc20} is a likely candidate for driving degradation in the absence of MPF and MAPK activities. Surprisingly, examination of the levels of Cdc20 in the presence of the inhibitors revealed that Cdc20 was also targeted for destruction, suggesting that APC/C^{Cdc20} is not responsible of the premature degradation of the D-box substrates. This result, however, needs to be considered preliminary because due to technical problems with the antibody, the Cdc20 Western was difficult to replicate. The non-involvement of APC/C^{Cdc20} in the premature destruction of the D-box substrates was further confirmed by Cdc20-morpholino knockdown, which failed to rescue securin stability upon MPF and MAPK inhibition.

It is well established that vertebrate Cdc20 does not contain a D-box, and in mammalian oocytes APC/C^{Cdh1} mediates its destruction through two independent degrons, the Ken-box and CRY-box (Pfleger and Kirschner, 2000; Reis et al., 2006b). Also, in contrast to Cdc20, the inhibition of MPF and MAPK stabilized the levels of Cdh1. Therefore, due to the absence of the Cdh1 inhibitor, MPF, the high levels of Cdh1 may be responsible for enhancing the prometaphase I activity of APC/C^{Cdh1}, thus making it capable of targeting the destruction of other substrates rather than just Cdc20. Nevertheless, Cdh1 knockdown using antisense morpholino failed to rescue the premature destruction of

securin upon MPF and MAPK inhibition, suggesting that this phenotype is not APC/C^{Cdh1}-dependent.

Additionally, simultaneous knockdown of both Cdc20 and Cdh1 induced an increase in the destruction of securin during prometaphase I in the presence and absence of MPF and MAPK inhibitors. These results, further suggest that Cdc20 and Cdh1 may not be necessary for the premature destruction of the D-box substrates upon MPF and MAPK inhibition. There are a number of possible explanations for this unexpected result: (i) another ubiquitin ligase may be active, (ii) the core APC/C may have upregulated acivity and (iii) the existence of other APC/C co-activators, which may be meiosis-specific and only become active when Cdh1 and Cdc20 are depleted.

Therefore, I turned to investigate the possible involvement of SCF (Skp1/Cullin/F-box protein). SCF was chosen in particular because: (i) it is the second major E3 ubiquitin ligase that is responsible of driving the meiotic cell cycle (Guardavaccaro et al., 2003); (ii) its action is tightly coupled to the phosphorylation status of its substrates (Guardavaccaro et al., 2003; Skowyra et al., 1997; Watanabe et al., 2004), and more importantly (iii) it is active during early meiosis, when securin and the other D-box substrates are targeted for destruction (Guardavaccaro et al., 2003; Marangos et al., 2007). Interestingly, inhibition of SCF using the dominant negative mutant of its F-box protein, β-TrCPΔ (Guardavaccaro et al., 2003; Marangos et al., 2007), also failed to rescue the premature degradation of securin, indicating that this effect is not $SCF^{\beta\text{-}TrCP}$-dependent.

Taken together, all these findings suggest that the instability of the D-box substrates upon MPF and MAPK inhibition might well be due to activation of the APC/C independently of its two major co-activators. However, it is not entirely clear how such activation may take place. The core APC/C with APC10/Doc1 acting as a D-box receptor is one possibility. Accordingly, it was recently reported that the D-box substrates could bind directly to the core APC/C in the absence of Cdc20 and Cdh1 (Passmore et al., 2003; Yamano et al., 2004). Such interaction is believed to be important in enhancing the processivity of the D-box substrates ubiquitination through increasing the affinity of the ligase towards its substrates (Carroll and Morgan, 2002). Also, it was found that mutagenesis of APC10/Doc1, the only APC/C subunit thus far implicated as a D-box receptor, not only decreases the binding of the D-box substrates to the APC/C *in vitro*, but also reduces the efficiency of the ligase in targeting the destruction of its substrates *in vivo*, thus delaying the mitotic cell cycle (Carroll et al., 2005).

Also, it is well established that the phosphorylation of the APC/C is required for its activation and association with Cdc20, through which the ligase is mainly regulated (Hershko et al., 1994; Kotani et al., 1998; Kraft et al., 2003). Therefore, it may be possible that in the absence of MPF and MAPK activities, the lack of association with the co-activator protein unleashes the core APC/C to target the destruction of the D-box substrates. This may explain the increase in securin destruction when Cdc20 and Cdh1 were simultaneously depleted in the presence and absence of MPF and MAPK inhibitors.

Alternatively, other APC/C co-activators, which are normally repressed by Cdh1 and Cdc20, may be involved. Meiosis-specific activators have been identified in many other organisms including yeast and flies (Asakawa et al., 2001; Blanco et al., 2001; Chu et al., 2001; Cooper et al., 2000; Jacobs et al., 2002). These co-activators are expressed exclusively during meiosis and have unique roles that are outside the functions of Cdc20 and Cdh1. In *Drosphila*, for example, the fizzy-like protein (cort) is only transcribed during oogenesis, and its knockout results in eggs that are terminally arrested at Met II (Chu et al., 2001; Lieberfarb et al., 1996; Page and OrrWeaver, 1996; Pesin and Orr-Weaver, 2007). Although, cort is present throughout meiosis alongside Cdc20, it only becomes active during meiosis II or meiosis I upon Cdc20 depletion (Pesin and Orr-Weaver, 2007; Swan and Schupbach, 2007). This suggests that during meiosis I, the activity of cort is some how repressed by Cdc20. Therefore, the same may be happening in mouse oocytes. So, in the absence of MPF and MAPK activities, the lack of phosphorylation of the APC/C and hence lack of association with Cdc20, may promote its activation by another Cdc20-like activator, which targets the D-box substrates for destruction by the proteasome.

In conclusion, the experiments reported here demonstrate that during prometaphase I, either the activity of MPF or MAPK is required for inhibiting the premature onset of the APC/C, thereby allowing the accumulation of the D-box containing substrates, such as securin and cyclin B1. This is believed to be achieved through the recruitment of Cdc20, which restrains the activity of the ligase during prometaphase through association with different inhibitory proteins, such as Emi1 and the components of the SAC. So, in the

absence of both MPF and MAPK activities, the APC/C is no longer phosphorylated and unable to recruit Cdc20. The loss of Cdc20 may paradoxically lead to unrestrained APC/C activity as a result of a loss of inhibition. However, it is not entirely clear how this activation may take place. The core APC/C with APC10/Doc1 as a D-box receptor is one possibility. Alternatively, this may be due to activation of some meiosis-specific APC/C activators, which are normally repressed by Cdc20 and Cdh1 and become active only when the two major co-activators are depleted.

Further work is needed to determine which, if any, of these mechanisms explains the findings described above. In the future, I am planning on using the dominant negative mutant of the APC10/Doc1 subunit to test if interfering with the binding of the D-box substrates to the core APC/C would rescue the premature destruction of securin and the other substrates upon MPF and MAPK inhibition. Additionally, It would be interesting to examine if other meiosis-specific Cdc20-like co-activators exist in mouse oocytes and if so their roles in controlling the meiotic cell cycle. It will also be important to determine whether inhibition of MPF and MAPK induces the oocytes to arrest at Met I or to exit the M-phase and enter interphase, given that those oocytes fail to extrude PB1 (data not shown). Finally, it will be crucial to establish if inhibition of both kinases also induces premature separation of chromosomes via separase activation, since its two main inhibitors (securin and cyclin B1) are targeted for destruction.

Chapter 6: Conclusions

The main aim of the experiments presented in this thesis is to better understand how the cell cycle is controlled during meiotic maturation of mouse oocytes. In what follows, I will summarise and discuss the main findings of the thesis.

Mammalian oocytes enter the meiotic cell cycle during the female embryonic life and become arrested in prophase of the first meiotic division for a period lasting from few months (mice) to many years (humans), until sexual maturity (Alberts et al., 2000; AUSTIN and Short, 1993; Bukovsky et al., 2005; Wolpert et al., 1998). At puberty and under the influence of hormones, these prophase I-arrested oocytes resume the first meiotic division, enter the second meiotic division and become arrested at the Met II stage until fertilization (Yanagimachi, 1994). During metaphase II arrest, the egg has the difficult task of ensuring the continued attachment of the sister chromatids, which is very important because any premature loss of cohesion might lead to aneuploid embryos upon fertilization of the egg by a sperm. The maintenance of the pairing of sister chromatids during this period is achieved through inhibition of the cohesin-protease, separase.

In vertebrates, it is well established that separase is controlled by securin and MPF, which are mutually exclusive at the molecular level, such that if one goes missing the other one can compensate for it (Ciosk et al., 1998; Gorr et al., 2005; Petronczki et al., 2003; Zou et al., 1999). Although, this mutuality has been proven in many systems including meiosis I in mouse oocytes (Herbert et al., 2003; Petronczki et al., 2003). I found here that securin

is the predominant inhibitor of separase during meiosis II **(Chapter 3)**. Thus, inhibition of CDK1 activity in Met II-arrested eggs using roscovitine failed to induce sister chromatid separation, even though it induced cytokinesis and PB2 extrusion **(Fig. 3.3)**. Moreover, antibody inhibition of CDK1/cyclin B1 binding to separase also failed to promote sister chromatid disjunction **(Fig. 3.4)**. Instead, securin morpholino knockdown induced sisters' separation, which could be rescued by injection of securin cRNA **(Fig. 3.5, 6)**.

Chromosomes segregation upon securin knockdown only occurred following PB1 extrusion and not during meiosis I **(Fig. 3.5)**, which further confirms the mutuality of securin and MPF activities during the first meiotic division. Therefore, it would be interesting to investigate why such mutuality only exists during meiosis I and not meiosis II, especially that securin levels at this stage are much lower than those observed during meiosis I **(Fig. 3.2)**. Thus, it will also be worth examining whether securin might have other functions during the first meiotic division, when its levels are significantly high **(Fig. 3.2)**. Indeed, it was reported by Mrangos and Carroll (2008) that the high levels of securin during prophase I arrest are important for preventing excessive loss of cyclin B1 and thus allowing progression through MI, by competing with cyclin B1 for APC^{cdh1} binding sites (Marangos and Carroll, 2008).

Moreover, it will be crucial to investigate if the maintenance of securin levels present problems for ageing eggs in keeping sister chromatids attachment, given that the maternal age effect was proven to be stronger for meiosis II errors when compared to meiosis I

non-disjunctions. For example, it was estimated by Yoon et al that the risk of MI errors causing trisomy 21 increases by 10-fold when comparing 25 year-old women to women over 40 years of age, whereas the same comparison for meiosis II errors shows a 60-fold increase (Yoon et al., 1995).

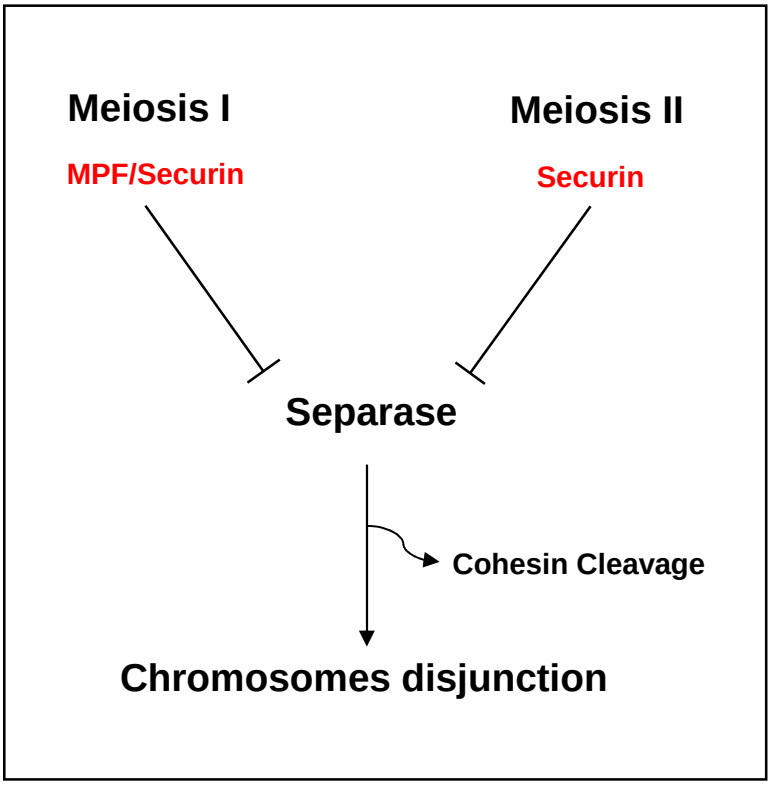

Fig. 6.1. Mechanisms of separase regulation during meiosis I and II. During meiosis I, early cleavage of cohesin and premature separation of chromosomes is prevented by both MPF and securin, which play equal roles in inhibiting the protease separase. During meiosis II, however, securin is the only inhibitor of separase.

