The Role Of Amp-Activated Protein Kinase In Mouse Oocyte Maturation And Subsequent Egg Activation

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THE ROLE OF AMP-ACTIVATED PROTEIN KINASE IN MOUSE OOCYTE MATURATION AND SUBSEQUENT EGG ACTIVATION

By

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ABSTRACT
THE ROLE OF AMP-ACTIVATED PROTEIN KINASE IN MOUSE OOCYTE MATURATION AND SUBSEQUENT EGG ACTIVATION

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Mammalian oogenesis begins during fetal development. Oocytes enter meiosis and arrest at prophase I before birth. Meiosis resumes after proper hormonal signaling, the oocyte completes meiosis I, and then ovulates in metaphase II, at which stage it arrests until fertilization occurs. Egg activation occurs upon sperm fertilization, which includes various physiological processes including calcium influx, release of cortical granules, and completion of meiosis II. However, egg activation can also occur without fertilization, which compromises the later embryonic development. The developmental period from prophase I to metaphase II is referred as oocyte maturation, and involves crucial dynamic change of the cytoskeleton network. The underlying mechanisms that control meiotic regulation still remain elusive. It is well established that a high cAMP level is required to maintain prophase I arrest, whereas mitogen activated protein kinase (MAPK) activity is needed for later metaphase II arrest of the oocyte. cAMP declines during meiotic resumption by the activation of phosphodiesterase (PDE), which converts cAMP into AMP. Elevated AMP activates AMP-activated protein kinase (AMPK). It was suggested that activation of AMPK provides an additional stimulus for meiotic resumption, and consistent with this idea, activation of AMPK mediates meiotic resumption both *in vivo* and *in vitro*. However, the role of AMPK in later process remained to be determined.

My research is focused on the role of AMPK after meiotic resumption. It is composed of three parts: (1) the effect of AMPK activation on completion of oocyte maturation; (2) the regulation of AMPK activity by spindle microtubules; and (3) AMPK regulation of egg activation. Results indicate that AMPK promotes anaphase onset and formation of the first polar body (PB). Meanwhile, the activity and localization of AMPK is dependent on spindle microtubule integrity. In addition, AMPK suppresses premature activation of oocytes by maintaining MAPK activity.

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ABBREVIATIONS

Ana: anaphase

CEO: cumulus cell-enclosed oocytes

DO: denuded oocyte

GV: germinal vesicle

GVB: germinal vesicle breakdown

MI: metaphase I

MII: metaphase II

PB: polar body

Enzymes and Proteins

ACC: acetyl-CoA carboxylase

AMPK: AMP-activated protein kinase

APC: anaphase promoting complex

CDK1: cyclin dependent kinase 1

CSF: cytostatic factor

PDE: phosphodiesterase

Emi2: early mitotic inhibitor 2

MAPK1/3: mitogen activated protein kinase1/3 (same as ERK1/2)

MPF: maturation promoting factor

MRLC: myosin regulatory light chain

NPPC: natriuretic peptide type C

Npr2: natriuretic peptide receptor 2

PKA: cAMP-dependent protein kinase

Hormones

FSH: follicle-stimulating hormone

hCG: human chorionic gonadotropin

LH: luteinizing hormone

PMSG: pregnant mare's serum gonadotropin

Others

AR: amphiregulin

B6SJL mice: C57BL/6J×SJL mice

Con: control

EGF: epidermal growth factor

LT oocyte: LT/SvEiJ mouse oocyte

MEM: minimum essential medium

MT: microtubule

MTOC: microtubule organizing center

ODPF: oocyte–derived paracrine factors

PGC: primordial germ cells

PT172 antibody: anti-phospho AMPK antibody

SAC: spindle assembly checkpoint

Chemicals

2-D-glucose: 2-deoxy-D-glucose

8-Br-Ado: 8-bromo-adenosine

AICAR (AIC): 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside

AraA: adenine 9-beta-d-arabinofuranoside

Cmpd C: compound C

dbcAMP: dibutyryl cAMP

EtOH: ethanol

IBMX: 3-isobutyl-1-methylxanthine

Ncdz: nocodazole

Pac: paclitaxel

PBS: phosphate-buffered saline

PC: palmitoyl carnitine

Puro: puromycin

BACKGROUND

Folliculogenesis and Follicle Structure

The follicle is the functional small unit of the ovary. Each follicle contains a single oocyte, which is enclosed by layers of somatic cells named granulosa cells. The oocyte continually exchange metabolite and communicate with this somatic compartment via gap junctions, transmembrane channels composed of connexins. Connexins are named after their molecular weight. Cx37 is specifically found at the interface between oocyte and surrounding somatic cells, while Cx42 is the major type between granulosa cells (Veitch et al., 2004).

It is difficult to understand oocyte behavior without knowing the structure of the surrounding environment. A cross section of ovary reveals all types of follicles that are at different developmental stages (Figure 1). The more developed the follicle is, the larger it becomes. The majority of follicles inside the ovary are primordial follicles. They are very small and contain only one layer of flattened granulosa cells. Most primordial follicles remain dormant throughout a woman's reproductive life but small percentage of them periodically become activated and grow into primary follicles. Although a primary follicle still has one layer of somatic granulosa cells, the size is almost doubled. This is mainly due to the change of granulosa shape from flat to cuboidal, the initiation of oocyte growth, and formation of zona pellucida that covers the oocyte (Oktem and Urman, 2010). A layer of connective tissue, which is called theca, covers the primary follicle. As the follicle grows, granulosa cells undergo mitosis and produce up to 6 layers of cells. Multiple granulosa layers mark the secondary follicle stage. While most secondary

follicles undergo atresia (apoptosis of follicle), a few further develop and become tertiary follicles. Up to this point (early secondary stage), it is generally thought that early follicle growth is independent of gonadotropin, follicle-stimulating hormone (FSH), since the receptor expression level is low in the granulosa cells (Oktay et al., 1997).

At the late secondary follicle stage, an antral cavity is formed and filled with the fluid that is secreted by granulosa cells. Major components of antral fluid are hormones, anticoagulants, enzymes and electrolytes. The inner granulosa cells around the oocyte are differentiated into cumulus cells, and those lining the follicle wall are called mural granulosa cells. Theca cells are also differentiated into theca interna and theca externa. Several secondary follicles are recruited during each cycle to further develop into tertiary, or Graafian follicles. This stage of growth is dependent on FSH. Graafian follicles have accumulated a large amount of antral fluid and become highly vascular in the theca layer. Selection of the dominant follicles occurs simultaneously. In the end, only the follicles that are sensitized to gonadotropin and have modulated steroidogenic activity are selected, while remaining follicles undergo atresia (Oktem and Urman, 2010). Normally only one Graafian follicle is selected to ovulate in human as opposed to several in rodents. Follicles at this stage are called preovulatory follicles (Jone and Lopez, 2006; Hutt and Abertini, 2007). After ovulation, the ruptured follicle forms the corpus luteum, which starts secreting progesterone, but degenerates soon if pregnancy does not occur (Jone and Lopez, 2006).

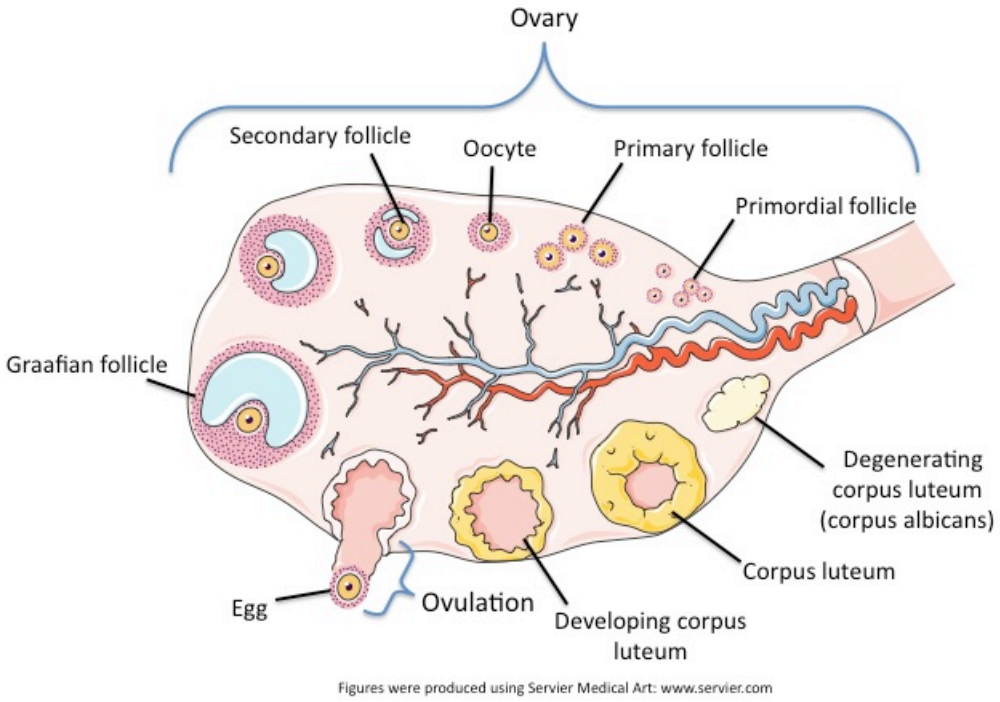

Figure 1 Folliculogenesis

Follicles at different developmental stages.

http://www.repropedia.org/folliculogenesis

Oogenesis

Unlike spermatogenesis, mammalian oogenesis is a discontinuous process. Primordial germ cells (PGC) originate in the epiblast by tissue-specific interactions during gastrulation, a process that requires bone morphogenic protein 4 (BMP4) signaling (Lawson et al., 1999). After the specification, PGCs migrate into the genital ridge. Germ cells, now called oogonia, undergo multiple rounds of proliferation before they enter meiosis and arrest at diplotene prophase I stage (Sasaki and Matsui, 2008).

Oocytes undergo incomplete cytokinesis in the last round of mitosis and remain in cluster before birth. Primordial follicles form in an event called cyst breakdown shortly after birth, during which the cytoplasmic bridges that link adjacent oocytes are destroyed and individual oocytes become enclosed by follicle cells (Tingen et al., 2009; Pepling and Spradling, 1998). Primary oocytes within the primordial follicles remain meiotically incompetent and arrested at the diplotene prophase I stage. Upon entering puberty, selected follicles start to develop, oocytes gradually gain meiotic competence, presumably due to the accumulation of maturation promoting factor (MPF), whose major components are cyclin B and cyclin-dependent kinase 1 (CDK1) (Mitra and Schultz, 1996).

After a surge of luteinizing hormone (LH), the oocyte inside each Graafian follicle resumes meiosis. The enlarged nucleus, which is called the germinal vesicle (GV), undergoes germinal vesicle breakdown (GVB). With the progression of meiosis, the first polar body (PB) is released. Normally, oocytes are ovulated at the PB stage (metaphase

II) and where they remain arrested until fertilization occurs (Figure 2). This process (GV-MII) is referred to as oocyte maturation (Downs, 2010).

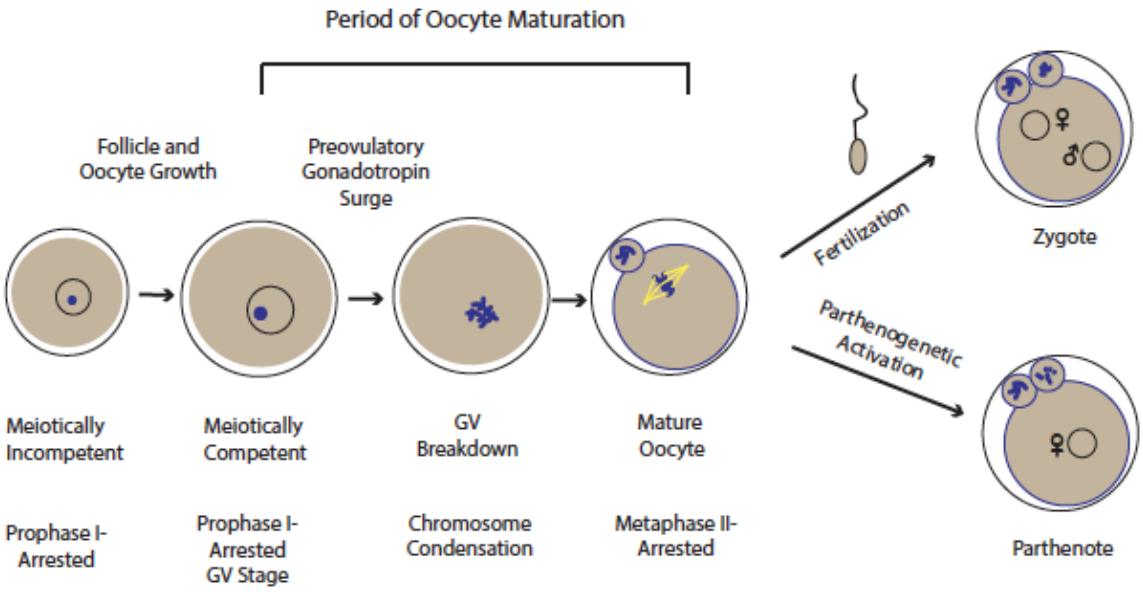

Figure 2 Overview of Oocyte Maturation

Meiotically incompetent oocyte gradually grows and acquires meiotic competence inside the follicle. After receiving a preovulatory gonadotropin surge, the oocyte resumes meiosis. The nuclear envelope (germinal vesicle) breaks down, the first polar body is released, and the oocyte arrests at metaphase II until fertilization occurs. Occasionally, oocyte undergoes parthenogenetic activation without sperm fertilization. The period from GV stage to formation of first PB is called oocyte maturation.

Regulation of Meiotic Arrest

We now have a better understanding of the signaling pathway that maintains prophase I arrest. High cAMP level within the oocyte is critical. Oocytes contain the constitutively active G-protein coupled receptor, Gpr3, which activates adenylyl cyclase (Mehlmann, 2005). cAMP activates protein kinase A (PKA) that suppresses MPF by phosphorylating MPF regulators, Wee1B and Cdc25B. The PKA mediated phosphorylation of Wee1 is activating, while that of Cdc25B is inhibitory. Active Wee1

negatively regulates the MPF component, CDK1. Cdc25B is a phosphatase, which removes inhibitory phosphorylation on CDK1. The phosphatase activity of Cdc25b is controlled by PKA: once phosphorylated by PKA, Cdc25B becomes inactive; meanwhile the phosphorylation of Wee1B by PKA keeps it in active form and blocks the MPF activity (Oh et al., 2010; Han et al., 2005). Thus, both phosphorylation events inhibit MPF activity (Figure 3), and ultimately, germinal vesicle breakdown is suppressed.

The somatic compartment within the follicle plays an important role in maintaining meiotic arrest. Thus, when a meiotically competent oocyte is released from the follicles, it undergoes spontaneous maturation. In oocytes, the cAMP level is balanced by its synthesis and degradation, which is controlled by adenylyl cyclase and oocyte specific phosphodiesterase (PDE), PDE3A, respectively. PDE activity is negatively regulated by cGMP (Masciarelli et al., 2004) that is produced in granulosa cells and transferred into the oocyte to maintain the arrest. Indeed, the cGMP level drops in the oocyte before meiotic resumption (Robinson et al., 2012).It was recently demonstrated that cGMP is the somatic compartment inhibitor that passes through the gap junctions and enters the oocyte to suppress PDE activity (Norris et al., 2008, 2009).

In addition to the granulosa cell regulation of oocyte meiotic arrest, the oocyte itself can also maintain its arrest by modifying gene expression in granulosa cells (Zhang et al., 2010). Oocyte derived paracrine factors (ODPF) such as BMP15, GDF-9 and Fgf8, upregulate natriuretic peptide receptor2 (Npr2) guanylate cyclase expression in cumulus cells. At the same time, the Npr2 receptor's ligand, NPPC, is spatially separated and highly expressed in the mural granulosa cells. Activation of Npr2 is thought to be a critical source of cGMP production in the somatic compartment (Figure 3).

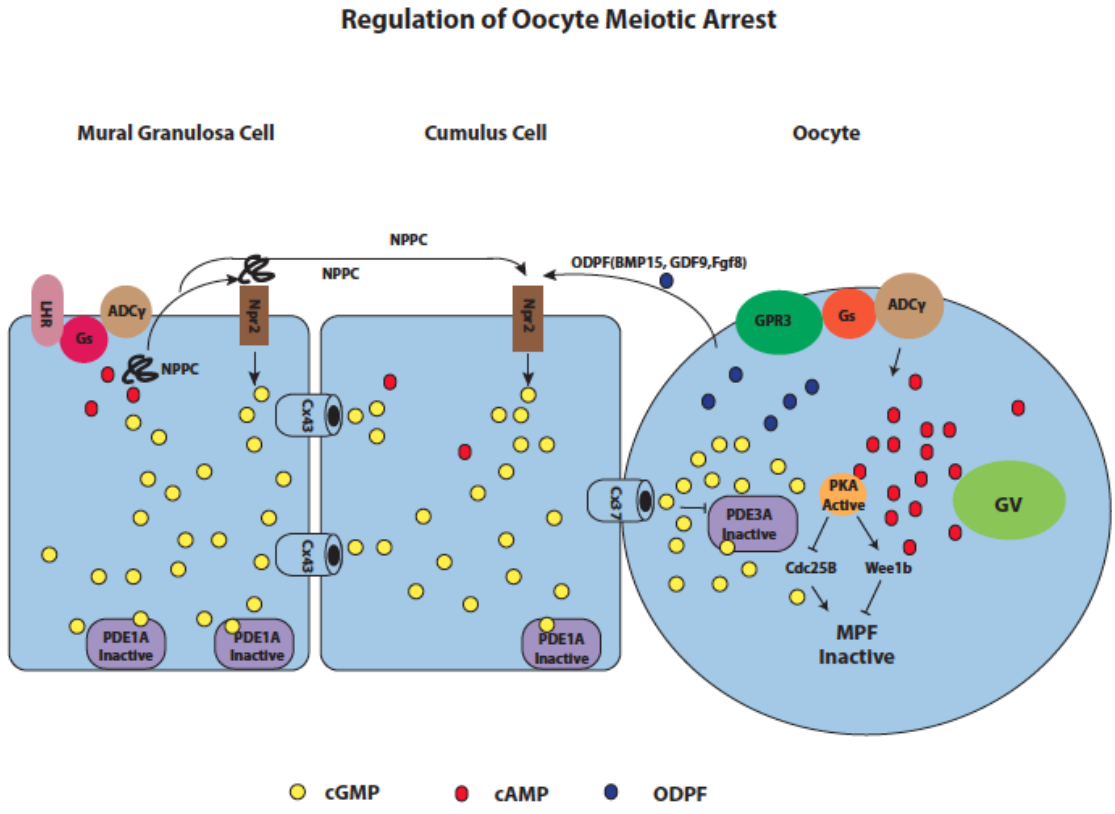

Figure 3 Regulation of Oocyte Meiotic Arrest

Diagram summarizing the control of meiotic arrest in the absence of LH signal. cGMP is mainly produced in the somatic compartment by membrane-bound guanylate cyclase, Npr2, which is activated by the ligand, NPPC. In addition, OOPFs also help increase the Npr2 activity. cGMP diffuses through the gap junction and transferred into the oocyte where cGMP inhibits PDE activity. Thus, cAMP level remains elevated and maintains meiotic arrest of oocytes (Conti et al., 2012; Downs, 2010; Zhang et al., 2010; Norris et al., 2010).

Regulation of Meiotic Resumption

LH initiates the meiotic resumption of oocytes *in vivo*. LH receptors are located on mural granulosa cells and theca cells. According to the current model, there are several ways for LH to convey the positive signal to the interior part of the follicle. One way is to activate PKA in granulosa cells that promotes secretion of EGF-like peptides. EGF-like peptides diffuse to bind receptors that activate the MAPK pathway in granulosa cells. The activation of MAPK induces closure of gap junctions (Norris et al., 2008), which decreases the amount of cGMP transferred to the oocyte. At the same time, the activation of MAPK exerts an unknown positive stimulus to the oocyte. In addition, LH inhibits Npr2 receptor activity and decreases cGMP production (Robinson et al., 2012). With less cGMP entering the oocyte, PDE becomes active, and reduces the cAMP level, leading to meiotic resumption (Figure 4).

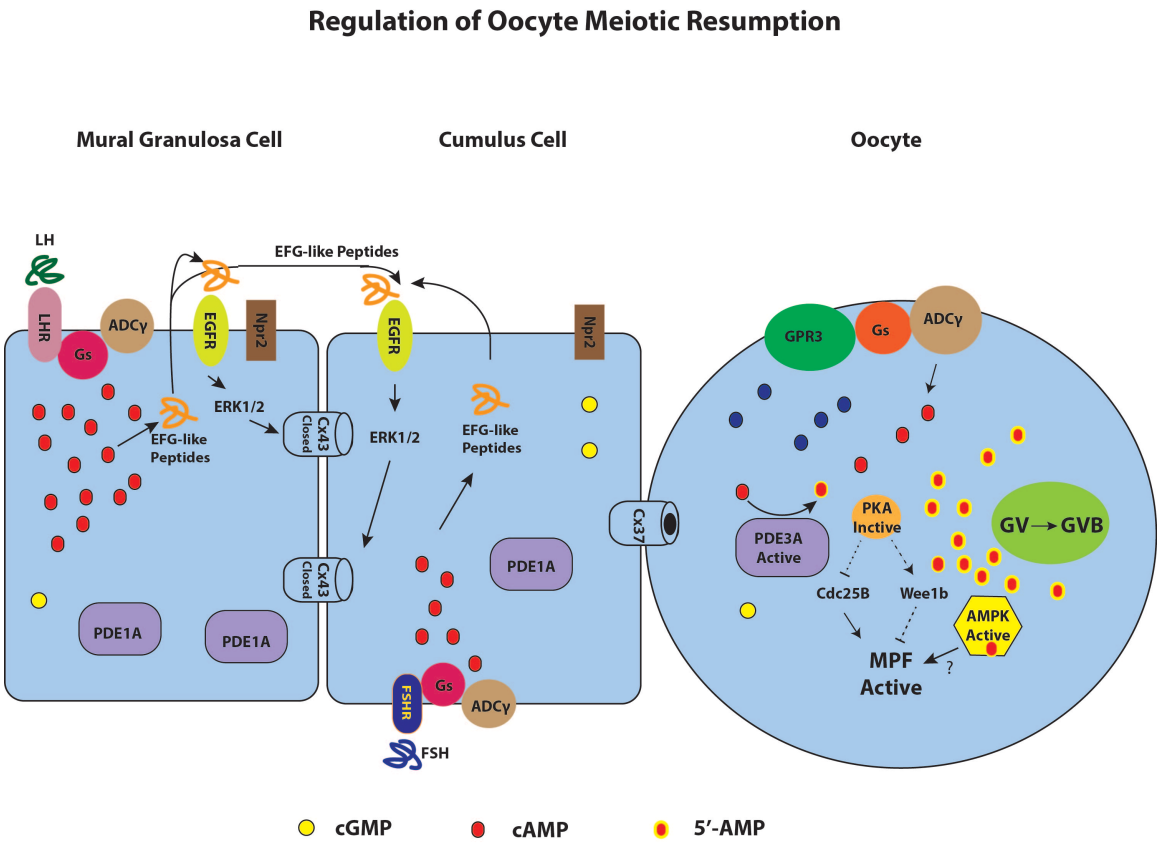

Figure 4 Signals for Meiotic Resumption

Summary of LH, FSH, and EFG-like peptide induced meiotic resumption. LH, FSH promotes EGF-like peptide production in the granulosa cells, and the activation of EGFR induces gap junction closure by phosphorylation of Cx43, at the same time, hormonal signaling decreases the guanylate cyclase activity. With less cGMP being transferred into the oocyte, PDE becomes active and cAMP is broken down into AMP. This relieves the inhibition of MPF and leads to meiotic resumption. It should be noted that AMPK is also activated by elevated AMP levels and exert a positive influence on GVB.

