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Role of *Xenopus* p21-activated kinase 2 (X-PAK2) in polar body formation during oocyte maturation

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Yanwei Xi

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Vertebrate oocytes are physiologically arrested in meiotic prophase until shortly before ovulation. Under the influence of gonadotropic hormones, these oocytes resume meiosis and progress to metaphase II, in which they await fertilization. The transition from prophase to metaphase II is termed oocyte maturation. During oocyte maturation, no mRNA synthesis is detectable. Instead, active protein translation from maternal mRNAs and post-translation modification are required for oocyte maturation. However, these protein translational events are by and large not known. In this study, I have studied the potential role of such a translational event: *de novo* synthesis of a protein kinase termed pEg3. Prophase oocytes contain low levels of pEg3 protein. *De novo* synthesis of pEg3 protein is evident during oocyte maturation. I employed morpholino oligos antisense to the translation initiation site of *Xenopus* pEg3 mRNA to block *de novo* synthesis of pEg3 during oocyte maturation. Inhibition of pEg3 translation did not interfere with oocyte maturation, as judged by emission of the first polar body and formation of a mature egg properly arrested in metaphase II. These oocytes also exhibited normal activation of several important maturation-specific protein kinases including p42 MAP kinase and maturation promoting factor. Therefore, *de novo* synthesis of pEg3 is not required for *Xenopus* oocyte maturation (Chapter 2).

In a related project (Chapter 3), I have analyzed the potential function of *Xenopus* p21-activated kinase 2 (X-PAK2) in oocyte maturation. X-PAK2 is phosphorylated during oocyte maturation but the significance of X-PAK2 phosphorylation is not clear. I demonstrated that overexpression of the N-terminal, autoinhibitory domain of X-PAK2

did not affect the initiation of oocyte maturation but specifically inhibited polar body formation. The significance of inhibition of polar body formation in oocyte maturation, an extreme form of asymmetric cell division, is discussed in relation to the role of Cdc42 (activator of PAKs) in other form of asymmetric cell division.

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List of Abbreviations

AC	adenylate cyclase
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CA	constitutively active
cAMP	adenosine 3',5'-cyclic monophosphate
DN	dominant negative
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
G_S	stimulatory G-protein
GV	germinal vesicle
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotropin
IP	immunoprecipitation
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MI	meiosis I
MII	meiosis II
MPF	maturation promoting factor
mPR	membrane progesterone receptor
PAK	p21-activated kinase
PKA	protein kinase A
PB	polar body
PBD	p21 GTPase (Rac or Cdc42) binding domain
PMSG	pregnant mare serum gonadotropin
PR	progesterone receptor
SH3	Src homology 3
WT	wild type
X-PAK	*Xenopus* p21-activated kinase
XPR	*Xenopus* progesterone receptor

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Chapter 1

General Introduction

1. Meiosis

Sperm and eggs are haploid gametes that unite at fertilization to generate a diploid zygote. Meiosis is a specialized form of cell division that generates sperm and eggs. In comparison with mitosis, meiosis consists of two successive cell divisions (M phases) without an intervening DNA replication (S phase), thus ensuring the chromosome number halved in the gametes. Errors in this process cause birth defects and infertility in humans (Nicolaidis and Petersen, 1998; Pellestor et al., 2003). In vertebrates, germ line is separated from somatic line in very early embryonic development. A basic dogma of reproductive biology is that females lose the capacity for oocyte production in fetal development and germ line is thought to be the sole source of oocytes. However it has been challenged recently by indicating that proliferative germ cells exist in postnatal mouse ovary and suggesting that oogenesis could also occur in mammalian adults (Johnson et al., 2004). After the specification and migration of the germ line, the diploid germ cells arrived at the embryonic testis or ovary. These germ cells undergo several times of mitosis and then many germ cells die; the remainders enter meiosis by replicating the genome once (S phase) and become primary oocytes. During oogenesis, which can last many years in some vertebrates, these oocytes arrest in prophase. The long period of meiotic prophase I arrest allows the oocyte to store enough components for early embryogenesis. After the female sexually matures, the prophase I arrested oocytes re-enter meiosis, stimulated by luteinizing hormone (LH). The resumption of meiosis is usually marked by germinal vesicle (nuclear envelope) breakdown (GVBD), followed by formation of the metaphase I spindle, separation of the homologous chromosomes,

extrusion of the first polar body. Without an interphase, the oocytes enter meiosis II immediately and arrest at metaphase II as mature eggs. At fertilization, the sperm binding initiates anaphase II, sister chromosomes separation, emittion of the second polar body and finally the completion of the whole process of meiosis. It is interesting that much of the basic cell cycle machinery employed in meiosis is analogous to that in mitosis (Marston and Amon, 2004). However, there must be some things unique to meiosis to explain the unique features of meiosis such as the lack of interphase between two metaphases.

2. Progesterone induces meiotic resumption of *Xenopus laevis* oocytes

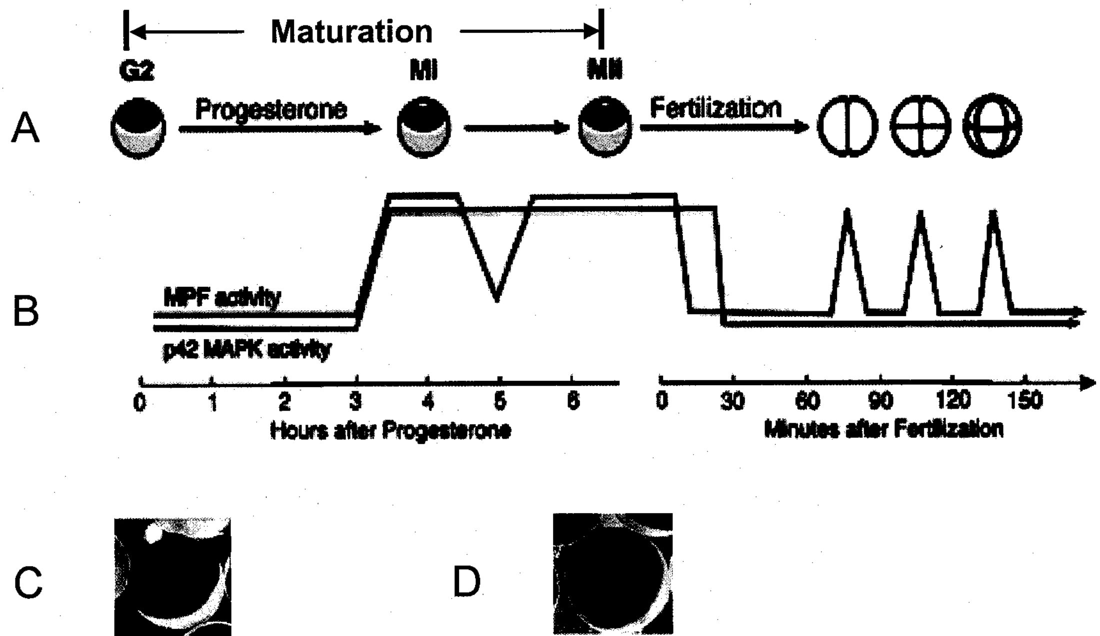
One of the best models for studying meiotic oocyte maturation is the oocytes from the African clawed toad (*Xenopus laevis*). It is easy to obtain a large number of oocytes by simply dissecting the frog. The size of the oocyte is large (1.2mm in diameter with a volume of 1μL) enough to be manipulated (microinjection of DNA, mRNA or proteins) under a dissecting microscope. Biochemical experiments using single cells are possible and routine.

Like oocytes of other vertebrates, fully grown frog oocytes are arrested in the meiotic prophase I (G2-like stage) with an intact nucleus named germinal vesicle (GV), and are enclosed in a follicle envelope before meiotic maturation and ovulation. Resumption of meiosis in G2 arrested frog oocytes needs progesterone, which is secreted by the follicle cells around the oocytes in response to gonadotropic hormones secreted by the pituitary

gland. Within several hours of progesterone stimulation, the GV migrates towards the animal pole of the oocyte and germinal vesicle breakdown (GVBD) occurs, which is indicated by a white spot on the otherwise dark pigmented animal pole of the oocyte (Figure 1.1) because of the dispersion of pigment granules underneath the animal hemisphere by the migrating GV. This white spot is normally used as a marker of initiation of oocyte maturation. After GVBD, maturation proceeds with chromosome condensation, metaphase I spindle formation, chromosome segregation, extrusion of the first polar body and finally reaching and arresting at metaphase II. The process from prophase I to metaphase II arrest is called oocyte maturation. At the same time of oocyte maturation, the maturating oocytes separate from the somatic cell layers (surface epithelium, theca, and follicle cell layer), acquire jelly coat and finally are released into the body cavity. This process is called ovulation. The mature oocytes (called eggs) remain at metaphase II until penetration by spermatozoa (Figure 1.1).

Extensive studies have been focused on frog oocyte maturation during the last thirty years since Dr. Yoshio Masui first discovered maturation promoting factor (MPF, a complex of Cdc2 kinase and cyclin B) in 1971 (Masui and Markert, 1971). What he found is: injection of a few nanoliters of cytoplasm, containing MPF activity, from mature oocyte into prophase arrested oocyte can initiate oocyte maturation. In addition, he discovered that progesterone can induce MPF activation in enucleated oocytes (oocytes whose nucleus had been physically removed) with the same kinetics as that in intact oocytes. This meant that transcription is not required in progesterone-induced MPF activation and

Figure 1.1 *Xenopus* oocyte maturation
(A) Schematic view of oocyte maturation in *Xenopus laevis*. A fully-grown *Xenopus* oocyte is arrested at the G2-like stage (prophase I) of the first meiotic cell cycle. After progesterone stimulation, the oocyte resumes meiosis, undergoes GVBD, emits the first polar body, and then enters meiosis II immediately and arrested at metaphase II. Sperm binding will release this meiosis II arrest and the egg emits the second polar body, fuses with sperm nucleus and then starts mitotic cell cycles as an embryo.
(B) Schematic view of maturation promotion factor (MPF) and MAP kinase (MAPK) activities change patterns during oocyte maturation.
(C) Typical light microscope picture of a prophase I-arrested oocyte. The darker side of the oocyte is the animal hemisphere, and the lighter side of the oocyte is the vegetal hemisphere.
(D) Typical light microscope picture of a MII-arrested oocyte. The white spot at the oocyte animal pole is the GVBD spot.
Modified from (Ferrell, 1999)



Maturation
G2
MI
MII
A
Progesterone
Fertilization
B
MPF activity
p42 MAPK activity
0
1
2
3
4
5
6
Hours after Progesterone
0
30
60
90
120
150
Minutes after Fertilization
C
D

it is the first and the best evidence for the non-genomic action of progesterone in the initiation of frog oocyte maturation.

2.1 Progesterone receptors

Although it is already demonstrated that the frog oocyte meiotic maturation is the best example of non-genomic action of progesterone, the mechanism by which progesterone stimulates meiotic maturation is not clear. Steroids can induce rapid, transcription-independent cellular signalling cascades through their classical receptors or their novel membrane receptors (Norman et al., 2004).

Two forms of the classical progesterone receptor (PR) have been cloned from *Xenopus laevis*, XPR-1 and XPR-2 (Bayaa et al., 2000; Tian et al., 2000). XPR-1 is mainly expressed in somatic follicle cells. Progesterone stimulation induces its degradation; XPR-2 is only expressed in oocytes and undergoes phosphorylation instead of degradation after progesterone induction (Liu et al., 2005). Overexpression of either XPR-1 or XPR-2 can increase the oocyte's sensitivity to progesterone and accelerate progesterone-induced oocyte maturation. This implied that the major form of PR (XPR-2) in the oocyte might be the long-sought PR responsible for progesterone induced frog oocyte maturation.

Recently, a putative membrane receptor for progestin (mPR) has been cloned from the ovary of sea trout, *Cynoscion nebulosus*, and is suggested to be responsible for 17,20*β*,

21-trihydroxy-4-pregnen-3-one (20β-S), a natural inducer of fish meiotic oocyte maturation (Zhu et al., 2003b). Its homolog in *Xenopus laevis* was also cloned (Zhu et al., 2003a). However, whether mPR plays any role in progesterone-induced oocyte maturation in the frog remains unknown.

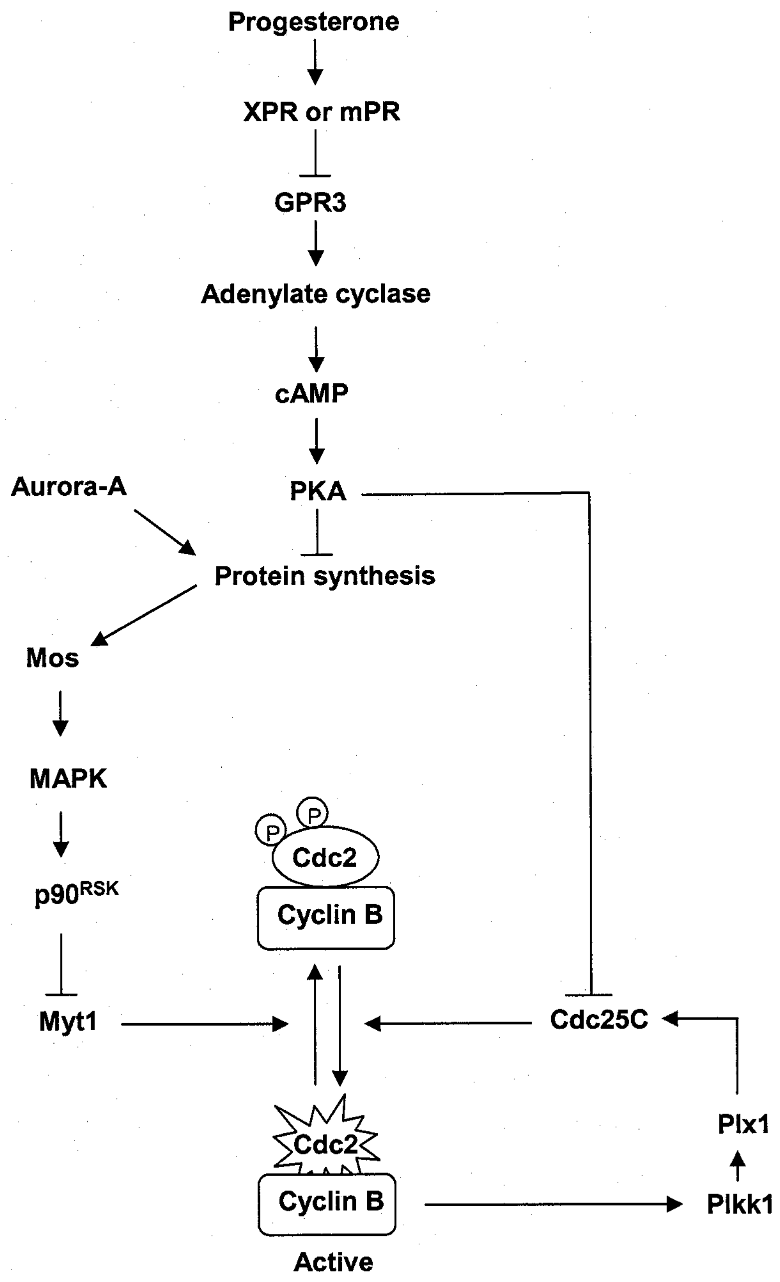
Regardless whether XPR, or mPR, or both are responsible for progesterone-induced frog oocyte maturation, progesterone appears to act by inhibition of stimulatory G protein (Gs) to reduce the levels of cAMP in the oocyte. Oocytes of both *Xenopus laevis* and mice undergo maturation when injected with an antibody that inhibits the Gs activity (Gallo et al., 1995; Kalinowski et al., 2004; Mehlmann et al., 2002). Because Gs by itself have no detectable constitutive activity (Iiri et al., 1994), it is likely that a Gs-linked receptor in the membrane keeps Gs active in the prophase I arrested oocyte. Mehlmann et al., consistent with this hypothesis, showed that an orphan Gs-linked receptor GPR3 is crucial in the regulation of meiosis in mice. Oocytes from GPR3 knock-out mice undergo spontaneous meiotic maturation independently of an increase in LH, and it could be rescued by injection of GPR3 mRNA into oocytes (Mehlmann et al., 2004).