As already mentioned in previous sections, securin is the best-known regulator of separase and therefore it is highly regulated by mechanisms responding to cell cycle progression. In addition to being expressed in a cell-cycle dependent manner, peaking in M phase and getting destroyed at anaphase onset (Nabti et al., 2008; Ramos-Morales et al., 2000), during the course of this study it has been reported that securin is subject to another layer of regulation, phosphoryaltion (Holt et al., 2008). This was demonstrated in yeast by Holt et al (2008), who found that securin stability during mitosis relies on its phosphorylation by MPF near the D-box motif (Holt et al., 2008). Securin phosphorylation was also observed upon entry into meiosis I in mouse oocytes **(Chapter 4)**. This phosphorylation was proven to be catalysed by both MPF and MAPK **(Fig. 4.2)**. I also found that inhibition of these two kinases induces the premature destruction of securin during proemetaphase of meiosis I **(Fig. 4.2, 3)**. However, although these results are consistent with earlier observation, this effect is not dependent on phosphorylation of securin, given that mutagenesis of CDK1 and MAPK phosphorylation sites on securin did not affect its stability **(Fig. 4.5)**. Therefore, it would be worth examining the reasons behind securin phosphorylation upon entry into meiosis I, other than regulating its stability and function, given that securin mutagenesis also had no effect on securin's ability to inhibit separase **(Fig. 4.6)**.

In a separate investigation, I found that CDK1 and MAPK inhibition also induces the premature degradation of other APC/C substrates, such as cyclin B1 and geminin **(Fig. 5.1)**. These findings suggest that this phenotype is not securin-specific and might be due to a general activation of the APC/C, whose activity is well known to be modulated by its

phosphorylation status (Hershko et al., 1994; Kotani et al., 1998; Kraft et al., 2003). Interestingly, the D-box containing substrates only and not the Ken-box substrates were subject to such effect **(Fig. 5.1, 2)**, implying that APC/C^{Cdc20} could be the mediator of this phenotype. Surprisingly, the prometaphase I instability of the D-box substrates was neither APC/C^{Cdc20} nor APC/C^{Cdh1}-dependent, given that Cdc20 was also targeted for destruction and morpholino-knockdown of Cdc20 and Cdh1 failed to rescue securin stability **(Fig. 5.3, 4)**. I also examined the possible involvement of the second major E3 ubiquitin ligase, $SCF^{\beta\text{-}TrCP}$. However, the inhibition of SCF using the dominant negative mutant of its F-box protein (β-TrCPΔ) could not restore securin levels, suggesting it is not $SCF^{\beta\text{-}TrCP}$-dependent **(Fig. 5.6)**.

Therefore, this effect could be attributed to activation of the APC/C independently of its two known co-activators. It is well established that APC/C phosphorylation is required for its activation and association with Cdc20, through which the ligase is mainly regulated (Hershko et al., 1994; Kotani et al., 1998; Kraft et al., 2003). Therefore, it may be possible that in the absence of MPF and MAPK activities, the lack of association with the co-activator protein unleashes the APC/C to target the destruction of the D-box substrates. However, it is not entirely clear how this activation may take place. The core APC/C with APC10/Doc1 as a D-box receptor is one possibility **(Fig. 6.2)**. Alternatively, this may be due to activation of some meiosis-specific APC/C activators **(Fig. 6.2)**, which are normally repressed by Cdc20 and Cdh1, and become active only when the two major co-activators are depleted.

Future work is needed to determine which, if any, of these two hypotheses explains the findings described above. Therefore, I am planning on genetically modifying the only APC/C subunit thus far implicated as a D-box receptor (APC10/Doc1), and investigating its effect on securin stability in the absence of MPF and MAPK activities. It will also be crucial to test whether the recruitment of the co-activators by the APC/C restrains its ligase activity from prematurely targeting the destruction of the D-box containing substrates. Moreover, It would be interesting to examine whether other meiosis-specific Cdc20-like co-activators exist in mouse oocytes and if so their roles in controlling the meiotic cell cycle. Finally, it will be worth examining whether the sustained-high activity of MAPK throughout the meiotic cell cycle plays any role in restraining the activity of the APC/C when MPF activity is low during the cell cycle transitions.

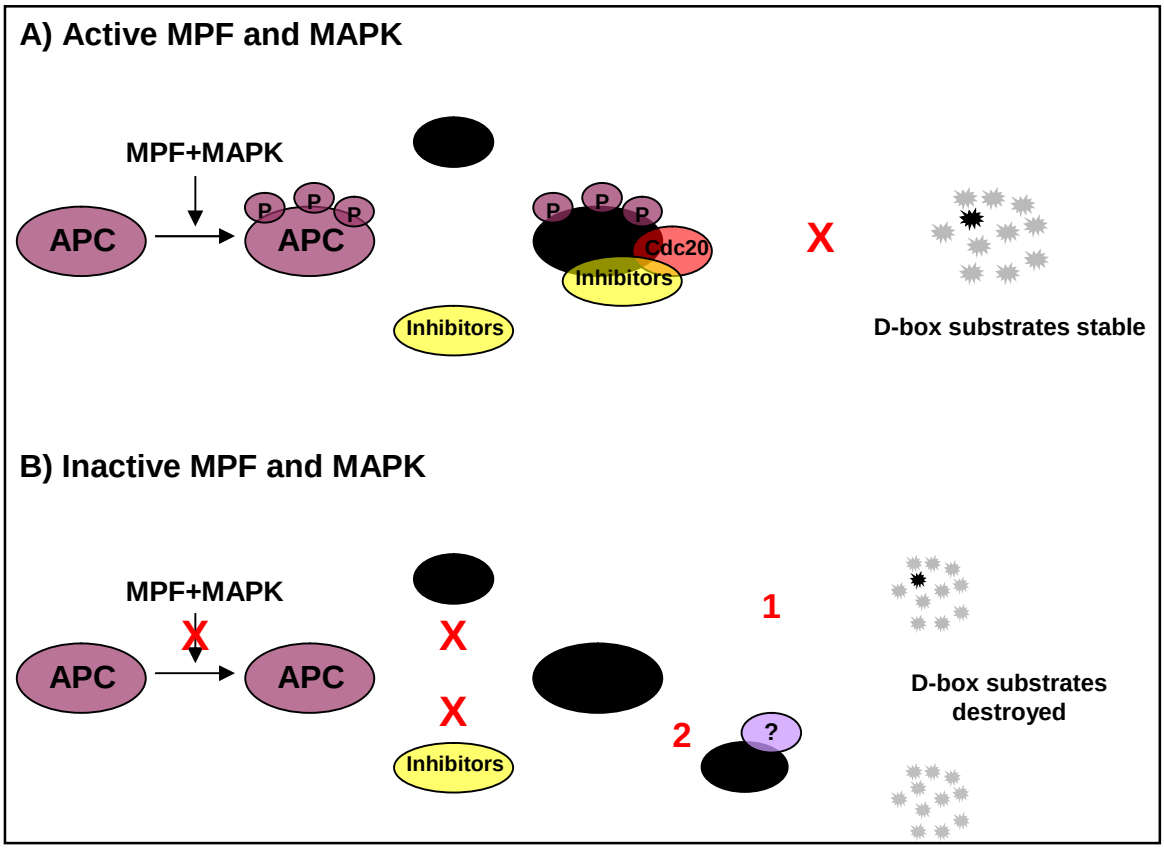

Fig. 6.2. The activity of MPF and MAPK is required for accumulation of the APC D-box substrates during MI. **(A)** In the presence of MPF and MAPK, the APC becomes phosphorylated, therefore promoting its association with Cdc20 and other inhibitory factors, such as Emi1 and the components of SAC. Binding of the inhibitors to APC/C^{Cdc20} prevents it from becoming active and prematurely targeting the D-box substrates for degradation. **(B)** In the absence of MPF and MAPK, the APC is no longer phosphorylated and therefore incapable of recruiting Cdc20 and the inhibitors. As a result, the APC becomes active and prematurely targets its D-box substrates for destruction. The degradation of these substrates could be: **(1)** mediated by the core APC with the APC10 subunit acting as a D-box receptor, or **(2)** through another co-activator, which only binds to and activates the APC in the absence of MPF and MAPK.

Reference List

Abrieu,A., Doree,M., and Picard,A. (1997). Mitogen-activated protein kinase activation down-regulates a mechanism that inactivates cyclin B-cdc2 kinase in G(2)-arrested oocytes. *Molecular Biology of the Cell* 8, 249-261.

Agarwal,R. and Cohen-Fix,O. (2002). Phosphorylation of the mitotic regulator Pds1/securin by Cdc28 is required for efficient nuclear localization of Esp1/separase. *Genes & Development* 16, 1371-1382.

Al,J.S., Wang,Q., Li,M., Shi,L.H., Ola,S.I., Xiong,B., Yin,S., Chen,D.Y., and Sun,Q.Y. (2008). Roles of Microtubules and Microfilaments in Spindle Movements During Rat Oocyte Meiosis. *Journal of Reproduction and Development* 54, 391-396.

Alberts,B., Johnson,A., Lewis,J., Raff,M., Roberts,K., and Walter,P. (2000). *The cell*. New York: Garland Science.

Anderson,E. and Albertini,D.F. (1976). Gap Junctions Between Oocyte and Companion Follicle Cells in Mammalian Ovary. *Journal of Cell Biology* 71, 680-686.

Aravind,L. and Koonin,E.V. (2002). Classification of the caspase-hemoglobinase fold: Detection of new families and implications for the origin of the eukaryotic separins. *Proteins-Structure Function and Genetics* 46, 355-367.

Asakawa,H., Kitamura,K., and Shimoda,C. (2001). A novel Cdc20-related WD-repeat protein, Fzr1, is required for spore formation in Schizosaccharomyces pombe. *Molecular Genetics and Genomics* 265, 424-435.

AUSTIN,C.R. (1951). Observations on the penetration of the sperm in the mammalian egg. *Aust. J. Sci. Res. (B)* 4, 581-596.

AUSTIN,C.R. (1952). The capacitation of the mammalian sperm. *Nature* 170, 326.

AUSTIN,C.R. and Short,R.V. (1993). *Reproduction in mammals: 1 Germ cells and fertilization*. Press syndicate of the university of Cambridge.

Ayabe,T., Kopf,G.S., and Schultz,R.M. (1995). Regulation of Mouse Egg Activation - Presence of Ryanodine Receptors and Effects of Microinjected Ryanodine and Cyclic Adp Ribose on Uninseminated and Inseminated Eggs. *Development* 121, 2233-2244.

Azoury,J., Lee,K.W., Georget,V., Rassinier,P., Leader,B., and Verlhac,M.H. (2008). Spindle Positioning in Mouse Oocytes Relies on a Dynamic Meshwork of Actin Filaments. *Current Biology* 18, 1514-1519.