Egg Activation

Binding of sperm triggers oocyte activation. The initial event includes an intracellular calcium spike, followed by subsequent repetitive calcium oscillations, mainly by activation of the IP3 receptor (Miyazaki et al., 1993). The egg releases cortical granules, which contain multiple enzymes that modify the zona pellucida and block polyspermy. The oocyte completes meiosis II, extrudes the second polar body and forms pronuclei. This entire process is referred to as egg activation.

Cytostatic factor (CSF) activity develops during metaphase II arrest. CSF is a multiple component complex, which contains MAPK and an anaphase promoting complex (APC) inhibitor, early mitotic inhibitor 2 (Emi2). High CSF activity maintains metaphase II arrest by inhibiting APC and keeping MPF activity high. During egg activation CSF activity decreases, thus removing the inhibition of APC and driving entry into anaphase II (Madgwick and Jones, 2007).

Some oocytes undergo spontaneous parthenogenetic activation without sperm fertilization (Figure 2). There are many contributing factors, such as the tenure of oocyte inside the oviduct and oocyte quality. Usually, the longer it is in the oviduct, the easier it gets activated (Kubiak, 1989). Genetic composition is another contributing factor. A well-known example is the LT/Sv strain of mice, which is genetically predisposed to parthenogenetic activation (Eppig et al., 1996). LT/Sv mice show a high incidence of ovarian teratomas, as a result of the oocytes that are directly activated and begin developing within the follicles. Once GV-stage oocytes are isolated and cultured *in vitro*, those oocytes undergo parthenogenetic activation as well, which is manifested by the formation of interphase pronuclei. In addition, mutations in the genes that are involved in maintaining MII arrest cause parthenogenetic activation. For instance, oocytes with a mutation in the MAPK upstream kinase, Mos, fail to arrest at metaphase II and display a spontaneous activation phenotype (Hashimoto et al., 1994).

Role of AMP-Activated Protein Kinase in Oocyte Maturation

During meiotic resumption, AMP produced from cAMP degradation is a potential activator for AMPK. AMPK is a heterotrimeric energy sensor protein, which contains a

catalytic α subunit and regulatory β,γ subunits (Figure 5). There are 2 isoforms for α subunit (α1,α2), 2 β subunits and 3 γ subunits. AMPK is activated by elevated AMP/ATP ratio and cellular stress (Salt et al., 1998). Phosphorylation of threonine 172 on the α catalytic subunit is the hallmark of AMPK activation. LKB1 is a tumor suppresser and shows an important role in establishing cell polarity, which is one of the upstream kinase of AMPK phosphorylation (Nakada et al., 2010; Williams and Brenman, 2008). In addition, calcium/calmodulin kinase kinase β (CaMKKβ) also activates AMPK in response to calcium signaling (Hardie et al., 2012). AMPK maintains cellular energy homeostasis by down-regulating energy consuming pathways (eg, protein synthesis) and up-regulating the pathways that generate ATP (eg, fatty acid oxidation). Acetyl-CoA carboxylase (ACC) is one of the AMPK targets and is phosphorylated upon AMPK activation. When ACC is active, it converts acetyl-coA into malonyl-CoA that inhibits carnitine palmitoyltransferase-1, an important carrier for transferring fatty-acyl CoA into mitochondria. Thus, without the transfer of fatty-acyl CoA into mitochondria, β-oxidation does not occur. However, when ACC is inhibited by AMPK phosphorylation, malonyl CoA levels are decreased, carnitine palmitoyltransferase-1 become active and fatty acid oxidation is promoted. (Downs et al., 2009). In fact, ACC phosphorylation level is often used as an indicator for AMPK activity. In addition to regulating energy homeostasis, AMPK is also implicated in many processes including cell growth, polarity establishment and transcription (Mihaylova and Shaw, 2011).

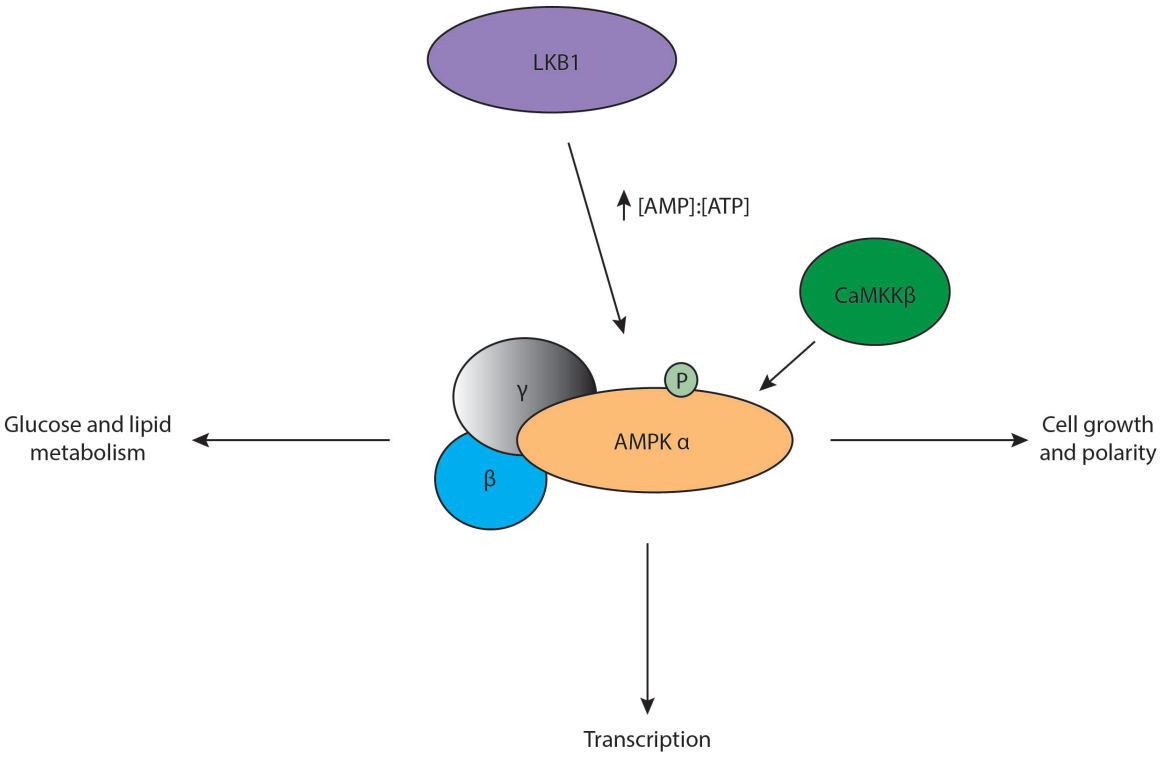

Figure 5 The general role of AMPK in the somatic cells

AMPK consists of the catalytic subunit α and two regulatory subunits β and γ. It is activated by the upstream kinase, LKB1, in the response to elevated AMP to ATP ratio. The activation of AMPK regulates energy homeostasis, cell growth and the establishment of polarity. In addition, it has a long-term impact on transcription as well.

Our current model of AMPK involvement in meiotic resumption is depicted in figure 6. During meiotic arrest, PKA is activated by cAMP, whereas during meiotic resumption, accumulation of AMP promotes maturation in mouse oocytes by turning on AMPK (Downs et al., 2002). One of the effects of AMPK on meiotic induction could be mediated by increasing fatty acid oxidation (Downs et al., 2009; Valsangkar and Downs, 2013).

Our lab has previously demonstrated the role of AMP-activated protein kinase (AMPK) in mouse oocyte maturation. AMPK is present in the oocyte and the activation

of AMPK precedes GVB (Chen et al., 2006; Chen and Downs, 2008). The AMPK activator, aminoimidazole carboxamide ribonucleotide (AICAR), induces GVB and the inhibitors, compound C and araA, block maturation. Moreover, GVB occurs when oocytes are challenged by different kinds of stress that are proven to activate AMPK (LaRosa and Downs, 2006).

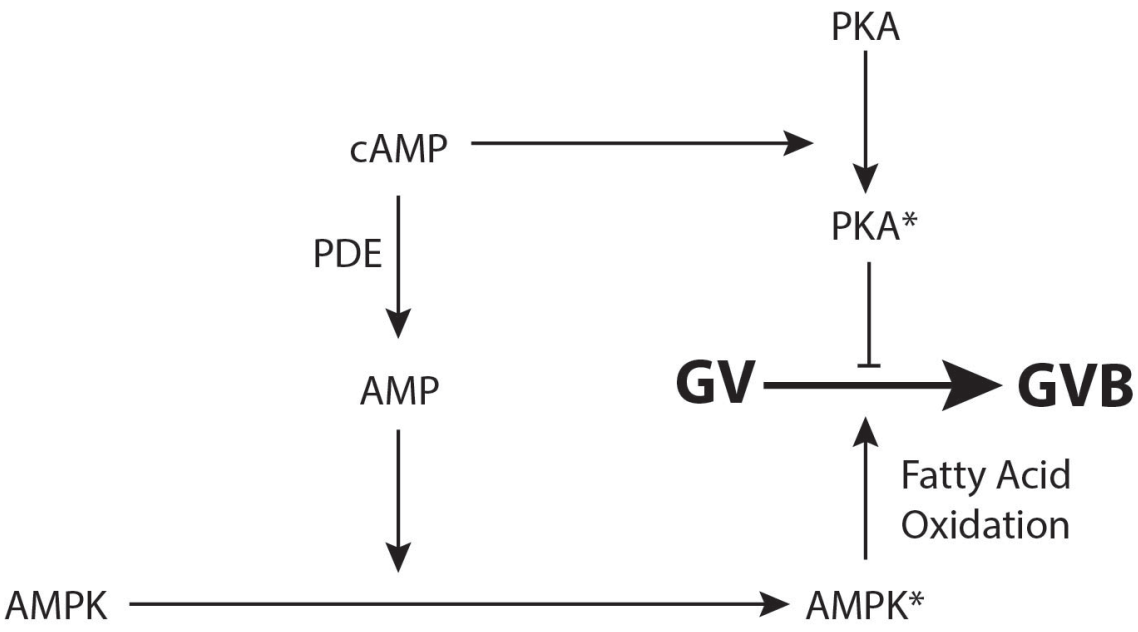

Figure 6 The proposed model of AMPK involvement in meiotic induction (adapted from Downs et al., 2002)

During meiotic arrest, PDE is inactive. Thus, oocytes contain a high level of cAMP that activates on PKA, which phosphorylates Wee1B and Cdc25B that prevents activation of MPF (see Figure 3). However, during meiotic induction, PDE becomes active and breaks down cAMP into AMP. The elevated AMP level activates AMPK, which acts as a positive stimulus to GVB.

Methodology - In Vitro Culture Systems

We use *in vitro* culture systems to study oocyte maturation. There are three different types of *in vitro* culture systems: (i) preovulatory follicle culture, (ii) cumulus-enclosed oocyte (CEO) culture and (iii) denuded oocyte (DO) culture (Downs, 2010).

Follicle culture is close to the physiological condition since the oocyte is in an intact functional unit. The oocyte is maintained in meiotic arrest within the follicle during the culture in control medium (ie, devoid of inhibitors or stimulants), because the follicle provides an inhibitory environment. The preovulatory follicle is responsive to luteinizing hormone (LH), follicle stimulating hormone (FSH) and epidermal growth-factor like peptides (EGF) (Downs and Chen, 2008; Norris et al., 2010), and the treatment with these hormones induces intrafollicular oocyte maturation.

CEOs are obtained by physically rupturing preovulatory follicles. However, once released from the follicle environment, meiotic arrest cannot be maintained and oocytes undergo spontaneous maturation when cultured in control medium. The inhibitory follicle environment can be mimicked *in vitro* by supplementing the medium with compounds that either increase the cAMP level or inhibit PDE activity within the oocyte. Commonly used compounds are the cAMP analogue, dibutyryl cAMP (dbcAMP), or the PDE inhibitors, 3-isobutyl-1-methylxanthine (IBMX) and milrinone. *In vitro*-arrested CEOs are responsive to FSH and EGF-like peptides but not LH, because LH receptors are localized at the mural granulosa cells, which are now uncoupled from CEOs.

DOs are obtained by removing surrounding cumulus cells. Like CEOs, they undergo spontaneous maturation in the medium, supporting the idea that cGMP produced

inside the somatic compartment is important for maintaining meiotic arrest. However, DOs do not respond to FSH, or EGF-like peptides, due to the fact that the receptors are located on the somatic cells (Figure 7). DO culture is often used to test the direct effect of a compound on the oocyte without potentially confounding contribution from cumulus or mural granulosa cells.

Since my work was focused on the event after GVB, spontaneous maturation of CEO or DO was mostly used for the experiments. The kinetics of GVB is faster in spontaneous maturation than meiotic induction. Most of the oocytes undergo GVB within 1h as opposed to 4-6h in meiotic induction. A well-aligned metaphase I stage can be observed at 8h after onset of the culture and extrusion of the PB can be easily observed starting at 10h after initiation of the culture. It is therefore a convenient culture system for investigating different aspects of oocyte maturation.

The purpose of my thesis work was to examine the potential role of AMPK in the events beyond germinal vesicle breakdown. Herein I demonstrate that AMPK is required at several different times during oogenesis. Firstly, AMPK promotes the completion of oocyte maturation by accelerating anaphase I onset and first PB formation. Secondly, the normal functioning of AMPK depends on spindle microtubule integrity. Finally, AMPK suppresses premature oocyte activation by maintaining MAPK activity.

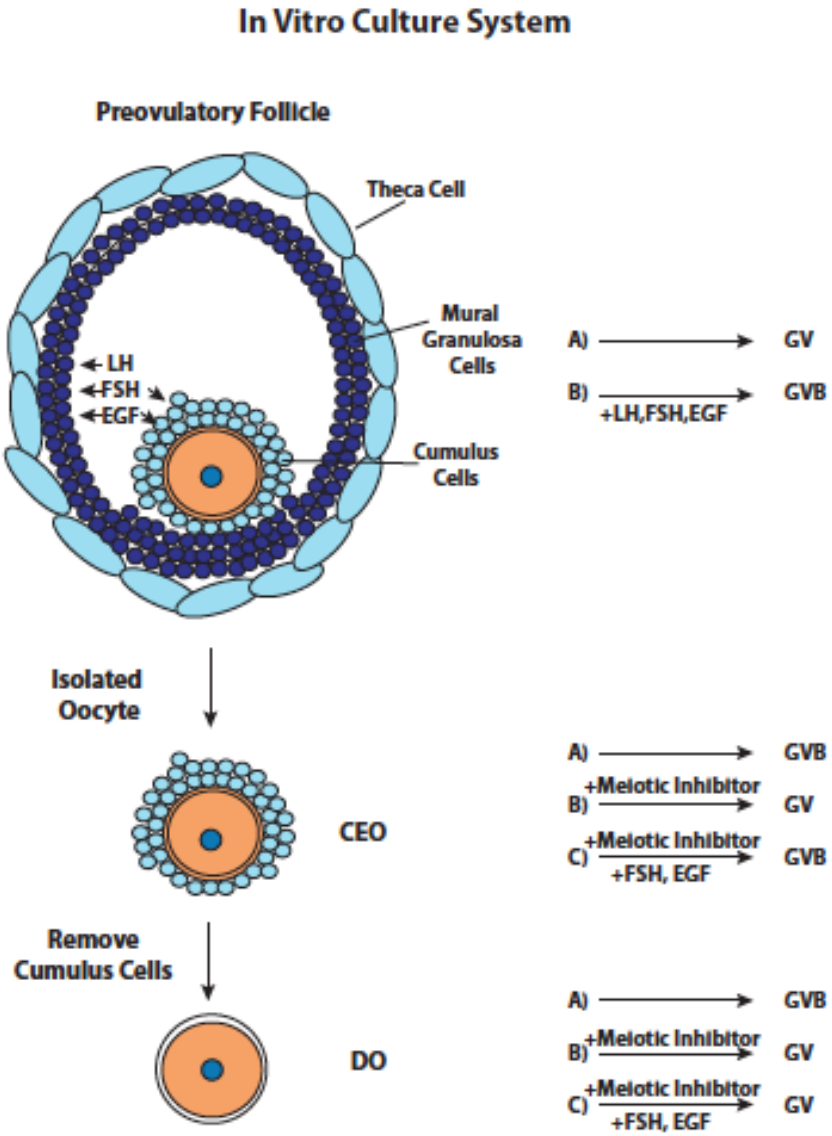

Figure 7 *In vitro* culture system for studying oocyte maturation (adapted from (Downs, 2010)

Oocytes remain at GV stage in the intact follicles when cultured in the control medium, but resume meiosis in response to LH, FSH and EGFs. Once released from the follicles, CEOs undergo spontaneous maturation in the medium without meiotic inhibitors. Arrested CEOs respond to FSH, EGFs. Similarly, DOs mature spontaneously in the control medium without meiotic inhibitors, but they are unresponsive to hormones when maintained arrest the in meiotic inhibitor.

CHAPTER I: A ROLE OF AMPK THOUGHOUT MEIOTIC MATURATION IN THE MOUSE OOCYTE: EVIDENCE FOR PROMOTION OF POLAR BODY FORMATION AND SUPPRESSION OF PREMATURE ACTIVATION

Summary

It has been shown that activation of AMPK is involved in the initiation of mouse oocyte meiotic resumption. The purpose of this study was to examine the role of AMPK in the entire oocyte maturation period after germinal vesicle breakdown. AMPK activators greatly promoted the rate and kinetics of polar body formation in both CEOs and DOs, whereas the AMPK inhibitors, compound C and Ara A, had opposite effects on polar body formation. Moreover, compound C stimulated the premature activation of CEOs but not in the DOs. In addition, oocytes isolated from the mice with a shorter hormonal priming period were predisposed to spontaneous activation, which can be either significantly increased by compound C or eliminated by the AMPK activator, AICAR treatment. Immunofluorescent staining showed that active AMPK was associated with the condensed chromosomes, spindle poles and the midbody during the maturation period. The α1 subunit of AMPK was colocalized with the chromosomes and the meiotic spindle. However, there was no specific staining pattern of the α2 subunit. Interestingly, a temporal disconnect is observed between the requirement of AMPK activity and its effect on PB formation. AMPK activity is required early during the maturation period for stimulation of PB. Altogether, these data suggest that AMPK is involved in the entire oocyte maturation period by promoting the formation of first polar body; meanwhile, it suppresses premature oocyte activation.

Introduction

AMPK is a cellular gauge that is activated by an elevated AMP to ATP ratio. Once activated, it turns on energy production and shuts down energy consuming processes (Hardie, 2003; Carling, 2004). AMPK consists of one catalytic α subunit and two regulatory subunits, β and γ. Notably both isoforms of catalytic subunits, α1 and α2, are expressed in the mouse oocytes (Downs et al., 2002). The role of this energy sensor is well studied in somatic cells. A small drop in ATP causes relatively big change in AMP to ATP ratio, which significantly increases AMPK activity (Mihaylova and Shaw, 2011). The activation of AMPK leads to adaptive changes in growth and differentiation. In somatic cells, activation of AMPK induces metabolic checkpoint that causes cell cycle arrest that is mediated by mammalian target of rapamycin (mTOR) (Gwinn et al., 2008).

Interestingly, AMPK seems to have the complete opposite effect on mouse oocyte meiotic induction. It has been demonstrated in the previous work that activation of AMPK precedes GVB and mediates hormone-induced maturation, which is demonstrated by increase in phospho-ACC level after FSH treatment and the appearance of active AMPK within the germinal vesicle. Blocking AMPK activity with the specific inhibitors, compound C and Ara A, inhibits meiotic resumption in both CEOs and DOs (Chen et al., 2006; Downs and Chen, 2006; Chen and Downs, 2008). In addition, meiotic resumption can also be induced by stress that activate AMPK (LaRosa and Downs, 2006, 2007).

While a role for AMPK in meiotic induction is well documented, its effect on the later maturation period (after GVB) remains unknown. Herein we demonstrate the

involvement of AMPK from GVB to metaphase II stage by using pharmacological activators or inhibitors of AMPK. To test the effect of AMPK modulators on later maturation events, spontaneous maturation system was mainly used instead of meiotic induction. Our data suggest that AMPK has a positive stimulation on PB formation and suppressive effect on premature activation. This involvement of AMPK is further suggested by the association of active AMPK with chromosomes, spindle poles, and midbody throughout maturation.

Materials and Methods

Oocyte Isolation and Culture Conditions

Immature, 19-23-day-old (C57B/6J X SJL/J) F_1 mice were used for all experiments. In most experiments, mice were primed with 5 IU equine chorionic gonadotropin (eCG) and 2 days later were killed; the ovaries were removed and placed in culture medium where cumulus cell-enclosed oocytes were released from the preovulatory follicles when poked with sterile needles. Some experiments utilized unprimed mice or mice primed only 1 d. Denuded oocytes were obtained by passage of CEO through mouth-operated small-bore pipets. Oocytes were washed free of other follicular tissue and transferred to plastic tubes containing 1 ml of the appropriate test medium. The culture medium used was Eagle's minimum essential medium with Earle's salts (GIBCO/Invitrogen; Carlsbad, CA) supplemented with 0.23 mM pyruvate, penicillin, streptomycin sulfate and 3 mg/ml crystallized lyophilized bovine serum albumin (ICN ImmunoBiologicals, Lisle, IL) and buffered with 26 mM bicarbonate.

Immunofluorescent Staining

Staining was performed on oocytes having undergone maturation either *in vitro* or *in vivo*. For the latter, 2-day-primed mice were administered 5 IU hCG and oocytes were retrieved from antral follicles at varying times post-hCG. Oocytes were fixed with 4% formaldehyde at 4°C and permeabilized with 0.1% triton in blocking buffer (0.05% saponin in PBS, pH 7.4, plus 10% sheep serum) for 30 min. Oocytes were then washed free of triton and continuously blocked for another 90 min at room temperature. Oocytes were incubated with rabbit primary antibodies (1:100) overnight at 4 °C, washed in

blocking buffer at room temperature, and incubated with FITC-conjugated sheep anti-rabbit antibody (1:1,000) at room temperature for 1 hr. For experiments involving alpha subunit blocking peptide, primary antibodies were incubated for 1 hr at room temperature with blocking peptide before adding oocytes for overnight incubation. After washing, oocytes were mounted on prewashed slides with vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA), and cover slips were sealed with nail polish.

Confocal Microscopy

Oocytes were viewed on a laser scanning confocal microscope (Carl Zeiss Co., Thornwood, NY) with a 63× objective. During imaging all settings were kept constant, -- that is, laser power, detector gain, amplifier offset, amplifier gain, and pinhole size. Digitally recorded images were exported by LSM Examiner (Carl Zeiss Co.).

Chemicals

Saponin, dbcAMP, 8-Br-adenosine, adenine-9-b-D-arabinofuranoside (araA), sheep serum, and FITC-labeled sheep anti-rabbit antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Compound C and AICAR were supplied by Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Anti-PT172 antibody was from Cell Signaling Technology (Beverly, MA), and anti- PT172, anti-α1, and anti-α2 blocking peptides were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Highly purified ovine FSH was purchased from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. A.F. Parlow.

Statistical Analysis

Oocyte maturation experiments were repeated at least three times with at least 30 oocytes per group per experiment. Data are reported as mean percentage GVB±SEM. Following arcsin transformation, maturation frequencies were analyzed statistically by ANOVA followed by Duncan's multiple range test. For all statistical analyses, a P-value <0.05 was considered significant.