2.2 Signalling cascades in oocyte maturation

It is generally though that the high level of cAMP is responsible for prophase I arrest (Maller et al., 1979). In arrested frog oocytes, α and/or $\beta\gamma$ subunits of Gs protein maintain the activity of adenylate cyclase (AC), which is required to maintain cAMP level (Finidori-Lepicard et al., 1981; Gallo et al., 1995; Lutz et al., 2000; Sadler and Maller,

1981; Sheng et al., 2005; Sheng et al., 2001; Smith, 1989). After progesterone stimulation, a reduction of cAMP results in the reduction of PKA (cAMP-dependent protein kinase A) activity (Maller and Krebs, 1977; Wang and Liu, 2004), which then leads to two parallel signal transduction cascades that ultimately converge on the activation of MPF (Figure 1.2). One branch involves the Mos/MAPK pathway. Progesterone induces Aurora-A dependent translation of maternal mRNAs such as Mos (Barkoff et al., 1998; Hake and Richter, 1994; Sarkissian et al., 2004), a mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK), which then induces activation of MEK1 (a MAPK kinase, MAPKK) and finally MAPK. Active MAPK release $p90^{Rsk}$ (ribosomal protein S6 kinase) to phosphorylate and inactivate Myt1, the kinase responsible for the inhibitory phosphorylation of Cdc2 on threonine 14 and tyrosine 15 (Palmer et al., 1998). In the other branch, PKA serves as the main negative regulator of Cdc25C phosphatase by phosphorylating Ser 287 and results in binding of 14-3-3, which directly inhibits Cdc25C phosphatase function in the cytoplasm. The progesterone-induced PKA inhibition releases Cdc25C phosphatase to dephosphorylate Cdc2 on threonine 14 and tyrosine 15 and finally activate MPF (Duckworth et al., 2002). Once MPF is activated, the small amount of active Cdc2 will initiate a positive feedback loop, in which Plx1 (Polo-like kinase 1) is involved and characterized by activating hyperphosphorylation of Cdc25C and inhibiting hyperphosphorylation of Myt1, and ultimately leads to complete activation of MPF (Abrieu et al., 1998; Karaiskou et al., 1999; Qian et al., 2001). Although the two-parallel-pathway model is supported by substantial experimental work, there are still large gaps. First, PKA lacking in catalytic activity can inhibit progesterone- or

Figure 1.2 Progesterone-induced *Xenopus* oocyte maturation
A schematic representation of signalling pathways involved in the progesterone-induced meiotic resumption in *Xenopus laevis*. Modified from (Kishimoto, 2003)


Progesterone
XPR or mPR
GPR3
Adenylate cyclase
cAMP
Aurora-A
PKA
Protein synthesis
Mos
MAPK
p90RSK
P
P
Cdc2
Cyclin B
Myt1
Cdc25C
Cdc2
Cyclin B
Active
Plx1
Plkk1

Mos-induced meiotic maturation, which indicates that PKA may also function downstream of Mos-MAPK pathway other than upstream of Cdc25C (Schmitt and Nebreda, 2002). Also protein synthesis is still required for meiotic maturation after downregulation of PKA activity. PKA thus appears to function in a very complicated way and has multiple physiological substrates during oocyte maturation. The identities of these substrates are still unknown (Wang and Liu, 2004). Second, protein synthesis is required for the activation of MPF but complete inhibition of Mos translation, via *mos*-specific antisense morpholino oligos, does not inhibit MPF activation (Dupre et al., 2002). These results imply that Mos does not represent the translation event for MPF activation or GVBD. What proteins, then, are required to be synthesized for MPF activation?

3. Meiosis I to Meiosis II transition

An important characteristic of meiosis is the absence of DNA replication between Meiosis I (MI) and Meiosis II (MII). This is necessary for the formation of a haploid gamete. Actually, there is no nucleus reformation between MI and MII in *Xenopus* oocytes. After ejection of the first polar body, the oocyte chromosomes remain condensed and do not form an interphase nucleus, preventing replication (Gerhart et al., 1984; Ohsumi et al., 1994).

3.1 Biochemical mechanisms controlling MI-MII transition

It is suggested that Cyclin B-Cdc2 kinase and Mos-MAPK pathway play key roles in the

mechanisms of inhibition of S phase during MI-MII transition. As discussed above, although Mos synthesis is not required for initiation of oocyte maturation, it is necessary for execution of meiosis II. In mouse, *Xenopus* and starfish oocytes, inhibition of Mos results in exit of meiosis I and entry into a series of embryonic mitotic cycles, accompanied by oscillations of Cdc2 activity and DNA replication (Dupre et al., 2002; Furuno et al., 1994; Tachibana et al., 2000). MAPK remains active throughout meiosis I and meiosis II. And it has been suggested that the high level of MAPK, together with residual cyclin B-Cdc2 activity (the activity of which might be supported by MAPK after exit from meiosis I), is responsible for the two consecutive M phases without S phase during oocyte maturation (Iwabuchi et al., 2000; Picard et al., 1996). Therefore, inhibition of the MAPK pathway by U0126 causes oocytes to enter S phase (rather than meiosis II) after meiosis I. A constitutively active form of $p90^{Rsk}$, the downstream effector of MAPK, can rescue this phenotype (Gross et al., 2000). These findings indicate that the Mos-MAPK-$p90^{Rsk}$ functions important in MI-MII transition during oocyte maturation.

During frog oocyte maturation, MPF exhibits a biphasic pattern of activation. The initial activation of MPF drives GVBD and metaphase I spindle formation. Then, MPF becomes mostly, but not completely, inactivated, and MPF is fully activated again prior to the formation of metaphase II spindle. Ubiquitination-mediated degradation of cyclin B is responsible for this partial inhibition in MPF activity (Murray, 1995). In frog oocytes, initiation of oocyte maturation does not require new B-type cyclin synthesis because of a large stockpile of cyclin B2 and B5, but new synthesis of cyclin B1 and B4 is required for

entry into MII. If new cyclin synthesis is inhibited by antisense oligonucleotides, the oocytes degenerated and failed to form a second metaphase spindle (Hochegger et al., 2001). Injection of an indestructible cyclin B into oocytes arrests the oocytes at the onset of anaphase I and prevents the re-increase of MPF activity (Huchon et al., 1993). Expression of a dominant negative mutant of Cdc2 kinase also results in nuclear reformation and DNA replication immediately after meiosis I (Furuno et al., 1994). In starfish oocytes, inhibition of the assembly of endogenous cyclin B-Cdc2 complex induces premature DNA replication (Picard et al., 1996). Furthermore, by using cell-free *Xenopus* meiotic extract, Iwabuchi et al. found that inactivation of the residual Cdc2 activity remained at the end of MI induced entry into S phase after MI. Moreover, the addition of more than a critical amount of Wee1, a mitotic inhibitor, to the extract induced Cdc2 inhibitory phosphorylation, also changed the M-M (metaphase to metaphase) transition into an M-S-M (metaphase to S-phase to metaphase) transition (Iwabuchi et al., 2000). Consistently, it was also indicated by others that the absence of Wee1 is important for the omission of S phase in the meiotic cell cycle (Murakami et al., 1999; Nakajo et al., 2000). Wee1 is also absent during meiosis I in mice, starfish and yeast, which might imply the absence of Wee1 is a conserved mechanism for inhibition of replication during meiosis.

In addition, other newly synthesized proteins are also required for MI-MII transition. For example, when exploring the function of Xkid, a *Xenopus* homolog of human kinesin-like protein, in chromosome alignment on the metaphase plate of spindles, Nebreda et al.

found that it is required for the MI-MII transition during oocyte maturation (Antonio et al., 2000; Perez et al., 2002). In the Xkid-depleted oocytes, cyclin B synthesis is suppressed and oocytes enter an interphase-like state after exit from meiosis I.

Interestingly, Aurora-A, the key kinase regulating protein synthesis (Mendez et al., 2000a; Mendez et al., 2000b), also exhibits biphasic activation pattern during oocyte maturation (Ma et al., 2003). Injection of a constitutively active form of Aurora-A could induce hormone-independent oocyte maturation. But the injected oocytes appeared to be arrested at meiosis I with condensed chromosomes but without organized microtubule spindles (Ma et al., 2003). Moreover, it was also demonstrated that injection of antibodies against the non-catalytic region of Aurora-A inhibits the normal "rotation" of metaphase I spindle and prevent polar body formation (Castro et al., 2003).

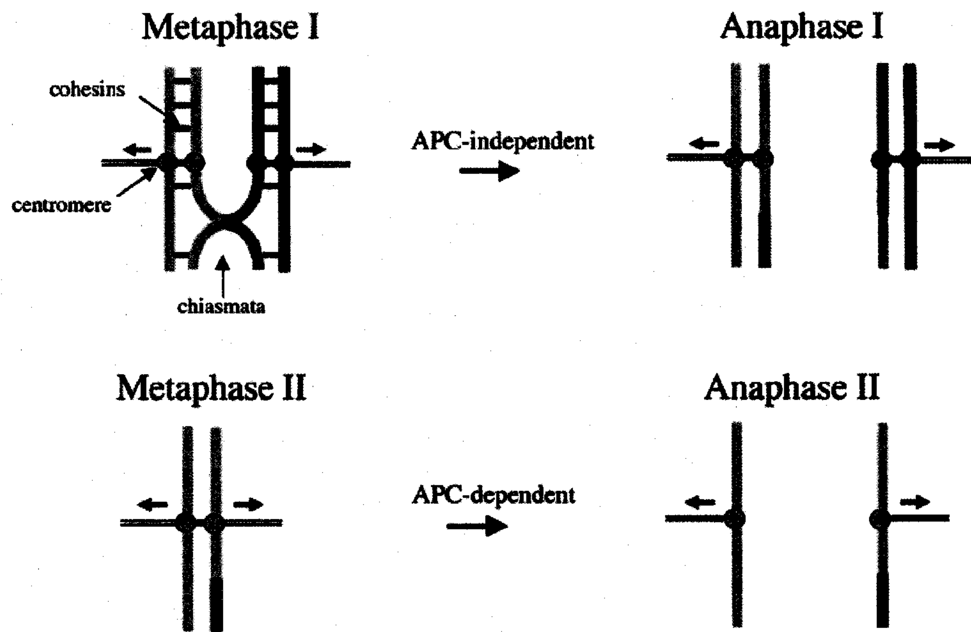
The Anaphase Promoting Complex/Cyclosome (APC/C) is an E3 ubiquitin ligase complex that polyubiquitinates key regulators of mitosis and targets them for degradation via the 26S proteasome (Irniger, 2002). In mitotic anaphase, the activated APC/C ubiquitinates cyclin B and securin, a protein that sequesters a protease named separase and keeps it inactive (Nasmyth, 2002; Peters, 2002). Once securin is degraded, separase is liberated and becomes active to cleave Scc1, a subunit of the cohesion complex holding sister chromatids together. Then the sister chromatids separate to the opposite spindle poles. There exists a well-characterized surveillance mechanism called spindle checkpoint, which monitors the alignment of sister chromatids on the mitotic spindle

during pro metaphase (Musacchio and Hardwick, 2002; Yu, 2002). Before metaphase, the Cdc20/fizzy, the activator of APC/C, is held in a ternary complex with the checkpoint protein Mad2 and APC/C, inhibiting APC/C activation. Once the sister chromatids are correctly aligned, Mad2 releases Cdc20/fizzy and activates APC/C, which results in the degradation of securin and segregation of sister chromatids (Fang et al., 1998). It is still controversial whether a similar mechanism operates to monitor homologous chromosomes in meiosis I.

Meiosis I is unique in its segregation of homologous chromosomes. Meiosis II, on the other hand, features separating sister chromatids, as is the case in mitosis (Figure 1.3). In order to finish homologous segregation, it is necessary for homologue pairs to be linked together. This is obtained by chiasmata, which is resulted from homologous recombination between non-sister chromatids during DNA replication. In addition, cohesion between sister chromatids, which is ensured by cohesins, should be maintained until anaphase II to prevent their premature dissociation. Once anaphase I is initiated, the cohesion between sister chromatid arms is released to allow homologous chromosomes to be separated, whereas cohesion of centromeric regions is maintained until anaphase II (Castro et al., 2001; Marston and Amon, 2004). How is the stepwise cohesion loss controlled? One possible mechanism is that the cohesion loss in meiosis I and meiosis II is regulated by different way: the cohesion loss in MI is separase-cleavage independent, whereas in MII is separase-cleavage dependent. For example, in *Xenopus* oocytes, the APC/C seems dispensable for the metaphase I-anaphase I transition, since inhibition of

Figure 1.3 Chromosome segregation during meiosis in *Xenopus* oocytes

In meiosis I, the cohesion between sister chromatid arms is lost in a APC-independent mechanism. However, the cohesion between sister chromatid centromeric regions is maintained until Anaphase II and is destructed in an APC-dependent mechanism. Homologous chromosomes are indicated by different colors (green and blue). Sister chromitids are in the same color (green or blue). Modified from (Castro et al., 2001)


Metaphase I
cohesins
centromere
chiasmata
APC-independent
Anaphase I
Metaphase II
APC-dependent
Anaphase II

APC/C fail to arrest oocyte in metaphase I but do arrest the oocyte in metaphase II (Peter et al., 2001; Taieb et al., 2001). On the other hand, functional studies of separase and its regulator, APC/C, indicates that they are both required in the segregation of homologues in meiosis I in yeast, or in worm or mouse oocytes (Buonomo et al., 2000; Davis et al., 2002; Herbert et al., 2003; Kitajima et al., 2003; Siomos et al., 2001; Terret et al., 2003). The significance of these apparent species differences is not yet clear.