Baker,D.J., Dawlaty,M.M., Galardy,P., and van Deursen,J.M. (2007). Mitotic regulation of the anaphase-promoting complex. *Cellular and Molecular Life Sciences* 64, 589-600.

Barros,C. and YANAGIMA.R (1971). Induction of Zona Reaction in Golden Hamster Eggs by Cortical Granule Material. *Nature* 233, 268-&.

Benmaamar,R. and Pagano,M. (2005). Involvement of the SCF complex in the control of Cdh1 degradation in S-phase. *Cell Cycle* 4, 1230-1232.

Berridge,M.J., Lipp,P., and Bootman,M.D. (2000). The versatility and universality of calcium signalling. *Nature Reviews Molecular Cell Biology* 1, 11-21.

Beyer,E.C., Kistler,J., Paul,D.L., and Goodenough,D.A. (1989). Antisera Directed Against Connexin-43 Peptides React with A 43-Kd Protein Localized to Gap-Junctions in Myocardium and Other Tissues. *Journal of Cell Biology* 108, 595-605.

Blanco,M.A., Pelloquin,L., and Moreno,S. (2001). Fission yeast mfr1 activates APC and coordinates meiotic nuclear division with sporulation. *Journal of Cell Science* 114, 2135-2143.

Bleil,J.D. and Wassarman,P.M. (1980). Structure and Function of the Zona Pellucida - Identification and Characterization of the Proteins of the Mouse Oocytes Zona Pellucida. *Developmental Biology* 76, 185-202.

Boelaert,K., Yu,R., Tannahill,L.A., Stratford,A.L., Khanim,F.L., Eggo,M.C., Moore,J.S., Young,L.S., Gittoes,N.J., Franklyn,J.A. et al. (2004). PTTG's C-terminal PXXP motifs modulate critical cellular processes in vitro. *J. Mol. Endocrinol.* 33, 663-677.

Bornslaeger,E.A., Mattei,P., and Schultz,R.M. (1986). Involvement of Camp-Dependent Protein-Kinase and Protein-Phosphorylation in Regulation of Mouse Oocyte Maturation. *Developmental Biology* 114, 453-462.

Bornslaeger,E.A. and Schultz,R.M. (1985). Regulation of Mouse Oocyte Maturation - Effect of Elevating Cumulus Cell Camp on Oocyte Camp Levels. *Biology of Reproduction* 33, 698-704.

Boulton,T.G., Nye,S.H., Robbins,D.J., Ip,N.Y., Radziejewska,E., Morgenbesser,S.D., Depinho,R.A., Panayotatos,N., Cobb,M.H., and Yancopoulos,G.D. (1991). Erks - A Family of Protein-Serine Threonine Kinases That Are Activated and Tyrosine Phosphorylated in Response to Insulin and Ngf. *Cell* 65, 663-675.

Bradbury,E.M., Inglis,R.J., and Matthews,H.R. (1974). Control of cell division by very lysine rich histone (F1) phosphorylation. *Nature* 247, 257-261.

Brandeis,M., Rosewell,I., Carrington,M., Crompton,T., Jacobs,M.A., Kirk,J., Gannon,J., and Hunt,T. (1998). Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. *Proc. Natl. Acad. Sci. U. S. A* 95, 4344-4349.

Brunet,S., Pahlavan,G., Taylor,S., and Maro,B. (2003). Functionality of the spindle checkpoint during the first meiotic division of mammalian oocytes. *Reproduction* 126, 443-450.

Bukovsky,A., Caudle,M.R., Svetlikova,M., Wimalasena,J., Ayala,M.E., and Dominguez,R. (2005). Oogenesis in adult mammals, including humans: a review. *Endocrine.* 26, 301-316.

Campbell,N.A., Reece,J.B., and Mitchell,L.G. (1999). *Biology*. California: Wesley Longman.

Carroll,C.W., Enquist-Newman,M., and Morgan,D.O. (2005). The APC subunit Doc1 promotes recognition of the substrate destruction box. *Current Biology* 15, 11-18.

Carroll,C.W. and Morgan,D.O. (2002). The Doc1 subunit is a processivity factor for the anaphase-promoting complex. *Nature Cell Biology* 4, 880-887.

Castro,A., rlot-Bonnemains,Y., Vigneron,S., Labbe,J.C., Prigent,C., and Lorca,T. (2002). APC/Fizzy-Related targets Aurora-A kinase for proteolysis. *Embo Reports* 3, 457-462.

Chang,D.C., Xu,N.H., and Luo,K.Q. (2003). Degradation of cyclin B is required for the onset of anaphase in mammalian cells. *Journal of Biological Chemistry* 278, 37865-37873.

Chang,H.Y., Levasseur,M., and Jones,K.T. (2004). Degradation of APC(cdc20) and APC(cdh1) substrates during the second meiotic division in mouse eggs. *Journal of Cell Science* 117, 6289-6296.

Chang,M.C. (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* 168, 697-698.

Chelysheva,L., Diallo,S., Vezon,D., Gendrot,G., Vrielynck,N., Belcram,K., Rocques,N., Marquez-Lema,A., Bhatt,A.M., Horlow,C. et al. (2005). AtREC8 and AtSCC3 are essential to the monopolar orientation of the kinetochores during meiosis. *Journal of Cell Science* 118, 4621-4632.

Cho,W.K., Stern,S., and Biggers,J.D. (1974). Inhibitory Effect of Dibutyryl Camp on Mouse Oocyte Maturation In-Vitro. *Journal of Experimental Zoology* 187, 383-386.

Choi,T.S., Fukasawa,K., Zhou,R.P., Tessarollo,L., Borror,K., Resau,J., and VandeWoude,G.F. (1996). The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. ***Proceedings of the National Academy of Sciences of the United States of America*** **93, 7032-7035.**

Chu,T., Henrion,G., Haegeli,V., and Strickland,S. (2001). Cortex, a Drosophila gene required to complete oocyte meiosis, is a member of the Cdc20/fizzy protein family. ***Genesis*** **29, 141-152.**

Chung,E.N. and Chen,R.H. (2003). Phosphorylation of Cdc20 is required for its inhibition by the spindle checkpoint. ***Nature Cell Biology*** **5, 748-753.**

Ciechanover,A., Heller,H., Katzetzion,R., and Hershko,A. (1981). Activation of the Heat-Stable Polypeptide of the Atp-Dependent Proteolytic System. ***Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*** **78, 761-765.**

Ciosk,R., Zachariae,W., Michaelis,C., Shevchenko,A., Mann,M., and Nasmyth,K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. ***Cell*** **93, 1067-1076.**

Cisek,L.J. and Corden,J.L. (1989). Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2. ***Nature*** **339, 679-684.**

Clarke,D.J., Segal,M., Mondesert,G., and Reed,S.I. (1999). The Pds1 anaphase inhibitor and Mec1 kinase define distinct checkpoints coupling S phase with mitosis in budding yeast. ***Current Biology*** **9, 365-368.**

Clute,P. and Pines,J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. ***Nature Cell Biology*** **1, 82-87.**

CohenFix,O., Peters,J.M., Kirschner,M.W., and Koshland,D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. ***Genes & Development*** **10, 3081-3093.**

Colledge,W.H., Carlton,M.B.L., Udy,G.B., and Evans,M.J. (1994). Disruption of C-Mos Causes Parthenogenetic Development of Unfertilized Mouse Eggs. ***Nature*** **370, 65-68.**

Conti,M., Andersen,C.B., Richard,F., Mehats,C., Chun,S.Y., Horner,K., Jin,C., and Tsafriri,A. (2002). Role of cyclic nucleotide signaling in oocyte maturation. ***Molecular and Cellular Endocrinology*** **187, 153-159.**

Cooper,K.F., Mallory,M.J., Egeland,D.B., Jarnik,M., and Strich,R. (2000). Ama1p is a meiosis-specific regulator of the anaphase promoting complex/cyclosome in yeast. ***Proceedings of the National Academy of Sciences of the United States of America*** **97, 14548-14553.**

Crews,C.M. and Erikson,R.L. (1992). Purification of A Murine Protein-Tyrosine Threonine Kinase That Phosphorylates and Activates the Erk-1 Gene-Product - Relationship to the Fission Yeast Byr1 Gene-Product. *Proceedings of the National Academy of Sciences of the United States of America* 89, 8205-8209.

Cuthbertson,K.S.R. and Cobbold,P.H. (1985). Phorbol Ester and Sperm Activate Mouse Oocytes by Inducing Sustained Oscillations in Cell Ca-2+. *Nature* 316, 541-542.

Cuthbertson,K.S.R., Whittingham,D.G., and Cobbold,P.H. (1981). Free Ca-2+ Increases in Exponential Phases During Mouse Oocyte Activation. *Nature* 294, 754-757.

Dalby,K.N., Morrice,N., Caudwell,F.B., Avruch,J., and Cohen,P. (1998). Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90(rsk) that are inducible by MAPK. *Journal of Biological Chemistry* 273, 1496-1505.

Dale,B., Defelice,L.J., and Ehrenstein,G. (1985). Injection of A Soluble Sperm Fraction Into Sea-Urchin Eggs Triggers the Cortical Reaction. *Experientia* 41, 1068-1070.

Darwiche,N., Freeman,L.A., and Strunnikov,A. (1999). Characterization of the components of the putative mammalian sister chromatid cohesion complex. *Gene* 233, 39-47.

Dawson,I.A., Roth,S., Akam,M., and Artavanistsakonas,S. (1993). Mutations of the Fizzy Locus Cause Metaphase Arrest in Drosophila-Melanogaster Embryos. *Development* 117, 359-376.

Dekel,N. (2005). Cellular, biochemical and molecular mechanisms regulating oocyte maturation. *Mol. Cell Endocrinol.* 234, 19-25.

Dekel,N. and Beers,W.H. (1978). Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins. *Proc. Natl. Acad. Sci. U. S. A* 75, 4369-4373.

deMoor,C.H. and Richter,J.D. (1997). The Mos pathway regulates cytoplasmic polyadenylation in Xenopus oocytes. *Molecular and Cellular Biology* 17, 6419-6426.

den Elzen,N. and Pines,J. (2001). Cyclin a is destroyed in prometaphase and can delay chromosome alignment and anaphase. *Journal of Cell Biology* 153, 121-135.

Deveaux,L.C. and Smith,G.R. (1994). Region-Specific Activators of Meiotic Recombination in Schizosaccharomyces-Pombe. *Genes & Development* 8, 203-210.

Doree,M. and Hunt,T. (2002). From Cdc2 to Cdk1: when did the cell cycle kinase join its cyclin partner? *J. Cell Sci.* 115, 2461-2464.

Ducibella,T. (1996). The cortical reaction and development of activation competence in mammalian oocytes. *Hum. Reprod. Update.* 2, 29-42.

Duckworth,B.C., Weaver,J.S., and Ruderman,J.V. (2002). G2 arrest in Xenopus oocytes depends on phosphorylation of cdc25 by protein kinase A. *Proc. Natl. Acad. Sci. U. S. A* 99, 16794-16799.

Dumont,J., Umbhauer,M., Rassinier,P., Hanauer,A., and Verlhac,M.H. (2005). p90Rsk is not involved in cytostatic factor arrest in mouse oocytes. *Journal of Cell Biology* 169, 227-231.

Dunbar,B.S., Avery,S., Lee,V., Prasad,S., Schwahn,D., Schwoebel,E., Skinner,S., and Wilkins,B. (1994). The Mammalian Zona-Pellucida - Its Biochemistry, Immunochemistry, Molecular-Biology, and Developmental Expression. *Reproduction Fertility and Development* 6, 331-347.