Results

Effects of AMPK Modulators on Polar Body Formation

Activators. To test the effect of AMPK activators on PB formation, a dose response experiment was carried out (Figure 1.1 A). CEOs were incubated with the increasing concentrations of different AMPK activators, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) or AMP for 16-17h before the formation of the first polar body was assessed. AICAR significantly increased the percentage of the PB formation by 26% at the most effective dose (0.2mM) tested *in vitro*. However, it was inhibitory on PB formation on the highest dose tested, 1mM. AMP gradually increased the PB percentage at all doses. The extent of stimulation within the two treatment groups was the same at the most effective concentrations. This suggests that stimulation of AMPK by activators promotes the completion of the maturation.

To test if the increase in PB rate was due to a fast PB formation kinetics, CEOs were cultured in either control or 0.2mM AICAR-containing medium, and the presence of PB was checked starting at 7h and every 3hours thereafter. The presence of PB was observed neither in control or AICAR-treated group after 7 h of culture. Sixty percent of the AICAR-treated oocytes showed PB at 10h, while this number was only 28% in the control oocytes and remained lower at the later time points (Figure 1.1 B). When CEOs were cultured in 2mM AMP, a similar acceleration of PB formation was observed (data not shown).

To determine if these agents can directly act on the oocyte, similar experiments were carried out with DOs. The presence of the PB was assessed 9h after initiation of the

culture, due to the faster kinetics in the DOs. As expected, AICAR dose dependently promoted PB formation in DOs, with the most effective dose of 0.1 mM and a 50% increase in PB rate. The highest dose of AICAR tested (0.5mM) was toxic to the DOs. In comparison to CEOs, AMP was ineffective on promoting PB in DOs, which may be caused by the impaired intake. We then tested another AMPK activator, 8-Br-adenosine (8-Br-Ado), which has been demonstrated to be effective on CEOs (Downs and Chen, 2006). The result showed a similar dose dependent increase in PB formation in DOs (Figure 1.1 C). In addition, AICAR treatment also significantly increased the PB formation kinetics in DOs (Figure 1.1D). This positive effect on DO implies that the activators are acting directly on oocyte itself.

Meiotic resumption in the mouse oocytes can be induced by different stresses that activate AMPK (LaRosa and Downs, 2006, 2007). Therefore, we decided to test whether stress can also bring about the similar effect on PB formation. An initial heat stress experiment was detrimental to oocyte development so we opted to challenge oocytes by altering their energy metabolism. Since oocyte maturation is an active process that constantly requires ATP, we decided to pulse oocytes in the energy deficient medium for short period of time and then followed by recovery in the regular medium. Oocytes were treated with 2-D-glucose, whose downstream metabolite, 6-phospho-2-D-Glucose, cannot be further utilized in glycolysis to generate ATP, which is one of the known methods of activating AMPK. For the experimental group, we incubated DOs in glucose- and pyruvate- free medium with 10mM 2-D-glucose for 3 h, and then washed and transferred the oocytes into the regular medium. For controls, one group of oocytes was similarly treated with the glucose- and pyruvate- free medium in the absence of 2-D-glucose for

the initial 3h before transfer to control medium; and another control group was cultured continuously in the 2-D-glucose containing medium in the absence of glucose and pyruvate for the entire culture period. As shown in figure 1.1E, only the group that was pulsed with 2-D-glucose showed an increased percentage of PB formation, whereas continuous exposure with 2-D-glucose inhibited the meiotic progression.

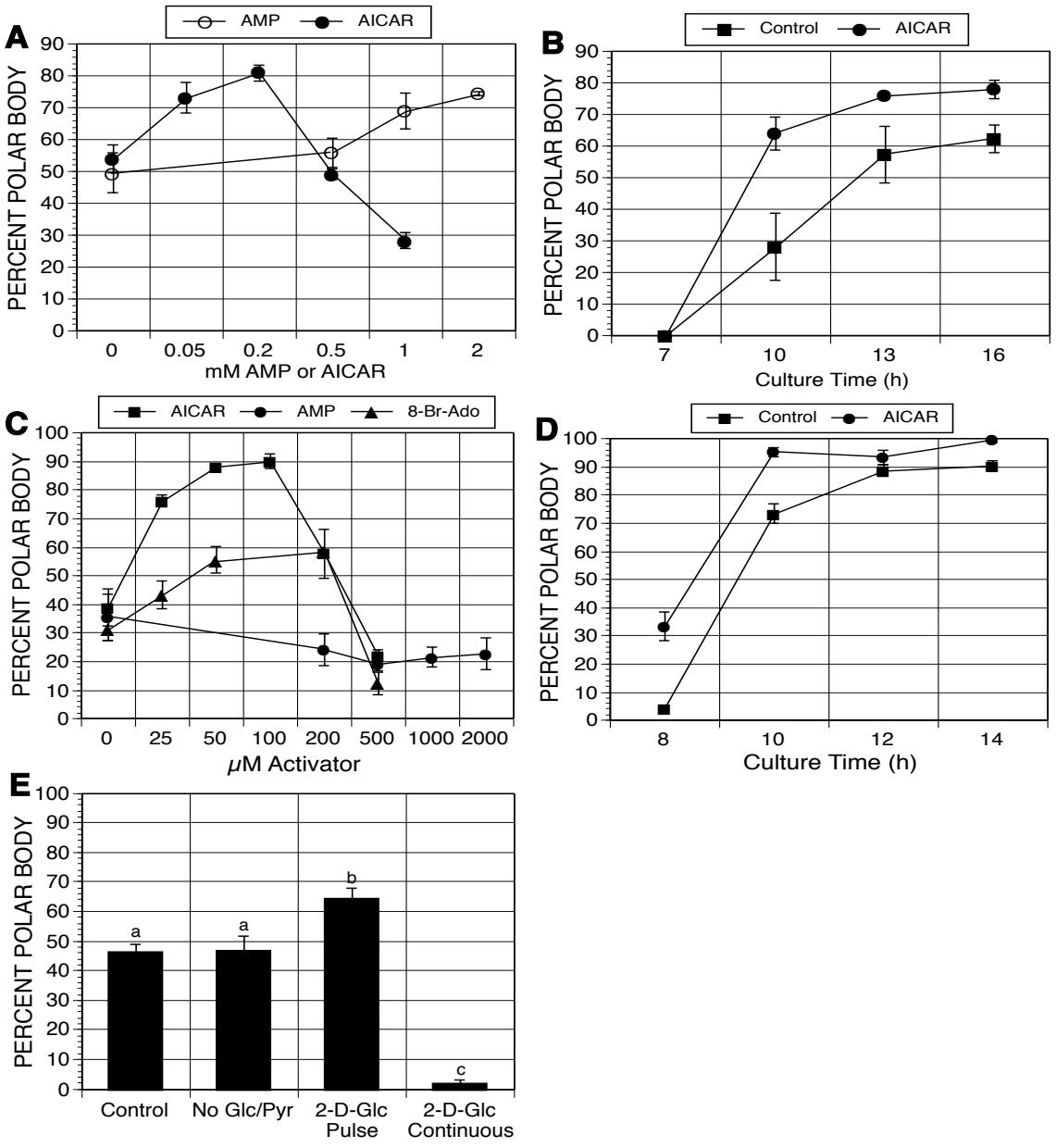

Figure 1. 1 Effect of AMPK on PB rate and kinetics

(A) CEOs were cultured in MEM/BSA for 16-17 hours in the presence of either AMP or AICAR. Both dose-dependently promoted polar body formation. (B) Kinetics of polar body formation in CEOs. CEOs were either cultured in control or in the medium containing 0.2mM AICAR, and oocytes were checked every 3 hours to assess the polar body rate. (C) The effect of AMPK stimulators on DOs. At the lower doses, both AICAR and 8-bromoadenosine increased the rate of polar body. No effect was observed on AMP. (D) Kinetics of PB rate in DOs. DOs were cultured either in control or 0.1mM AICAR. The frequency of polar body in AICAR group was higher at every time points tested. (E) Short term pulsing with 2-D-Glucose significantly promoted PB formation.

Inhibitors. Next we tested the effect of AMPK inhibitors on PB formation. Consistent with the activator data, treatment with AMPK inhibitors, compound C (Zhou et al., 2001) and Ara A (Henin et al., 1996) , dose dependently inhibited PB formation in both CEOs and DOs, with the effect of Ara A less pronounced in DOs. Interestingly, treatment with compound C induced a higher percentage of oocyte activation in CEOs (19%) than DOs (9.5%, data not shown), which was never observed with Ara A treatment (Figure 1.2 A and B). Activated oocytes displayed a predominant pronucleus and underwent limited cleavage after further culture.

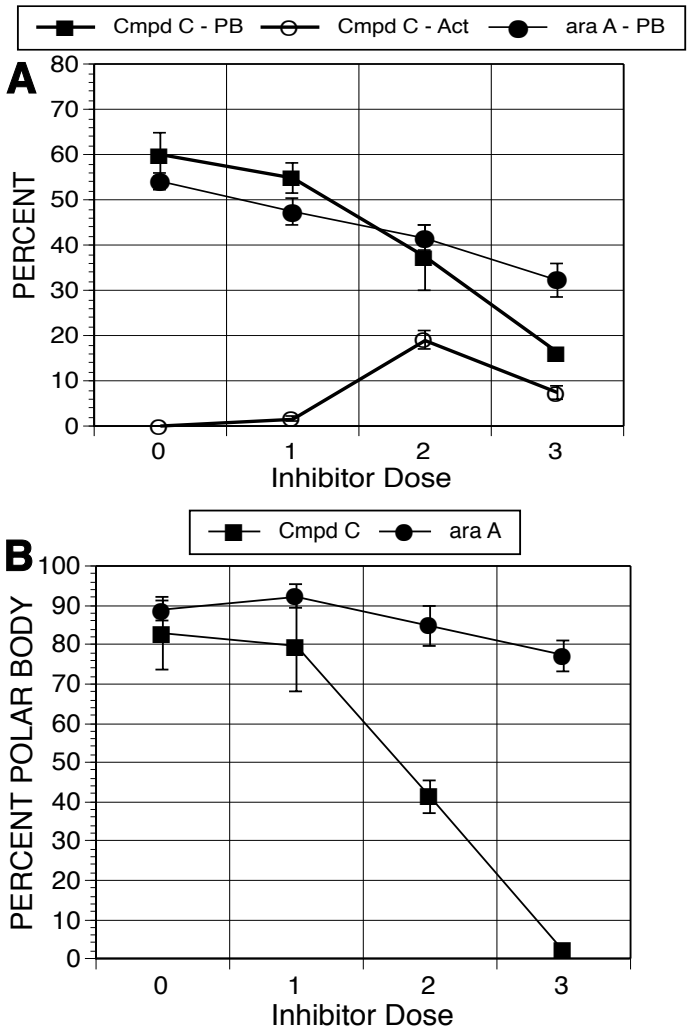

Figure 1. 2 Effect of AMPK inhibitors on PB formation

(A) CEOs and (B) DOs were cultured for 17h in increasing concentrations of compound C (0,1,2.5,5μM) and Ara A(0,1,1.5,2 mM). Compound C induced 19% activation of CEOs at 2.5μM.

Effect of Timing the Exposure to AICAR and Compound C on PB Formation and Activation

In order to determine the window that is susceptible to AMPK activation on PB stimulation, the AICAR treatment for CEOs was delayed for 0, 2 or 4h. As shown in figure 1.3, delaying AICAR treatment decreased the extent of PB stimulation, suggesting an early requirement for AMPK activation on first PB formation. Consistent with this

data, when compound C treatment was delayed, the inhibition of PB extrusion was also reduced. When compound C treatment was delayed by 4h, the rate of PB formation was not significantly different from the control group. Interestingly, the activation rate induced by compound C was not affected by the delayed exposure, which may indicate the sensitive window for stimulatory effect of AMPK on PB formation precedes later inhibitory effect on oocyte activation.

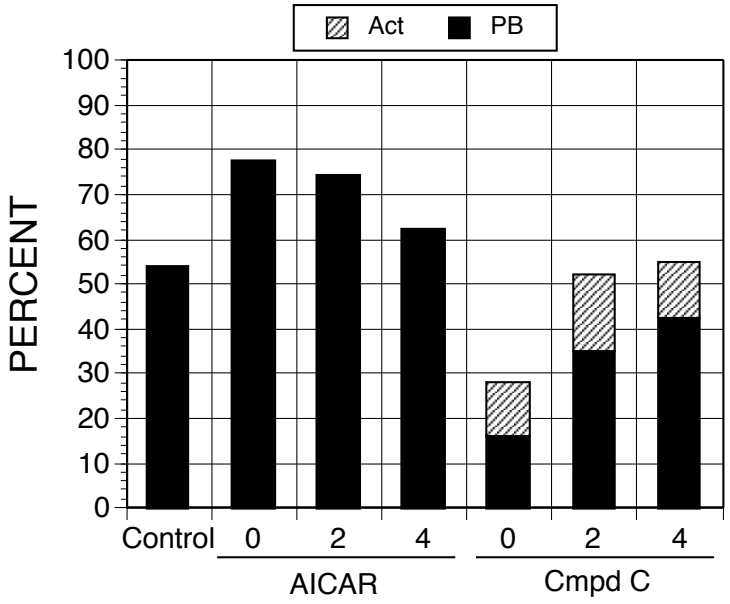

Figure 1. 3 Effect of delaying AICAR or compound C exposure on PB and activation

CEOs were treated with 0.2mM AICAR or 2.5 μM compound C 0, 2, or 4h after initiation of culture. Percentages of PB and activation were assessed after a total of 16-17h of culture.

Effects of Hormone Priming on Polar Body Formation and Oocyte Activation

Normally, mouse follicles grow into preovulatory follicle stages 2 days after receiving PMSG injection. We have shown that oocytes isolated from the mice that were

injected with PMSG for 1 day (hereafter designated as 1-day primed mice), show a significantly higher activation rate than those from 2-day primed mice (Downs, 1990). Here, we further analyzed the effect of AMPK modulators on PB formation and egg activation. CEOs from unprimed, 1-day primed or 2-day primed mice were cultured for 21-22 h in control medium or medium containing either 0.2 mM AICAR or 2.5 μM compound C. The PB rates in 2-day primed and 1-day primed oocytes were comparable, but this number was reduced in CEOs from unprimed mice, suggesting a deficiency in meiotic competence. Consistent with the previous findings, the percentages of oocytes undergoing spontaneous activation increased as the priming periods decreased, confirming a protective effect of priming on spontaneous activation (Downs, 1990). AICAR increased PB formation in all three groups and eliminated the spontaneous activation in unprimed and 1-day primed groups. Compound C treatment induced spontaneous activation in the oocytes from 2-day primed animals and augmented the activation rates in 1-day primed and unprimed groups (Figure 1.4 A). Together, these data support the idea that activation of AMPK promotes completion of the maturation while blocking premature activation.

A kinetics experiment was carried out to determine the timing of oocyte activation as it relates to PB formation. CEO from 1-day-primed mice were cultured for varying periods of time from 15 h to 24 h in the presence or absence of 2.5 μM compound C and assessed for PB formation and activation. In control oocytes, the percentage of oocytes at MII peaked at 18 h (55%) and then declined to 35% at 24 h (Figure. 1.4B). A similar pattern was observed in compound C-treated oocytes, but at reduced rates, with PB frequency peaking at 26% and decreasing 6 h later to 9%. In control oocytes, activation

was initiated between 18 and 21 h of culture, at the same time PB number started to decrease. The percentage of activated oocytes at 24 h (17%) was similar to the 20% decrease in MII oocytes at this time. Activation in compound C-treated oocytes was initiated as early as 15 h (5%) and increased rapidly thereafter, to 48% at 24 h. These data suggest that a significant portion of the activated oocytes derived from MII-stage oocytes.

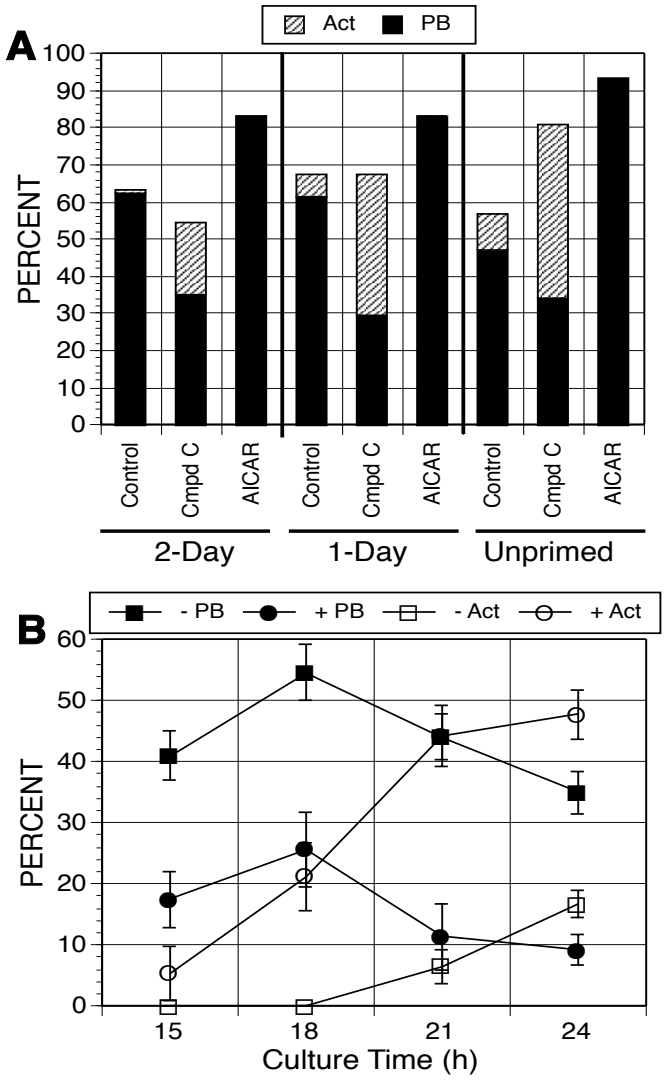

Figure 1. 4 Effect of priming on PB formation and oocyte activation

(A) CEO were derived from unprimed mice or mice primed for 1 or 2 days, cultured 21–22 h in control medium or medium containing 200 mM AICAR or 2.5 mM compound C (Cmpd C), and assessed for polar body formation and activation; (B) CEO from 1-day-primed mice were cultured 15–24 h in either the absence (-) or presence (+) of 2.5 mM Cmpd C and assessed every 3h for polar body formation (PB) and activation (Act).

Immunostaining of Active AMPK Throughout Meiotic Maturation

In order to determine the cellular localization of active AMPK, oocytes were isolated at different times post-hCG treatment and stained for antibody against the phospho-threonine 172 site on the catalytic α subunit. As shown in figure 1.5, active

AMPK was associated with condensed chromosomes after GVB, relocated to spindle poles during metaphase I, concentrated in the midbody region during anaphase I and appeared at the spindle poles again during metaphase II.

When we looked at the distribution of AMPK in *in vitro* matured oocytes, the same staining patterns were observed regardless of maturation conditions: (1) spontaneous maturation of CEO; and meiotic induction in (2) dbcAMP-arrested DO stimulated with AICAR and (3) dbcAMP-arrested CEO stimulated with FSH (Figure 1.6); these results served to validate our *in vitro* maturation system.

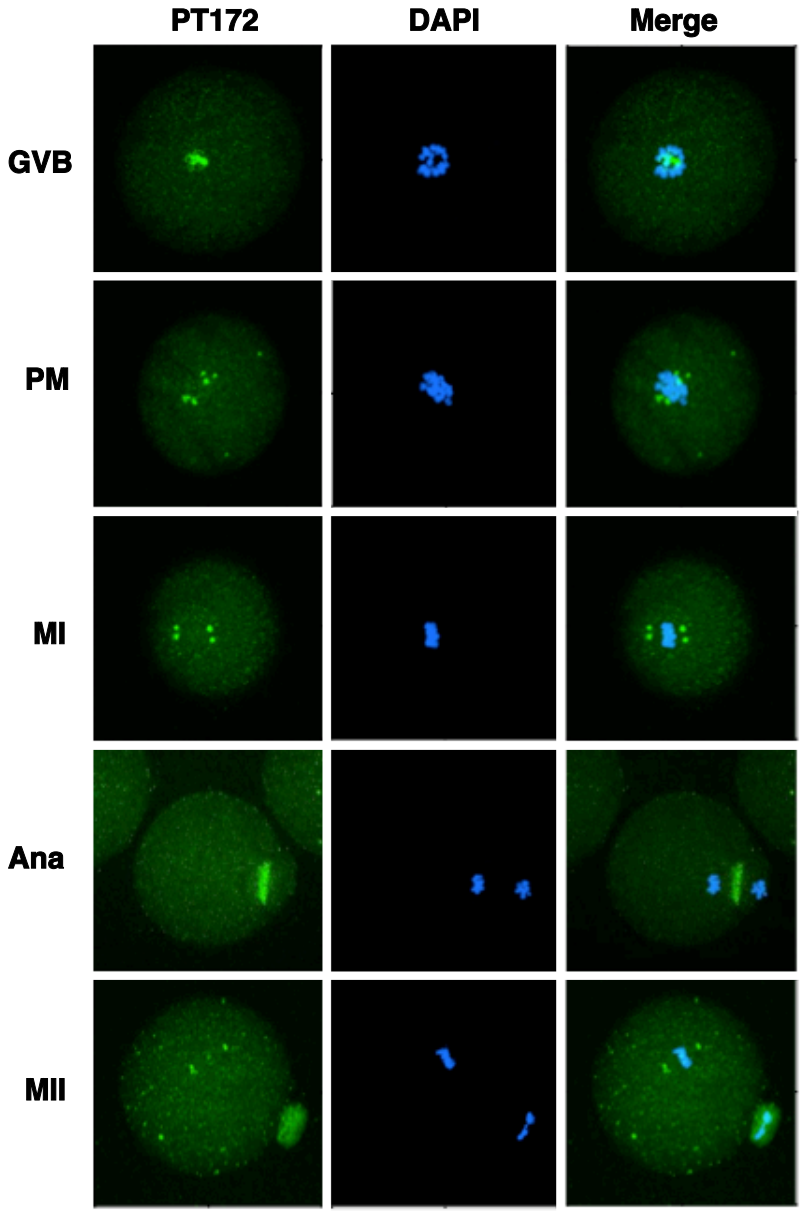

Figure 1. 5 Immunolocalization of active AMPK throughout oocyte maturation

Oocytes were isolated at varying stages of meiosis after hCG administration to 2-day-primed mice, fixed and stained with anti-PT172 antibody for active AMPK (green) and DAPI for chromatin (blue). GVB, germinal vesicle breakdown; PM, prometaphase; MI, metaphase I; Ana, anaphase; MII, metaphase II.