3.2 Chromosomes and spindle dynamics during MI-MII transition

In addition to the reductive feature of chromosome behavior (one round of DNA replication followed by two cell divisions, resulting in a haploid gamete), another unique characteristic of meiotic oocyte maturation is that two polar bodies and one gamete (egg) are produced after two cell divisions (meiosis I and meiosis II). The polar bodies contain hardly any cytoplasm, whereas the egg contains nearly the entire volume of cellular constituents. It is a biological necessity for the egg to preserve the vast maternal cytoplasmic components to initiate and support embryonic development, especially in amphibians whose embryos develop externally.

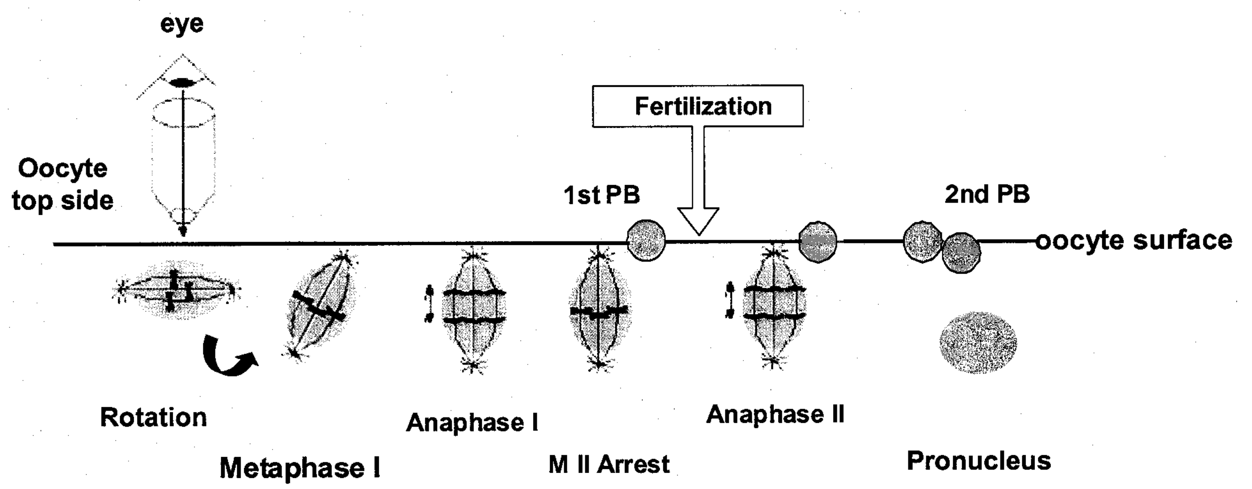
These large amounts of cytoplasmic materials are accumulated during oogenesis. It includes enzymes for DNA, RNA and protein synthesis; mRNAs; organelles; morphogenetic factors that control early embryogenesis; and so on (Gurdon et al., 1979). Frog eggs are highly asymmetrical, and this asymmetry along the animal-vegetal axis is specified during oogenesis. It has been shown that the yolk proteins are all transported to

the vegetal half of oocyte during oogenesis although they are absorbed uniformly around the surface of the oocyte (Danilchik and Gerhart, 1987). The organelles, mRNAs and other components are also moved through cytoskeleton to certain regions of the oocyte, where they will function in future (Forristall et al., 1995; Kloc and Etkin, 1995; Kloc et al., 1998). Therefore, it must be strictly regulated as to when, where and how the polar bodies are formed, not only to maintain the vast maternal cytoplasm but also to maintain asymmetry of the eggs.

3.3 Contact between the properly positioned spindle and oocyte cortex is required for anaphase I initiation

In *Xenopus* oocytes, following GVBD, the metaphase I spindle assembles directly underneath the animal pole. Due to the relative dimensions of a frog oocyte (1.2mm in diameter) and that of the spindle (26μm in length), the newly assembled bipolar metaphase spindle is virtually parallel to the overlaying oocyte cortex (Figure 1.4). Then the spindle rotates for some degree in order for one pole to attach to the nearest animal pole cortex. The attachment of the spindle pole to the animal pole cortex induces anaphase I, cytokinesis and the first polar formation. Without a delay, the metaphase II spindle is formed, and the mature egg is arrested with its metaphase II spindle attached to the animal pole cortex (Gard, 1992). At fertilization, anaphase II is initiated, sister chromatids are separated and the second polar body is formed. Clearly, the formation of the first polar body requires the proper positioning of the metaphase I spindle, as metaphase plane determines the positioning of the contractile ring that drives cytokinesis

Figure 1.4 Chromosome and spindle dynamics during *Xenopus* oocyte maturation
In *Xenopus* oocytes, following GVBD, the metaphase I spindle assembles and parallel to the oocyte cortex. It seems necessary for the spindle to rotate for some degree to attach the nearest animal pole cortex to initiate anaphase I, cytokinesis and the first polar formation. Immediately after meiosis I, the metaphase II spindle is formed, and the mature egg is arrested with its metaphase II spindle attached to the animal pole cortex. Until fertilization, anaphase II is initiated, sister chromatids are separated and the second polar body is formed. Modified from (Castro et al., 2003)


eye
Fertilization
Oocyte
top side
1st PB
2nd PB
oocyte surface
Rotation
Metaphase I
Anaphase I
M II Arrest
Anaphase II
Pronucleus

(Canman et al., 2003). It has been shown that in *Xenopus* oocytes treated with cytochalasin B, an inhibitor of actin polymerization, the bipolar metaphase spindle fails to attach to the animal pole cortex, and no polar body forms (Gard et al., 1995). Similarly in cytochalasin B-treated mouse oocytes, metaphase I spindle remains in the centre of the oocyte and not attached to the oocyte surface (in mouse oocytes, metaphase spindle migrates to the oocyte cortex instead of rotation, possibly because mouse oocytes are not polarized) (Leader et al., 2002; Louvet-Vallee et al., 2005; Wassarman et al., 1976). Also, injection of antibodies against the non-catalytic region of Aurora-A inhibits the normal "rotation" of metaphase I spindle and prevents polar body formation (Castro et al., 2003). Theses findings strongly suggest that attachment of the metaphase I spindle to the oocyte cortex is required for the initiation of anaphase I and polar body formation.

Actually, Gard et al. also revealed that the oocyte cortex exhibits animal-vegetal polarization, which is required to support spindle rotation and polar body formation, and further suggested there is a defined site on the oocyte cortex (the animal pole of the oocyte) which is functionally polarized with a high concentration of F-actin and is thought to anchor the spindle through interactions with microtubules (Gard et al., 1995; Kim et al., 2000; Sardet et al., 2002; Sun et al., 2001). Webter et al. did an elegant study showing that inhibition of myosin-10 (an actin-based myosin that can also associate with microtubules) function disrupts spindle rotation, indicating that myosin-10 has a critically important role in spindle anchoring during frog oocyte maturation by acting as the linkage between microtubules and F-actin (Weber et al., 2004). Although it is well

established that anaphase I is initiated after one pole of the bipolar metaphase I spindle attaches to the oocyte cortex, the detail signalling cascades controlling this process is not known.

4. *De novo* protein synthesis during *Xenopus laevis* oocyte maturation

Yoshio Masui already demonstrated that progesterone can induce MPF activation in enucleated oocytes, suggesting oocyte maturation requires no transcription activation (Masui and Markert, 1971). Also, transcription activity is undetectable in fully grown oocyte or during oocyte maturation, fertilization, and in early embryos (until midblastula) (Newport and Kirschner, 1982). The oocytes rely solely on the proteins already present in the cells and proteins newly translated from the stockpile of maternal mRNAs, which are accumulated largely during the long period of oogenesis and which are not actively translated due to a very short polyadenylate tail. These newly synthesized proteins are necessary in multiple steps during frog oocyte maturation. The most obvious example is Mos protein kinase, which is an activator of MAPK pathway and functions throughout the whole maturation process. In the presence of protein synthesis inhibitors, such as cycloheximide, frog oocytes can not undergo progesterone-induced GVBD (Allende et al., 1988). Inhibition of protein synthesis between GVBD and the onset of metaphase I induces exit from M-phase and enters an "interphase-like" state (Huchon et al., 1993). The identities of these *de novo* synthesized proteins are still not well known. But clearly protein synthesis is required for multiple steps during oocyte maturation.

Chapter 2

De novo synthesis and hyperphosphorylation of pEg3 during meiotic maturation of *Xenopus laevis* oocytes

1. Introduction

During oocyte maturation, the timely activation of protein translation is critically important. One mechanism controlling translational activation of maternal mRNAs is the level of cytoplasmic polyadenylation (Wickens et al., 1997). During oogenesis, mRNAs are synthesized with short poly(A) tail. Before translation can occur, some mRNA undergo cytoplasmic polyadenylation (Mendez and Richter, 2001; Vassalli and Stutz, 1995; Wormington, 1994). Based on the mRNA poly(A) length change, Paris and Philippe performed a differential screening of a cDNA library in order to isolate important maternal mRNAs for early embryonic development. They isolated 11 cDNAs, 9 of which (Egl to Eg9) are polyadenylated in eggs and deadenylated in midblastula embryos (Paris and Philippe, 1990).

This search has revealed several important proteins involved in cell cycle control. Egl, highly homologous to $p34^{cdc2}$ (catalytic subunit of MPF) and renamed CDK2, associates with cyclin E and controls G1/S transition (Koff et al., 1991; Ohtsubo et al., 1995; Paris et al., 1991). Protein kinase Eg2 not only participates in mitotic spindle formation and stabilization but is also important for frog oocyte maturation by regulating polyadenylation and spindle rotation during MI-MII transition (Castro et al., 2003; Ma et al., 2003; Mendez et al., 2000b; Roghi et al., 1998). Eg5, a kinesin-like protein, is phosphorylated by Eg2 and also involved in mitotic spindle formation and centrosome separation (Giet et al., 1999; Le Guellec et al., 1991; Sawin et al., 1992). Eg7, also named XCAP-D2, is a subunit of the condensin complex and involved in mitotic chromosomes

condensation (Cubizolles et al., 1998; Uzbekov et al., 2003).

Protein kinase Eg3 is a member of the KIN1/PAR-1/MARK protein family, which have a general function in cell cycle control and cell polarity establishment (Tassan and Le Goff, 2004) including: 1) KIN1 kinase in cell growth and cell morphology (Drewes and Nurse, 2003; La Carbona et al., 2004; Levin and Bishop, 1990); 2) PAR-1 in anterior-posterior (A/P) axis formation and asymmetric distribution of cell fate determinants (Guo and Kemphues, 1995; Kemphues, 2000; Pellettieri and Seydoux, 2002); 3) MARK in distablization of microtubules and establishment of neuronal polarity (Biernat et al., 2002; Drewes et al., 1997)(Table 2.1). The N-terminal domain of Eg3 is a well conserved catalytic kinase domain, which is separated from the other conserved C-terminal domain by a medium domain. *Xenopus* Eg3 share 63% and 65% of identity with the overall sequences of mouse and human orthologs, respectively. The kinase domain and KA1 (kinase association domain 1) in *Xenopus* Eg3 are especially highly conserved with members of KIN1/PAR-1/MARK family (Blot et al., 2002)(Figure 2.1). Human pEg3 is found to associate with and phosphorylate CDC25B phosphatase, which indicates a potential role for pEg3 in cell cycle regulation (Davezac et al., 2002). In addition, ectopic expression of *Xenopus* pEg3 in wild type fission yeast will affect cell growth and cell polarity by delocalization of F-actin all around the cell cortex. Specific localization of F-actin has already been demonstrated to be functionally important for spindle anchoring and rotation during oocyte maturation (Gard et al., 1995; Tassan and Le Goff, 2004). All these indicate that *Xenopus* pEg3 might have a potential role during oocyte

Table 2.1 KIN1/PAR-1/MARK protein family

Organism	Gene	Localization	Main function
S. pombe	kin1	Cell cortex	Cell growth and cell polarity
S. cerevisiae	KIN1 KIN2	Cell cortex	Encytosis
C. elegans	PAR-1	Cell cortex	Anterior-posterior (A/P) axis formation and asymmetric distribution of cell fate determinants
D. melanogaster	PAR-1	Cell cortex	A/P axis establishment and cell signalling
Mammals	MARK1/2	—	Distabilisation of microtubules and establishment of neuronal polarity
	C-TAK1	—	Cell cycle and signal transduction
	Eg3/MELK	—	Cell cycle dependent and cell polarity

A general function of the KIN1/PAR-1/MARK kinases is to control cell cycle and establish cell polarity.

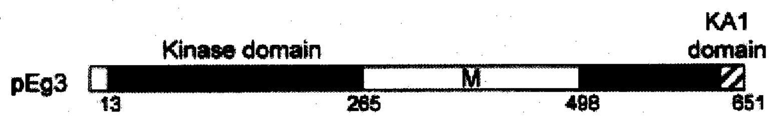
Figure 2.1 pEg3 is a member of the KIN1/PAR-1/MARK protein family

(A) Schematic representation of pEg3 primary structure. The N-terminal domain (N) of pEg3 is a conserved catalytic kinase domain and the C-terminal domain (C) is another conserved domain. Between them, there is a medium domain (M). The hatched red box corresponds to the KA1 domain (Kinase Associated domain 1). Numbers indicate amino acid positions in pEg3 sequence.