Espey,L.L. (1980). Ovulation as an inflammatory reaction--a hypothesis. *Biol Reprod.* 22, 73-106.

Evans,J.P. and Florman,H.M. (2002). The state of the union: the cell biology of fertilization. *Nat. Cell Biol* 4 Suppl, s57-s63.

Evans,T., Rosenthal,E.T., Youngblom,J., Distel,D., and Hunt,T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 33, 389-396.

Fan,H.Y., Tong,C., Chen,D.Y., and Sun,Q.Y. (2002). Roles of MAP kinase signaling pathway in oocyte meiosis. *Chinese Science Bulletin* 47, 1157-1162.

Fang,G.W., Yu,H.T., and Kirschner,M.W. (1998b). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Molecular Cell* 2, 163-171.

Fang,G.W., Yu,H.T., and Kirschner,M.W. (1998a). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes & Development* 12, 1871-1883.

Fattaey,A. and Booher,R.N. (1997). Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. *Prog. Cell Cycle Res.* 3, 233-240.

Fay,D.S., Keenan,S., and Han,M. (2002). fzr-1 and lin-35/Rb function redundantly to control cell proliferation in C-elegans as revealed by a nonbiased synthetic screen. *Genes & Development* 16, 503-517.

Fisher,D.L., Brassac,T., Galas,S., and Doree,M. (1999). Dissociation of MAP kinase activation and MPF activation in hormone-stimulated maturation of Xenopus oocytes. *Development* 126, 4537-4546.

Florman,H.M. and Wassarman,P.M. (1985). O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* 41, 313-324.

Floyd,S., Pines,J., and Lindon,C. (2008). APC/C-Cdh1 Targets Aurora Kinase to Control Reorganization of the Mitotic Spindle at Anaphase. *Current Biology* 18, 1649-1658.

Fujimoto,S., Yoshida,N., Fukui,T., Amanai,M., Isobe,T., Itagaki,C., Izumi,T., and Perry,A.C.F. (2004). Mammalian phospholipase C zeta induces oocyte activation from the sperm perinuclear matrix. *Developmental Biology* 274, 370-383.

Fulka,J., Motlik,J., Fulka,J., and Jilek,F. (1986). Effect of Cycloheximide on Nuclear Maturation of Pig and Mouse Oocytes. *Journal of Reproduction and Fertility* 77, 281-285.

Funabiki,H., Kumada,K., and Yanagida,M. (1996a). Fission yeast cut1 and cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *Embo Journal* 15, 6617-6628.

Funabiki,H., Yamano,H., Kumada,K., Nagao,K., Hunt,T., and Yanagida,M. (1996b). Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature* 381, 438-441.

Furuno,N., Nishizawa,M., Okazaki,K., Tanaka,H., Iwashita,J., Nakajo,N., Ogawa,Y., and Sagata,N. (1994). Suppression of Dna-Replication Via Mos Function During Meiotic Divisions in Xenopus-Oocytes. *Embo Journal* 13, 2399-2410.

Garcia-Higuera,I., Manchado,E., Dubus,P., Canamero,M., Mendez,J., Moreno,S., and Malumbres,M. (2008). Genomic stability and tumour suppression by the APC/C cofactor Cdh1. *Nature Cell Biology* 10, 802-811.

Gardner,R., Putnam,C.W., and Weinert,T. (1999). RAD53, DUN1 and PDS1 define two parallel G(2)/M checkpoint pathways in budding yeast. *Embo Journal* 18, 3173-3185.

Gautier,J., Minshull,J., Lohka,M., Glotzer,M., Hunt,T., and Maller,J.L. (1990). Cyclin is a component of maturation-promoting factor from Xenopus. *Cell* 60, 487-494.

Gautier,J., Solomon,M.J., Booher,R.N., Bazan,J.F., and Kirschner,M.W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell* 67, 197-211.

Gebauer,F. and Richter,J.D. (1997). Synthesis and function of Mos: The control switch of vertebrate oocyte meiosis. *Bioessays* 19, 23-28.

Gebauer,F., Xu,W.H., Cooper,G.M., and Richter,J.D. (1994). Translational Control by Cytoplasmic Polyadenylation of C-Mos Messenger-Rna Is Necessary for Oocyte Maturation in the Mouse. *Embo Journal* 13, 5712-5720.

Geley,S., Kramer,E., Gieffers,C., Gannon,J., Peters,J.M., and Hunt,T. (2001). Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin a starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *Journal of Cell Biology* 153, 137-147.

Gilbert,S.F. (2006). *Developmental Biology*. USA: Sianaur associates.

Glotzer,M., Murray,A.W., and Kirschner,M.W. (1991). Cyclin Is Degraded by the Ubiquitin Pathway. *Nature* 349, 132-138.

Gomez,R., Valdeolmillos,A., Parra,M.T., Viera,A., Carreiro,C., Roncal,F., Rufas,J.S., Barbero,J.L., and Suja,J.A. (2007). Mammalian SGO2 appears at the inner centromere domain and redistributes depending on tension across centromeres during meiosis II and mitosis. *Embo Reports* 8, 173-180.

Gore-Langton,R.E. and Armstrong,D.T. The physiology of reproduction (Follicular steroidogenesis and its control). 1998. New York, Raven Press.
Ref Type: Serial (Book,Monograph)

Gorr,I.H., Boos,D., and Stemmann,O. (2005). Mutual inhibition of separase and Cdk1 by two-step complex formation. *Molecular Cell* 19, 135-141.

Gorr,I.H., Reis,A., Boos,D., Wuhr,M., Madgwick,S., Jones,K.T., and Stemmann,O. (2006). Essential CDK1-inhibitory role for separase during meiosis I in vertebrate oocytes. *Nature Cell Biology* 8, 1035-U120.

Gotoh,Y. and Nishida,E. (1995). Activation Mechanism and Function of the Map Kinase Cascade. *Molecular Reproduction and Development* 42, 486-492.

Gotoh,Y., Nishida,E., Matsuda,S., Shiina,N., Kosako,H., Shiokawa,K., Akiyama,T., Ohta,K., and Sakai,H. (1991). Invitro Effects on Microtubule Dynamics of Purified Xenopus M-Phase-Activated Map Kinase. *Nature* 349, 251-254.

Gross,S.D., Schwab,M.S., Lewellyn,A.L., and Maller,J.L. (1999). Induction of metaphase arrest in cleaving Xenopus embryos in by the protein kinase p90(Rsk). *Science* 286, 1365-1367.

Gross,S.D., Schwab,M.S., Taieb,F.E., Lewellyn,A.L., Qian,Y.W., and Maller,J.L. (2000). The critical role of the MAP kinase pathway in meiosis II in Xenopus oocytes is mediated by p90(Rsk). *Current Biology* 10, 430-438.

Gu,Y., Rosenblatt,J., and Morgan,D.O. (1992). Cell cycle regulation of CDK2 activity by phosphorylation of Thr16 and Tyr15. *EMBO J.* 11, 3995-4005.

Guacci,V., Koshland,D., and Strunnikov,A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in S-cerevisiae. *Cell* 91, 47-57.

Guardavaccaro,D., Kudo,Y., Boulaire,J., Barchi,M., Busino,L., Donzelli,M., Margottin-Goguet,F., Jackson,P.K., Yamasaki,L., and Pagano,M. (2003). Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Developmental Cell* 4, 799-812.

Gwatkin,R.B.L., Williams,D.T., Hartmann,J.F., and Kniazuk,M. (1973). Zona Reaction of Hamster and Mouse Eggs - Production In-Vitro by A Trypsin-Like Protease from Cortical Granules. *Journal of Reproduction and Fertility* 32, 259-265.

Haas,A.L., Warms,J.V.B., Hershko,A., and Rose,I.A. (1982). Ubiquitin-Activating Enzyme - Mechanism and Role in Protein-Ubiquitin Conjugation. *Journal of Biological Chemistry* 257, 2543-2548.

Haccard,O., Lewellyn,A., Hartley,R.S., Erikson,E., and Maller,J.L. (1995). Induction of Xenopus Oocyte Meiotic Maturation by Map Kinase. *Developmental Biology* 168, 677-682.

Hagting,A., den Elzen,N., Vodermaier,H.C., Waizenegger,I.C., Peters,J.M., and Pines,J. (2002). Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *Journal of Cell Biology* 157, 1125-1137.

Halet,G., Marangos,P., Fitzharris,G., and Carroll,J. (2003). Ca2+ oscillations at fertilization in mammals. *Biochemical Society Transactions* 31, 907-911.

Hames,R.S., Wattam,S.L., Yamano,H., Bacchieri,R., and Fry,A.M. (2001). APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box. *Embo Journal* 20, 7117-7127.

Han,S.J., Chen,R., Paronetto,M.P., and Conti,M. (2005). Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse. *Current Biology* 15, 1670-1676.

Hansen,D.V., Loktev,A.V., Ban,K.H., and Jackson,P.K. (2004). Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCF beta TrCP-dependent destruction of the APC inhibitor Emi1. *Molecular Biology of the Cell* 15, 5623-5634.

Hansen,D.V., Tung,J.J., and Jackson,P.K. (2006). CaMKII and Polo-like kinase 1 sequentially phosphorylate the cytostatic factor Emi2/XErp1 to trigger its destruction and meiotic exit. *Proceedings of the National Academy of Sciences of the United States of America* 103, 608-613.

Harper,J.W., Burton,J.L., and Solomon,M.J. (2002). The anaphase-promoting complex: it's not just for mitosis any more. *Genes & Development* 16, 2179-2206.

Hartman,T., Stead,K., Koshland,D., and Guacci,V. (2000). Pds5p is an essential chromosomal protein required for both sister chromatid cohesion and condensation in Saccharomyces cerevisiae. *Journal of Cell Biology* 151, 613-626.

Hartwell,L.H., Culotti,J., and Reid,B. (1970). Genetic Control of Cell-Division Cycle in Yeast .1. Detection of Mutants. *Proceedings of the National Academy of Sciences of the United States of America* 66, 352-&.

Hartwell,L.H., Mortimer,R.K., Culotti,J., and Culotti,M. (1973). Genetic-Control of Cell-Division Cycle in Yeast .5. Genetic Analysis of Cdc Mutants. *Genetics* 74, 267-286.

Hashimoto,N. and Kishimoto,T. (1986). Cell cycle dynamics of maturation-promoting factor during mouse oocyte maturation. *Tokai J Exp Clin. Med* 11, 471-477.

Hashimoto,N. and Kishimoto,T. (1988). Regulation of Meiotic Metaphase by A Cytoplasmic Maturation-Promoting Factor During Mouse Oocyte Maturation. *Developmental Biology* 126, 242-252.

Hashimoto,N., Watanabe,N., Furuta,Y., Tamemoto,H., Sagata,N., Yokoyama,M., Okazaki,K., Nagayoshi,M., Takeda,N., Ikawa,Y. et al. (1994). Parthenogenetic Activation of Oocytes in C-Mos-Deficient Mice. *Nature* 370, 68-71.

Hassold,T., Hall,H., and Hunt,P. (2007). The origin of human aneuploidy: where we have been, where we are going. *Human Molecular Genetics* 16, R203-R208.

Hassold,T. and Hunt,P. (2001). To ERR (meiotically) is human: The genesis of human aneuploidy. *Nature Reviews Genetics* 2, 280-291.

Heikinheimo,O. and Gibbons,W.E. (1998). The molecular mechanisms of oocyte maturation and early embryonic development are unveiling new insights into reproductive medicine. *Mol. Hum. Reprod.* 4, 745-756.

Heller,D.T., Cahill,D.M., and Schultz,R.M. (1981). Biochemical-Studies of Mammalian Oogenesis - Metabolic Cooperativity Between Granulosa-Cells and Growing-Mouse Oocytes. *Developmental Biology* 84, 455-464.