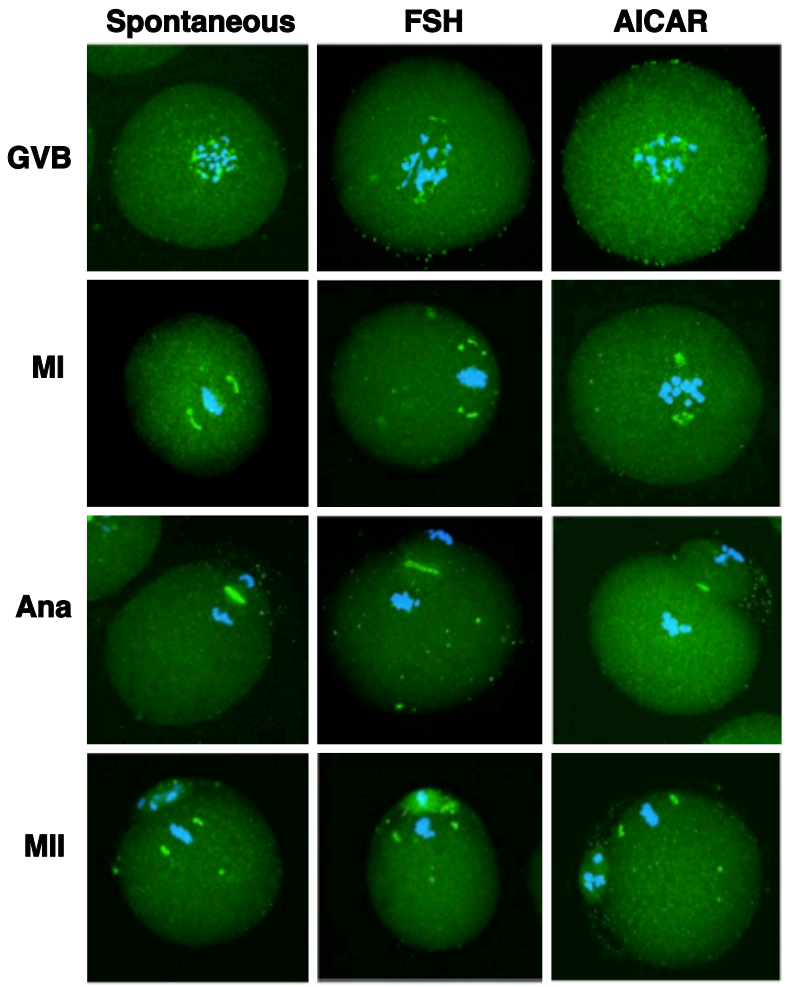

Figure 1. 6 Localization of active AMPK in maturing oocytes *in vivo*

From left to right: oocytes were matured in the following maturation conditions (1) spontaneous maturation of CEO; (2) FSH-induced maturation of CEOs; (3) AICAR-induced maturation of DOs. Oocyte were fixed and stained with anti-PT172 antibody for active AMPK (green) and DAPI for chromatin (blue). GVB, germinal vesicle breakdown; MI, metaphase I; Ana, anaphase; MII, metaphase II.

Immunostaining of isoforms of the catalytic subunit of AMPK

Since there are two different isoforms of alpha catalytic subunit of AMPK in the mouse oocytes (Downs et al., 2002), we stained each isoform using specific antibodies. There was no discrete staining of the α2 subunit (data not shown), whereas α1 subunit showed specific staining with chromosomes after GVB, associated with the spindle at the later stages (Figure 1.7). This specific staining was eliminated either by neutralizing antibody with blocking peptide or omitting the primary antibody. Blocking peptide for α2 subunit did not affect the specific spindle staining for α1. Interestingly, this specific staining pattern of α1 subunit did not coincide with the active AMPK.

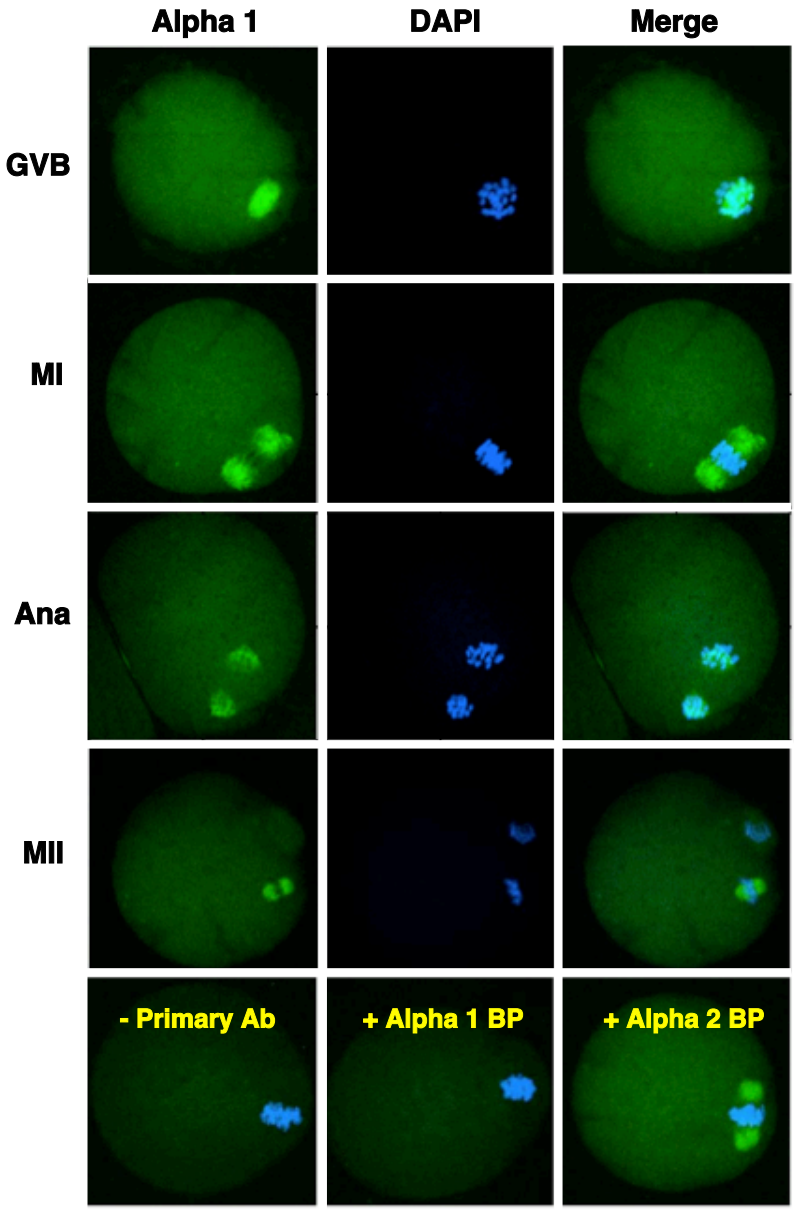

Figure 1. 7 Immunolocalization of the alpha 1 catalytic subunit of AMPK

Oocytes were fixed at varying stages of meiosis following hCG injection to 2-day-primed mice and stained with anti- α 1 antibody (green) and DAPI for chromatin (blue). Controls in the bottom row were of MI-stage oocytes and were either not exposed to primary antibody or anti- α 1 antibody was first treated with α 1 blocking peptide or α 2 blocking peptide. GVB, germinal vesicle breakdown; MI, metaphase I; Ana, anaphase; MII, metaphase II.

Discussion

Our data suggest that AMPK is involved in the entire mouse oocyte maturation process. Activation of AMPK promotes the completion of maturation and suppresses premature egg activation, while inhibition of its activity by AMPK inhibitors, compound C and AraA, had opposite effects. Compound C, but not AraA, induced spontaneous activation in CEOs and augmented the activation rate in oocytes from 1-day and unprimed mice, while AICAR eliminated the activation. Active AMPK associated with chromosomes during GVB, spindle poles at metaphase I and II, midbody during anaphase. The α1 catalytic subunit is localized with chromosomes and the meiotic spindle throughout the maturation period. Altogether, these data indicate a supportive role of AMPK in the entire oocyte maturation.

Previous work from our lab demonstrated activation of AMPK precedes meiotic resumption and mediates hormone induced maturation (Chen and Downs, 2008). In this study we presented evidence for participation of AMPK in later progression of meiosis to metaphase II. AMPK activators, AICAR and AMP, dose dependently promoted the frequency and the kinetics of PB formation in CEOs. In fact, this effect of AMPK activators on PB formation kinetics is mainly due to the early onset of anaphase I through inactivating spindle checkpoint activity (see chapter III for details). Although AMP is ineffective in DOs, another activator, 8-Br-Ado, effectively increased PB formation in DOs. It is unclear why AMP was ineffective in DOs, but cumulus cells are likely required for the regular uptake. Moreover, when oocytes were pulsed with 2-D-Glucose, which is known to activate AMPK by depleting ATP (Hurley et al., 2005; Zhu et al., 2005; Park et al., 2009; Fujimoto et al., 2009; Pradelli et al., 2010), the PB formation was significantly

increased. It should be noted that although AMPK can also be activated by heat stress, which induced meiotic resumption in our previous research (LaRosa and Downs, 2007), it was not effective in stimulating PB in our current study. This is expected, since it has been demonstrated that heat stress impairs both nuclear and cytoplasmic maturation of the oocytes and also impairs later developmental competence (Nabenishi et al., 2012; Wang et al., 2009)

Additional support of the role of AMPK on PB formation was provided by the inhibitor data. The rate of PB was suppressed by compound C, but to a lesser extent by Ara A in DOs, where the uptake may have been hindered by removing the cumulus cells. Compound C treatment induced premature activation of the CEOs, but not as effectively in DOs. This is consistent with the previous observation that cumulus cells facilitate activation (Miao et al., 2005). In an earlier study it has been shown that longer period of hormone priming decreased the susceptibility of oocytes to undergo spontaneous activation (Downs, 1990). Therefore, we compared the effects of AMPK modulators on oocytes from differently primed animals. We increased the *in vitro* culture period because *in vitro* aging reduces the ability of oocytes to maintain MPF activity, making them more susceptible to spontaneous and induced activation (Fissore et al., 2002). Results showed that compound C treatment augmented oocyte susceptibility to activation from 1-day and unprimed mice, whereas AICAR eliminated the oocyte activation and promoted the PB formation, suggesting a protective role of AMPK on premature oocyte activation.

It has been characterized that while most oocytes undergo parthenogenetic activation after reaching metaphase II stage, some oocytes activate directly from metaphase I. The well-known examples are LT/Sv and related strains, which display high

percentage of spontaneous activation (Kaufman and Howlett, 1986; Eppig et al., 1996; Hirao and Eppig, 1997). To investigate at what stage oocytes become activated after compound C treatment, a careful kinetics experiment was conducted on oocytes retrieved from 1-day primed mice. Oocytes were cultured in both the presence and absence of compound C and the formation of PB and activation rates were assessed. In the control group, the presence of PB declined after 18h culture, and the decreased number of PB was offset by the increased percentage of pronucleus formation. This piece of data indicated that the activated oocytes were derived from metaphase II stage oocytes in the control group. However, the same situation did not apply to the compound C-treated group, where the percentage of activation was almost 3 times greater than the decrease in PB rate. In fact, later immunofluorescent staining of compound C-treated oocytes with DAPI and tubulin cauptured a portion of them in an anaphase-like state with condensed chromosomes within the PB, but with chromosomes inside the oocyte that had already become decondensed (Unpublished data, not shown). This suggested that the activated oocytes after compound C treatment originated directly from metaphase I, which is also consistent with the fact that majority of the oocytes arrest at metaphase I after the compound C treatment (data not shown).

It is well established that metaphase II arrest in oocytes is maintained by high MPF and MAPK activity. Recently, it has also been shown that the inorganic metal, Zinc, regulates activity of the APC inhibitor, Emi2, and is a new member of CSF components (Bernhardt et al., 2011) . 6-dimethylaminopurine (6-DMAP), a general kinase inhibitor, is widely used to activate oocytes *in vitro*, and the site of its action is mainly on interrupting the MPF activity, which is regulated by MAPK (Fan and Sun, 2004; Szollosi et al., 1993;

Moses and Masui, 1994). Our data suggest that interrupting AMPK activity in the mouse oocytes interferes with the second meiotic arrest point and leads to activation. In fact, a later portion of my thesis work determined that AMPK regulates MAPK activity to prevent premature activation (Chapter III).

Interestingly, AMPK activity is required very early during maturation (<4h after initiation of the culture) to promote PB, because there was a progressive loss of stimulation or inhibition of PB formation when AICAR or compound C treatment was delayed. However, oocyte activation induced by compound C treatment was refractory to the delayed compound C exposure, at least to the latest delay tested (4h). This suggests that targets of AMPK for promoting PB formation and oocyte activation are differ in stages during cell cycle progression.

In fact, AMPK was revealed as an important kinase that knocking down of AMPK, impaired Drosophila cell cycle progression (Bettencourt-Dias et al., 2004). However, the relationship between AMPK activation and cell cycle progression in the somatic cells is opposite to what we have observed in the mouse oocytes. A study by Jones et al., (2005) showed that a glucose deficiency in mouse primary embryonic fibroblasts caused a AMPK mediated metabolic checkpoint. Furthermore, cells expressing constitutively active AMPK α2 subunits arrested at G1/S stage. The same observation has been confirmed in variety of cell types (Imamura et al., 2001; Igata et al., 2005; Motoshima et al., 2006; Baumann et al., 2007; Gwinn et al., 2008; Fogarty and Hardie, 2009; Ishii et al., 2009; Zang et al., 2009). The AMPK upstream kinase, LKB1, which phosphorylates threonine 172 site on the catalytic α subunit. LKB1 mutation has been shown to cause an inherited cancer, Peutz-Jeghers syndrome, in humans, which is

characterized by high incidence of cancer (Kyriakis, 2003; Hemminki et al., 1998; Jenne et al., 1998). In addition, LKB1 was also implicated in regulating the polarity of drosophila embryo and human epithelial cell (Alessi et al., 2006; Lee et al., 2007; Mirouse et al., 2007). Independently, LKB1 was found directly associated with the meiotic spindle in the mouse oocytes (Szczepańska and Maleszewski, 2005). It would be interesting to know whether AMPK also exerts same effects in mouse oocytes.

Consistent with the above observations in the somatic cells, AMPK activation suppresses GVB in pigs (Mayes et al., 2007), cows (Tosca et al., 2007; Bilodeau-Goeseels, S Sasseville et al., 2007), and nemertean worms (Stricker et al., 2010), and it is ineffective in inducing meiotic resumption in the rat oocytes (Downs, 2011). Herein, our data indicated a stimulatory role of AMPK in mouse oocyte meiotic progression at both G2/M and the first polar body extrusion. Mouse oocytes are different form the somatic cells in a way that glucose is not the primary energy substance. Instead, oocytes are mainly dependent on pyruvate that is supplied by the somatic compartment. Increased glucose concentration suppressed GVB, while lowering glucose levels promotes meiotic induction, which may relates to AMPK activation (Downs and Mastropolo, 1994; Downs, 1995). Thus, the major discrepancy between mouse oocytes and these from other species in response to AMPK stimulation might also be due to the differential response to energy substrates; at least this is the case in the rat oocytes (Downs, 2011).

The involvement of AMPK in the entire maturation process is further supported by the active AMPK localization. We have previously showed the positive staining of active AMPK in the germinal vesicle before oocytes undergo GVB. Here we have shown that active AMPK is associated with the chromosomes following GVB, on the spindle

poles during metaphase I and metaphase II, and the midbody during anaphase I. This localization pattern is identical to that reported in somatic cells (Vazquez-Martin et al., 2009). Furthermore, active AMPK coincides with numerous other kinases that are important in meiotic maturation--for instances, MEK1/2 (Sun et al., 2008), phospho-protein kinase C delta (Ma and Koch, 2008), aurora kinase (Yao et al., 2004; Shuda et al., 2009) and polo-like kinase (Wianny et al., 1998; Pahlavan et al., 2000; Tong et al., 2002; Fan et al., 2003). Many kinases regulate of spindle microtubule dynamics during mouse oocyte maturation. I have examined this potential relationship between AMPK and microtubules in chapter II.

It was of interest to determine the localization patterns of both α 1 and 2 subunits, since both are present in the mouse oocyte (Downs et al., 2002). There was no discrete staining pattern of α2 subunit, whereas, the α1 subunit was associated with chromosomes initially and with the meiotic spindle thereafter. In contrast, the α2 subunit exhibited distinct localization in the somatic cells (Salt et al., 1998). It is possible that having a different pattern is the key to opposite effect on cell cycle progression. These results suggest that unlike the situation in somatic cells, α1, and not α2, may play a more important role in regulating the maturation of mouse oocytes.

Chapter II: PERTURBING MICROTUBLE INTEGRITY BLOCKS AMP-ACTIVATED PROTEIN KINASE-INDUCED MEIOTIC RESUMPTION IN CULTURED MOUSE OOCYTES

Summary

The oocyte meiotic spindle is comprised of microtubules (MTs) that bind chromatin and regulate both metaphase plate formation and karyokinesis during meiotic maturation; however, little is known about the role of MTs in meiosis reinitiation. This study was conducted to determine if microtubule (MT) integrity is required for meiotic induction and how it impacts activation of AMP-activated protein kinase (AMPK), an important participant in the meiotic induction process. Treatment with microtubule-disrupting agents nocodazole and vinblastine dose-dependently suppressed meiotic resumption in both arrested cumulus cell-enclosed oocytes (CEOs) stimulated with FSH and arrested denuded oocytes (DOs) stimulated with the AMPK activator, AICAR. This coincided with suppression of AMPK activation as determined by western blotting and germinal vesicle immunostaining. Treatment with the MT stabilizer paclitaxol also suppressed meiotic induction. Targeting actin filament polymerization had only a marginal effect on meiotic induction. Immunolocalization experiments revealed that active AMPK colocalized with gamma tubulin during metaphase I and II, while it localized at the spindle midzone during anaphase. This discrete localization pattern was dependent on MT integrity. Treatment with nocodazole led to disruption of proper spindle pole localization of active AMPK, while paclitaxel induced excessive polymerization of spindle MT and formation of ectopic asters with accentuated AMPK colocalization. Although oocytes stimulated by AMPK activator increased the rate of

GVB, spindle formation and PB extrusion, blocking AMPK activity did not influence peripheral movement of the spindle. These data suggest that the meiosis-inducing action and localization of AMPK are regulated by MT spindle integrity during mouse oocyte maturation.

Introduction

Mammalian oocyte maturation refers to the developmental period between germinal vesicle breakdown and formation of the first polar body and involves dynamic changes within the cytoskeletal network. Initially, the meiotic spindle forms at a position slightly off-center, where the germinal vesicle (GV) was originally located. After germinal vesicle breakdown (GVB), microtubules start polymerizing around the condensing chromosomes, and as the chromosomes congress a bipolar metaphase I spindle is established, with chromosomes correctly aligned at the metaphase plate. Meiotic spindle migration occurs after its formation and this process relies on a dynamic microfilament meshwork (Azoury et al., 2008), actin filament nucleator, Formin-2 (Dumont et al., 2007), and RhoGTPase (Na and Zernicka-Goetz, 2006). The peripheral spindle migration is a crucial process that assures conservation of ooplasm through asymmetrical cell division of the polar body.

The meiotic spindle association of AMPK is intriguing, because numerous proteins showing a similar localization pattern are known to regulate meiotic spindle assembly and function; AMPK has also been shown to have a role in maintenance of genome integrity and establishment of cell polarity in Drosophila embryos and other somatic cells (see Discussion). It is well understood that normal cytoskeleton function is required for later oocyte maturation processes, since it was directly related to spindle positioning, chromosome segregation. However, less is known about the role of the cytoskeleton requirement on meiotic induction. So it was of interest to test how microtubule and spindle integrity was related to AMPK function in mouse oocytes.

Specifically, we are interested in answering two questions; 1) how does perturbation of the microtubule integrity affect AMPK mediated meiotic induction in mouse oocytes? 2) And what is the relation between AMPK and spindle microtubule integrity? Our data indicate that disruption of microtubule integrity blocks hormone-induced maturation in meiotically arrested oocytes while coincidentally blocking AMPK activity. Moreover, active AMPK colocalization with γ-tubulin at microtubule organization centers (MTOC) during metaphase I and II is dependent on spindle integrity. However, treatment of oocytes with AMPK inhibitor did not prevent spindle formation, despite suppressing the completion of meiotic maturation.

Materials and Methods

Oocyte Isolation and Culture Condition

All procedures were approved by the Marquette University Institutional Animal Care and Use Committee. Immature, 19-23-day-old (C57B/ 6J× SJL/J) F_1 female mice were used for all experiments. Mice were primed with 5IU pregnant mare serum gonadotropin (PMSG) 2 days before the experiments. Mice were killed by cervical dislocation and ovaries were removed and placed in the petri dish containing culture medium. CEOs were isolated by puncturing the follicles with sterile needles. DOs were obtained by stripping cumulus cell by using mouth operated small-bore pipette. Both CEOs and DOs were washed twice and transferred to plastic culture tubes containing 1ml of the appropriate test medium. The culture medium used was Eagle minimum essential medium (Sigma, St Louis, MO) supplemented with penicillin, streptomycin sulfate, 0.23mM sodium pyruvate and 3mg/ml bovine serum albumin (MP Biomedicals, Solon, OH).

Immunofluorescent Staining

Oocytes were fixed with microtubule stabilizing buffer as previously described (Messinger and Albertini, 1991) at 4°C overnight. Oocytes were permeabilized 30 min with 0.1% triton-100 in blocking solution, which contained 10% donkey serum and 0.5 mg/ml saponin in PBS, followed by 1h in blocking solution minus triton-100. Oocytes were incubated with antibody recognizing active AMPK (1:100) overnight at 4°C, and washed 4 times at room temperature in blocking buffer. Oocytes were coincubated with FITC-conjugated anti-α-tubulin antibody (1:100) and Cy3-conjugated secondary antibody

(1:100) 1h at room temperature. After washing, oocytes were placed at slides and mounted with medium containing DAPI (Vector Laboratories, Burlingame, CA).

Confocal Microscopy

Oocytes were viewed on laser scanning confocal microscope (Carl Zeiss Co., Thornwood, NY) with a 63X objective. During scanning, all settings were kept constant: i.e., laser power, detector gain, amplifier offset, amplifier gain, and pinhole size. Digitally recorded images were exported by LSM Examiner software (Carl Zeiss Co.).

Western Blot Analysis

Oocytes samples were washed with PBS/PVP, then twice with the protease inhibitor cocktail (Roche, Indianapolis, IN). Samples were treated with Laemmli's Buffer with 20% beta-mercaptoethanol at 95°C for 5 minutes. Electrophoresis was carried out using NuPAGE 3-8% Tris-Acetate Gels (Invitrogen, Carlsbad, CA), and proteins were transferred to nitrocellulose. Membranes were blocked with 5% non-fat milk, followed by incubation with primary antibody pACC (1:250, Cell Signaling) at 4°C overnight. Blots were rinsed with TBS (pH 7.4) and TBS-Tween-20 (0.05%), and incubated with HRP-conjugated goat anti-rabbit antibody (1:2000 in 5% non-fat milk, Pierce) at room temperature for 60 min. After washing, protein signals were detected by Super Signal West Pico Chemiluminescent Substrate (Pierce). Blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific; Rockford, IL) and reprobed with anti-ACC1 antibody (1:250; Cell signaling) as a loading control. pACC/ACC1 ratio was quantified with ImageJ software based on protein band density.

Chemicals

Saponin, dbcAMP, donkey serum, nocodazole, vinblastine, paclitaxel and FITC-labeled mouse anti-α tubulin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Cy3-conjugated donkey anti-rabbit antibody was supplied by Jackson ImmmoResearch (West Grove, PA). AICAR and compound C were obtained from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Anti-phospho AMPK (PT172) antibody was purchased from Cell Signaling Technology (Beverly, MA). Highly purified ovine FSH was from the National Hormone and Peptide Program (NHPP) and Dr. A.F. Parlow.

Statistical Analysis

All experiments were repeated at least three times and data presented as mean ± SEM. Percentages of GVB or nuclear stain underwent arcsin transformation and data were analyzed statistically by ANOVA followed by Duncan's multiple range test. A *P*-value <0.05 was considered significant.