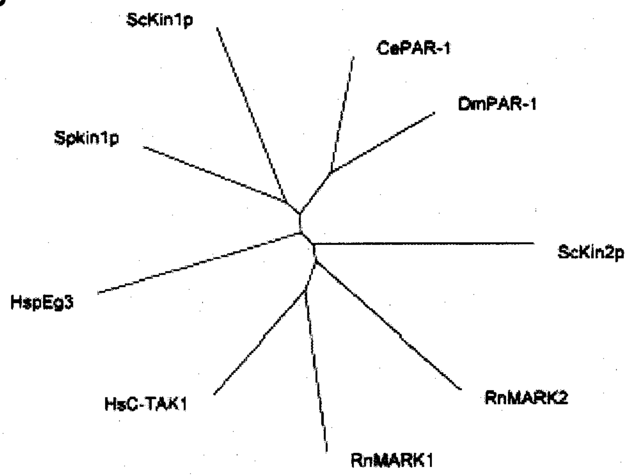
(B) A phyllogenic tree based on sequence homology of kinases of the KIN1/PAR-1/MARK family. Dm: D. melanogaster, Ce: C. elegans, Hs: H. sapiens, Rn: R. norvegicus, Sc: S. cerevisiae, Sp: S. pombe.

Modified from (Tassan and Le Goff, 2004)

A



B



maturation, especially in establishing cell polarity for polar body formation.

In *Xenopus*, phosphorylation and kinase activity of Eg3 are cell cycle dependent (Blot et al., 2002). After progesterone stimulation, Eg3 begins to be synthesized and accumulated. When GVBD occurs, all Eg3 proteins (including maternal and newly synthesized Eg3) are hyperphosphorylated. However, whether *de novo* synthesis of pEg3 has any functional role in oocyte maturation is not known. We employed antisense morpholino oligos to pEg3 to address this question.

2. Materials and Methods

2.1 Chemicals and antibodies

pEg3 antisense morpholino oligos (ACTCTTCATAATCATCCACAGCCAT) was purchased from Gene Tools. Sytox Green was purchased from Molecular Probes. Collagenase was purchased from Roche Diagnostics. Calcium ionophore A23187, human chorionic gonadotropin (hCG), Heochst 33258, and pregnant mare serum gonadotropin (PMSG) were purchased from Sigma.

Rabbit antibody against pEg3 was kindly provided by Jean-Pierre Tassan (Universite′ de Rennes 1, Rennes, France). Rabbit anti-MAPK serum was produced in house with a coupled peptide kindly provided by J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, Washington). Sheep anti-cyclin B2 serum was kindly provided by J. L. Maller (University of Colorado School of Medicine, Denver, Colorado). Mouse antibody against myc tag and HA tag were produced in house. Rat anti-HA antibody was purchased from Roche Diagnostics. Peroxidase-conjugated donkey anti-sheep antibody was from Jacksom Immnoresearch Laboratories. Peroxidase-linked sheep anti-mouse and anti-rat antibodies were obtained from Amersham Biosciences.

2.2 Animal and oocyte manipulation

Sexually mature *Xenopus laevis* females were purchased from NASCO and maintained between 18-20°C. Frogs were gonadotropin primed (PMSG, 50IU per frog) 3-7 days before operations. Ovarian fragments were removed surgically under hypothermia. stage

VI oocytes were manually defolliculated from follicles using Dumont No.5 forceps in OR2 medium (83mM NaCl, 2.5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 1mM Na_2HPO_4, 5mM HEPES pH 7.8) minus calcium. Oocytes isolated by this method still contain a layer of follicle cells. Collagenase treatment was also used to obtain follicle-cell free oocytes for chromosome staining experiments. Ovary sections were treated in collagenase solution (2mg/ml in calcium-free OR2 medium, with 1mg/ml soybean trypsin inhibitor) for 3 hour to release oocytes. Stage VI oocytes were then individually selected and grouped (Smith et al., 1991).

2.3 Expression plasmids

All the expression plasmids used in this study were kindly provided by Jean-Pierre Tassan (Universite´ de Rennes 1, Rennes Cedex, France) except Eg3-N was made by myself. The major expression vector used is PT7TS+6myc vector, which contains a T7 promoter, 6xmyc tag before object sequence and PstI restriction site for linearization. Another expression vector used is pCS2+HA vector, which contains a SP6 promoter, a HA tag before object sequence, a SV40 poly (A) sequence and a NotI restriction site for linearization. The information about these plasmids is summarized here:

1) PT7TS+6myc-Eg3-WT: Encodes the full length of wild type pEg3 (1-651 amino acid).

2) PT7TS+6myc-Eg3-MC: Encodes the middle and C terminal fragment (252-651 amino acid) of pEg3.

3) PT7TS+6myc-Eg3-K/R: Encodes the full-length pEg3 with a point mutation of lysine 42 replaced by arginine.

4) pCS2+HA-Eg3-N: Encodes the N-terminal (amino acids 1-264) of pEg3.

Messenger RNAs were *in vitro* transcripted by using mMESSAGE mMACHINE® T7/SP6 Kit (Ambion). The synthesized mRNA was dissolved in nuclease-free water, aliquoted, and stored in -80°C until injection.

Oocyte injection of mRNA or morpholino oligos was conducted in calcium-free OR2 medium and usually 20nl of mRNA or 10nl of appropriately diluted morpholino oligos was injected into each oocyte. Typically, injected oocytes would be treated with progesterone (1μM) after overnight incubation. GVBD was assessed by the first appearance of a white spot (maturation spot) on the pigmented animal pole after 3-6 hours (depending on the donor frog and seasons) following the addition of progesterone. Degradation of cyclin B and inactivation of MPF was usually evident 1 hour following the first appearance of GVBD. Three hours after GVBD, oocyte maturation completed and the mature eggs were arrested at metaphase II. Artificially activation of oocytes was performed by incubating metaphase II arrested oocytes in OR2 plus calcium containing 0.5μg/mL calcium ionophore A23187 for 60 seconds. Oocytes were then fixed in methanol at different time for microscopy analysis.

2.4 Oocyte extracts preparation and western blotting

Oocytes were lysed in ice-cold extraction (EB) buffer (20mM HEPES [pH 7.3], 80mM glycerophosphate, 20mM EGTA, 15mM $MgCl_2$, 1mM dithiothreitol, 10μM ATP, 150μM

NaF, 10μg of leupeptin per ml, 200μM phenylmethylsulfonyl fluoride, 25μg of benzamidine per ml, 10μl lysis buffer per oocyte). Following centrifugation (13000g for 5 min, 4°C), the clarified extract was removed, mixed with 2X SDS sample buffer plus beta-mercaptoethanol (β-Me), and stored in -20°C for further western blotting. Or else, clarified extract was directly used or immunoprecipitated for kinase assay.

Boiled cell lysate samples (100°C for 4min) were analyzed by SDS-PAGE on 15% polyacrylamide gel. Usually, half an oocyte was loaded on gel except for anti-*Xenopus* MAPK blot, in which one fourth of oocyte was enough. The fractionated proteins were transferred onto Protran pure nitrocellulose membrane (Scheicher & Schuel, Keene, NH) (voltage 9V for 1h). Nonspecific binding was minimized by blocking the membrane in Blotto (5% skim milk in TBST (TBS supplemented with 0.02% Tween-20)) for 30 min before incubating with primary antibody. The membrane was incubated overnight with appropriate primary antibody depending on the experiment. Then the membrane was washed in TBST at least 3 times (10min each) to remove nonspecific antibody binding. The blot was then incubated for 1h with appropriate secondary antibody depending on the experiment. The blot was washed again at least 3 times (10min each) in TBST. Immunoreactive proteins were detected by ECL western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, England) followed by exposure to film.

For re-probing, the blot was washed for twice in TBST and stripped in 10ml stripping

buffer (1M Tris-HCl 625 μl, pH 6.8, 10% SDS 2ml, β-Me 79.72 μl) at 65°C for 30 min to remove primary and secondary antibodies. The blot was then washed twice in TBST (10min each) and re-blocked with 5% milk in TBST for 30-60min. The blot was then re-probed by appropriate primary and secondary antibody depending on the experiment.

2.5 MPF kinase assay

MPF assay was performed by following the protocol described by Nebreda and Hunt (Nebreda and Hunt, 1993). Briefly, 8μl of the clarified extract was added to 4μl of the same EB buffer containing 2μg histone H1, 5μCi of [^{32}P] γ-ATP (Amersham) and 100μM ATP. Kinase reactions were carried out at room temperature for 20 min and stopped by adding 12μl of 2X SDS sample buffer. Proteins were separated on a 15% SDS-PAGE, dried and visualized by autoradiography.

2.6 In vitro translation

1μl object mRNA was incubated with 35μl rabbit reticulocyte lysate, 5μl [^{35}S] methionine and 1μl amino acid (no Met) at 30°C for 1h. Then 2μl of sample was added into 20μl 2X SDS sample buffer and boiled for at least 3 min. The mixture was loaded onto a 15% SDS-polyacrylamide running gel overlaid with a 5% SDS-polyacrylamide stacking gel. The mixture was separated by electrophoresis until the dye-front just ran out of the gel (200V for 45-60 min). The stacking gel was removed and the running gel was stained and fixed in 45% methanol, 10% acetic acid, 2.5mg/ml coomassie brilliant blue and water for 15-20 min. Excess stain was removed by washing in a series of solution containing 45%

methanol, 10% acetic acid and water until the protein bands were seen. The gel then was dried on filter paper under vacuum (70°C, 3h) and visualized by autoradiography.

2.7 Chromosome DNA imaging in whole frog oocyte

Although GVBD spot is easily scored, chromosomes dynamics is only visible after cytological or immunological processing. For only visualizing chromosomal DNA, a simplified protocol was used. Briefly oocytes were fixed in methanol for 30 minutes, rehydrated in 50% (v/v) methanol/TBS, and then transferred to TBS containing a fluorescence DNA-binding dye (1:10000 diluted Sytox Green or 1:1000 Heochst 33258 dye) for 10 minutes. The meiotic spindle beneath the GVBD spot and was viewed under a dissecting fluorescence microscope (Leica, MZ FLIII), equipped with Qimaging Retiga 1300 digital camera (W. Nuhsbaum Inc) and Openlab 3.0.4 software (Improvision Inc). Fluorescence was from Ebq-100 light source with GFP/UV (FITC) filter.

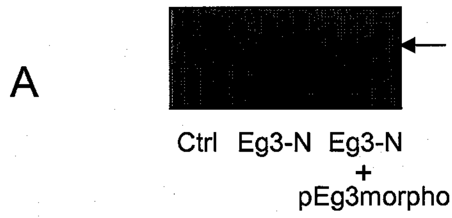
3. Results

3.1 pEg3morpho can effectively and specifically block pEg3 translation

It has been reported that progesterone induces pEg3 accumulation during frog oocyte maturation (Blot et al., 2002). To determine the possible function of pEg3 synthesis, we employed antisense morpholino oligos to inhibit *de novo* synthesis of pEg3. We first determined whether pEg3morpho can inhibit pEg3 synthesis *in vitro*. We constructed an expression vector containing the N-terminus (amino acids 1-264) of pEg3 (Eg3-N). Following in vitro transcription, the corresponding mRNA was subjected to in vitro translation in the presence or absence of pEg3morpho. Fig. 2.2A shows that pEg3morpho can significantly inhibit *in vitro* synthesis of pEg3. We then determined the effective concentrations of morpholino oligos in inhibiting *de novo* protein synthesis in frog oocytes. Fig. 2.2B shows that injection of as little as 18ng pEg3morpho per oocyte (yielding ~2μM internal concentration, given 1μL as the internal oocyte volume) abolished progesterone-induced pEg3 accumulation. For the following experiments, pEg3morpho was injected at 18ng per oocyte. By using Eg2-M (specific antisense morpholino oligos to Aurora-A) and M_B (specific antisense morpholino oligos to Aurora-B) as controls, Fig. 2.2C clearly shows that pEg3morpho effectively and specifically blocked pEg3 accumulation during oocyte maturation. However, the pEg3morpho did not affect the apparent stability, the progesterone-induced hyperphosphorylation of the existing pEg3 (Fig. 2.2C).

Figure 2.2 pEg3morpho can effectively and specifically block pEg3 translation
(A) In vitro translation with Eg3-N mRNA (1μl) or Eg3-N mRNA+pEg3morpho (1μl Eg3-N mRNA + 0.2μg pEg3morpho).
(B) Control, unjected oocytes (0) or oocytes injected with the indicate amounts of pEg3morpho were incubated overnight in the absence (-) or presence (+) of progesterone. Groups of 10 oocytes were lysed and the extracts were analyzed by immunoblotting using antibodies against *Xenopus* pEg3.
(C) Control oocytes or oocytes injected with pEg3morpho (Eg3-M, 18ng per oocyte), Aurora-Amorpho (Eg2-M, 21ng per oocyte) or Aurora-Bmorpho (M_B, 21ng per oocyte) were incubated overnight in the absence (lane 1) or presence of progesterone (all lanes except 1). Extracts were prepared and analyzed by immunoblotting using antibodies against *Xenopus* pEg3 (upper panel) or *Xenopus* Eg2 (lower panel).