Herbert,M., Levasseur,M., Homer,H., Yallop,K., Murdoch,A., and McDougall,A. (2003). Homologue disjunction in mouse oocytes requires proteolysis of securin and cyclin B1. *Nature Cell Biology* 5, 1023-1025.

Hershko,A. and Ciechanover,A. (1998). The ubiquitin system. *Annual Review of Biochemistry* 67, 425-479.

Hershko,A., Ciechanover,A., and Rose,I.A. (1981). Identification of the Active Amino-Acid Residue of the Polypeptide of Atp-Dependent Protein Breakdown. *Journal of Biological Chemistry* 256, 1525-1528.

Hershko,A., Ganoth,D., Sudakin,V., Dahan,A., Cohen,L.H., Luca,F.C., Ruderman,J.V., and Eytan,E. (1994). Components of A System That Ligates Cyclin to Ubiquitin and Their Regulation by the Protein-Kinase Cdc2. *Journal of Biological Chemistry* 269, 4940-4946.

Hershko,A., Heller,H., Elias,S., and Ciechanover,A. (1983). Components of Ubiquitin-Protein Ligase System - Resolution, Affinity Purification, and Role in Protein Breakdown. *Journal of Biological Chemistry* 258, 8206-8214.

Hirano,T., Funahashi,S.I., Uemura,T., and Yanagida,M. (1986). Isolation and characterization of Schizosaccharomyces pombe cutmutants that block nuclear division but not cytokinesis. *EMBO J* 5, 2973-2979.

Holland,A.J. and Taylor,S.S. (2006). Cyclin-B1-mediated inhibition of excess separase is required for timely chromosome disjunction. *Journal of Cell Science* 119, 3325-3336.

Holt,L.J., Krutchinsky,A.N., and Morgan,D.O. (2008). Positive feedback sharpens the anaphase switch. *Nature* 454, 353-357.

Homer,H.A., McDougall,A., Levasseur,M., Murdoch,A.P., and Herbert,M. (2005a). Mad2 is required for inhibiting securin and cyclin B degradation following spindle depolymerisation in meiosis I mouse oocytes. *Reproduction* 130, 829-843.

Homer,H.A., McDougall,A., Levasseur,M., Yallop,K., Murdoch,A.P., and Herbert,M. (2005b). Mad2 prevents aneuploidy and premature proteolysis of cyclin B and securin during meiosis I in mouse oocytes. *Genes & Development* 19, 202-207.

Horner,K., Livera,G., Hinckley,M., Trinh,K., Storm,D., and Conti,M. (2003). Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Developmental Biology* 258, 385-396.

Hornig,N.C.D., Knowles,P.P., McDonald,N.Q., and Uhlmann,F. (2002). The dual mechanism of separase regulation by securin. *Current Biology* 12, 973-982.

Huang,W.D., Kessler,D.S., and Erikson,R.L. (1995). Biochemical and Biological Analysis of Mek1 Phosphorylation Site Mutants. *Molecular Biology of the Cell* 6, 237-245.

Huang,X.X., Hatcher,R., York,J.P., and Zhang,P.M. (2005). Securin and separase phosphorylation act redundantly to maintain sister chromatid cohesion in mammalian cells. *Molecular Biology of the Cell* 16, 4725-4732.

Inoue,D., Ohe,M., Kanemori,Y., Nobui,T., and Sagata,N. (2007). A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of Xenopus laevis eggs. ***Nature*** **446, 1100-1104.**

Inoue,N., Ikawa,M., Isotani,A., and Okabe,M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. ***Nature*** **434, 234-238.**

Jacobs,H., Richter,D., Venkatesh,T., and Lehner,C. (2002). Completion of mitosis requires neither fzr/rap nor fzr2, a male germline-specific Drosophila Cdh1 homolog. ***Curr. Biol*** **12, 1435-1441.**

Jallepalli,P.V., Waizenegger,I.C., Bunz,F., Langer,S., Speicher,M.R., Peters,J.M., Kinzler,K.W., Vogelstein,B., and Lengauer,C. (2001). Securin is required for chromosomal stability in human cells. ***Cell*** **105, 445-457.**

Jaspersen,S.L., Charles,J.F., and Morgan,D.O. (1999). Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. ***Current Biology*** **9, 227-236.**

Jeganathan,K.B., Baker,D.J., and van Deursen,J.M. (2006). Securin associates with APC(Cdh1) in prometaphase but its destruction is delayed by Rae1 and Nup98 until the metaphase/anaphase transition. ***Cell Cycle*** **5, 366-370.**

Jeganathan,K.B., Malureanu,L., and van Deursen,J.M. (2005). The Rae1-Nup98 complex prevents aneuploidy by inhibiting securin degradation. ***Nature*** **438, 1036-1039.**

Jensen,S., Segal,M., Clarke,D.J., and Reed,S.I. (2001). A novel role of the budding yeast separin Esp1 in anaphase spindle elongation: Evidence that proper spindle association of Esp1 is regulated by Pds1. ***Journal of Cell Biology*** **152, 27-40.**

Jones,K.T. (2004). Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. ***Mol. Hum. Reprod.*** **10, 1-5.**

Jones,K.T. (2005). Mammalian egg activation: from Ca2+ spiking to cell cycle progression. ***Reproduction*** **130, 813-823.**

Jones,K.T. and Nixon,V.L. (2000). Sperm-induced Ca2+ oscillations in mouse oocytes and eggs can be mimicked by photolysis of caged inositol 1,4,5-trisphosphate: Evidence to support a continuous low level production of inositol 1,4,5-trisphosphate during mammalian fertilization. ***Developmental Biology*** **225, 1-12.**

Kalab,P., Kubiak,J.Z., Verlhac,M.H., Colledge,W.H., and Maro,B. (1996). Activation of p90rsk during meiotic maturation and first mitosis in mouse oocytes and eggs: MAP kinase-independent and -dependent activation. ***Development*** **122, 1957-1964.**

Kanatsu-Shinohara,M., Schultz,R.M., and Kopf,G.S. (2000). Acquisition of meiotic competence in mouse oocytes: Absolute amounts of p34(cdc2), cyclin b1, cdc25C, and wee1 in meiotically incompetent and competent oocytes. *Biology of Reproduction* 63, 1610-1616.

Kim,J.H., Machaty,Z., Cabot,R., Han,Y.M., and Prather,R.S. (1998). Development of pig oocytes activated by stimulation of an exogenous G-protein coupled receptor. *Theriogenology* 49, 154.

Kitagawa,R., Law,E., Tang,L., and Rose,A.M. (2002). The Cdc20 homolog, FZY-1, and its interacting protein, IFY-1, are required for proper chromosome in Caenorhabditis elegans. *Current Biology* 12, 2118-2123.

Kitajima,T.S., Hauf,S., Ohsugi,M., Yamamoto,T., and Watanabe,Y. (2005). Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting shugoshin localization. *Current Biology* 15, 353-359.

Kitajima,T.S., Kawashima,S.A., and Watanabe,Y. (2004). The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. *Nature* 427, 510-517.

Kitajima,T.S., Sakuno,T., Ishiguro,K., Iemura,S., Natsume,T., Kawashima,S.A., and Watanabe,Y. (2006). Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature* 441, 46-52.

Klein,F., Mahr,P., Galova,M., Buonomo,S.B., Michaelis,C., Nairz,K., and Nasmyth,K. (1999). A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. *Cell* 98, 91-103.

Knobil,E. and Neil,J.D. Physiology of reproduction. 2006. New York, Elsevier. Ref Type: Serial (Book,Monograph)

Knott,J.G., Kurokawa,M., Fissore,R.A., Schultz,R.M., and Williams,C.J. (2005). Transgenic RNA interference reveals role for mouse sperm phospholipase C in triggering Ca2+ oscillations during fertilization. *Biology of Reproduction* 72, 992-996.

Kobayashi,H., Minshull,J., Ford,C., Golsteyn,R., Poon,R., and Hunt,T. (1991). On the Synthesis and Destruction of A-Type and B-Type Cyclins During Oogenesis and Meiotic Maturation in Xenopus-Laevis. *Journal of Cell Biology* 114, 755-765.

Kops,G.J.P.L., Weaver,B.A.A., and Cleveland,D.W. (2005). On the road to cancer: Aneuploidy and the mitotic checkpoint. *Nature Reviews Cancer* 5, 773-785.

Kosako,H., Gotoh,Y., and Nishida,E. (1994). Requirement for the Map Kinase Kinase Map Kinase Cascade in Xenopus-Oocyte Maturation. *Embo Journal* 13, 2131-2138.

Kraft,C., Herzog,F., Gieffers,C., Mechtler,K., Hagting,A., Pines,J., and Peters,J.M. (2003). Mitotic regulation of the human anaphase-promoting complex by phosphorylation. *Embo Journal* 22, 6598-6609.

Kudo,N.R., Wassmann,K., Anger,M., Schuh,M., Wirth,K.G., Xu,H.L., Helmhart,W., Kudo,H., Mckay,M., Maro,B. et al. (2006). Resolution of chiasmata in oocytes requires separase-mediated proteolysis. *Cell* 126, 135-146.

Kumada,K., Nakamura,T., Nagao,K., Funabiki,H., Nakagawa,T., and Yanagida,M. (1998). Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. *Current Biology* 8, 633-641.

Lahavbaratz,S., Sudakin,V., Ruderman,J.V., and Hershko,A. (1995). Reversible Phosphorylation Controls the Activity of Cyclosome-Associated Cyclin-Ubiquitin Ligase. *Proceedings of the National Academy of Sciences of the United States of America* 92, 9303-9307.

Langan,T.A., Gautier,J., Lohka,M., Hollingsworth,R., Moreno,S., Nurse,P., Maller,J., and Sclafani,R.A. (1989). Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. *Mol. Cell Biol* 9, 3860-3868.

Larsen,W.J., Wert,S.E., and Brunner,G.D. (1986). A Dramatic Loss of Cumulus Cell Gap-Junctions Is Correlated with Germinal Vesicle Breakdown in Rat Oocytes. *Developmental Biology* 113, 517-521.

Ledan,E., Polanski,Z., Terret,M.E., and Maro,B. (2001). Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation. *Dev. Biol* 232, 400-413.

Lee,J., Kitajima,T.S., Tanno,Y., Yoshida,K., Morita,T., Miyano,T., Miyake,M., and Watanabe,Y. (2008). Unified mode of centromeric protection by shugoshin in mammalian oocytes and somatic cells. *Nature Cell Biology* 10, 42-U29.

Leismann,O., Herzig,A., Heidmann,S., and Lehner,C.F. (2000). Degradation of Drosophila PIM regulates sister chromatid separation during mitosis. *Genes & Development* 14, 2192-2205.

Lieberfarb,M.E., Chu,T.Y., Wreden,C., Theurkauf,W., Gergen,J.P., and Strickland,S. (1996). Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. *Development* 122, 579-588.

Lincoln,A.J., Wickramasinghe,D., Stein,P., Schultz,R.M., Palko,M.E., De Miguel,M.P., Tessarollo,L., and Donovan,P.J. (2002). Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation. *Nat Genet.* 30, 446-449.

Lindon,C. and Pines,J. (2004). Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. *Journal of Cell Biology* 164, 233-241.

Listovsky,T., Oren,Y.S., Yudkovsky,Y., Mahbubani,H.M., Weiss,A.M., Lebendiker,M., and Brandeis,M. (2004). Mammalian Cdh1/Fzr mediates its own degradation. *Embo Journal* 23, 1619-1626.

Littlepage,L.E. and Ruderman,J.V. (2002). Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. *Genes & Development* 16, 2274-2285.

Liu,C.Y., Litscher,E.S., Mortillo,S., Sakai,Y., Kinloch,R.A., Stewart,C.L., and Wassarman,P.M. (1996). Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proceedings of the National Academy of Sciences of the United States of America* 93, 5431-5436.