Results

Effect of Cytoskeletal Disrupting Agents on Maturation and AMPK Activity

We have shown that AMPK is involved in hormone-induced maturation and its activation precedes GVB in mouse oocytes (Chen and Downs, 2008). We therefore determined how perturbing microtubule integrity would affect AMPK activity and meiotic induction. dbcAMP-arrested CEOs were treated with 0.1µg/ml FSH to induce meiotic resumption, exposed to increasing concentrations of nocodazole, and assessed for GVB after 17-18 h. As shown in Figure 2.1A, FSH increased the percentage of GVB by 62%, while supplementation with nocodazole significantly blocked this increase in a dose-dependent manner, by 62% at the highest dose tested. DMSO alone, the nocodazole vehicle, had no effect on meiotic resumption (data not presented). To test for a direct effect of nocodazole on the oocyte, dbcAMP-arrested DOs were treated with 250µM AICAR, an AMPK activator, and scored for GVB. AICAR stimulated an increase in GVB from 23% to 89%. This stimulation was abolished by nocodazole, which significantly decreased the percentage of maturation to 8% (Figure 2.1B). This inhibition was completely reversible, thereby demonstrating that the drug did not act through toxic means.

FSH-treated CEOs were cultured for 4 h and AICAR-treated DOs were cultured for 2.5 h before fixation and immunofluorescent staining for active AMPK, using an antibody against phospho-threonine 172 on the alpha catalytic subunit of AMPK. As previously shown, FSH and AICAR increased phospho-AMPK staining in the germinal vesicle prior to GVB. This staining pattern is characterized by homogeneous staining

throughout the germinal vesicle with absence of label within nucleoli (Chen and Downs, 2008). Since nocodazole blocked AICAR- and FSH-induced GVB, it was important to determine if this GV staining pattern was present after nocodazole treatment of these oocytes. As shown in Figure 2.1C, 21% of control CEOs arrested in dbcAMP showed nuclear staining, with a 20% increase following FSH treatment, and exposure to 10 μg/ml nocodazole eliminated this increase. A similar pattern was evident in DOs: GV staining was detected in 26% of control oocytes, with this number increasing to 57% after culture in AICAR, and nocodazole again prevented the response (20% with active AMPK accumulation in the GV; Figure. 2.1D). These data support the idea that activity of AMPK is greatly influenced by microtubule integrity. This was confirmed by western blot analysis of nocodazole-treated oocytes that showed suppression of AICAR-induced AMPK activity, using phospho-acetyl CoA carboxylase as a marker for AMPK activity (Figure 2.1E, F).

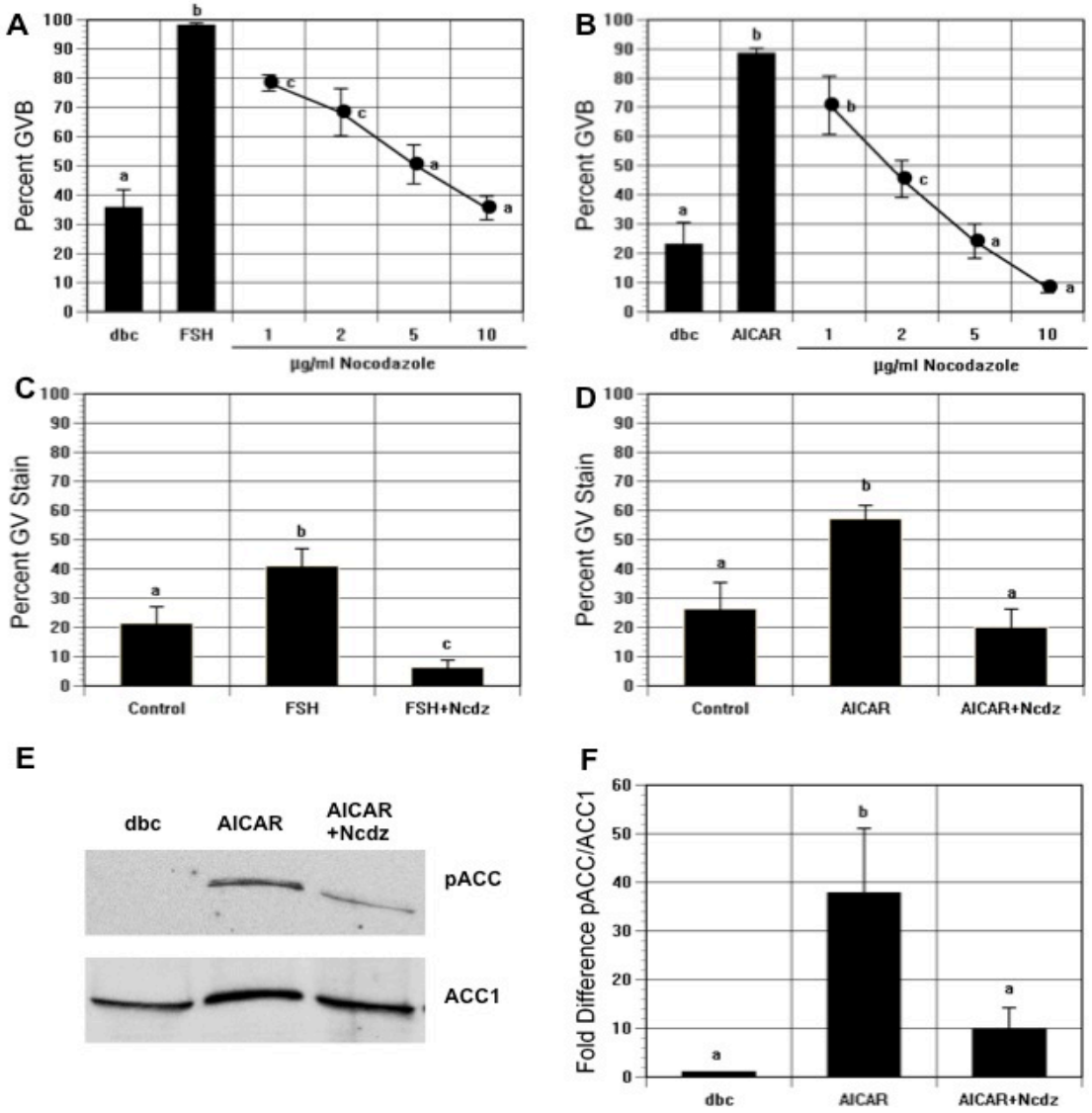

Figure 2. 1 Effects of nocodazole treatment on meiotic resumption and AMPK activation

(A) CEOs were cultured in medium containing 300 µM dbcAMP (dbc) plus FSH in the presence of increasing concentrations of nocodazole. Germinal vesicle breakdown (GVB) was assessed 17-18h later. (B) DOs were cultured with 300 µM dbcAMP plus 250 µM AICAR and treated with increasing doses of nocodazole. GVB was assessed after 4h of culture. (C) CEOs were cultured in 300 µM dbcAMP alone (control) or dbcAMP plus FSH with or without 10 µg/ml nocodazole; 4h later oocytes were fixed and processed for PT172 staining. (D) DOs were cultured for 2.5 h in the same medium as CEOs but with FSH replaced by 250 µM AICAR; oocytes were then processed for PT172 staining. In both C and D, nocodazole reduced the frequency of the GV stain to control levels. (E) Western blot analysis of pACC of oocytes that were treated as described in B; ACC1 was used as loading control. (F) Quantification of pACC and ACC1 from western blot. pACC and ACC1 ratio were normalized to the control dbcAMP-treated group. Groups with no common letter are significantly different.

When CEOs were treated with another microtubule-destabilizing agent, vinblastine, meiotic induction was again suppressed, with a 31% decrease at the highest concentration tested (Figure 2.2A). Inhibition was even more robust in AICAR-treated DOs, with 20μg/ml vinblastine reducing the maturation percentage to control levels (Figure 2.2B).

To test how stabilizing microtubules affects the meiotic induction, the microtubule-stabilizing agent, paclitaxel was utilized. CEOs were cultured 17-18h and treated with FSH plus increasing doses of paclitaxel. Similar to the finding with microtubule disrupting agents, FSH-induced GVB was blocked in dose-dependent fashion by paclitaxel (Figure 2.2C), and the agent was even more potent in AICAR-treated DOs (Figure 2.2D). When FSH- and paclitaxel-treated oocytes were fixed after 4 h and stained with anti-PT172 antibody, no label was observed in the GV; however, punctate staining was present throughout the cytoplasm (data not shown). When paclitaxel was added to AICAR-treated DOs, punctate staining of active AMPK was observed near or within the GV, but with no homogeneous staining within the GV. The increased punctate staining of active AMPK after paclitaxel treatment may correspond to excessive stabilization of microtubule polymers.

To determine whether inhibiting actin filament polymerization plays a role in meiotic induction, cytochalasin D and latrunculin A were added to either FSH-stimulated CEO cultures or AICAR- stimulated DO cultures. There was no effect of cytochalasin D on GVB. Latrunculin A showed a modest reduction (29%) of GVB in FSH-treated CEOs (Figure. 2.2E), but it was toxic to AICAR-treated DOs.

We decided to test if activating the AMPK downstream pathway, fatty acid oxidation, would bypass the inhibitory effect of nocodazole on meiotic induction. Palmitoyl carnitine, the fatty acid derivative that was shown to induce maturation by increasing fatty acid oxidation, was added to the CEO culture (Downs et al., 2009). Consistent with the early findings, nocodazole blocked FSH-induced maturation in CEOs; however, direct stimulation of fatty acid oxidation by palmitoyl acid significantly reversed this inhibition (Figure 2.2F). These data indicate that the fatty acid oxidation pathway is still active during microtubule depolymerization, suggesting nocodazole treatment does not cause a generalized toxicity in the oocytes.

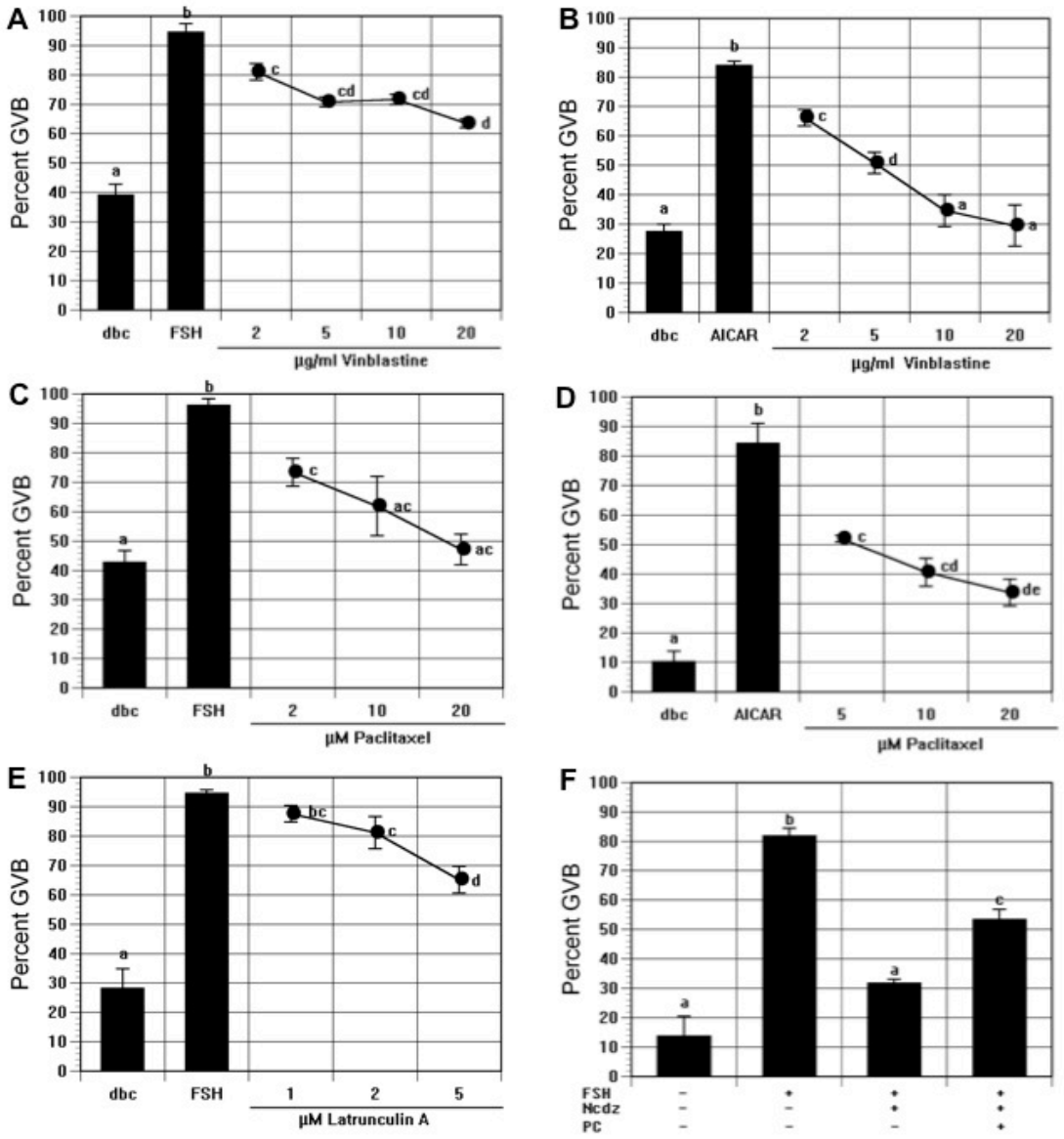

Figure 2. 2 Effect of additional microtubule-targeted agents on FSH- and AICAR-induced maturation *in vitro*

(A, C) CEOs were cultured 17-18 h in medium containing 300 µM dbcAMP plus FSH or (B, D) DOs were cultured 4 h in 300 µM dbcAMP plus 250 µM AICAR, in the presence of increasing concentrations of vinblastine (A, B) or paclitaxel (C, D). (E) CEOs were cultured 17-18 h in 300 µM dbcAMP plus FSH and increasing concentrations of latrunculin A before GVB assessment. (F) dbcAMP-arrested CEOs were induced to undergo maturation with FSH, and this stimulation was suppressed by further treatment with nocodazole. Palmitoyl carnitine (50 µM) was added to the latter group to trigger GVB via activation of fatty acid oxidation. Groups with no common letter are significantly different.

Association of Active AMPK with Microtubules and Effect of Microtubule Perturbants on Localization

Since studies in other labs have demonstrated that the meiotic spindle-associated localization of numerous proteins is dependent on MT integrity, the next series of experiments were conducted to confirm this relationship for AMPK. Oocytes undergoing *in vivo* maturation were fixed 8 h after human chorionic gonadotropin (hCG) injection to obtain metaphase I-stage oocytes and stained with antibodies to active AMPK and γ-tubulin. As expected, AMPK colocalized with γ-tubulin at the spindle poles during MI (Figure. 2.3A).

To test if active AMPK localization is dependent on spindle microtubule integrity, microtubule-targeted agents were tested on CEOs during *in vitro* culture. Isolated CEOs were arrested in 300μM dibutyryl cAMP (dbcAMP) and then treated with 0.1 μg/ml FSH to induce maturation. CEOs were cultured for 17 h before addition of the microtubule-depolymerization agent, nocodazole (0.05 μg/ml), or the microtubule-stabilizing agent, paclitaxel (20 μM), and oocytes were fixed 1 h later and stained for active AMPK and α-tubulin. Nocodazole depolymerized the spindle, and as a result, chromosomes became scattered and, not surprisingly, normal spindle pole localization of active AMPK was disrupted (Figure. 2.3B). When oocytes were treated with paclitaxel, microtubules were excessively polymerized and spindles were larger than normal; formation of a double meiotic spindle was occasionally observed, with 2 points of active AMPK localization at each pole. Many small asters also appeared in the cytoplasm with colocalized AMPK (Figure 2.3B). To test whether reestablishment of a bipolar spindle could bring active AMPK localization back to its normal localization, in-vitro matured MII-stage CEOs

were subjected to nocodazole (5 μg/ml) for 10 minutes; the nocodazole was then washed out and oocytes were allowed to recover for 1 h to allow reformation of a bipolar MII spindle before fixation and processing for immunoflorescent staining. The MII spindle completely disappeared after 10 min treatment with nocodazole, and active AMPK was randomly dispersed as before (data not shown). Upon spindle repolymerization, active AMPK localization returned to the spindle poles (Figure 2.3B), thereby demonstrating reversibility of the nocodazole effect and a microtubule-dependent localization of active AMPK in mouse oocytes.

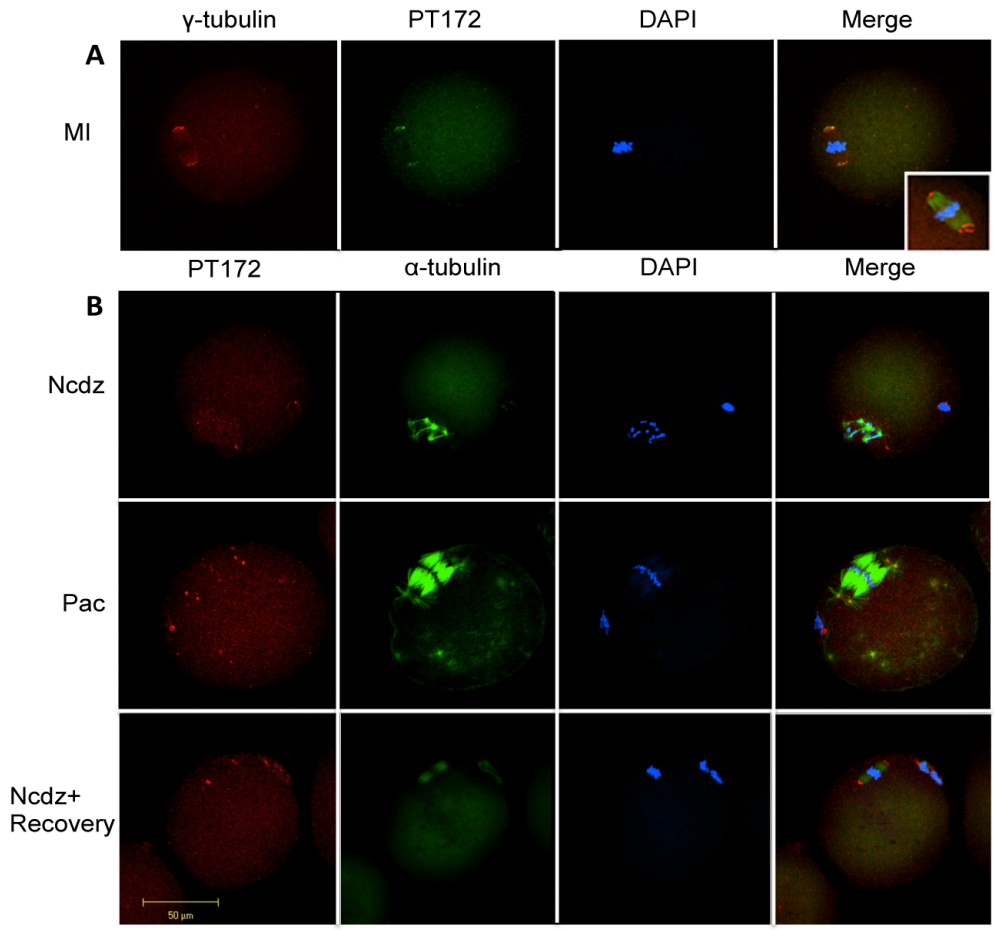

Figure 2. 3 Immunofluorescent staining of active AMPK and tubulin

(A) Oocytes were collected 8 h after administration of hCG to PMSG-primed mice to obtain oocytes at MI. Active AMPK (green) is colocalized at the spindle poles with g-tubulin (red). Chromatin, blue. INSET: MI spindle from triple-stained oocyte showing a-tubulin, green; active AMPK, red; chromatin, blue. (B) Effect of microtubule-targeted agents on active AMPK localization. CEOs were cultured 17 h in the presence of 300 µM dbcAMP plus FSH; then either the microtubule-destabilizing agent, nocodazole (Ncdz, 0.05 µg/ml), or microtubule-stabilizing agent, paclitaxel (Pac, 20 µM), was added and oocytes were fixed 1h later and processed for immunostaining for active AMPK (PT172, red) and a-tubulin (green). Nocodazole treatment disrupted the normal spindle pole localization of active AMPK. When treated with paclitaxel, microtubules were excessively polymerized, and this was associated with increased AMPK staining. After CEOs were cultured 17 h in the presence of 300 µM dbcAMP plus FSH, oocytes were exposed to nocodazole (5 µg/ml) for 10 min. The nocodazole was then washed out and oocytes were allowed to recover for 1h before fixation and immunostaining. Upon microtubule repolymerization, active AMPK reestablished its normal spindle pole localization. Red, active AMPK; green, α –tubulin; blue, chromatin. Scale bar, 50 µm.

Effect of AMPK Modulators on Cytoskeleton Dynamics

Our data suggest microtubule integrity is critical for localization and activity of AMPK. We next tested if the reverse regulation was true; that is, if AMPK regulates spindle dynamics. AMPK stimulators, AICAR, AMP and 8-Br-adenosine accelerate PB formation of spontaneously maturing CEOs (Downs et al., 2010), and it is possible that this is mediated by accelerated spindle formation. First, CEOs were matured spontaneously in MEM/BSA control medium in the presence or absence of 200 μM AICAR and the kinetics of GVB were determined. After 45 minutes of AICAR treatment, the percentage of GVB was increased from 35% to 52%; thereafter, maturation percentages were comparable up to105 min of culture (Figure. 2.4A). To examine the impact of AICAR on spindle formation, CEOs that were cultured in the presence or absence of AICAR were fixed at discrete time points and processed for α-tubulin immunofluorescent staining. Polymerization of microtubules into a barrel-shaped spindle was the criterion used for assessment. AICAR significantly fastened spindle formation at the early time point (an increase from 12% to 24% at 3 h), which is roughly 1 hour after GVB (Figure. 2.4B). This trend toward increased spindle formation in AICAR-treated oocytes continued for the next 2 h, but differences were not significant.

Spindle migration is important for asymmetric cell division in oocytes, which helps minimize loss of cytoplasm during polar body extrusion. We determined whether blocking AMPK activity could influence the migration of the meiotic spindle. Spontaneously maturing CEOs were supplemented with the AMPK inhibitor, compound C, and position of the spindle was checked at 8,12 and 16 h after initiation of culture. Spindles were considered to have migrated to the periphery if they were located more

than one-half the radius of the oocyte from its center. As shown in Figure 2.4C, by 8h culture 79% of the control oocytes had a peripherally-located spindle; this number was 68% in compound C-treated oocytes, which was not significantly different from controls. By 16 h, most CEOs in control medium had released the first polar body and 95% of the oocyte spindles were peripherally localized. The supplementation of compound C significantly blocked PB formation, but did not interfere with the peripheral movement of the spindle.

Finally, we tested whether inhibition of AMPK activity would perturb actin microfilament dynamics, especially the polymerization status of the cortical region. Extensive actin polymerization occurs in the cortical area and facilitates attachment of the outer-most spindle pole to the cortex (Azoury et al., 2008). When CEOs were cultured in control MEM or medium containing 2.5 μM compound C for 9.5 h and 11.5 h and processed for phalloidin staining, normal F actin polymerization was observed in both groups (data not shown). Thus, compound C treatment did not interfere with polar body formation by blocking actin microfilament polymerization.