A



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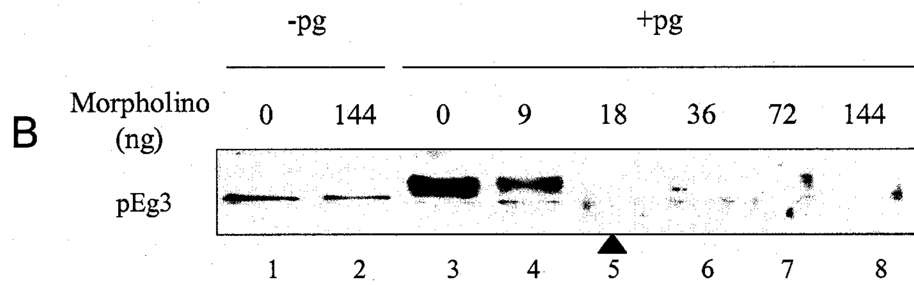
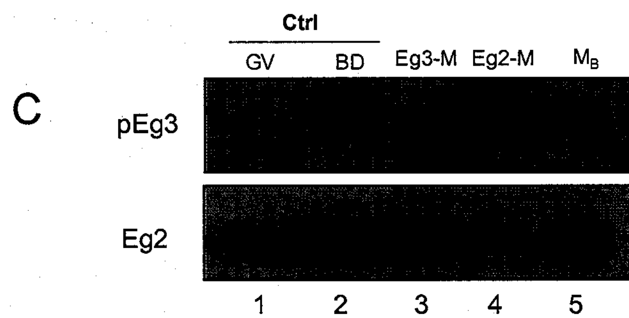


C



3.2 Inhibiting *de novo* synthesis of pEg3 does not affect maturation-specific kinase activation

Control oocytes and oocytes injected with pEg3morpho showed similar time course when GVBD response was measured (Fig. 2.3A). Control and pEg3morpho-injected oocytes were stimulated by progesterone (1μM). 2.5h after progesterone addition, GVBD spot (white spot) began to emerge on the animal pole of oocytes in both groups. Within 1.5 hours, both groups had completed GVBD. This clearly indicated that inhibition of *de novo* synthesis of pEg3 protein did not affect progesterone-induced GVBD in frog oocytes. Two of the best studied protein kinases in oocyte maturation are MAP kinase and maturation promoting factor (MPF). MAP kinase is activated at GVBD and remains activated thereafter. MPF is similarly activated at GVBD, but then undergoes a transient inactivation following GVBD before reactivation prior to metaphase II (Ferrell, 1999; Kishimoto, 2003). As shown in Fig. 2.3B, pEg3morpho inhibited *de novo* synthesis of pEg3 during oocyte maturation (compare lane 4 and lane 8). However, oocytes injected with pEg3morpho exhibited patterns of MAP kinase and MPF activation that were identical to those in control oocytes. Therefore, blocking *de novo* synthesis of pEg3 has no influence on MAPK and MPF activities, suggesting that "cytoplasmic maturation" in pEg3morpho-injected oocyte is normal.

Figure 2.3 *De novo* synthesis of pEg3 is not required for oocyte GVBD or activation of MAPK/MPF

(A) Uninjected oocytes (control) or oocytes injected with pEg3morpho (18ng per oocyte) were incubated 2 hr before the addition of progesterone. The number of GVBD oocytes of each group is counted every half an hour since the addition of progesterone. Shown is a representative of five independent experiments.

(B) Control oocytes or pEg3morpho-injected oocytes (18ng per oocyte) were incubated in the absence (lanes 1 and 5) or presence (lanes except 1 and 5) of progesterone. Oocytes were individually withdrawn as the maturation spot first appeared (GVBD). Group of 3 oocytes were lysed at GVBD, or 1 hour or overnight following GVBD. The extracts were analyzed by immunoblotting using antibodies against *Xenopus* pEg3 or MAP kinase or Cyclin B2, or analyzed for MPF activity by using histone H1 as a substrate.

A

GVBD(%)

100
80
60
40
20
0

0 0.5 1 1.5 2 2.5 3 3.5 4 ON

Time(hr)

CTRL
pEg3morpho

B

Ctrl | Eg3-M

GV GVBD 1hr O/N GV GVBD 1hr O/N

pEg3

MAPK

cyclinB_2

MPF

Histone H1

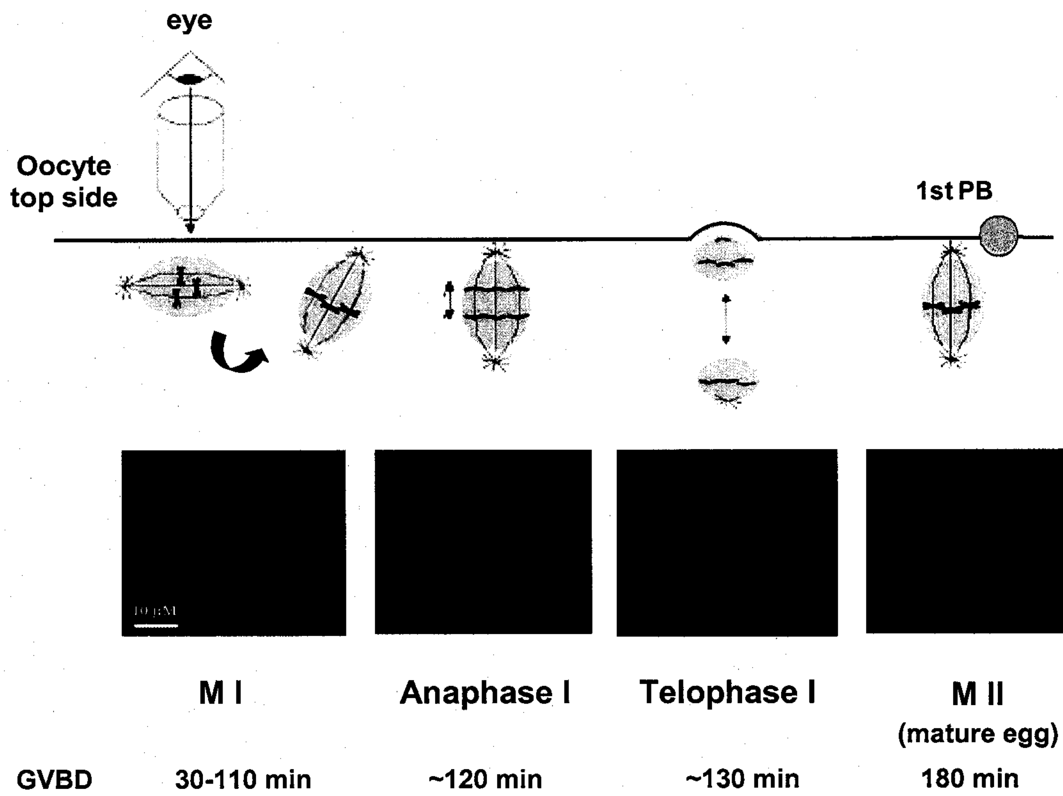
1 2 3 4 5 6 7 8

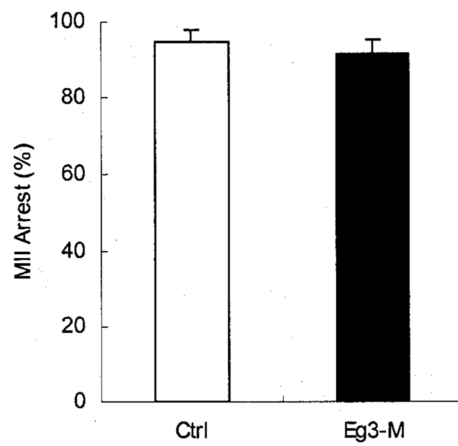
3.3 Inhibition of *de novo* pEg3 synthesis has no effect on the first polar body formation

Although the pEg3morpho-injected oocytes exhibited normal patterns of MAP kinase and MPF activation, it was possible that *de novo* synthesis of pEg3 might function in chromosomes dynamics during MI-MII transition. We determine chromosomes dynamics during oocyte maturation by staining fixed oocyte with Sytox Green DNA dye followed by fluorescence microscopy. Fig. 2.4A depicts typical images of oocytes in various stages. Following GVBD, metaphase I spindle forms, rotates and aligns perpendicular to the oocyte surface. The attachment of the spindle pole to the animal pole cortex initiates anaphase I. After telophase I and cytokinesis, the first polar body is formed. The oocytes enter meiosis II and arrest at metaphase II with a vertical metaphase II spindle to the oocyte cortex and the 1st PB. Based on this criterion, the chromosomes dynamics of pEg3morpho-injected oocytes were examined. Fig. 2.4B shows each group had similar percentage of oocytes (>90%) were arrested at metaphase II with "flower" pattern chromosomes and the 1st PB. This indicates that inhibition of *de novo* synthesis of pEg3 by morpholino had no effect on the first polar body formation.

Figure 2.4 pEg3morpho has no effect on the first polar body formation

Control oocytes or oocytes injected with pEg3morpho (Eg3-M) were incubated for at least 2 hours before the addition of progesterone. Following overnight incubation, some oocytes were lysed and subjected to immunoblotting with antibody against *Xenopus* pEg3; the rest oocytes were fixed and stained with Sytox Green and viewed from the animal pole under a dissecting fluorescence microscope. (A) shows typical images of chromosomes in various stages during oocyte maturation. Modified from (Castro et al., 2003) (B) shows that control and pEg3morpho-injected group had similar percentage of oocytes (>90%) were arrested at metaphase II. Shown here are results from six independent experiments.

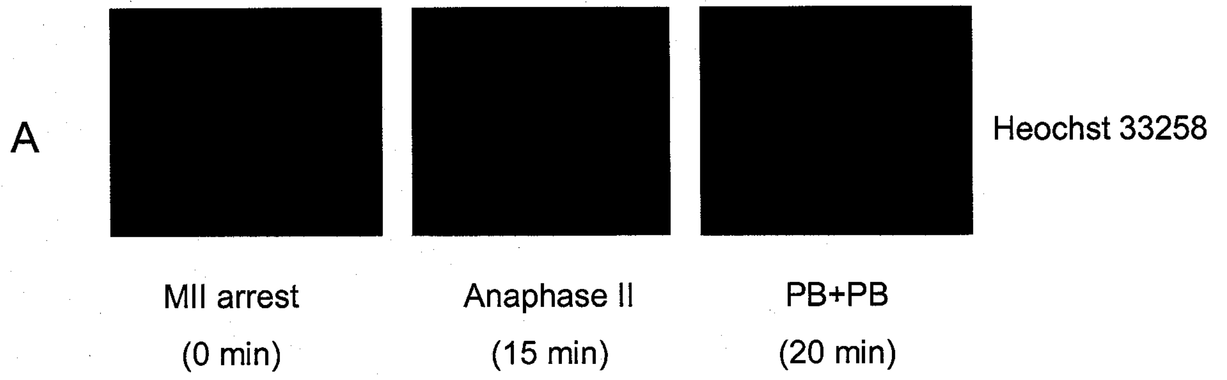

eye
Oocyte
top side
1st PB
A
M I
Anaphase I
Telophase I
M II
(mature egg)
GVBD
30-110 min
~120 min
~130 min
180 min

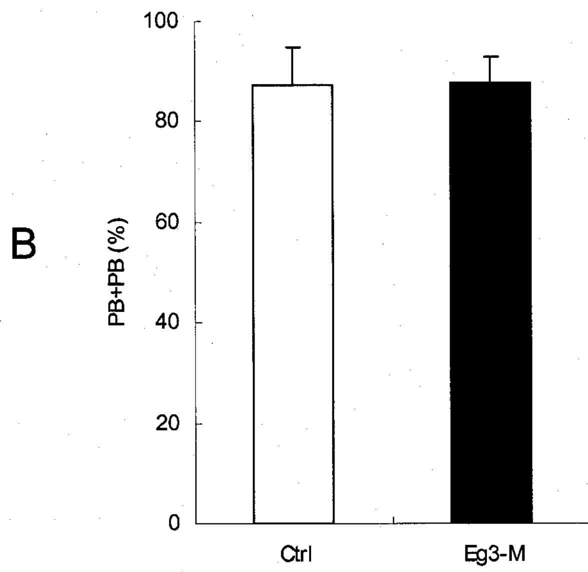

B
100
80
60
40
20
0
MII Arrest (%)
Ctrl
Eg3-M

3.4 Inhibition of *de novo* pEg3 synthesis has no effect on the second polar body formation

Although *de novo* synthesis of pEg3 is not required for proper oocyte maturation, we wished to test whether mature eggs can eject the second polar body in the absence of *de novo* synthesis of pEg3. Calcium ionophore A23187 has been shown to trigger parthenogenetic activation of mature amphibian eggs, resulting in both biochemical characteristics and chromosome behaviors similar to those induced by sperm binding (Belanger and Schuetz, 1975; Masui et al., 1977; Peter et al., 2001). Metaphase II arrested eggs were artificially activated by calcium ionophore A23187 treatment (0.5μg/mL, for 60s). Normally, 15 minutes after treatment, anaphase II began, and another 5 minutes later, meiosis II completed and the 2nd PB formed (Fig. 2.5A). Control and pEg3morpho-injected oocytes (mature in the absence of *de novo* pEg3 synthesis) were fixed 30 min after calcium ionophore treatment, and then briefly stained with a fluorescence DNA dye, Heochst 33258, and checked under a dissecting fluorescence microscope. Fig. 2.5B clearly shows eggs that mature in the absence of pEg3 synthesis (Eg3-M) responded to calcium ionophore A23187 similarly to control eggs in that both groups exhibited high percentage of 2nd PB formation (~87%). These results suggest that inhibition of *de novo* pEg3 synthesis during frog oocyte maturation has no influence on the second polar body formation upon egg activation.

Figure 2.5 pEg3morpho has no effect on the second polar body formation
Control oocytes or oocytes injected with pEg3morpho (Eg3-M) were incubated overnight in the presence of progesterone. Metaphase II arrested oocytes were treated by calcium ionophore A23187 (0.5μg/mL, for 60s). (A) shows three representative images of the normal process of oocyte after calcium ionophore treatment. 30 min after calcium ionophore treatment, some oocytes were lysed and subjected to immunoblotting with antibody against *Xenopus* pEg3; the rest oocytes were fixed, and then briefly stained with Heochst 33258, and checked under a dissecting fluorescence microscope. (B) shows that the pEg3morpho-injected group had almost the same percentage of oocytes (~87%) formed the 2nd PB 30 min after calcium ionophore A23187 treatment as control group. Shown here are results from three independent experiments.