Liu,J.J. and Maller,J.L. (2005). Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CaMK II to release metaphase arrest by cytostatic factor. *Current Biology* 15, 1458-1468.

Llano,E., Gomez,R., Gutierrez-Caballero,C., Herran,Y., Sanchez-Martin,M., Vazquez-Quinones,L., Hernandez,T., de Alava,E., Cuadrado,A., Barbero,J.L. et al. (2008). Shugoshin-2 is essential for the completion of meiosis but not for mitotic cell division in mice. *Genes & Development* 22, 2400-2413.

Lohka,M.J., Hayes,M.K., and Maller,J.L. (1988). Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. *Proc. Natl. Acad. Sci. U. S. A* 85, 3009-3013.

Lorca,T., Castro,A., Martinez,A.M., Vigneron,S., Morin,N., Sigrist,S., Lehner,C., Doree,M., and Labbe,J.C. (1998). Fizzy is required for activation of the APC/cyclosome in Xenopus egg extracts. *Embo Journal* 17, 3565-3575.

Lorca,T., Cruzalegui,F.H., Fesquet,D., Cavadore,J.C., Mery,J., Means,A., and Doree,M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs. *Nature* 366, 270-273.

Lorca,T., Galas,S., Fesquet,D., Devault,A., Cavadore,J.C., and Doree,M. (1991). Degradation of the proto-oncogene product p39mos is not necessary for cyclin proteolysis and exit from meiotic metaphase: requirement for a Ca(2+)-calmodulin dependent event. *EMBO J.* 10, 2087-2093.

Lorenz,A., Wells,J.L., Pryce,D.W., Novatchkova,M., Eisenhaber,F., McFarlane,R.J., and Loidl,J. (2004). S. pombe meiotic linear elements contain proteins related to synaptonemal complex components. *J Cell Sci.* 117, 3343-3351.

Losada,A., Hirano,M., and Hirano,T. (1998). Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. *Genes & Development* 12, 1986-1997.

Losada,A., Hirano,M., and Hirano,T. (2002). Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes & Development* 16, 3004-3016.

Lozano,J.C., Perret,E., Schatt,P., Arnould,C., Peaucellier,G., and Picard,A. (2002). Molecular cloning, gene localization, and structure of human cyclin B3. *Biochemical and Biophysical Research Communications* 291, 406-413.

Lundgren,K., Walworth,N., Booher,R., Dembski,M., Kirschner,M., and Beach,D. (1991). mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell* 64, 1111-1122.

Madgwick,S., Hansen,D.V., Levasseur,M., Jackson,P.K., and Jones,K.T. (2006). Mouse Emi2 is required to enter meiosis II by reestablishing cyclin B1 during interkinesis. *Journal of Cell Biology* 174, 791-801.

Madgwick,S. and Jones,K.T. (2007). How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor. *Cell Div.* 2, 4.

Madgwick,S., Nixon,V.L., Chang,H.Y., Herbert,M., Levasseur,M., and Jones,K.T. (2004). Maintenance of sister chromatid attachment in mouse eggs through maturation-promoting factor activity. *Developmental Biology* 275, 68-81.

Mailhes,J.B., Hilliard,C., Fuseler,J.W., and London,S.N. (2003). Okadaic acid, an inhibitor of protein phosphatase 1 and 2A, induces premature separation of sister chromatids during meiosis I and aneuploidy in mouse oocytes in vitro. *Chromosome Research* 11, 619-631.

Maller,J.L. and Krebs,E.G. (1977). Progesterone-Stimulated Meiotic Cell-Division in Xenopus Oocytes - Induction by Regulatory Subunit and Inhibition by Catalytic Subunit of Adenosine 3'-5'-Monophosphate-Dependent Protein-Kinase. *Journal of Biological Chemistry* 252, 1712-1718.

Mao,Y.H., Abrieu,A., and Cleveland,D.W. (2003). Activating and silencing the mitotic checkpoint through CENP-E-dependent activation/inactivation of BubR1. *Cell* 114, 87-98.

Marangos,P. and Carroll,J. (2008). Securin regulates entry into M-phase by modulating the stability of cyclin B. *Nature Cell Biology* 10, 445-U151.

Marangos,P., Verschuren,E.W., Chen,R., Jackson,P.K., and Carroll,J. (2007). Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC(Cdh1). *Journal of Cell Biology* 176, 65-75.

Masui,Y. (2001). From oocyte maturation to the in vitro cell cycle: the history of discoveries of Maturation-Promoting Factor (MPF) and Cytostatic Factor (CSF). *Differentiation* 69, 1-17.

Masui,Y. and Markert,C.L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* 177, 129-145.

Mattioli,M., Galeati,G., Bacci,M.L., and Barboni,B. (1991). Changes in maturation-promoting activity in the cytoplasm of pig oocytes throughout maturation. *Mol. Reprod. Dev.* 30, 119-125.

McGowan,C.H. and Russell,P. (1993). Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. *EMBO J.* 12, 75-85.

Mcgrew,J.T., Goetsch,L., Byers,B., and Baum,P. (1992). Requirement for Esp1 in the Nuclear Division of Saccharomyces-Cerevisiae. *Molecular Biology of the Cell* 3, 1443-1454.

McGuinness,B.E., Hirota,T., Kudo,N.R., Peters,J.M., and Nasmyth,K. (2005). Shugoshin prevents dissociation of cohesin from centromeres during mitosis in vertebrate cells. *Plos Biology* 3, 433-449.

Mehlmann,L.M. (2005a). Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction* 130, 791-799.

Mehlmann,L.M. (2005b). Oocyte-specific expression of Gpr3 is required for the maintenance of meiotic arrest in mouse oocytes. *Developmental Biology* 288, 397-404.

Mehlmann,L.M., Chattopadhyay,A., Carpenter,G., and Jaffe,L.A. (2001). Evidence that phospholipase c from the sperm is not responsible for initiating Ca2+ release at fertilization in mouse eggs. *Developmental Biology* 236, 492-501.

Mehlmann,L.M., Jones,T.L.Z., and Jaffe,L.A. (2002). Meiotic arrest in the mouse follicle maintained by a G(s) protein in the oocyte. *Science* 297, 1343-1345.

Mehlmann,L.M., Saeki,Y., Tanaka,S., Brennan,T.J., Evsikov,A.V., Pendola,F.L., Knowles,B.B., Eppig,J.J., and Jaffe,L.A. (2004). The G(s)-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science* 306, 1947-1950.

Mei,J.J., Huang,X.X., and Zhang,P.M. (2001). Securin is not required for cellular viability, but is required for normal growth of mouse embryonic fibroblasts. *Current Biology* 11, 1197-1201.

Michaelis,C., Ciosk,R., and Nasmyth,K. (1997). Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91, 35-45.

Millar,J., McGowan,C., Jones,R., Sadhu,K., Bueno,A., Richardson,H., and Russell,P. (1991). cdc25 M-phase inducer. *Cold Spring Harb. Symp. Quant. Biol* 56, 577-584.

Miller,D.J., Gong,X., Decker,G., and Shur,B.D. (1993a). Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy. *J. Cell Biol* 123, 1431-1440.

Miller,D.J., Gong,X., and Shur,B.D. (1993b). Sperm require beta-N-acetylglucosaminidase to penetrate through the egg zona pellucida. *Development* 118, 1279-1289.

Miller,J.J., Summers,M.K., Hansen,D.V., Nachury,M.V., Lehman,N.L., Loktev,A., and Jackson,P.K. (2006). Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. *Genes & Development* 20, 2410-2420.

Miyazaki,S., Shirakawa,H., Nakada,K., and Honda,Y. (1993). Essential Role of the Inositol 1,4,5-Trisphosphate Receptor/Ca2+ Release Channel in Ca2+ Waves and Ca2+ Oscillations at Fertilization of Mammalian Eggs. *Developmental Biology* 158, 62-78.

Mochida,S. and Hunt,T. (2007). Calcineurin is required to release Xenopus egg extracts from meiotic M phase. *Nature* 449, 336-+.

Mollinari,C., Kleman,J.P., Saoudi,Y., Jablonski,S.A., Perard,J., Yen,T.J., and Margolis,R.L. (2005). Ablation of PRC1 by small interfering RNA demonstrates that cytokinetic abscission requires a central spindle bundle in mammalian cells, whereas completion of furrowing does not. *Molecular Biology of the Cell* 16, 1043-1055.

Moore,D.P. and Orr-Weaver,T.L. (1998). Chromosome segregation during meiosis: building an unambivalent bivalent. *Curr. Top. Dev. Biol* 37, 263-299.

Moos,J., Xu,Z., Schultz,R.M., and Kopf,G.S. (1996). Regulation of nuclear envelope assembly disassembly by MAP kinase. *Developmental Biology* 175, 358-361.

Moshe,Y., Boulaire,J., Pagano,M., and Hershko,A. (2004). Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. *Proceedings of the National Academy of Sciences of the United States of America* 101, 7937-7942.

Mueller,P.R., Coleman,T.R., Kumagai,A., and Dunphy,W.G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* 270, 86-90.

Murray,A.W., Solomon,M.J., and Kirschner,M.W. (1989). The Role of Cyclin Synthesis and Degradation in the Control of Maturation Promoting Factor Activity. *Nature* 339, 280-286.

Musacchio,A. and Salmon,E.D. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology* 8, 379-393.

Nabti,I., Reis,A., Levasseur,M., Stemmann,O., and Jones,K.T. (2008). Securin and not CDK1/cyclin B1 regulates sister chromatid disjunction during meiosis II in mouse eggs. *Dev. Biol.* 321, 379-386.

Nagase,T., Seki,N., Ishikawa,K., Tanaka,A., and Nomura,N. (1996). Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA0161-KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res.* 3, 17-24.

Nasmyth,K., Peters,L.M., and Uhlmann,F. (2000). Splitting the chromosome: Cutting the ties that bind sister chromatids. *Science* 288, 1379-1384.

Nishiyama,T., Ohsumi,K., and Kishimoto,T. (2007a). Phosphorylation of Erp1 by p90rsk is required for cytostatic factor arrest in Xenopus laevis eggs. *Nature* 446, 1096-1099.

Nishiyama,T., Yoshizaki,N., Kishimoto,T., and Ohsumi,K. (2007b). Transient activation of calcineurin is essential to initiate embryonic development in Xenopus laevis. *Nature* 449, 341-+.

Normann,N.W. and Liteack,G. Hormones. 1997. New York, Academic Press. Ref Type: Serial (Book,Monograph)

Norris,R.P., Freudzon,M., Mehlmann,L.M., Cowan,A.E., Simon,A.M., Paul,D.L., Lampe,P.D., and Jaffe,L.A. (2008). Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development* 135, 3229-3238.

Norris,R.P., Ratzan,W.J., Freudzon,M., Mehlmann,L.M., Krall,J., Movsesian,M.A., Wang,H.C., Ke,H.M., Nikolaev,V.O., and Jaffe,L.A. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* 136, 1869-1878.

Nuccitelli,R., Yim,D.L., and Smart,T. (1993). The Sperm-Induced Ca2+ Wave Following Fertilization of the Xenopus Egg Requires the Production of Ins(1,4,5)P(3). *Developmental Biology* 158, 200-212.

Ohe,M., Inoue,D., Kanemori,Y., and Sagata,N. (2007). Erp1/Emi2 is essential for the meiosis I to meiosis II transition in Xenopus oocytes. *Developmental Biology* 303, 157-164.

Okeefe,S.J., Kiessling,A.A., and Cooper,G.M. (1991). The C-Mos Gene-Product Is Required for Cyclin-B Accumulation During Meiosis of Mouse Eggs. *Proceedings of the National Academy of Sciences of the United States of America* 88, 7869-7872.