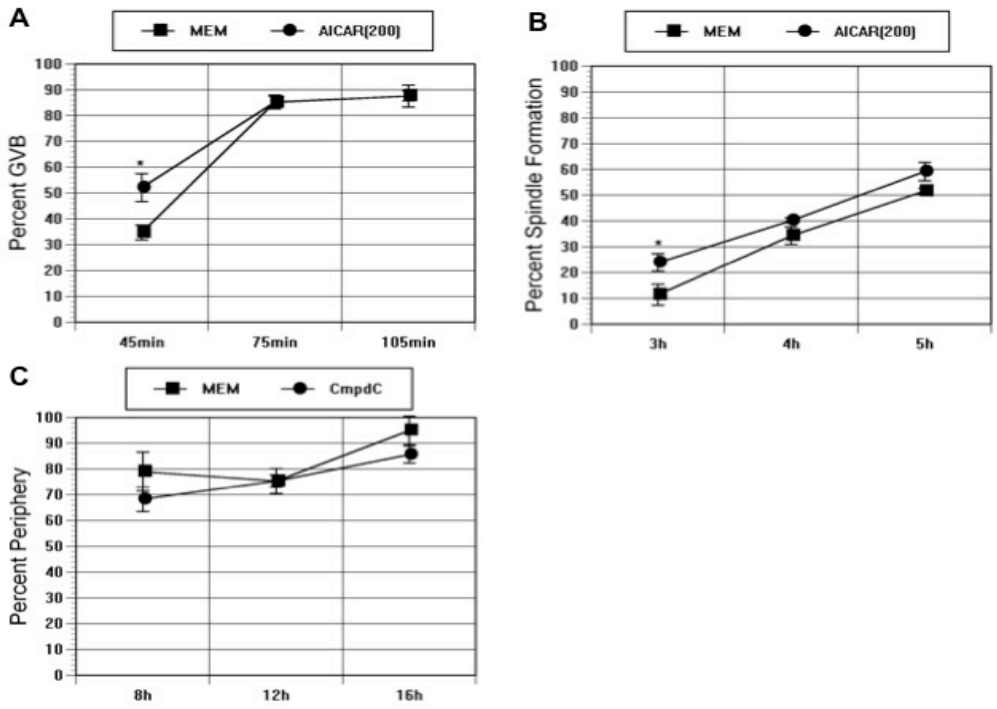

Figure 2. 4 Effect of AMPK modulators on spontaneous maturation, early spindle formation and spindle periphery movement

(A) Effect of AMPK activator, AICAR, on spontaneous maturation. CEOs were cultured in control MEM or medium supplemented with 250 µM AICAR, and percentage of GVB was checked at 45, 75 and 105 min after initiation of the culture. (B) Effect of AICAR on early spindle formation. CEOs were cultured in MEM or MEM plus 250 µM AICAR for 3, 4, and 5 h before spindle formation was assessed by immunofluorescent staining. (C) Effect of the AMPK inhibitor, compound C, on spindle periphery movement. CEOs were cultured in control medium or medium containing 2.5 µM compound C. Spindle periphery movement was examined at the indicated time points. An asterisk denotes a significant difference from the corresponding control.

Discussion

In this study, we show that perturbing microtubule integrity in mouse oocytes reversibly interferes with meiotic induction of CEOs by FSH and DOs by AICAR, which is accompanied by the loss of AMPK activity. It also disrupts the normal localization of active AMPK during maturation. On the other hand, microfilament perturbants had little effect on maturation. While AMPK stimulation accelerated both GVB and spindle formation, blocking its activity with compound C did not affect actin polymerization or interfere with peripheral spindle migration, though karyokinesis was suppressed.

Disruption of microtubules with nocodazole and vinblastine, as well as their stabilization with paclitaxel, blocked both AICAR-induced maturation in DO and FSH-induced maturation in CEO. This was associated with suppression of AMPK activation, as determined by Western blotting of phospho-ACC as well as loss of pAMPK staining in the germinal vesicle that occurs prior to meiotic resumption (Chen and Downs, 2008). Compartmentalization is one of the many ways of controlling signal transduction and cell cycle (Pines, 1999). Best-known examples in oocyte meiosis are MPF and its two regulatory components Cdc25B and Wee1. MPF is more concentrated in the cytoplasm during GV arrest, whereas both Cdc25B phosphatase and MPF adopt nuclear localization shortly before GVB, where Cdc25B keeps MPF activated; at the same time, Wee1 is exported from the nucleus to relieve the inhibition on MPF (Oh et al., 2010). MAPK, which is required for GVB, also translocates to the germinal vesicle just before GVB in the pig oocytes (Inoue et al., 1998). That nocodazole and paclitaxel suppressed GV staining of pAMPK in FSH- and AICAR-treated oocytes, coincident with a reduction in meiotic resumption, suggests a microtubule-dependent step within the oocyte in AMPK

regulation of meiosis that involves nuclear localization. Nuclear transport is gated by the pore complexes that span the nuclear membrane (Adams and Wente, 2013; Stewart, 2007). Cytoskeleton is required for successful nuclear imports of some proteins, such as p53 and parathyroid hormone-related protein (Salman et al., 2005; Rathinasamy and Panda, 2008; Giannakakou et al., 2000; Lam et al., 2002). It has been proposed that MPF is translocated to the periphery of the nucleus by microtubule dependent transport before it is imported into the nucleus, and this is thought to be critical for meiotic resumption (Van-Blerkom, 1991; Albertini, 1992). Perhaps a similar mechanism applies for AMPK even though we cannot rule out the possibility that disrupting microtubule dynamics interferes with the regular special organization of other proteins that help to drive meiotic resumption in response to FSH and AICAR. Consistent with this idea, nocodazole-suppressed AMPK activity, as assessed by western analysis.

Nocodazole was shown to block GVB in spontaneously maturing rat oocytes (Albertini, 1987), but we did not observe a similar inhibitory action on spontaneously maturing mouse oocytes (data not shown), results consistent with those of others (Wassarman et al., 1976; Van-Blerkom and Bell, 1986). Supplementation with taxol also did not affect the GVB of spontaneously maturing mouse oocytes (data not shown) and this has been shown by others for both rat and mouse oocytes; however, like the effects with nocodazole, taxol impairs later meiotic progression, resulting in failure of karyokinesis in mouse oocytes (Albertini, 1987; Combelles and Albertini, 2001). These distinct effects of microtubule perturbants provide another piece of evidence on the differences between the two species regarding meiotic regulation (cf, Downs, 2011). In addition, these data suggest that the differences exist between spontaneous maturation

and meiotic induction of the oocytes, the latter is more sensitive to perturbation of the microtubule integrity.

In a previous study (Downs *et al*, 2010), we demonstrated that active AMPK associates with chromosomes after GVB, localizes to the spindle pole during Metaphase I and II, and moves to the spindle midzone during anaphase. Here we show that active AMPK colocalizes with γ-tubulin, indicating an association with spindle organizing centers during formation of metaphase I spindles. This distribution pattern of pAMPK during oocyte maturation is similar to that occurring during mitosis of somatic cells (Vazquez-Martin *et al*, 2009) and is consistent with a reported role for AMPK in cell cycle progression (Bettencourt-Dias et al., 2004; Alessi et al., 2006; Koh and Chung, 2007; Williams and Brenman, 2008). The translocation of AMPK throughout maturation suggests a dynamic process at work. The poleward transport of active AMPK might be dependent on the minus-end directed motor protein, dynein, which has been shown to be critical for spindle pole transport of proteins such as NuMA and Eg5 (Merdes et al., 2000; Uteng et al., 2008).

Since AMPK shows a close association with microtubule containing structures, it is not surprising that perturbation of microtubules dramatically affected the localization of AMPK within the oocyte. Nocodazole destruction of spindle structure led to dispersal of active AMPK in a punctate staining pattern that coincided with staining of microtubule asters, indicating that the localization pattern of active AMPK is dependent on the integrity of the meiotic spindle and presence of microtubule organizing centers (MTOCs). Mouse oocytes have multiple acentriolar MTOCs that can be grouped into two subsets: one is associated with the meiotic spindle and the other is cortical (Maro et al., 1985;

Schatten et al., 1986; Messinger and Albertini, 1991). Active AMPK was associated with both types of MTOCs. Active AMPK is more concentrated around MTOCs that is associated with spindle, since stabilization of microtubules with paclitaxel treatment induced further accumulation of AMPK at MTOCs and enlarged spindles.

As discussed in the previous chapter, the upstream kinase of AMPK, LKB1, is implicated in establishing the cell polarity in numerous types of cells including mouse oocytes (Zhang et al., 2008; Mirouse et al., 2007; Bonaccorsi et al., 2007; Szczepańska and Maleszewski, 2005). Thus, it was of interest to test whether AMPK is also involved in a similar capacity in mouse oocytes. In oocytes, the spindle forms at the center of the oocyte after GVB, and then migrates toward the oocyte periphery where the PB is extruded, a process crucial to asymmetric cell division and differentiation of cortex into a cortical granule-free domain (Sun and Schatten, 2006).

Inhibiting AMPK activity in spontaneously maturing CEO with compound C did not perturb the peripheral movement of the spindle. Though some recent studies also suggest the participation of microtubules (Ai et al., 2008), spindle movement in the oocyte is known to be highly dependent on a dense actin microfilament meshwork (Azoury *et al*, 2008). Our results suggest that interfering with AMPK activity does not affect the integrity of the cortical actin network, because the extensive F actin polymerization in the cortex near the spindle appeared unperturbed by compound C treatment. Myosin regulatory light chain (MRLC) is an important component of myosin II that participates in actomyosin-mediated cytokinesis at the cleavage furrow (Dumont et al., 2007), and becomes phosphorylated in somatic cells in response to AMPK activation (Lee *et al*, 2007). However, it is unclear whether MRLC is a physiological substrate for

AMPK under energy depleted conditions (Bultot et al., 2009). It will be interesting to determine if AMPK influences the interaction of actin and myosin at the time of cytokinesis.

As we discovered in the previous chapter, AMPK activators accelerate the kinetics of the first polar body formation, which is likely due to the positive effect of AMPK activation during the early meiotic events. Here we show that both spontaneous GVB and the meiotic spindle formation rates are increased by AICAR treatment, which indicate that AMPK manifests an early effect during maturation to shorten the overall maturation time. This is consistent with the previous finding that AICAR is required within 4h after the initiation of the culture to promote later polar body formation. Although we observed a positive effect of AICAR treatment on spindle formation, it is likely simply due to the accelerated kinetics of GVB.

Numerous proteins exhibit a similar localization pattern and have important roles in spindle organization and function. For instance, aurora-A kinase induces GVB in Xenopus oocyte and has to be dephosphorylated for the MI-MII transition (Ma et al., 2003). In mouse oocytes, aurora-A is critical for spindle assembly, centrosome maturation and chromosome segregation. Furthermore, its role is not limited to oocyte maturation, as it also contributes to early embryo spindle organization (Yao et al., 2004; Ding et al., 2011). Another aurora kinase family member, Aurora-B, is one of the chromosome passenger proteins and specifically localizes to the metaphase chromosomes, where it mediates chromosome segregation and spindle kinetochore attachment (Uzbekova et al., 2008; Shuda et al., 2009). Aurora C kinase localization is similar to that of active AMPK; moreover, injection of deficient kinase induced failure of

cytokinesis and disrupted proper localization of kinetochore proteins, BubR1 and Bub (Yang et al., 2010). Astrin is associated with meiotic spindle microtubules with particular concentration at the spindle poles, and perturbation of its function greatly compromises normal spindle integrity and chromosome segregation (Yuan et al., 2009). The apoptosis inhibitor protein, survivin, mimics AMPK localization in mouse oocytes, and its depletion adversely affects chromosome alignment and alters normal polar body extrusion (Sun et al., 2009). Another similarly localized kinase, Polo-like kinase-1 (Plk1), was implicated in spindle assembly in oocytes and later mitosis in the embryo after fertilization (Wianny et al., 1998; Tong et al., 2002). In addition to regulating spindle organization, Plk1 also promotes M phase entry by participating in the MPF amplification loop (Karaiskou et al., 2004), and blocking its activity with antibody reduces the GVB rate (Tong et al., 2002). Of particular relevance to the present study is the recent finding that pharmacological inhibition of Plk1 blocks AMPK activation and cytokinesis in HeLa cells (Vazquez-Martin et al., 2011). Another kinase, protein kinase C delta (PKCδ), associates with γ-tubulin and pericentrin, and disruption of its function leads to spindle disorganization and misalignment of chromosomes (Ma and Koch, 2008). PKC has also been shown to be involved in the MI-to-MII transition (Viveiros et al., 2001). Finally, phospho-MEK1/2, the upstream kinase for MAPK activation, displays a prominent presence at the spindle poles in mouse oocytes, and blocking MAPK leads to meiotic spindle abnormalities and poor chromatin condensation (Sun et al., 2008). It is plausible that AMPK interacts with one or more of these proteins to influence meiotic progression. An alternate scenario is that the kinase docks at MTOCs without contributing to spindle assembly or function.

In conclusion, both AMPK localization and activity in the mouse oocyte are dependent on intact microtubules. Further, while perturbation of microtubule integrity blocks meiotic induction at the level of the oocyte, it has no effect on spontaneous maturation. This demonstrates that additional microtubule-sensitive modalities are required to overcome induction of maturation in meiotically arrested oocytes. Once isolated from follicles, oocytes undergo spontaneous maturation due to the loss of cGMP within oocytes. However, it is suggested that an additional unknown stimulus is generated for meiotic induction. This was supported by the fact that the frequency of the GVB in CEO after FSH stimulation was greater than the control arrested DO. If the effect of FSH on GVB is simply mediated by uncoupling of the somatic compartment, one should expect to see the same level of GVB as DO (Downs et al., 1988). It is possible that this additional component is sensitive to microtubule perturbants. Altogether, our data lend further support to the idea that these are distinct processes.

CHAPTER III: SUPPRESSION OF CHEMICALLY INDUCED AND SPONTANEOUS MOUSE OOCYTE ACTIVATION BY AMP-ACTIVATED PROTEIN KINASE

Summary

Oocyte activation is an important process triggered by sperm fertilization that initiates embryonic development. However, parthenogenetic activation can occur either spontaneously or with chemical treatments. The LT/Sv mouse strain is genetically predisposed to spontaneous activation. LT/Sv oocytes have a cell cycle defect and are ovulated at the metaphase I stage instead of metaphase II. A thorough understanding of the female meiosis defects in this strain remains elusive. We have shown in chapter I that AMPK has an important role in stimulating meiotic resumption and promoting completion of meiosis I while suppressing premature parthenogenetic activation. Here we show that early activation of AMPK during the oocyte maturation period blocked chemically induced activation in B6SJL oocytes and spontaneous activation in LT/SvEiJ oocytes. This inhibitory effect was associated with high levels of MAPK 1/3 activity. Furthermore, stimulation of AMPK partially rescued the meiotic defects of LT/SvEiJ mouse oocytes in concert with correction of abnormal spindle pole localization of AMPK and prolonged spindle assembly checkpoint activity. Altogether, these results confirm a role for AMPK in sustaining the MII arrest in mature oocytes and suggest that dysfunctional AMPK contributes to meiotic defects in LT/Sv oocytes.

Introduction

Mammalian oocytes initiate meiosis during embryonic development and become arrested at the dictyate stage of prophase I around the time of birth. Following a mid-cycle gonadotropin stimulus, oocytes within preovulatory follicles resume meiosis, complete maturation and are ovulated at the metaphase II (MII) stage, where they remain arrested until fertilization. Germinal vesicle breakdown is driven by M-phase promoting factor (MPF), a dimer containing catalytic CDK1 and regulatory cyclin B1 subunits; MPF activity drops during anaphase onset, then rises and stays elevated during MII arrest (Jones, 2004). Binding of sperm triggers activation of the mature ovum, a series of events that includes calcium oscillations, completion of meiosis II, release of cortical granules, and initiation of embryonic development (Horner and Wolfner, 2008; Wakai et al., 2011). Calcium signaling is a universal component of fertilization in the animal kingdom (Stricker, 1999); in vertebrates, calcium oscillations are an essential feature of egg activation and trigger release from MII arrest (Malcuit et al., 2006; Ducibella and Fissore, 2008). Cytostatic factor (CSF) is important for maintaining metaphase II arrest, which involves stabilization of MPF by the c-Mos/MAPK1/3 pathway and suppression of the anaphase promoting complex (APC) by early mitotic inhibitor 2 (Emi2) (Madgwick and Jones, 2007). Interrupting the c-Mos/MAPK1/3 pathway or mutation of *mos* leads to loss of MPF activity and premature egg activation (Colledge et al., 1994; Phillips et al., 2002; Hashimoto et al., 1994); in addition, knockdown of Emi2 relieves the inhibition of APC that allows degradation of cyclin B and drives eggs into anaphase (Shoji et al., 2006; Madgwick et al., 2006), mimicking the effect of sperm-induced calcium oscillations (Nixon et al., 2002; Suzuki et al., 2010).

Oocyte aging, chemical treatment, and the intrinsic quality of the oocyte are factors that can lead to mouse egg activation in the absence of sperm (Kaufman, 1983). It is well known that freshly ovulated oocytes are resistant to activation but become prone to activation with increased age and tenure in the oviducts (Marston and Chang, 1964; Kubiak, 1989; Xu et al., 1997). Chemical treatments such as calcium ionophore (Nakagawa et al., 2001), phorbol ester (Cuthbertson and Cobbold, 1985; Sun et al., 1999), protein synthesis inhibitors (Siracusa et al., 1978; Fulka Jr et al., 1994), ethanol (Kaufman, 1982; Dyban and Khozhai, 1980; Cuthbertson, 1983) and heavy metals (O'Neill et al., 1991; Ma et al., 2005) are well characterized inducers of parthenogenesis in oocytes matured both *in vivo* and *in vitro*. There are strain-dependent differences in oocyte susceptibility to spontaneous activation (Cheng et al., 2012), while altering metabolism or length of hormonal priming can induce parthenogenesis in strains that normally exhibit very low rates of activation (Downs, 1990; Downs et al., 2010).

The LT/Sv strain is an inbred recombinant mouse line derived from C58 and BALB strains whose oocytes fail to complete meiosis, leading to arrest at metaphase I (Kaufman and Howlett, 1986; Maleszewski and Yanagimachi, 1995; Ciemerych and Kubiak, 1998), due to persistent MPF activity and a poor anaphase I trigger (Hirao and Eppig, 1997, 1999). This MI arrest is associated with premature activation of oocytes within the ovary and a high incidence of ovarian teratomas (Stevens and Varnum, 1973; Eppig et al., 1977; Artzt et al., 1987). The parthenotes advance to the egg cylinder stage but further development is disrupted, with formation of a disorganized mass of differentiated cell types (Stevens and Varnum, 1973). A significant number of LT/Sv oocytes are ovulated at the metaphase I stage, and this arrest appears necessary, but not

sufficient, to induce parthenogenetic activation (Eppig et al., 1996; Everett et al., 2004). Apparently the protracted MI arrest brings about changes within the oocyte that predisposes it to activation. The exact mutation(s) of the LT/Sv mouse remains unknown, but evidence suggests that the phenotype of LT/Sv oocytes is controlled by multiple alleles (Artzt et al., 1987; Eppig et al., 1996).

In this series of experiments we further examined the relationship between AMPK and oocyte activation. We report that AMPK activators suppress both chemically induced activation in oocytes from C57BL/6J×SJL F_1 (B6SJL) mice and spontaneous activation in oocytes from LT/Sv mice during maturation *in vitro*, but are less effective in ovulated B6SJL oocytes. This protection by AMPK was manifest during the oocyte maturation period and was associated with high levels of MAPK1/3 activity and the accelerated loss of the spindle assembly checkpoint protein, MAD2. Stimulation of AMPK in LT/Sv oocytes rescued the "LT phenotype" by reducing the rate of parthenogenetic activation and promoting anaphase onset and PB formation. These data support a multifunctional role of AMPK in mouse oocytes and suggest that AMPK dysfunction may contribute to meiotic defects in LT/Sv oocytes.

Materials and Methods

Chemicals

Unless otherwise noted, chemicals were supplied by Sigma-Aldrich Co. (St. Louis, MO). AICAR was from Toronto Research Chemicals, Inc. (North York, Ontario, Canada) and amphiregulin from R&D Systems (Minneapolis, MN). Highly purified ovine FSH was obtained from the National Hormone and Peptide Program (NHPP) and Dr. A.F. Parlow. Cy3-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmmoResearch (West Grove, PA), Anti-phospho AMPK (PT172) antibody from Cell Signaling Technology (Beverly, MA), and rabbit anti-ERK1/2 and pERK1/2 (MAPK1/3) antibodies from Sigma Chemical Co. Anti-MAD2 antibody was a kind gift from Dr. E.D. Salmon (Univ. North Carolina).

Oocyte Isolation and Culture Condition

All procedures were approved by the Marquette University Institutional Animal Care and Use Committee. Immature, 20-23-day-old C57BL/6J× SJL/J F_1 (B6SJL) and LT/SvEiJ female mice were used for all experiments. Mice were primed with 5 IU pregnant mare serum gonadotropin (PMSG) and oocytes were isolated 2 d later. For superovulation, 5 IU human gonadotropin (hCG) was administered IP 44-48 h post-PSMG and oocytes were isolated at varying times thereafter. Mice were killed by cervical dislocation and either ovaries or oviducts were removed and placed in a petri dish containing culture medium. Cumulus cell-enclosed oocytes (CEO) were isolated by puncturing the follicles with sterile needles. Denuded oocytes (DO) were obtained by stripping cumulus cells with a mouth operated small-bore pipette. Oviducts were

punctured with sterile needles to release the expanded cumulus masses, which were then treated briefly with hyaluronidase to remove cumulus cells.

Both CEOs and DOs were washed twice and transferred to plastic culture tubes containing 1ml of the appropriate test medium. The culture medium used was Eagle minimum essential medium (MEM) supplemented with penicillin, streptomycin sulfate, 0.23 mM sodium pyruvate and 3 mg/ml lyophilized crystallized bovine serum albumin (MP Biomedicals, Solon, OH). Calcium was omitted from the medium when activation was stimulated with strontium or calcium ionophore A23187.

Chemical Activation

Figure 1 depicts the three activation schemes used in this study. Spontaneous maturation system was used in this research. CEOs or DOs were cultured 17 h in control MEM prior to treatment with chemical activators. Cumulus cells were removed from CEOs at the end of this initial culture period so that only DOs received the activation stimulus. The three activation regimens are as follows:

1. *Strontium*. 2.5 h in calcium-free MEM containing 1 mM strontium, wash and 3.5 h culture in control MEM.

2. *A23187/puromycin*. 5 min in control MEM containing 5 μM calcium ionophore A23187, wash and 6 h culture in control MEM containing 10μg/ml puromycin.

3. *Ethanol*. 7 min in control MEM containing 7% EtOH, wash and 6 h culture in control MEM.

CEOs or DOs were exposed to AMPK activators (0.2 mM AICAR, 2mM AMP, or 2 μM RSVA405) during meiotic maturation (pre-maturation) or during/after the activation procedure (post-maturation). CEOs were hormonally stimulated with 0.1 μg/ml FSH or 50 μg/ml amphiregulin during meiotic maturation. Oocytes were considered to be activated if they contained a pronucleus, two polar bodies with or without pronucleus, or had undergone cleavage.

Immunofluorescent Staining

Cumulus cell-free oocytes were fixed with 4% formaldehyde in PBS at 4°C overnight. They were then permeabilized 30 min with 0.1% triton-100 in blocking solution, which contained 10% donkey serum and 0.5 mg/ml saponin in PBS, followed by 1h in blocking solution. Oocytes were incubated with primary antibody (1:100) overnight at 4°C (either rabbit anti-pPRKA^{Thr172} or rabbit anti-MAD2 antibody), and washed 4 times at room temperature in blocking buffer. This was followed by 1 h incubation with FITC-conjugated mouse anti-tubulin antibody (1:100) and Cy3-conjugated donkey anti-rabbit IgG antibody (1:100) at room temperature. Some oocytes were stained for actin using Alexa 568 Fluor phalloidin (Life Technologies, Grand Island, NY) during the secondary antibody culture period. Oocytes were washed four times, placed on slides and mounted with medium containing DAPI (Vector Laboratories, Burlingame, CA).