A
Heochst 33258
MII arrest
(0 min)
Anaphase II
(15 min)
PB+PB
(20 min)


B
PB+PB (%)
100
80
60
40
20
0
Ctrl
Eg3-M

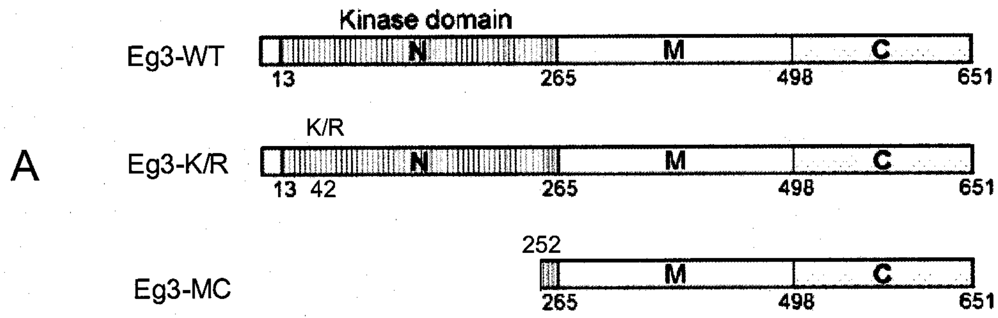
3.5 Overexpression of WT, K/R or MC mutant of pEg3 has no effect on oocyte maturation

Our results appeared to indicate that *de novo* synthesis of pEg3 is dispensable for oocyte maturation. To further investigate the possible functional role of pEg3 during frog oocyte maturation, we employed wild type pEg3 (WT) and two mutants (Fig. 2.6A). Kinase dead pEg3 (K/R) is the full-length pEg3 with no kinase activity due to a point mutation changing lysine 42 to arginine. The noncatalytic domain of pEg3 (MC) is the middle and C terminal fragment (252-651 amino acid) of pEg3, which is confirmed to be phosphorylated during oocyte maturation (Blot et al., 2002). mRNAs (WT, K/R or MC) were synthesized in vitro and injected into oocytes individually. After 5hr or overnight incubation to allow mRNA translation, the injected oocytes were stimulated with progesterone. As shown in Figure 2.6B, each group had similar percentage of oocytes (~90%) that properly matured (metaphase II). We also compared GVBD response time course of these oocytes by using the time when 50% oocytes of each group had gone GVBD. Fig. 2.6B also shows that: it took about the same time (~4.7h) for oocytes from each group to reach 50% GVBD after progesterone stimulation. Fig. 2.6C shows that all pEg3 constructs were overexpressed in oocytes. These results indicate that overexpression of WT, K/R or MC mutant of pEg3 has no effect on oocyte maturation.

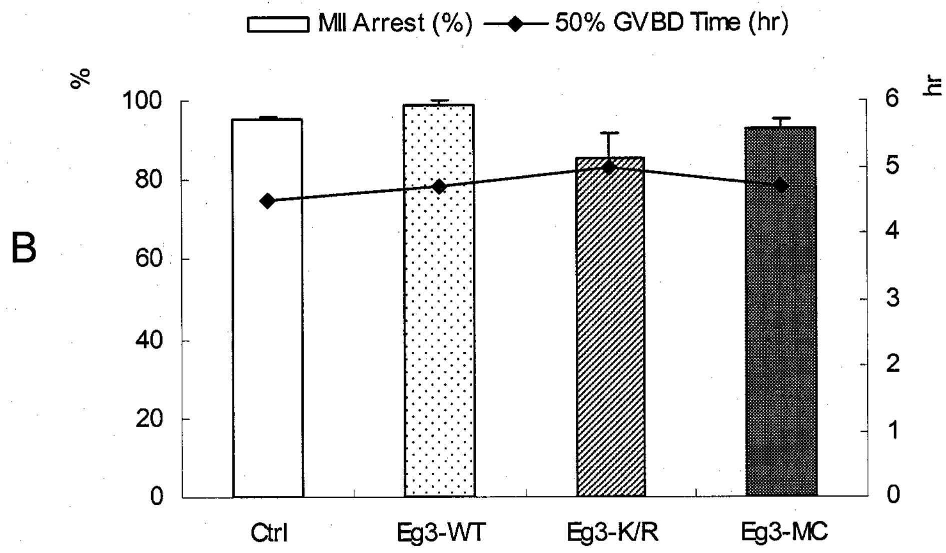
Figure 2.6 Overexpression of WT, K/R or MC mutant of pEg3 did not affect oocyte maturation

(A) Schematic representation of pEg3 mutants (WT, K/R and MC). Control oocytes or oocytes injected with different mRNAs (WT, K/R and MC) were incubated for 5hr before addition of progesterone. Following overnight incubation, some oocytes were lysed and subjected to immunoblotting with antibody against *Xenopus* pEg3 or anti-myc antibody (C); the rest oocytes were fixed, stained with Sytox Green and viewed from the animal pole under a dissecting fluorescence microscope. (B) shows that: it took about the same time (~4.7h) for oocytes from each group to undergo 50% GVBD after progesterone stimulation. Shown here is a representative from three independent experiments. And all groups had similar percentage of oocytes (~90%) were arrested at metaphase II. Shown here are results from three independent experiments.

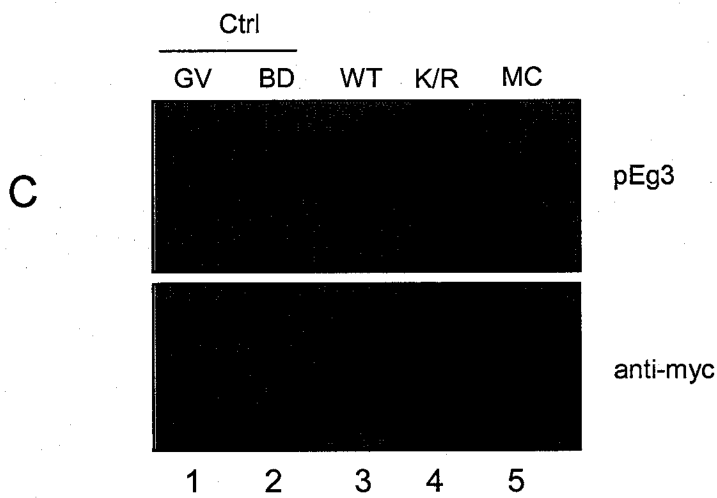
A



B



C



4. Discussion

In this study, we have employed morpholino oligos antisense to the translation initiation site of pEg3 to inhibit *de novo* synthesis of pEg3 during progesterone-induced oocyte maturation. The inhibition appears efficient, although it is not possible to conclude that we have completely blocked *de novo* synthesis of pEg3 in intact oocytes. As oocytes accumulate pEg3, the protein (both existing pEg3 and the newly synthesized) also undergoes hyperphosphorylation resulting in electrophoretic mobility shift (Blot et al., 2002). In the presence of morpholino oligos, *de novo* protein synthesis may be inhibited, but the existing protein appears stable and undergoes hyperphosphorylation. Therefore it is very difficult, if possible, to confirm that we have completely inhibited *de novo* protein synthesis. Nonetheless, the inhibition is always very significant. The morpholino strategy also appears to be very specific. The presence of morpholino antisense to pEg3 does not affect *de novo* synthesis of another oocyte protein, Eg2 (also called Aurora A). The reverse is also true. Despite this near complete or complete inhibition of pEg3 *de novo* synthesis, we have not observed any defect in oocyte maturation. This conclusion is based on both biochemical characterization of two maturation specific protein kinases (MAP kinase and MPF) but also based on chromosome behavior, more specifically the emission of the first polar body and proper metaphase II arrest.

But as pEg3 accumulation is progressive during oocyte maturation, reaching peak levels in fully mature oocytes (Blot et al., 2002), it is possible that the *de novo* synthesized pEg3 may function in a later stages (e.g. during fertilization or even during subsequent

embryogenesis). Although we have carried out "parthenogenetic" activation (mimicking fertilization) of the eggs matured in the presence of the morpholino oligos and have found no defect in emission of the second polar body, no further conclusion can be made as the eggs "activated" in such a fashion do not undergo further embryonic division. Therefore, other experimental approaches will be required to further examine the possible role of maturation-specific *de novo* synthesis of pEg3 in development of the embryos. One such approach involves transplanting the in vitro matured eggs back into an ovulating female to allow the acquisition of the capacity for fertilization and embryonic development (Heasman et al., 1991). Unfortunately, this is beyond the scope of my research project.

Chapter 3

X-PAK2 is involved in the first polar body formation during meiotic maturation of *Xenopus laevis* oocytes

1. Introduction

Polar body formation during oocyte meiotic maturation is an extreme case of asymmetric cell division, ensuring the reservation of maternal cytoplasmic stores in oocyte while halving the genome. The mechanism regulating the formation of polar body is poorly understood. As discussed above, the polarization of cortical actin patches and the attachment of metaphase I spindle to the animal pole cortex are necessary for anaphase I initiation and the first polar body formation. The p21-activated kinases (PAKs) and their activator, small GTPase Cdc42, have an evolutionary conserved function in the establishment of actin polarity and asymmetric cell division from yeast to mammals (Etienne-Manneville, 2004; Hofmann et al., 2004). For example, in the bud formation process of budding yeast, another type of asymmetric cell division, it is also necessary for one pole of the mitotic spindle (the daughter pole) to attach the bud cortex to initiate cytokinesis. The PAK kinases (Cla4 and Ste20) also play an essential role in establishing the actin polarity and promoting budding and cytokinesis together with their activator, Cdc42 (D'Amours and Amon, 2004; Hofken and Schiebel, 2002; Holly and Blumer, 1999; Schuyler and Pellman, 2001). Loss or inhibition of PAKs function leads to a complete loss of polarization and prevents bud formation (Chiroli et al., 2003; Holly and Blumer, 1999). In our lab, Ma et al. have already demonstrated that in frog oocyte, inhibition of Cdc42 completely blocked the first polar body formation (Ma et al., submitted). So here I tested the hypothesis that *Xenopus* PAK kinases (X-PAKs) might also be involved in the first polar body formation during frog oocyte maturation.

The p21-activated kinases (PAKs) are a highly conserved family of protein kinases whose activities are stimulated by binding of active Rac and Cdc42 GTPases. They have some common functions in activation of MAPK cascades and regulation of cell polarity and motility by acting on the actin and tubulin cytoskeletons (Bokoch, 2003; Dan et al., 2001; Hofmann et al., 2004; Jaffer and Chernoff, 2002). PAKs have a similar primary structure, which contains an N-terminal p21 GTPase (Rac or Cdc42) binding domain (PBD or CRIB) and a C-terminal protein kinase domain (Fig. 3.1A), except that some PAKs (Cla4, Skm1 and Pak2) from yeast contain an N-terminal pleckstrin homology (PH) domain. Based on the structural organization and activity regulation, the PAK family is divided into two subfamilies in higher eukaryotes: group A (PAKs 1-3) and group B (PAKs 4-6). PAKs of Group A are serine/threonine protein kinases with a significantly conserved sequence homology in their catalytic domains. They contain several putative Src homology 3 (SH3)-binding motifs in the N terminus, a PBD and a C-terminal kinase domain. They bind both Cdc42 and Rac, and are strongly activated by the bindings. Group B PAKs contain a PBD at the extreme N terminus and a C-terminal kinase domain. They bind Cdc42 and Rac, but are not appreciably activated upon binding. It is thought that binding by Cdc42 is more important for their localization rather than activation of group B PAKs.

PAKs of group A exist as a homodimer. Cdc42 or Rac binding disrupts dimerization, releases autoinhibition and obtains kinase activity by phosphorylation at several sites (Bokoch, 2003). For example, PAK1 exists as a homodimer in a trans-inhibited

Figure 3.1 Schematic diagram of PAK structure and its activation by Cdc42

(A) Domain structure of PAK. The structure of PAK normally contains an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain. The protein Nck binding site, Rac and Cdc42 binding domain (CRIB) and Pix family of proteins binding site are the N-terminal to the kinase domain. A $G\beta\gamma$ subunit complex binding site exists at the extreme C terminus.

(B) Activation mechanism of PAK1. PAK1 forms dimer in its inactive state through intramolecular interaction between the N-terminus and the kinase domain. Cdc42, in their GTP-bound state, binds to CRIB domain, dissociating the dimer and releasing both kinase domains. Phosphorylation of Thr423 fully activates the enzyme. Further phosphorylation of a series of Ser in the regulatory domain will prevent reversal of these steps.

Modified from (Bokoch, 2003)

Nck CRIB Pix Kinase domain Gβγ

A PAK

B

Kinase (N-lobe)
Kinase (C-lobe)
T423
CRIB
GTP
Cdc42
PS
PT423

conformation where the N-terminal autoinhibitory domain of one PAK1 molecule binds and inhibits the C-terminal kinase domain of the other (Fig. 3.1B). Binding of active Cdc42 or Rac leads to a conformation change, dissociates the dimer and unlocks both kinase domains, as the PBD overlaps the autoinhibitory domain. Phosphorylation of several sites both in C and N termini fully activates the kinase and prevents reversal of these steps (Lei et al., 2000). Phosphorylation of Thr423 in the kinase domain is very important for full catalytic function toward exogenous substrates (Yu et al., 1998; Zenke et al., 1999). PAK2 also has similar activation mechanism (Gatti et al., 1999; Wu and Wang, 2003).

In *Xenopus*, four PAKs have been discovered. X-PAKs 1-3 belong to group A, whereas X-PAK5 belongs to group B (Cau et al., 2001; Cau et al., 2000; Faure et al., 1997; Souopgui et al., 2002). Earlier studies indicated that over-expression of X-PAK1-Cter could prevent progesterone-induced oocyte maturation (Faure et al., 1997; Faure et al., 1999). However, X-PAK1 was inactive in G2 oocytes and remained inactive through oocyte maturation and appeared not responsive to Cdc42 in *Xenopus* oocytes. In contrast, X-PAK2 was active in G2 oocytes but became inactive at GVBD due to its phosphorylation. And it was suggested to be involved in the control of G2/M transition as the downstream effector of Cdc42 (Cau et al., 2000). X-PAK3 was undetectable in oocyte or eggs and began to be expressed at late gastrula stages of embryonic development in the neuroectoderm (Souopgui et al., 2002). X-PAK5 was found in the embryos to bind to actin and microtubule networks and regulate convergent extension movements during

gastrula in a calcium-dependent way (Cau et al., 2001; Faure et al., 2005). Therefore, it seems that only X-PAK2 is involved in oocyte maturation and can response to its activator, Cdc42, in oocytes. So I focused mainly on the functions of X-PAK2 in the first polar body formation during frog oocyte maturation.

2. Materials and Methods

2.1 Chemicals and antibodies

Histone H2B was purchased from Roche Diagnostics. MBP (myelin basic protein) was purchased from Sigma. p-γPAK (Thr402) antibody was purchased from Santa Cruz Biotechnology, Inc. Rabbit antibody against xFizzy was kindly provided by Thierry Lorca (CNRS, Montpellier, France).

2.2 Expression plasmids

X-PAK2-WT and X-PAK2-CA constructs used in this study were kindly provided by Stephen Hammes (UT SouthWestern). $Cdc42^{N17}$ and $Cdc42^{L61}$ were kindly provided by Nathalie Lamarche-Vane (McGill University, Canada). The other contructs were made by myself. The major expression vector is pGEM vector, which contains a T7 promoter, a HA tag before object sequence and a NheI restriction site for linearization. The other expression vector is pCS2+HA vector. The information about these plasmids is summarized here:

1) pCS2+HA-Cdc42-WT: Encodes the wild type *Xenopus* Cdc42 (1-191 amino acid).