Okuma,A., Kuraoka,A., Iida,H., Inai,T., Wasano,K., and Shibata,Y. (1996). Colocalization of connexin 43 and connexin 45 but absence of connexin 40 in granulosa cell gap junctions of rat ovary. *Journal of Reproduction and Fertility* 107, 255-264.

Olsiewski,P.J. and Beers,W.H. (1983). Camp Synthesis in the Rat Oocyte. *Developmental Biology* 100, 287-293.

Page,A.W. and OrrWeaver,T.L. (1996). The Drosophila genes grauzone and cortex are necessary for proper female meiosis. *Journal of Cell Science* 109, 1707-1715.

Palmer,A., Gavin,A.C., and Nebreda,A.R. (1998). A link between MAP kinase and p(34cdc2) cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *Embo Journal* 17, 5037-5047.

Panizza,S., Tanaka,T.U., Hochwagen,A., Eisenhaber,F., and Nasmyth,K. (2000). Pds5 cooperates with cohesin in maintaining sister chromatid cohesion. *Current Biology* 10, 1557-1564.

Papi,M., Berdougo,E., Randall,C.L., Ganguly,S., and Jallepalli,P.V. (2005). Multiple roles for separase auto-cleavage during the G2/M transition. *Nature Cell Biology* 7, 1029-U144.

Papkoff,J., Verma,I.M., and Hunter,T. (1982). Detection of A Transforming Gene-Product in Cells Transformed by Moloney Murine Sarcoma-Virus. *Cell* 29, 417-426.

Parisi,S., McKay,M.J., Molnar,M., Thompson,M.A., van der Spek,P.J., van Drunen-Schoenmaker,E., Kanaar,R., Lehmann,E., Hoeijmakers,J.H., and Kohli,J. (1999). Rec8p, a meiotic recombination and sister chromatid cohesion phosphoprotein of the Rad21p family conserved from fission yeast to humans. *Mol. Cell Biol* 19, 3515-3528.

Passmore,L.A., McCormack,E.A., Au,S.W.N., Paul,A., Willison,K.R., Harper,J.W., and Barford,D. (2003). Doc1 mediates the activity of the anaphase-promoting complex by contributing to substrate recognition. *Embo Journal* 22, 786-796.

Pei,L. (2000). Activation of mitogen-activated protein kinase cascade regulates pituitary tumor-transforming gene transactivation function. *Journal of Biological Chemistry* 275, 31191-31198.

Pei,L. and Melmed,S. (1997). Isolation and characterization of a pituitary tumor-transforming gene (PTTG). *Molecular Endocrinology* 11, 433-441.

Peng,X.R., Hsueh,A.J.W., Lapolt,P.S., Bjersing,L., and Ny,T. (1991). Localization of Luteinizing-Hormone Receptor Messenger-Ribonucleic-Acid Expression in Ovarian Cell-Types During Follicle Development and Ovulation. *Endocrinology* 129, 3200-3207.

Pesin,J.A. and Orr-Weaver,T.L. (2007). Developmental role and regulation of cortex, a meiosis-specific anaphase-promoting complex/cyclosome activator. *Plos Genetics* 3, 2208-2220.

Peter,M., Nakagawa,J., Doree,M., Labbe,J.C., and Nigg,E.A. (1990). In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61, 591-602.

Peters,J.M. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol. Cell* 9, 931-943.

Peters,J.M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nature Reviews Molecular Cell Biology* 7, 644-656.

Petronczki,M., Siomos,M.F., and Nasmyth,K. (2003). Un menage a quatre: The molecular biology of chromosome segregation in meiosis. *Cell* 112, 423-440.

Pfleger,C.M. and Kirschner,M.W. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes & Development* 14, 655-665.

Phillips,K.P., Petrunewich,M.A.F., Collins,J.L., Booth,R.A., Liu,X.J., and Baltz,J.M. (2002). Inhibition of MEK or cdc2 kinase parthenogenetically activates mouse eggs and yields the same phenotypes as Mos(-/-) parthenogenotes. *Developmental Biology* 247, 210-223.

Pincus,G. and Enzmann,E.V. (1935). THE COMPARATIVE BEHAVIOR OF MAMMALIAN EGGS IN VIVO AND IN VITRO : I. THE ACTIVATION OF OVARIAN EGGS. *J Exp Med* 62, 665-675.

Pines,J. (1999). Four-dimensional control of the cell cycle. *Nat. Cell Biol* 1, E73-E79.

Pines,J. and Hunter,T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* 58, 833-846.

Pirino,G., Wescott,M.P., and Donovan,P.J. (2009). Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes. *Cell Cycle* 8, 665-670.

Polanski,Z., Ledan,E., Brunet,S., Louvet,S., Verlhac,M.H., Kubiak,J.Z., and Maro,B. (1998). Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes. *Development* 125, 4989-4997.

Posada,J. and Cooper,J.A. (1992). Requirements for Phosphorylation of Map Kinase During Meiosis in Xenopus Oocytes. *Science* 255, 212-215.

Prinz,S., Hwang,E.S., Visintin,R., and Amon,A. (1998). The regulation of Cdc20 proteolysis reveals a role for the APC components Cdc23 and Cdc27 during S phase and early mitosis. *Current Biology* 8, 750-760.

Racowsky,C., Baldwin,K.V., Larabell,C.A., Demarais,A.A., and Kazilek,C.J. (1989). Down-Regulation of Membrana Granulosa-Cell Gap-Junctions Is Correlated with Irreversible Commitment to Resume Meiosis in Golden Syrian-Hamster Oocytes. *European Journal of Cell Biology* 49, 244-251.

Raff,J.W., Jeffers,K., and Huang,J.Y. (2002). The roles of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B in space and time. *Journal of Cell Biology* 157, 1139-1149.

Ramos-Morales,F., Dominguez,A., Romero,F., Luna,R., Multon,M.C., Pintor-Toro,J.A., and Tortolero,M. (2000). Cell cycle regulated expression and phosphorylation of hpttg proto-oncogene product. *Oncogene* 19, 403-409.

Rankin,T., Familari,M., Lee,E., Ginsberg,A., Dwyer,N., BlanchetteMackie,J., Drago,J., Westphal,H., and Dean,J. (1996). Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. *Development* 122, 2903-2910.

Rauh,N.R., Schmidt,A., Bormann,J., Nigg,E.A., and Mayer,T.U. (2005). Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature* 437, 1048-1052.

Reimann,J.D.R., Freed,E., Hsu,J.Y., Kramer,E.R., Peters,J.M., and Jackson,P.K. (2001a). Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. *Cell* 105, 645-655.

Reimann,J.D.R., Gardner,B.E., Margottin-Goguet,F., and Jackson,P.K. (2001b). Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins. *Genes & Development* 15, 3278-3285.

Reis,A., Chang,H.Y., Levasseur,M., and Jones,K.T. (2006a). APC(cdh1) activity in mouse oocytes prevents entry into the first meiotic division. *Nature Cell Biology* 8, 539-540.

Reis,A., Levasseur,M., Chang,H.Y., Elliott,D.J., and Jones,K.T. (2006b). The CRY box: a second APC(cdh1)-dependent degron in mammalian cdc20. *Embo Reports* 7, 1040-1045.

Reis,A., Madgwick,S., Chang,H.Y., Nabti,I., Levasseur,M., and Jones,K.T. (2007). Prometaphase APC(cdh1) activity prevents non-disjunction in mammalian oocytes. *Nature Cell Biology* 9, 1192-1198.

Richter,J.D. (1999). Cytoplasmic polyadenylation in development and beyond. *Microbiology and Molecular Biology Reviews* 63, 446-+.

Riedel,C.G., Katis,V.L., Katou,Y., Mori,S., Itoh,T., Helmhart,W., Galova,M., Petronczki,M., Gregan,J., Cetin,B. et al. (2006). Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature* 441, 53-61.

Rieder,C.L., Cole,R.W., Khodjakov,A., and Sluder,G. (1995). The Checkpoint Delaying Anaphase in Response to Chromosome Monoorientation Is Mediated by An Inhibitory Signal Produced by Unattached Kinetochores. *Journal of Cell Biology* 130, 941-948.

Rieder,C.L. and Maiato,H. (2004). Stuck in division or passing through: What happens when cells cannot satisfy the spindle assembly checkpoint. *Developmental Cell* 7, 637-651.

Rieder,C.L., Schultz,A., Cole,R., and Sluder,G. (1994). Anaphase Onset in Vertebrate Somatic-Cells Is Controlled by A Checkpoint That Monitors Sister Kinetochore Attachment to the Spindle. *Journal of Cell Biology* 127, 1301-1310.

Runft,L.L., Jaffe,L.A., and Mehlmann,L.M. (2002). Egg activation at fertilization: Where it all begins. *Developmental Biology* 245, 237-254.

Sagata,N., Daar,I., Oskarsson,M., Showalter,S.D., and Vandewoude,G.F. (1989a). The Product of the Mos Proto-Oncogene As A Candidate Initiator for Oocyte Maturation. *Science* 245, 643-646.

Sagata,N., Oskarsson,M., Copeland,T., Brumbaugh,J., and Vandewoude,G.F. (1988). Function of C-Mos Proto-Oncogene Product in Meiotic Maturation in Xenopus Oocytes. *Nature* 335, 519-525.

Sagata,N., Watanabe,N., Vandewoude,G.F., and Ikawa,Y. (1989b). The C-Mos Proto-Oncogene Product Is A Cytostatic Factor Responsible for Meiotic Arrest in Vertebrate Eggs. *Nature* 342, 512-518.

Salah,S.M. and Nasmyth,K. (2000). Destruction of the securin Pds1p occurs at the onset of anaphase during both meiotic divisions in yeast. *Chromosoma* 109, 27-34.

Saunders,C.M., Larman,M.G., Parrington,J., Cox,L.J., Royse,J., Blayney,L.M., Swann,K., and Lai,F.A. (2002). PLC zeta: a sperm-specific trigger of Ca2+ oscillations in eggs and embryo development. *Development* 129, 3533-3544.

Schmidt,A., Duncan,P., Rauh,N., Sauer,G., Nigg,E.A., and Mayer,T.U. (2004). Xenopus polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. *Molecular Biology of the Cell* 15, 256A.

Schmidt,A., Duncan,P.I., Rauh,N.R., Sauer,G., Fry,A.M., Nigg,E.A., and Mayer,T.U. (2005). Xenopus polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. *Genes & Development* 19, 502-513.

Schwab,M., Lutum,A.S., and Seufert,W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* 90, 683-693.

Schwab,M., Neutzner,M., Mocker,D., and Seufert,W. (2001). Yeast Hct1 recognizes the mitotic cyclin Clb2 and other substrates of the ubiquitin ligase APC. *Embo Journal* 20, 5165-5175.

Sela-Abramovich,S., Edry,I., Galiani,D., Nevo,N., and Dekel,N. (2006). Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology* 147, 2280-2286.

Sheets,M.D., Wu,M., and Wickens,M. (1995). Polyadenylation of C-Mos Messenger-Rna As A Control Point in Xenopus Meiotic Maturation. *Nature* 374, 511-516.

Shoji,S., Yoshida,N., Amanai,M., Ohgishi,M., Fukui,T., Fujimoto,S., Nakano,Y., Kajikawa,E., and Perry,A.C.F. (2006). Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit via Cdc20. *Embo Journal* 25, 834-845.

Sigrist,S., Jacobs,H., Stratmann,R., and Lehner,C.F. (1995). Exit from Mitosis Is Regulated by Drosophila Fizzy and the Sequential Destruction of Cyclin-A, Cyclin-B and Cyclin-B3. *Embo Journal* 14, 4827-4838.

Sigrist,S.J. and Lehner,C.F. (1997). Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* 90, 671-681.

Skowyra,D., Craig,K.L., Tyers,M., Elledge,S.J., and Harper,J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* 91, 209-219.