Confocal Microscopy

Oocytes were observed on a laser scanning confocal microscope (Carl Zeiss Co., Thornwood, NY) with a 63X objective. During scanning, all settings were kept constant:

i.e., laser power, detector gain, amplifier offset, amplifier gain, and pinhole size. Digitally recorded images were exported by LSM Examiner software (Carl Zeiss Co.).

Western Blot Analysis

Oocyte samples were washed with PBS/PVP, then twice with protease inhibitor cocktail (Roche, Indianapolis, IN). Samples were lysed by Laemmli's buffer with 20% beta-mercaptoethanol at 95°C for 5 minutes. Electrophoresis was carried out using NuPAGE 4-12% Bis-Tris Gels (InVitrogen, Carlsbad, CA), and proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk, followed by incubation with rabbit anti-pERK1/2 (pMAPK1/3) antibody (1:1000) at 4°C overnight. Blots were rinsed twice with TBS (pH 7.4) and once with TBS-Tween-20 (0.05%), followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody (1:5000 in 5% non-fat milk; Thermo Scientific Pierce, Rockford, IL) at room temperature for 1 h. After washing, protein signals were detected by super signal west pico chemiluminescent substrate (Thermo Scientific Pierce). Blots were stripped with restore western blot stripping buffer (Thermo Scientific Pierce) at room temperature for 15 min and reprobed with rabbit anti-MAPK1/3 antibody (1:1000). pMAPK1/3:MAPK1/3 ratios were quantified with ImageJ software based on protein band density.

Statistical Analysis

All experiments were repeated at least three times and data presented as mean ± SEM. Percentages of activation, polar body, or MAD2 staining underwent arcsin transformation and data were analyzed statistically by ANOVA followed by Duncan's

multiple range test. Students T-test was used to compare paired treatments in some experiments. A *P*-value <0.05 was considered significant.

Results

Effects of AMPK activators on chemically induced activation in B6SJL oocytes

We have previously shown that the AMPK activator, AICAR, prevents premature activation in spontaneously activating oocytes from B6SJL mice when present during meiotic maturation (Downs et al., 2010). To test if AMPK stimulation would also block activation induced by chemical treatments, we adopted three different protocols depending on the agent(s) used to stimulate activation: strontium, A23187/puromycin or ethanol. For all three treatments, CEOs or DOs were cultured 17 h in control MEM to allow for meiotic maturation. Remaining cumulus cells were then removed from CEOs, and these cumulus cell-free oocytes as well as the mature DOs were exposed to chemical activating conditions (see Figure 3.1).

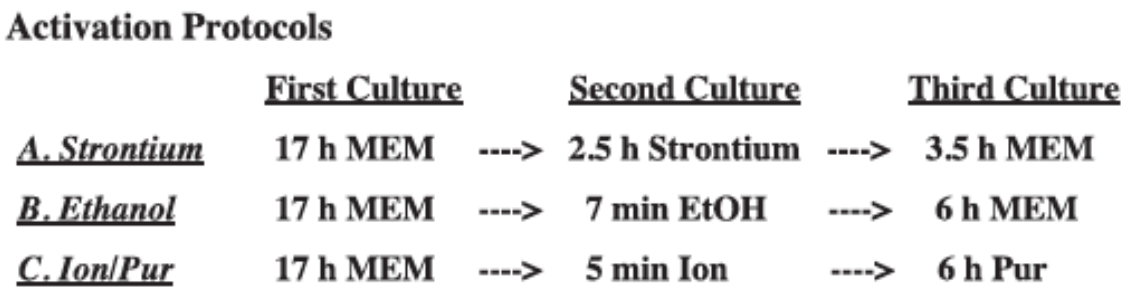

Figure 3. 1 Schematic diagram of activation protocols

For all treatment regimens, CEOs or DOs were first cultured 17 h in control medium to permit completion of spontaneous maturation. When appropriate, cumulus cells were then removed from CEOs, and DOs were exposed to chemical activating conditions. (A). Oocytes were treated with strontium in calcium-free MEM for 2.5 h, washed and then transferred to control MEM for 3.5 h. (B). Oocytes were exposed to 7% ethanol for 7 min, washed and then transferred to control MEM for 6 h. (C). Oocytes were treated with A23187 for 5 min, washed and then transferred to puromycin for 6 h.

Strontium-induced activation. The first series of experiments utilized the activating agent strontium, and cultures were carried out in calcium-free medium, since the absence of calcium has previously been demonstrated to augment activation by this agent (Whittingham and Siracusa, 1978). After removing cumulus cells from matured CEO, oocytes were exposed to 1 mM strontium in calcium-free MEM for 2.5 h, followed by 3.5 h incubation in the calcium-containing medium. In control oocytes not exposed to strontium, only 4% were activated compared to 70% activation in strontium-treated oocytes (Figure. 3.2A). Supplementation of AICAR during the initial maturation period significantly reduced this number to 15%. Interestingly, later addition of AICAR only showed a modest reduction of activation (57%, Figure 3.2A). In the next experiment, we tested the direct effects of AICAR on strontium activation of matured DOs, utilizing the

same protocol that was used with CEOs. In the absence of cumulus cells, no activation was observed in oocytes that were only exposed to calcium-free medium, but strontium treatment only induced 34.8% of DOs to activate. AICAR blocked strontium-induced activation, and the effect of exposure during meiotic maturation was comparable to that when exposure followed maturation (Figure. 3.2A).

When the experiment was repeated with native AMP, similar results were obtained. Exposure to AMP during first culture period significantly reduced the activation rate in CEOs from 71% to 23% (compared to 4% in controls), whereas exposure to AMP post-activation did not prevent the activation (Figure. 3.2B). When the experiment was repeated in DOs, a similar suppression of activation was obtained if AMP treatment occurred during the maturation period; however, surprisingly, treatment post-maturation with AMP increased the level of activation (Figure. 3.2B).

We repeated the experiment using a newly described resveratrol analog, RSVA405(Vingtdeux et al., 2011), which activates AMPK like AICAR, and was proven to be effective in promoting GVB and PB formation in the mouse oocytes (data not shown). Consistent with the AICAR and AMP results, RSVA405 suppressed strontium-induced activation in both CEOs and DOs only when present during the initial maturation period (Figure 3.2C).

FSH and the EGF-like peptide, amphiregulin (AR), are both potent stimulators of meiotic maturation of mouse CEOs *in vitro*, with downstream activation of oocyte AMPK preceding GVB (Chen and Downs, 2008). We predicted that, because of their AMPK-stimulating capability, treatment of CEOs with either of these peptides would

block strontium-induced activation. For this experiment, FSH and AR were present only during the initial maturation period. These results are shown in Figure. 3.2D. As expected, FSH and AR significantly reduced the activation rate from 77% to 38% and 33%, respectively.

Since the data suggest that AMPK activation during the maturation period is important to prevent later strontium-induced activation, an experiment was carried out to determine the window of requirement for AMPK activation to achieve protective effect by delaying AICAR treatment by 0,6 or 8 h (Figure 3.2E). There was no loss of protection when AICAR treatment was delayed by 6 h; however, AICAR started losing its protective effect when the exposure was delayed by 8 h. This suggests that maximum protection requires exposure within 6h of the initiation of culture, a time point corresponding to the early spindle formation.

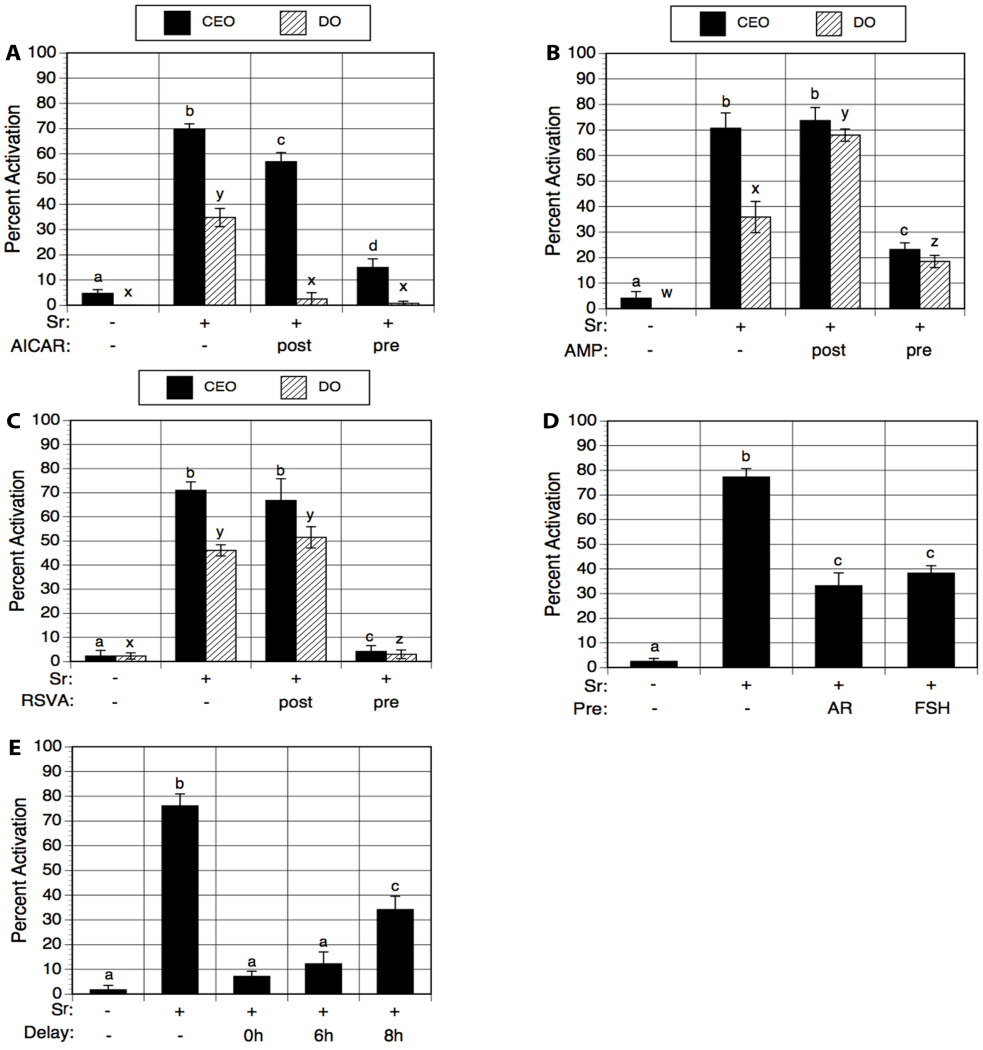

Figure 3. 2 Effect of AMPK stimulation and hormones on strontium-induced activation

Treatment with AICAR (A), AMP (B) or RSVA405 (C) during oocyte maturation (pre-maturation) prevented strontium-induced activation in both CEO and DO, but supplementation after the 17-h maturation period (post-maturation) was relatively ineffective. (D). Amphiregulin (AR) or FSH suppressed strontium-induced activation in CEO when present during the initial maturation period. (E). Effect of delaying AICAR treatment on strontium-induced activation. The inhibitory effect of AICAR on activation in CEO was decreased when AICAR treatment was initiated 8 h, but not 6 h after the start of culture. All data are presented as mean percent activated ± SEM. Groups with no common letter are significantly different (CEO: a-d; DO, w-z).

Since the cMos-MAPK1/3 pathway is important for maintaining MII arrest (Colledge et al., 1994; Hashimoto et al., 1994; Phillips et al., 2002), we tested the effect of AMPK activation in CEO on MAPK1/3 activity following strontium activation. Oocytes were collected for western blot at 17 h (after maturation), 19.5 h (post-strontium treatment) and 23 h (after final recovery). As shown in Figure 3.3, control oocytes that were treated with calcium-free medium alone maintained a high level of MAPK1/3 activity at all three time points. In contrast, oocytes that were treated with strontium exhibited decreased MAPK1/3 activity after the second and third culture periods. Consistent with their preventive effect on activation, both AMP- and AICAR-treated oocytes maintained high MAPK1/3 activity at all time points tested.

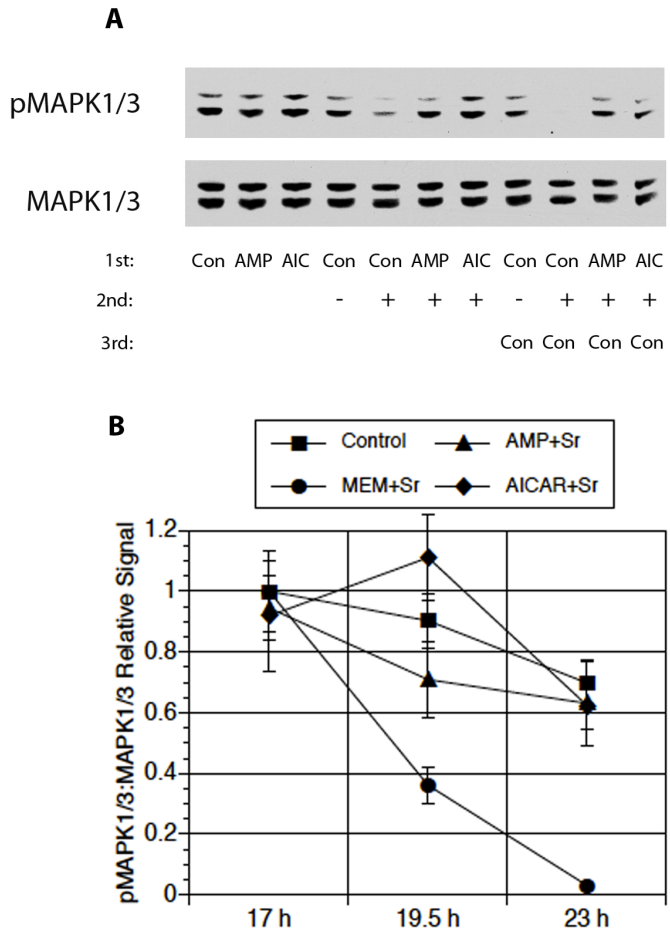

Figure 3. 3 Maintenance of MAPK1/3 activity in oocyte treated with AICAR(AIC) or AMP during maturation

(A). Representative western blot analysis of p-MAPK1/3 in oocytes treated as described in Figure. 1A. Denuded oocyte extracts were subjected to western blot analysis at the end of each of the three treatment phases. +/- in the 2nd culture indicates the presence or absence of strontium. Con, control. (B). Quantitative analysis of pMAPK1/3 signals from western blot, presented as a ratio of the band intensity of pMAPK1/3 to that of non-phosphorylated MAPK1/3. Mean ± SEM of three blots. Treatments with either AICAR or AMP maintained high pMAPK1/3 levels that were comparable to the control group. Data are presented as mean ratio ± SEM.

A23187/Puromycin-Induced Activation. CEOs were used to test the ability of AICAR to reverse the activation resulting from sequential treatment of calcium ionophore A23187 followed by the protein synthesis inhibitor, puromycin. Mature oocytes were cultured 5 min in 5 μM calcium ionophore A23187 followed by 6 h in 10μg/ml puromycin (Figure. 3.1). In the control group treated with ionophore alone, only 12% of the oocytes underwent activation (Figure. 3.4A). When ionophore-treated oocytes were subsequently exposed to puromycin, the activation rate was increased to 66%. If oocytes were exposed to AICAR during puromycin exposure, there was no significant reduction in activation (43%); however, a significant decrease occurred when AICAR was present during the initial maturation period (31% activation; Figure. 3.4A).

Ethanol-Induced Activation. CEOs were used to test the effect of AICAR on ethanol-induced activation. When oocytes were exposed for 7 min to 7% ethanol followed by 6 h culture in control medium (Figure. 1), 78% of the oocytes underwent activation compared to 1% of controls (Figure. 3.4B). If CEOs were treated with AICAR during the initial maturation period, ethanol-induced activation was reduced by 54%, while restricting AICAR treatment to the last 6 h had no effect.

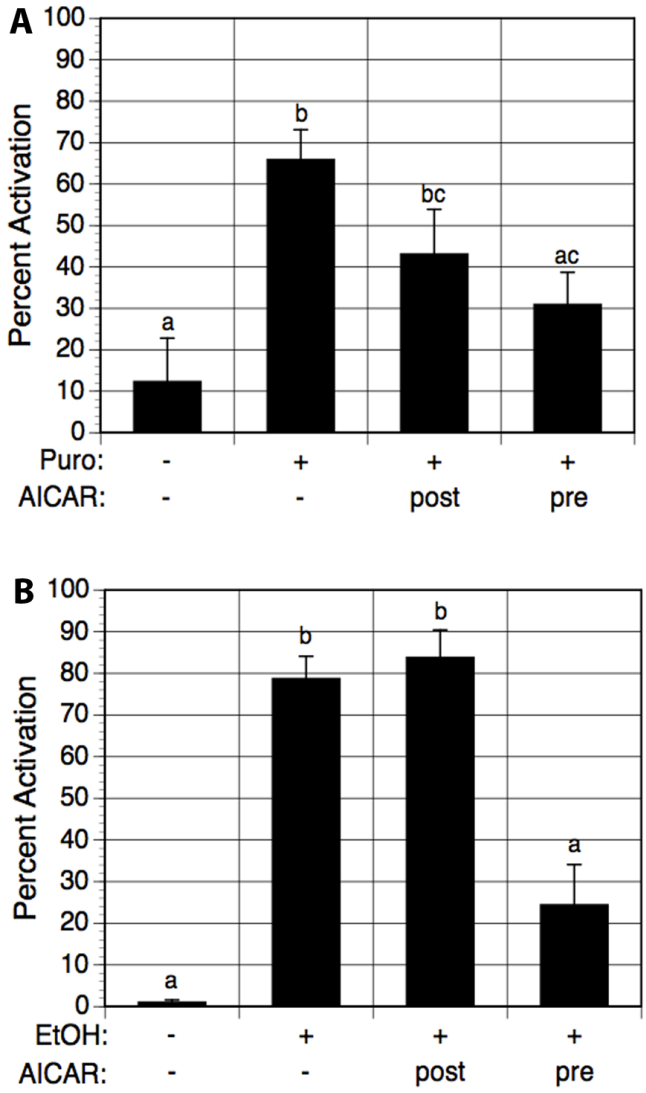

Figure 3. 4 Treatment with AICAR on A23187/puromycin- and ethanol-induced activation

Treatment with AICAR during, but not following, oocyte maturation blocked A23187/puromycin-induced (A) and ethanol-induced (B) activation in CEO. Data are presented as mean percent activated ± SEM. Groups with no common letter are significantly different.

Effect of AMPK activation on in vivo aged B6SJL oocytes

It has been well documented that increased aging of ovulated oocytes within the oviduct increases their susceptibility to spontaneous activation (Marston and Chang, 1964; Kubiak, 1989; Xu et al., 1997). This was confirmed when eggs were retrieved from the oviducts of superovulated B6SJL mice 16, 20 and 24 h post-hCG and cultured 6 h in control medium. The percentage of activation increased in direct relationship to the time post-hCG the eggs were retrieved (2, 12 and 32% activation, respectively); there was a modest trend for suppression of activation at all time points when AICAR was present during 6 h culture (0, 8 and 22% activation, respectively; Figure. 3.5).

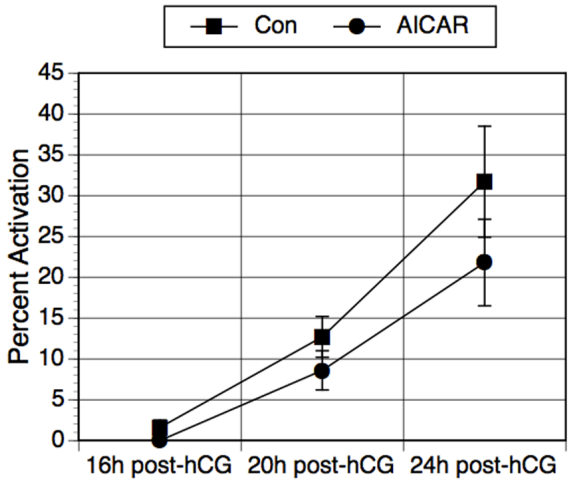

Figure 3. 5 Effect of AMPK on activation of *in vivo* matured oocytes

Oocytes were retrieved from oviducts 16, 20 and 24 h post-hCG and cultured 6 h with or without AICAR. Although there was a trend toward less activation in AICAR-treated oocytes, differences were not significant. Data are presented as mean percent activated ± SEM.

Effects of AMPK activators on oocyte activation in the LT/SvEiJ strain

Oocytes from the LT/Sv mouse strain exhibit a high rate of spontaneous activation (Eppig et al., 1977). It was therefore important to determine if AMPK activators would also suppress this type of oocyte activation. We initially compared spontaneous activation rates in oocytes from B6SJL and LT/SvEiJ (hereafter designated LT) mice following 30 h culture in the presence or absence of AICAR. In the absence of AICAR, activation rates were 2 and 21%, respectively, while AICAR reduced these rates to 0 and 4% (Figure. 3.6A). When LT CEOs were treated with RSVA405, a similar reduction in activation was observed (Figure. 3.6B).

To determine the critical window for AMPK activity required to convey this suppressive effect on LT oocyte activation, oocytes were first cultured in either MEM or

medium containing AICAR for 17 h, then were transferred to MEM or AICAR containing medium for another 13 h before activation was assessed. As shown in Figure 3.6C, LT oocytes showed high levels of activation when they were cultured in control/control and control/AICAR medium. However, the activation rates were significantly reduced when oocytes were cultured in AICAR/AICAR and AICAR/MEM. These results are consistent with earlier experiments in B6SJL oocytes showing a protective effect of AICAR during the initial period of oocyte maturation.

In addition to high rates of spontaneous activation, LT oocytes also fail to progress past metaphase I both *in vivo* and during spontaneous maturation *in vitro* (Kaufman and Howlett, 1986; Hirao and Eppig, 1997; O'Neill and Kaufman, 1987). Thus, we tested whether AMPK stimulation could overcome this meiotic defect. CEO from B6SJL and LT mice were cultured in either control or AICAR-containing medium for 17 h and assessed for polar body formation. In B6SJL oocytes, 53% formed polar bodies in control medium, while AICAR increased this number to 78% (Figure.3. 6D). No polar bodies were observed in LT oocytes cultured in control medium, but 22% of AICAR-treated oocytes extruded a polar body.

When LT oocytes were stained for active AMPK after 17 h culture in control medium, we observed a high incidence of abnormal localization. Instead of staining at both spindle poles, which is the typical staining pattern in B6SJL oocytes, in most LT oocytes the localization was either shifted away from the poles or was absent at one or both of the poles (data not presented). Since LT oocytes are arrested at MI for a prolonged period of time, to eliminate the possibility that this phenotype was due to an aging effect, we stained oocytes for active AMPK after 8 h in both B6SJL and LT strains,

when MI has been freshly established, and compared MI localization patterns between the two strains. Seventy-five percent of B6SJL MI-stage oocytes cultured in control medium exhibited active AMPK at both spindle poles (Figs 3.6E, 3.7A). However, only 28% of LT MI-stage oocytes had similar bipolar staining (Figs. 3.6E, 3.7B,C). Treatment with AICAR had no effect in B6SJL oocytes, but doubled the percentage of LT oocytes with normal AMPK staining (57%).

A comparison of meiotic status in these same oocytes showed that 97% of B6SJL oocytes cultured 8 h in control medium reached prometaphase or metaphase I, with 3% in anaphase (Figure. 3.6F). Similarly, 100% of LT oocytes were at metaphase I or prometaphase. The addition of AICAR accelerated meiotic progression, such that 71% of B6SJL oocytes were at anaphase or beyond after 8 h of culture. In addition, 33% of LT oocytes were at anaphase after exposure to AICAR, which, along with the polar body data above, demonstrates AICAR-induced rescue from metaphase arrest in a significant percentage of the oocytes.