2) pCS2+HA-$Cdc42^{N17}$: Encodes the full length of *Xenopus* Cdc42 with a point mutation of threonine 17 replaced by asparagine.

3) pCS2+HA-$Cdc42^{L61}$: Encodes the full length of *Xenopus* Cdc42 with a point mutation of glutamine 61 replaced by leucine.

4) pGEM-HA-X-PAK2-WT: Encodes the wild type of *Xenopus* PAK2 (1-517 amino acid).

5) pGEM-HA-X-PAK2-CA: Encodes the full length of *Xenopus* PAK2 with double mutations of two conserved histidine to leucine (H74, 77L).

6) pCS2+HA-X-PAK2-DN: Encodes the full length of *Xenopus* PAK2 with a point mutation of lysine 271 replaced by arginine.

7) pCS2+HA-X-PAK2-NT: Encodes the N terminal fragment of *Xenopus* PAK2 (1-217 amino acid).

8) pCS2+HA-X-PAK2-NTm: Encodes the N terminal fragment of *Xenopus* PAK2 (1-217 amino acid) with double mutations of two histidine to leucine (H74, 77L).

2.3 Immunoprecipitation

Extracts (prepared in EB lysis buffer as described above) from 20–30 control oocytes or oocytes injected with HA tagged mRNAs were incubated with 50μl of the anti-HA beads for 90 min at 4°C. The beads were washed three times with EB lysis buffer. We eluted bound proteins by boiling them in SDS sample buffer and analyzed them by immunoblotting with anti-HA and anti-p-γPAK (Thr402) antibodies.

2.4 X-PAK2 kinase assay

X-PAK2 kinase assay was performed according to the protocol described (Cau et al., 2000). Briefly, either whole cell lysis or proteins immunoprecipitated with mouse anti-HA antibodies was incubated for 20 min at room temperature in 15μl of kinase buffer (25mM HEPES [pH 7.5], 25mM $MgCl_2$, 25mM β-glycerophosphate, 2mM dithiothreitol, and 0.1mM orthovanadate) containing 1μl of histone H2B (5mg/ml) and 2.5μl MBP

(2mg/ml), 1μl of 1mM ATP and 0.5μl of [γ-^{32}P] ATP (3000Ci/mmol) to reach a final [γ-^{32}P] ATP specific activity of 6Ci/mmol. Kinase reactions were stopped by the addition of 30μl 2X SDS sample buffer. Then samples were subjected to 15% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

2.5 Other materials and methods

Refer to Chapter 2 materials and methods

3. Results

3.1 An endogenous X-PAK is phosphorylated at GVBD

Autophosphorylation of Thr423 in PAK1 (equivalent to Thr402 in PAK2) was considered to be a key event for full activation of the enzyme (Walter et al., 1998; Zenke et al., 1999). We employed a phospho-specific antibody (p-γPAK (Thr402)) to examine the phosphorylation and activity status of endogenous X-PAK during oocyte maturation. p-γPAK (Thr402) antibody should recognize all endogenous group A X-PAKs, which were phosphorylated at the equivalent site to Thr402 in PAK2, due to the highly conservative sequence around Thr402 in PAKs of human, mouse, rabbit and *Xenopus* (Fig. 3.2A). By using this phospho-specific antibody, we discovered that a possible endogenous X-PAK was phosphorylated at GVBD and remained phosphorylated until metaphase II arrest. This suggested that the endogenous X-PAK was inactive in GV oocytes, but fully activated upon GVBD and remained active throughout the rest of oocyte maturation process (Fig. 3.2B). Then we examined whether the endogenous X-PAK was responsive to Cdc42. Fig 3.2C shows that inactivation of Cdc42 by injecting a DN mutant of Cdc42 ($Cdc42^{N17}$) had no effect on the endogenous X-PAK, which remained phosphorylated and active. In control experiments, we have demonstrated that $Cdc42^{N17}$ blocks the 1st PB formation (Ma et al., submitted).

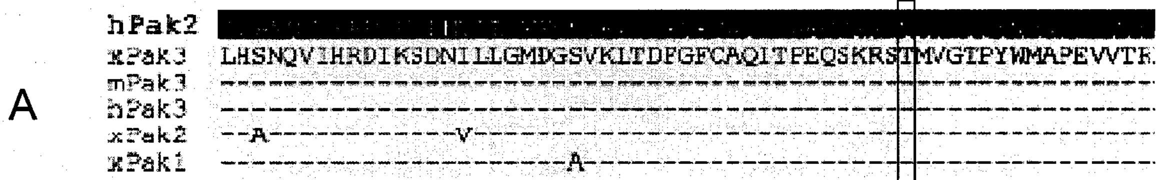
Figure 3.2 An endogenous well conserved X-PAK was phosphorylated at GVBD

(A) The sequence around Thr402 in X-PAK2 and in mouse and human PAKs.

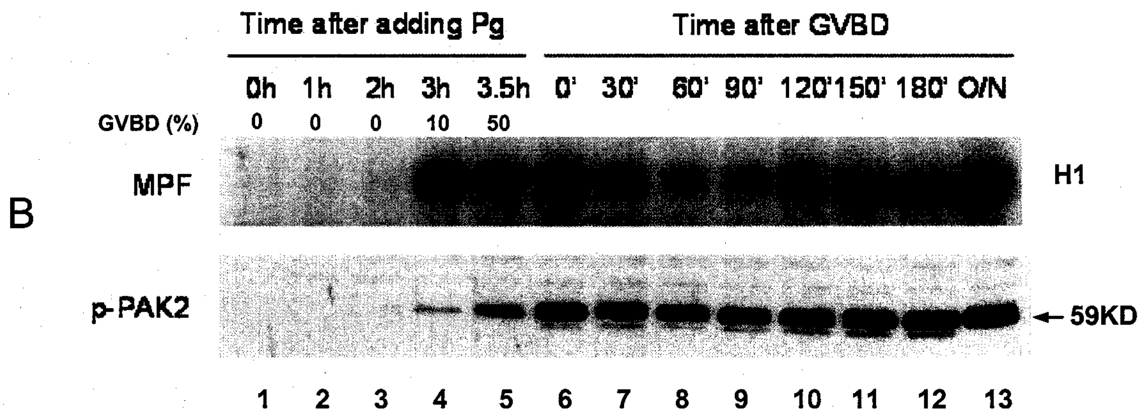
(B) Time course of the endogenous X-PAK (recognized by p-γPAK antibody) phosphorylation during oocyte maturation. After the addition of progesterone, three oocytes were picked randomly at different time and lysed, before 50% oocytes underwent GVBD. After 50% oocytes underwent GVBD, a group of oocytes that underwent GVBD within 5 minutes was picked as synchronized oocytes. And single oocytes were picked from that group at different time after GVBD and individually lysed. The extracts were subjected to MPF kinase assay and immunoblotting with p-PAK2 antibody. Each lane represents a different oocyte. Shown is a representative of three independent experiments.

(C) Unjected oocytes (ctrl, lanes 1 to 4) and oocytes injected with $Cdc42^{N17}$ mRNA (lanes 5 to 8) were incubated overnight before the addition of progesterone. Individual oocytes were lysed at GVBD or at the indicated times following GVBD. GV oocytes (without progesterone) were lysed at the same time as the O/N oocytes. Extracts were subjected to MPF assays or immunoblotting with p-PAK2 antibody. Shown is a representative of three independent experiments.

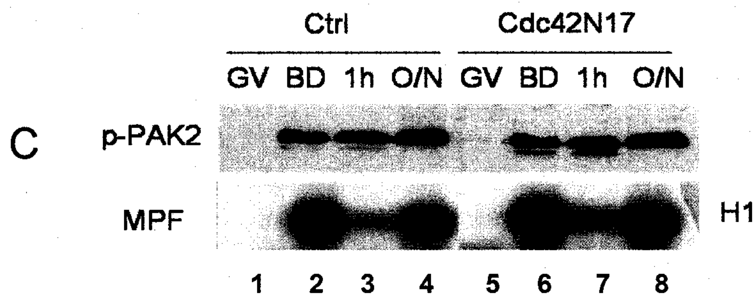
A



B



C



3.2 Overexpression of X-PAK2 WT, CA or DN mutant has no influence on the first polar body formation

To investigate the possible functional role of X-PAK2 during frog oocyte maturation, three X-PAK2 constructs were employed in oocyte injection experiments (Fig. 3.3A). Wild type X-PAK2 (WT) is the full length X-PAK2 without any mutations. Constitutively active X-PAK2 (CA) is the full length X-PAK2 with double mutations of two conserved histidine to leucine (H74, 77L). This double mutation has been shown to disrupt the interaction of PAK2 with Cdc42 and activate PAK2 kinase (Daniels et al., 1999; Sells et al., 1997). Dominant negative X-PAK2 (DN) is the full length X-PAK2 with no kinase activity due to a point mutation of lysine 271 replaced by arginine in the catalytic domain. mRNAs (WT, CA or DN) were synthesized in vitro and individually injected into oocytes. The oocytes were incubated for at least 5hr before the addition of progesterone. Following overnight incubation, oocytes were fixed, stained with Sytox Green and viewed from the animal pole under a fluorescence microscope. Fig. 3.3B shows each group had similar percentage of oocytes (~91%) that properly matured and arrested at metaphase II.

Given that p-γPAK (Thr402) antibody recognized a possible endogenous X-PAK that became phosphorylated at GVBD, we wished to determine whether X-PAK2 derived from mRNA injection also exhibited similar phosphorylation. But as shown in Fig. 3.3C, bottom panel, although X-PKA2 (lane 2) or its CA mutant (lane 4) were expressed in oocytes and underwent electrophoretic mobility shift (lanes 3 and 5 for WT and CA

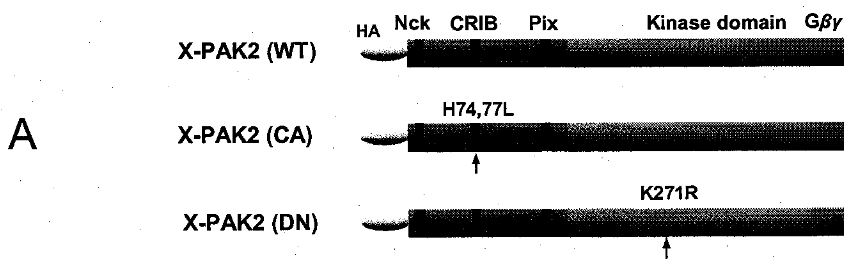
X-PAK2 respectively), no appreciable signal was detected by p-γPAK antibody beyond that of the endogenous protein (compare lanes 1, 3 and 5, Fig. 3.3C, top panel). To further confirm the above conclusion, we further analyzed these same extracts by anti-HA immunoprecipitation followed by immunoblotting using p-γPAK antibody (Fig. 3.3C, lanes 6-10). These immunoprecipitates contained the expected HA-tagged protein derived from the injected mRNA (bottom panel) but no signals were observed in any by p-γPAK antibody (top panel). These results strongly suggested that the exogenous X-PAK2 was not phosphorylated on Thr402 in frog oocytes. These immunoprecipitates were also subjected to in vitro kinase assays in the presence of two frequently used PAK substrates, myelin basic protein (MBP) and histone H2B (Cau et al., 2000; Wilkes et al., 2003) (Fig. 3.3D). Although we consistently observed increased kinase activity in immunoprecipitates derived from GVBD oocytes extracts (lanes 2, 4 and 6) compared to those from GV oocytes extracts (lanes 1, 3 and 5), we concluded that the increased activities likely represented protein kinases non-specifically associated with these immunoprecipitates, as the increase was observed regardless whether oocytes were injected with mRNA. Finally, we investigated the ability of Cdc42 dominant negative mutant ($Cdc42^{N17}$) and constitutively active mutant ($Cdc42^{L61}$) to influence phosphorylation of X-PAK2. X-PAK2 exhibited the typical electrophoretic mobility shift in mature (BD) oocytes (Fig. 3.3E, lane 4) even in the presence of $Cdc42^{N17}$, indicating that phosphorylation of X-PAK2 was not mediated by Cdc42. On the other hand, in the presence of $Cdc42^{L61}$, progesterone failed to induce GVBD, as reported previously (Cau et al., 2000). Inhibition of GVBD was accompanied by the lack of X-PAK2

phosphorylation (top panel, lane 6) and lack of phosphorylation of the endogenous X-PAK (bottom panel, lane 6). These results indicated that phosphorylation of both the endogenous X-PAK and exogenous X-PAK2 were related to progesterone-induced GVBD and not with the state of Cdc42 activity.

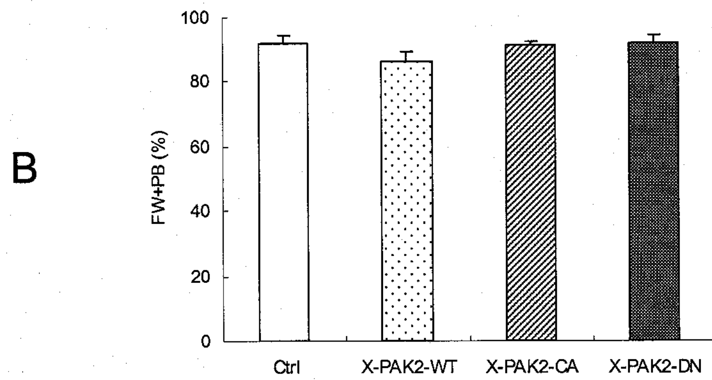
Figure 3.3 Overexpression of exogenous X-PAK2 WT, CA or DN mutant had no influence on the first polar body formation

(A) Schematic representation of X-PAK2 WT and CA, DN mutants. Control oocytes or oocytes injected with indicated mRNAs were incubated for 5hr before addition of progesterone. Following overnight incubation, some oocytes were lysed and subjected to immunoblotting with indicated antibodies (B, C and E) or immunoprecipitated with anti-HA antibody and analyzed for X-PAK2 activity (D); the rest oocytes were fixed, stained with Sytox Green and viewed from the animal pole under a dissecting fluorescence microscope. "FW+PB" means the oocytes that properly matured and arrested at metaphase II with flower-like pattern of chromosomes (FW) and the first polar body (PB). Shown here are results from three independent experiments.