Smythe,T.L. and Stricker,S.A. (2005). Germinal vesicle breakdown is not fully dependent on MAPK activation in maturing oocytes of marine nemertean worms. *Molecular Reproduction and Development* 70, 91-102.

Stemmann,O., Zou,H., Gerber,S.A., Gygi,S.P., and Kirschner,M.W. (2001). Dual inhibition of sister chromatid separation at metaphase. *Cell* 107, 715-726.

Stewart,S. and Fang,G.W. (2005). Destruction box-dependent degradation of Aurora B is mediated by the anaphase-promoting complex/cyclosome and Cdh1. *Cancer Research* 65, 8730-8735.

Stratmann,R. and Lehner,C.F. (1996). Separation of sister chromatids in mitosis requires the Drosophila pimples product, a protein degraded after the metaphase anaphase transition. *Cell* 84, 25-35.

Streb,H., Irvine,R.F., Berridge,M.J., and Schulz,I. (1983). Release of Ca-2+ from A Nonmitochondrial Intracellular Store in Pancreatic Acinar-Cells by Inositol-1,4,5-Trisphosphate. *Nature* 306, 67-69.

Stricker,S.A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol* 211, 157-176.

Sumara,I., Vorlaufer,E., Gieffers,C., Peters,B.H., and Peters,J.M. (2000). Characterization of vertebrate cohesin complexes and their regulation in prophase. *Journal of Cell Biology* 151, 749-761.

Sumara,I., Vorlaufer,E., Stukenberg,P.T., Kelm,O., Redemann,N., Nigg,E.A., and Peters,J.M. (2002). The dissociation of cohesin from chromosomes in prophase is regulated by polo-like kinase. *Molecular Cell* 9, 515-525.

Sun,Q.Y., Breitbart,H., and Schatten,H. (1999). Role of the MAPK cascade in mammalian germ cells. *Reproduction Fertility and Development* 11, 443-450.

Sun,Q.Y. and Schatten,H. (2006). Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction* 131, 193-205.

Swan,A. and Schupbach,T. (2007b). The Cdc20 (Fzy)/Cdh1-related protein, Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in Drosophila. *Development* 134, 891-899.

Swan,A. and Schupbach,T. (2007a). The Cdc20 (Fzy)/Cdh1-related protein, Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in Drosophila. *Development* 134, 891-899.

Swann,K. (1990). A Cytosolic Sperm Factor Stimulates Repetitive Calcium Increases and Mimics Fertilization in Hamster Eggs. *Development* 110, 1295-1302.

Taieb,F.E., Gross,S.D., Lewellyn,A.L., and Maller,J.L. (2001). Activation of the anaphase-promoting complex and degradation of cyclin B is not required for progression from Meiosis I to II in Xenopus oocytes. *Current Biology* 11, 508-513.

Takenaka,K., Gotoh,Y., and Nishida,E. (1997). MAP kinase is required for the spindle assembly checkpoint but is dispensable for the normal M phase entry and exit in Xenopus egg cell cycle extracts. *Journal of Cell Biology* 136, 1091-1097.

Tang,Z.Y., Sun,Y.X., Harley,S.E., Zou,H., and Yu,H.T. (2004). Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis. *Proceedings of the National Academy of Sciences of the United States of America* 101, 18012-18017.

Tatemoto,H. and Muto,N. (2001). Mitogen-activated protein kinase regulates normal transition from metaphase to interphase following parthenogenetic activation in porcine oocytes. *Zygote* 9, 15-23.

Terret,M.E., Wassmann,K., Waizenegger,I., Maro,B., Peters,J.M., and Verlhac,M.H. (2003). The meiosis I-to-meiosis II transition in mouse oocytes requires separase activity. *Current Biology* 13, 1797-1802.

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

Tornell,J., Billig,H., and Hillensjo,T. (1990). Resumption of Rat Oocyte Meiosis Is Paralleled by A Decrease in Guanosine 3',5'-Cyclic-Monophosphate (Cgmp) and Is Inhibited by Microinjection of Cgmp. *Acta Physiologica Scandinavica* 139, 511-517.

Toth,A., Ciosk,R., Uhlmann,F., Galova,M., Schleiffer,A., and Nasmyth,K. (1999). Yeast Cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes & Development* 13, 320-333.

Tung,J.J., Hansen,D.V., Ban,K.H., Loktev,A.V., Summers,M.K., Adler,J.R., and Jackson,P.K. (2005). A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of Xenopus eggs. *Proceedings of the National Academy of Sciences of the United States of America* 102, 4318-4323.

Tunquist,B.J. and Maller,J.L. (2003). Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes & Development* 17, 683-710.

Uhlmann,F. (2001). Secured cutting: controlling separase at the metaphase to anaphase transition. *Embo Reports* 2, 487-492.

Uhlmann,F., Lottspeich,F., and Nasmyth,K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature* 400, 37-42.

Uhlmann,F., Wernic,D., Poupart,M.A., Koonin,E.V., and Nasmyth,K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. *Cell* 103, 375-386.

Van Beneden,E. (1883). Recherches sur la maturation de l'oeuf et la fecondation. Ascaris Megalocephala. *Arch Biol* 4, 265-640.

van Heemst,D. and Heyting,C. (2000). Sister chromatid cohesion and recombination in meiosis. *Chromosoma* 109, 10-26.

Verlhac,M.H., Depennart,H., Maro,B., Cobb,M.H., and Clarke,H.J. (1993). Map Kinase Becomes Stably Activated at Metaphase and Is Associated with Microtubule-Organizing Centers During Meiotic Maturation of Mouse Oocytes. *Developmental Biology* 158, 330-340.

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

Visconti,P.E., Galantino-Homer,H., Moore,G.D., Bailey,J.L., Ning,X., Fornes,M., and Kopf,G.S. (1998). The molecular basis of sperm capacitation. *J. Androl* 19, 242-248.

Visintin,R., Prinz,S., and Amon,A. (1997). CDC20 and CDH1: A family of substrate-specific activators of APC-dependent proteolysis. *Science* 278, 460-463.

Vivarelli,E., Conti,M., Defelici,M., and Siracusa,G. (1983). Meiotic Resumption and Intracellular Camp Levels in Mouse Oocytes Treated with Compounds Which Act on Camp Metabolism. *Cell Differentiation* 12, 271-276.

Waizenegger,I.C., Gimenez-Abian,J.F., Wernic,D., and Peters,J.M. (2002). Regulation of human separase by securin binding and autocleavage. *Current Biology* 12, 1368-1378.

Waizenegger,I.C., Hauf,S., Meinke,A., and Peters,J.M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell* 103, 399-410.

Wang,Z.Y., Yu,R., and Melmed,S. (2001). Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. *Molecular Endocrinology* 15, 1870-1879.

Wassarman,P.M. The physiology of reproduction (The mammalian ovum). 1988. New York, Raven Press.
Ref Type: Serial (Book,Monograph)

Wassarman,P.M. (1990). Profile of a mammalian sperm receptor. *Development* 108, 1-17.

Wassarman,P.M. (2005). Contribution of mouse egg zona pellucida glycoproteins to gamete recognition during fertilization. *J. Cell Physiol* 204, 388-391.

Wassmann,K., Niault,T., and Maro,B. (2003). Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes. *Current Biology* 13, 1596-1608.

Watanabe,N., Arai,H., Nishihara,Y., Taniguchi,M., Watanabe,N., Hunter,T., and Osada,H. (2004). M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCF beta-TrCP. *Proceedings of the National Academy of Sciences of the United States of America* 101, 4419-4424.

Watanabe,Y. and Nurse,P. (1999). Cohesin Rec8 is required for reductional chromosome segregation at meiosis. ***Nature*** **400, 461-464.**

Webb,R.J., Marshall,F., Swann,K., and Carroll,J. (2002). Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase A in mammalian oocytes. ***Developmental Biology*** **246, 441-454.**

Wilson,E. (1925). ***The cell in development and heredity.*** **New York: Oxford university press.**

Wolpert,L., Beddington,R., Brockes,J., Jessel,T., Lawrence,P., and Meyeroitz,E. (1998). ***Principles of development.*** **New York: Oxford University press.**

Wu,J.Q., Hansen,D.V., Guo,Y.X., Wang,M.Z., Tang,W.L., Freel,C.D., Tung,J.J., Jackson,P.K., and Kornbluth,S. (2007). Control of Emi2 activity and stability through Mos-mediated recruitment of PP2A. ***Proceedings of the National Academy of Sciences of the United States of America*** **104, 16564-16569.**

Yamamoto,A., Guacci,V., and Koshland,D. (1996). Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. ***Journal of Cell Biology*** **133, 85-97.**

Yamano,H., Gannon,J., Mahbubani,H., and Hunt,T. (2004). Cell cycle-regulated recognition of the destruction box of cyclin B by the APC/C in Xenopus egg extracts. ***Molecular Cell*** **13, 137-147.**

Yanagimachi,R. The physiology of reproduction (mammalian fertilization). 1994. New York, Raven Press.
Ref Type: Serial (Book,Monograph)

Yokobayashi,S., Yamamoto,M., and Watanabe,Y. (2003). Cohesins determine the attachment manner of kinetochores to spindle microtubules at meiosis I in fission yeast. ***Molecular and Cellular Biology*** **23, 3965-3973.**

Yoon,H.J., Feoktistova,A., Wolfe,B.A., Jennings,J.L., Link,A.J., and Gould,K.L. (2002). Proteomics analysis identifies new components of the fission and budding yeast anaphase-promoting complexes. ***Current Biology*** **12, 2048-2054.**

Yoon,P.W., Freeman,S., Taft,L., Flanders,D., Khoury,M., Sherman,S., and Hassold,T. (1995). Advanced Maternal and Paternal Ages and the Risk of Down-Syndrome Characterized by the Origin of the Chromosomal Error - A Population-Based Study. ***American Journal of Epidemiology*** **141, S3.**

Yoon,S.Y., Jellerette,T., Salicioni,A.M., Lee,H.C., Yoo,M.S., Coward,K., Parrington,J., Grow,D., Cibelli,J.B., Visconti,P.E. et al. (2008). Human sperm devoid of PLC, zeta 1 fail to induce Ca2+ release and are unable to initiate the first step of embryo development. ***Journal of Clinical Investigation*** **118, 3671-3681.**

Yu,H.T. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. *Current Opinion in Cell Biology* 14, 706-714.

Zachariae,W., Schwab,M., Nasmyth,K., and Seufert,W. (1998a). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* 282, 1721-1724.

Zachariae,W., Shevchenko,A., Andrews,P.D., Ciosk,R., Galova,M., Stark,M.J.R., Mann,M., and Nasmyth,K. (1998b). Mass spectrometric analysis of the anaphase-promoting complex from yeast: Identification of a subunit related to cullins. *Science* 279, 1216-1219.

Zachariae,W., Shin,T.H., Galova,M., Obermaier,B., and Nasmyth,K. (1996). Identification of subunits of the anaphase-promoting complex of Saccharomyces cerevisiae. *Science* 274, 1201-1204.

Zhang,D., Ma,W., Li,Y.H., Hou,Y., Li,S.W., Meng,X.Q., Sun,X.F., Sun,Q.Y., and Wang,W.H. (2004). Intra-oocyte localization of MAD2 and its relationship with kinetochores, microtubules, and chromosomes in rat oocytes during meiosis. *Biology of Reproduction* 71, 740-748.

Zhao,X., Singh,B., and Batten,B.E. (1991). The Role of C-Mos Proto-Oncoprotein in Mammalian Meiotic Maturation. *Oncogene* 6, 43-49.

Zou,H., McGarry,T.J., Bernal,T., and Kirschner,M.W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* 285, 418-422.

Zur,A. and Brandeis,M. (2001). Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. *Embo Journal* 20, 792-801.

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