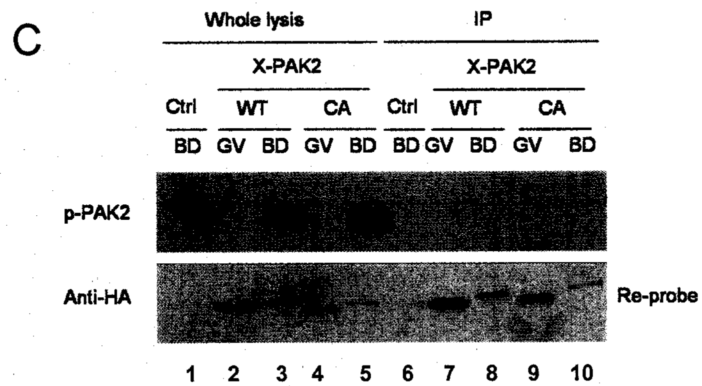
A



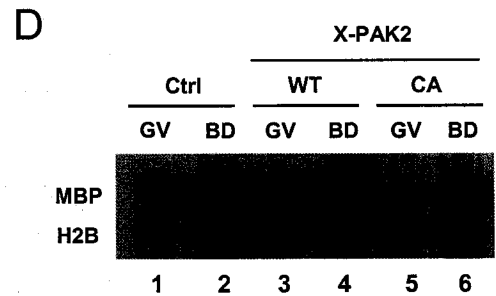
B



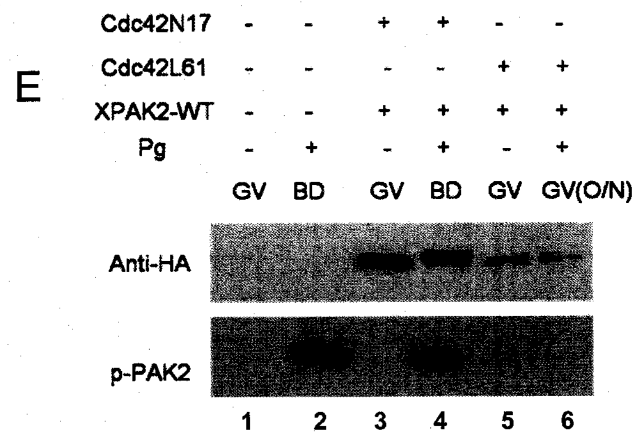
C



D



E



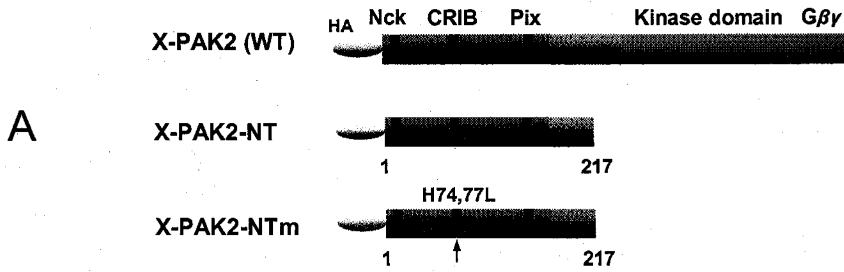
3.3 The N-terminal, autoinhibitory domain of X-PAK2 inhibits first polar body formation during oocyte maturation

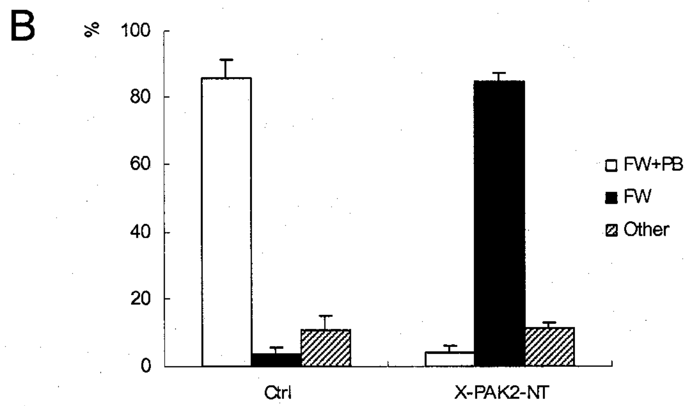
The release of inhibition by binding of small GTPase to the PBD sequence within the autoinhibitory domain of PAKs is necessary for its full activation (Bokoch, 2003). So there exists another dominant-negative form of PAKs, the N-terminus of PAK, which can bind to the C-terminal kinase domain and inhibit its phosphorylation (Chu et al., 2004; Rudel and Bokoch, 1997). We therefore constructed an expression vector containing the N-terminal autoinhibitory domain of *Xenopus* X-PAK2 (X-PAK2-NT). Oocytes injected with X-PAK2-NT mRNA were treated with progesterone. X-PAK2-NT did not affect progesterone-induced GVBD (see the light pictures in Fig. 3.4C). However, upon fixing and staining with the fluorescent DNA dye Sytox Green, it was evident that while control oocytes had properly matured and arrested in metaphase II (with a polar body), oocytes injected with X-PAK2-NT had not emitted the first polar body but contained a single metaphase spindle (Fig. 3.4, B and C). As X-PAK2-NT is known to bind to the effector domain of active, GTP-bound Cdc42, our observation could simply mean that X-PAK2-NT inhibited Cdc42 function, whether endogenous X-PAK2 had a function in polar body formation or not. To eliminate this possibility, we substituted two histidine residues within X-PAK2-NT (H74, H77) with leucine, generating X-PAK2-NTm. This change is known to abolish the ability of X-PAK2-NT to bind Cdc42 but does not affect the ability of X-PAK2-NT to function as a dominant negative mutant of X-PAK2 (Zenke et al., 1999). X-PAK2-NTm inhibited first PB formation as efficiently as X-PAK2-NT (Fig. 3.4D). Similar levels of X-PAK2-NT and X-PAK2-NTm were observed in

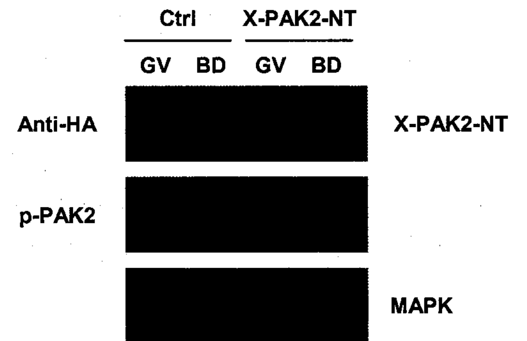
mRNA-injected oocytes (Fig. 3.4D). These results strongly suggest that X-PAK2-NT inhibit the first PB formation by binding and inhibiting endogenous X-PAK2 kinase domain. In other words, endogenous X-PAK2 may be required for polar body formation.

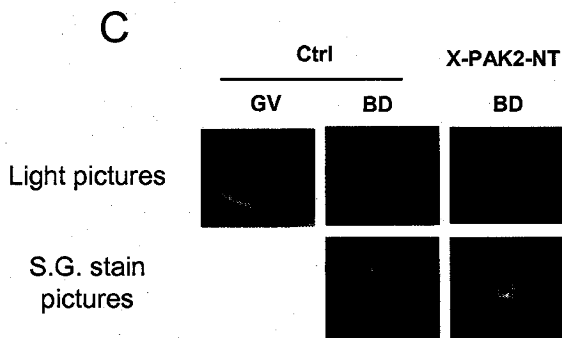
Figure 3.4 The N-terminal, autoinhibitory domain of X-PAK2 blocked the first polar body formation during oocyte maturation

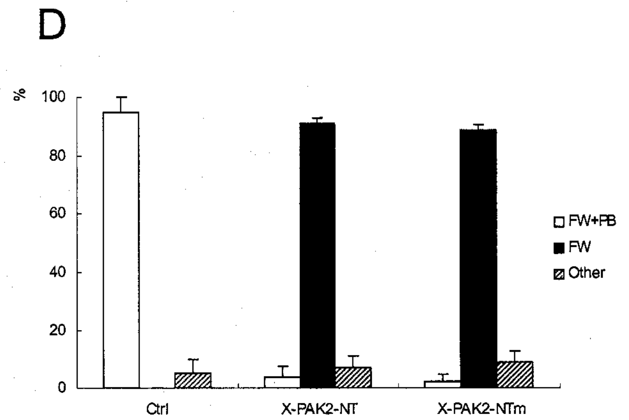
(A) Schematic representation of X-PAK2 WT and NT, NTm mutants. Control oocytes or oocytes injected with indicated mRNAs were incubated for 5hr before addition of progesterone. Following overnight incubation, some oocytes were lysed and subjected to immunoblotting with indicated antibodies; the rest oocytes were fixed, stained with Sytox Green and viewed from the animal pole under a dissecting fluorescence microscope (B, D). (C) shows representative images of control oocytes and oocytes without the formation of the first polar body. "FW+PB" means the oocytes that properly matured and arrested at metaphase II with a flower-like pattern of chromosomes (FW) and the first polar body (PB). "FW" means the oocytes that the first polar body formation was blocked. And only a flower-like pattern of chromosomes (FW) could be seen. "Other" means the oocytes that were dead or abnormal and belonged to neither "FW+PB" group nor "FW" group. Shown here are results from three independent experiments.

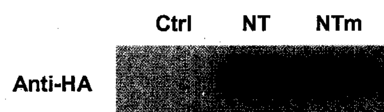

A
X-PAK2 (WT)
HA
Nck
CRIB
Pix
Kinase domain
Gβγ
X-PAK2-NT
1
217
X-PAK2-NTm
H74,77L
1
217


B
%
100
80
60
40
20
0
FW+PB
FW
Other
Ctrl
X-PAK2-NT


Ctrl
X-PAK2-NT
GV
BD
GV
BD
Anti-HA
X-PAK2-NT
p-PAK2
MAPK


C
Ctrl
X-PAK2-NT
GV
BD
BD
Light pictures
S.G. stain
pictures


D
%
100
80
60
40
20
0
FW+PB
FW
Other
Ctrl
X-PAK2-NT
X-PAK2-NTm


Ctrl
NT
NTm
Anti-HA

4. Discussion

The most important finding presented in this chapter is the ability of a known autoinhibitory domain of X-PAK2, X-PAK2-NT, to inhibit first polar body formation. The inhibition is very specific in the sense that part of the maturation process including germinal vesicle breakdown, formation of the metaphase I spindle and spindle migration and anchoring to the cortex is not affected. The cell cycle arrest is also very efficient (>80%) and highly reproducible. Furthermore, we have demonstrated that the ability of X-PAK2-NT to inhibit polar body formation is not dependent on its ability to bind Cdc42 (Fig. 3.4D), essentially eliminating the possibility that X-PAK2-NT inhibits polar body formation by non-specific inhibition of Cdc42. Taken together, these results strongly suggest that endogenous X-PAK2 play an important role in polar body formation.

Interestingly, Cla4, a PAK kinase in yeast, is known to play an important role in budding (like polar body formation, budding is another form of asymmetric cell division). Cla4 is thought to directly phosphorylate septins and promote septin ring formation which is required for cytokinesis during bud formation.(Cvrckova et al., 1995). It is currently not known whether septins and septin ring are similarly required for cytokinesis during polar body formation. In addition to phosphorylating substrates, X-PAK2 may also function in a kinase activity-independent manner in polar body formation. For example, it is known that mammalian PAK1 is known to function as a scaffold protein, bringing specific GEF (guanine nucleotide exchange factor) to activate Cdc42 (Li et al., 2003). As we have shown that Cdc42 activity is essential for polar body formation (Ma et al., submitted),

X-PAK2 may function similarly as a scaffold protein during oocyte maturation, bring a specific GEF to the site of polar body formation where Cdc42 is present (Ma et al., submitted).

Clearly, there are many unanswered questions in the above interpretation. First, what is the endogenous protein that is recognized by p-γPAK (Thr402) antibody? The molecular mass of this protein (59KD) is consistent with it being X-PAK2. Indeed, it co-migrated with X-PAK2 derived from mRNA injection (Fig. 3.3C). However, X-PAK2 derived from mRNA injection did not exhibit similar reactivity to the antibody. It is possible that full length X-PAK2 derived from mRNA injection is mis-localized and therefore is not subjected to the same activation-specific modification (i.e. Thr402 phosphorylation) as the endogenous X-PAK2. Although the exogenous X-PAK2 exhibited electrophoretic mobility shift similar to that of the endogenous protein, both at GVBD, the phosphorylation of exogenous X-PAK2 may be non-specific and therefore has no functional consequence. Consistent with this interpretation, anti-HA immunoprecipitates derived from mRNA injected oocytes showed no appreciable in vitro kinase activity, whether samples were derived from oocytes that had undergone GVBD or not (Fig. 3.3D). Second, whereas a previous study has indicated that endogenous X-PAK2 is active in immature oocytes and that GVBD is accompanied by inhibition of X-PAK2 (Cau et al., 2000), our results suggest that endogenous X-PAK2 is phosphorylated at Thr402, and therefore activated, at GVBD. The conclusion of Cau et al. is based on in vitro kinase assays using artificial substrates, which may or may not reflect the ability of X-PAK2 to

phosphorylate physiological substrates in intact oocytes. Furthermore, we have failed to demonstrate any in vitro kinase activity using the same artificial substrate, despite the clear presence of HA-tagged X-PAK2 in the immunoprecipitates. Therefore, whether the kinase activity of endogenous X-PAK2 is regulated by progesterone in intact oocytes remains unsettled.

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Functional analysis of Eg proteins accumulation during *Xenopus* oocyte maturation
Presented at Cell Cycle Regulation of Meiosis (Sep 13-15th, 2004; Newcastle, UK)

Yanwei Xi, Jean-Pierre Tassan, and X. Johné Liu
De novo synthesis and hyperphosphorylation of pEg3 during meiotic maturation of *Xenopus laevis* oocytes
Presented at the 38th Annual Meeting of the Society for the Study of Reproduction (July 24-27th, 2005; Quebec City, Canada)

Publication

Yanwei Xi, Jing Zhang, Rulin Liu, Fenglai Liang
Demulsification capacity of the lipopeptide from *Bacillus subtilis*.
Journal of Chemical Industry and Engineering (China). Under revisions

Yanwei Xi, Fenglai Liang, Rulin Liu
Demulsification of water-in-oil emulsions by a *Rhodococcus* sp.
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Chunqi Ma, Helene A. Benink, Veronique Montplaisir, Ling Wang, **Yanwei Xi**, Pei-Pei Zheng, William M. Bement, X. Johné Liu
Cdc42 activation cordinates spindle positioning and the first polar body formation in oocyte maturation. (Curr Bio, in press)

Patent

Yanwei Xi, Jing Zhang, Fenglai Liang, Rulin Liu
A new type of demulsifier with high efficiency
In application for the national patent of China