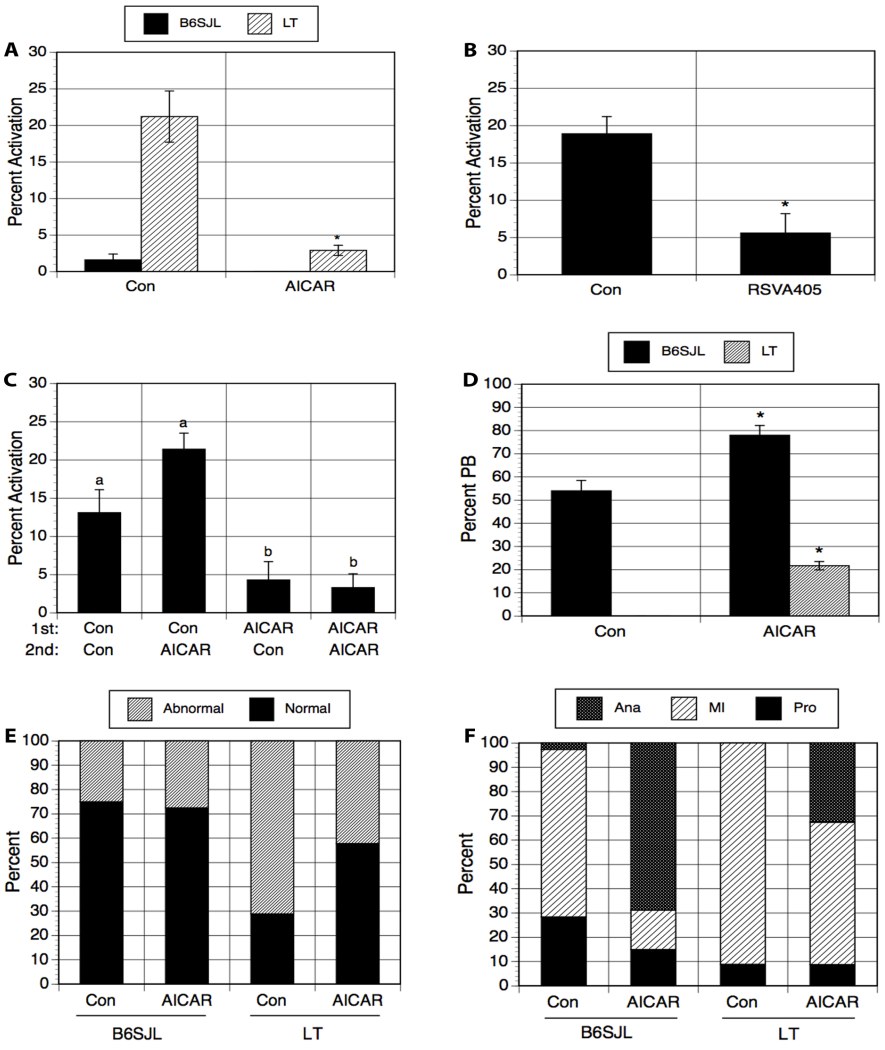

Figure 3. 6 Effects of AMPK stimulation on LT/SvEiJ oocytes

(A). AICAR blocked spontaneous activation in LT oocytes during 30 h cultures. (B). RSVA405 had a similar inhibitory action on LT activation. Asterisks in A and B denote a significant difference from controls (Con). (C). AICAR blocked activation in LT oocytes when present during, but not after, oocyte maturation. LT/Sv oocytes were first cultured in control MEM (Con) or AICAR for 17 h, and then transferred to control or AICAR-supplemented medium for another 13 h before activation was assessed. A different letter denotes a significant difference. (D). AICAR stimulated PB formation in both B6SJL and LT oocytes during 17 h cultures. An asterisk denotes a significant difference from controls (Con). Data in A-D are presented as percent activation or PB ± SEM. (E) and (F). pPRKA (PT172) localization (E) and stage of meiosis (F) in MI stage oocytes after 8 h of culture. AICAR increased the percentage of normal PT172 spindle pole localization in LT oocytes and increased the percentage of anaphase I (Ana) in oocytes from both the B6SJL and LT strains. Pro, prometaphase.

Interestingly, in addition to abnormal active AMPK localization, some AICAR-treated LT oocytes underwent karyokinesis without cytokinesis after 17h of culture. The chromosomes were segregated, and MII spindle was established. Actin filament staining by phalloidin did not detect PB, although the oocytes showed the presence of actin cap, which is the site close to future PB extrusion and with excessive polymerization of actin filaments (Figure 3.7D, E). These data suggest that despite the fact that AMPK stimulation can promote anaphase onset and PB formation, the LT oocytes still manifest the defect in cell cycle progression.

AICAR Promotes the Loss of Chromosomal MAD2 Staining

It has been previously reported that prolonged spindle assembly checkpoint (SAC) activity contributes to MI arrest in LT oocytes (Hupalowska et al., 2008). To test the hypothesis that stimulation of AMPK promotes anaphase onset by overcoming SAC activity, oocytes were stained for MAD2, one of the major components of SAC (Waters et al., 1998). We first determined the kinetics of centromeric MAD2 loss in B6SJL CEO (Figure. 3.7F). Although MAD2 staining intensity was reduced in control oocytes by 6 h, 95% of them still remained positive. Between 6 and 8h the number of MAD2-positive oocytes decreased by 50%. AICAR treatment accelerated the loss of MAD2, with only 61% and 23% positively staining oocytes at 6 and 8h, respectively. In LT CEO, 98% stained positive for MAD2 after 8 h of culture, whereas in the AICAR-treated group, it was reduced to 41% (Figure 3.7G, H, I).

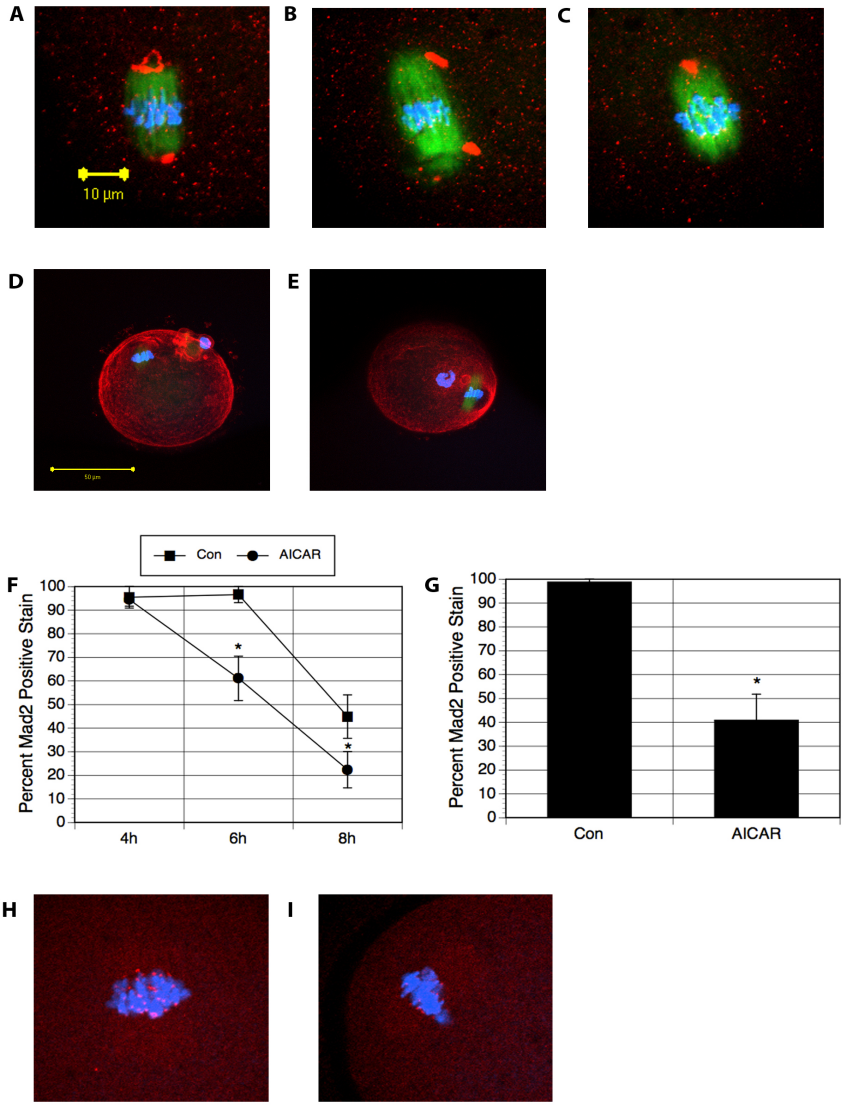

Figure 3. 7 Immunofluoresecent staining in LT oocytes

(A-C). Immunofluorescent staining of active AMPK in LT oocytes. (A). Normal spindle pole localization of active AMPK. (B). Shifted spindle pole localization. (C). Missing spindle pole localization. Green, a-tubulin; Red, pPRKA^{Thr172}; Blue, DAPI. (D) and (E). 3D projection of LT oocytes stained with actin (red) and tubulin (green). Representative staining of oocytes with (D) and without PB (E). (F-I). AICAR treatment accelerated loss of MAD2 in kinetochores. (F). Kinetics of centromeric MAD2 loss in B6SJL oocytes in the presence or absence of AICAR. (G). AICAR treatment eliminated prolonged MAD2 kinetochore localization in LT oocytes. The data in (F) and (G) are presented as percent positive MAD2 stain± SEM; the asterisk denotes a significant difference from –AICAR controls. (H) and (I). MAD2 stain in LT oocytes that were cultured 8 h in control (H) or AICAR-supplemented (I) medium. Red, MAD2; Blue, DAPI.

Discussion

In this study, we demonstrate the suppressive effects of AMPK activators on parthenogenetic activation in mouse oocytes. Stimulation with the AMPK activators AICAR, AMP or RSVA405 during oocyte maturation prevented both chemically induced activation in B6SJL mice and spontaneous activation in LT mice, while exposure of oocytes to these agents after maturation had little effect on either type of activation. Stimulating AMPK in ovulated, *in vivo*-matured B6SJL eggs was relatively ineffective in preventing activation, likely due to the fact that the eggs were already mature at the time of treatment. Additional effects of AMPK activation on LT oocytes included changes in active AMPK localization, release from metaphase arrest and a reduction in spindle checkpoint activity.

Treatment of mouse oocytes with strontium produces repetitive calcium oscillations that mimic the effect of sperm binding, making it one of the most effective chemical activating agents (Whittingham and Siracusa, 1978; Bos-Mikich et al., 1995; Alberio et al., 2001). Nevertheless, exposure to AICAR, AMP or RSVA405 was sufficient to block strontium-induced activation in both CEO and DO, indicating a direct action on the oocyte. Twice as many oocytes were activated in CEO compared to DO, confirming that the presence of cumulus cells during the maturation period promotes activation (Eppig, 1982; Eppig et al., 2000). The protection by AMP and AICAR was associated with maintenance of MAPK1/3 phosphorylation, which suggests AMPK may prevent activation by blocking dephosphorylation of MAPK1/3 and keeping MPF activity high (Madgwick and Jones, 2007). At this point, we are not sure how this interaction between AMPK and MAPK happens. Inappropriate activation of the cMos/MAPK1/3

pathway in Xenopus oocytes can induce a metaphase cell cycle arrest (MacNicol et al., 1995; Daar et al., 1991), while treating oocytes with inhibitors of MAP2K1 (MEK1), the immediate upstream MAPK1/3 kinase, alleviates metaphase arrest and stimulates activation (Hashimoto et al., 1994; Tong et al., 2003). Because treatment with AMPK activator and strontium were temporally separated, it is possible that this action of AMPK may reflect an indirect upstream effect, preventing an activation stimulus that acts on the MAPK1/3 pathway. Strontium-induced oocyte activation involves the interaction of phospholipase and InsP3 receptor (Zhang et al., 2005) and it is possible that AMPK activation also interferes with these processes (see also Ross et al., 2008).

Calcium ionophore A23187 only produces a single calcium spike. A23187 and protein synthesis inhibitors alone each modestly stimulate oocyte activation, but when oocytes are treated sequentially with these two agents, synergistic activation is observed (Bos-Mikich et al., 1995; Nakasaka et al., 2000; Nakagawa et al., 2001; Lu et al., 2006). Brief exposure to ethanol is also an effective trigger for parthenogenetic activation, likewise acting through simple calcium transients (Alberio et al., 2001; Cuthbertson et al., 1981; Winston et al., 1995). Nevertheless, both of these treatments produced activation frequencies comparable to those achieved with strontium. More importantly, AMPK activators suppressed both A23187/puromycin- and ethanol-induced activation with a potency resembling that for strontium-treated oocytes. These results are important because they demonstrate a consistent activation-suppressing response to AMPK stimulators regardless of the type of chemical activating stimulus.

Hormonal stimulation was protective for oocytes against chemically induced activation. This protective effect might be mediated by AMPK, since we have previously

demonstrated that these ligands activate the AMPK pathway within the oocytes (Chen and Downs, 2008). However, it should be noted that the strontium- induced activation rate in FSH-treated CEOs was reduced to the level of DO. It is also possible that hormone-induced uncoupling of the oocytes from cumulus cells decreases the pro-activation effect of cumulus cells (Eppig, 1982), thereby rendering it a physiologically denuded oocyte that is less sensitive to strontium treatment.

AMPK activation during oocyte maturation was essential for optimal protection from chemically induced activation; later treatment with AMPK activators during either the activation or recovery periods was largely ineffective. We observed this relationship consistently for CEOs. However, for DOs, while the exposure of AMPK stimulators during the maturation period was always inhibitory to activation, the results for recovery period treatments varied among the groups: RSVA405 had no effect, AICAR was inhibitory, and, curiously, AMP was stimulatory. This may be due to the fact that the potency of the activators are different between CEOs and DOs, or in the case of AMP, there may be an additional effect on the oocyte. This early requirement for AMPK activation for preventing activation is consistent with our *in vivo* oocyte maturation data; AICAR was less effective in preventing spontaneous activation when oocytes were already matured at the time of the treatment. Further, the AICAR timing experiments showed that treatment with kinase activator could be delayed up to 6 h after the initiation of culture and still achieve full protection against activation, a time point that roughly corresponds to metaphase spindle formation. After 6 h, physiological changes begin to manifest in the oocyte that make it more susceptible to activating conditions that AMPK activators cannot overcome. Interestingly, this temporal sensitivity is different than that

for AMPK promotion of PB formation, which requires earlier exposure to AMPK activators, around the time of GVB (less than 4h) (Downs et al., 2010). Thus, AMPK not only induces initiation of meiotic resumption and promotes PB, but also suppresses oocyte activation, though the data suggest its action is required at different time points during the maturation period to achieve these effects.

Most LT oocytes have a deficit in cell cycle progression, arresting and ovulating as metaphase I stage as opposed to metaphase II (Kaufman and Howlett, 1986; Maleszewski and Yanagimachi, 1995; Ciemerych and Kubiak, 1998), and this metaphase I arrest is associated with the spontaneous activation phenotype (Eppig et al., 1996), though the exact link is obscure. Exposure of AMPK activators during the initial maturation period not only prevented chemically induced maturation in B6SJL, but also inhibited the spontaneous activation of LT oocytes. Based on the Western blot analysis of MAPK1/3 activity in B6SJL, it is reasonable to propose that AMPK also acts as the same way in preventing LT oocyte activation. Hirao and Eppig suggested a role for CSF in the prolonged metaphase I arrest in LT oocytes based on the fact that high levels of MAPK1/3 activity is maintained in the LT oocytes (Hirao and Eppig, 1997, 1999; Maleszewski and Yanagimachi, 1995); however, in wild type oocytes high CSF activity did not initiate the metaphase I arrest, but only delayed the metaphase-to-anaphase transition, which suggested that CSF is not the primary cause of metaphase I arrest in this strain, though it may help maintain it.

If the metaphase I arrest in LT oocytes is, indeed, linked to parthenogenesis, then it is not surprising that AMPK also stimulated meiotic progression past this arrest point. Recently, it was reported that prolonged spindle assembly checkpoint (SAC) activity is

involved in the metaphase I arrest of the LT oocytes (Hupalowska et al., 2008; Maciejewska et al., 2009). To further address possible downstream mediators of AMPK, we investigated whether AMPK altered spindle assembly checkpoint dynamics. SAC is a surveillance mechanism that monitors the integrity of the mitotic spindle and proper attachment of chromosomes to spindle microtubules and controls activation of the anaphase-promoting complex (APC) (Chen et al., 1996; Li and Benezra, 1996; Waters et al., 1999). The same surveillance mechanism also exists in meiotic cells. MAD2 is one of the components of the SAC complex in mouse and rat oocytes that localizes to unattached kinetochores, sequestering the main component of APC, Cdc20, thereby inhibiting APC activity and keeping MPF activity high (Zhang et al., 2004; Wassmann et al., 2003a). MAD2 is required in mouse oocytes for maintaining MI and preventing premature anaphase (Wassmann et al., 2003b; Homer et al., 2005; Wang et al., 2009). Interestingly, as LT oocytes (Hupalowska et al., 2008) or mice (Hoffmann et al., 2012) age, the MI arrest attenuates, coincident with declining SAC activity. We have shown herein that AICAR activation stimulated the loss of chromatin MAD2 staining, in concert with promoting anaphase onset. It is therefore likely that AMPK activator-induced loss of MAD2 kinetochore binding contributes to both its acceleration of meiosis in B6SJL oocytes and rescue of LT oocytes from MI arrest and activation. The exact mechanism on how AMPK regulates MAD2 dynamics remains to be determined.

Ciemerych and Kubiak proposed the major cause for LT phenotype is due to a lack of an appropriate anaphase I trigger (Ciemerych and Kubiak, 1998). In our study, activating AMPK provided such a trigger by altering the kinetochore binding dynamics of MAD2, presumably by affecting SAC activity. Our data suggest that completion of

maturation driven by AMPK stimulation has a protective role on premature activation. This is also consistent with the previous compound C data from Chapter I, which blocked oocyte maturation at metaphase I and induced oocyte activation. In fact, upon close observation, treating LT oocytes with AICAR reduced activation by 18% (Figure. 3.6A), which was offset by the number of oocytes that progress to MII (21%; Figure. 3.6D). Hence, it is tempting to speculate that the LT oocytes that would have activated in the absence of AICAR are those same oocytes stimulated to reach MII in its presence and that the nearly complete suppression of activation is due to meiotic escape from MI. Why the remaining MI-arrested oocytes do not activate is not clear, but it appears that only about one-fifth of the LT oocytes are prone to activation in our culture conditions. Also, it would be interesting to know if AMPK stimulation affects the ploidy of the resulting secondary oocytes, since AICAR treatment accelerated the MAD2 loss from the kinetochore and the AICAR-treated oocytes enter anaphase earlier than the controls.

Curiously, the percentage of AMPK-treated LT oocytes that entered anaphase (33%) was higher than the number extruding a PB (21%). This suggested that karyokinesis occurred in the absence of cytokinesis in a small population of these oocytes. Indeed, when we stained actin filaments with phalloidin, 4% of AICAR-treated oocytes after 17 h of culture contained separated homologous chromosomes with a MII spindle, yet no sign of a PB or contractile ring. The normal polymerization of the actin cap close to the meiotic spindle suggests that the initial steps of cytokinesis happened successfully in AICAR-treated LT oocytes, while it is most likely that the downstream formation of contractile ring was affected. This is supported by the fact that myosin regulatory light chain (MRLC), an important protein involved in the regulation of contractile ring

formation, has recently been shown to be a downstream target of AMPK (Lee et al., 2007; Thaiparambil et al., 2012). If abnormal functioning of AMPK is a major contributing factor of the LT phenotype, it would be interesting to see if MRLC localization and function is affected in LT oocytes and how it is related to the partial rescue of meiosis I arrest by AICAR. It is also possible that a small number of oocytes had segregated homologous chromosomes that reaggregated after anaphase onset (Dumont et al., 2007; Pfender et al., 2011). To distinguish these possibilities, a further observation under time-lapse microscopy is needed.

When LT oocytes were stained for active AMPK after 8 h of culture, abnormal localization was frequently observed. This was not due to an aging effect following prolonged MI arrest, because it was observed in oocytes with newly formed meiotic spindles. Although some control B6SJL oocytes also showed abnormal spindle pole localization, it was much less frequent. Importantly, supplementation of AICAR significantly lowered the incidence of AMPK mislocalization in LT oocytes, which suggests that LT oocytes may have insufficient AMPK function. It has been reported that metaphase I-arrested LT oocytes have more organized centrosomal material on one spindle pole than the other (Albertini and Eppig, 1995). This could help explain the missing or shifted spindle pole localization of active AMPK in these oocytes, since active AMPK colocalizes with microtubule organizing centers (Chapter II).

Our results support the possibility that defective control of AMPK activity contributes to the LT phenotype. Genes for the two AMPK catalytic subunits, *Prkaa1* and *Prkaa2*, are located on mouse chromosomes 15 and 4, respectively. However, a whole genome scan determined that regions in chromosome 1 and 9 were likely

responsible for LT oocyte defects (Everett et al., 2004). It may be that dysregulation of AMPK is a downstream consequence of abnormal genetic control in these regions. Regardless, it is apparent that AMPK has a profound influence on meiotic progression and regulation of parthenogenetic activation in both B6SJL and LT oocytes. Up to this point, our experiments have depended largely upon pharmacological manipulation of AMPK, and genetic ablation experiments are needed for confirmation. Both isoforms of the catalytic subunit have been knocked out in mice and each is viable and able to reproduce; however, the double knockout is an embryonic lethal (Viollet et al., 2009). It will be important to eliminate both isoforms in the oocyte to determine if AMPK is a requisite player or merely serves to fine tune these physiological processes.

SUMMARY AND CONCLUSION

The data presented herein suggest an involvement of AMPK in the entire sequence of events, involving mouse oocyte maturation and subsequent activation. It has been demonstrated that the activation of AMPK mediates hormone-induced meiotic resumption in mouse oocytes, and most significantly, the activation precedes GVB. As a continuation of these study, I investigated the role of AMPK in the completion of maturation and subsequent oocyte activation process. Surprisingly, AMPK not only had a role in the meiotic resumption, it also promoted the first polar body formation and suppressed premature activation. The subsequent study revealed the potential sites of action for AMPK in meiosis: the positive effect on PB formation is associated with the accelerated anaphase onset by regulating the dynamics of MAD2 protein localization at the kinetochores, whereas the inhibitory effect on oocyte activation was linked to the high levels of MAPK activity, which is critical for maintaining metaphase II arrest.

All evidence suggests a multifunctional role of AMPK in the mouse oocytes (Figure 4.1). Interestingly, there is a temporal disconnect between the requirement of AMPK activity and its effects on PB extrusion and oocyte activation. In both cases, the activity is required early during the maturation period. Consistent with the sequential events of PB formation and oocyte activation, AMPK activity is required within 4h after initiation of maturation for optimal promotion of PB and the AICAR treatment can be delayed up to 6h without losing the maximum protection against oocyte activation. These data suggest that AMPK is most likely has multiple targets during oocyte maturation. According to our experimental data, AMPK affects SAC activity to promote anaphase

onset, and prevents MAPK dephosphorylation to maintain metaphase II arrest, although it is not clear how these interactions occur. It is safe to speculate that AMPK may induce GVB by promoting MPF activity; PB formation by regulating MRLC, which is required for cytokinesis.

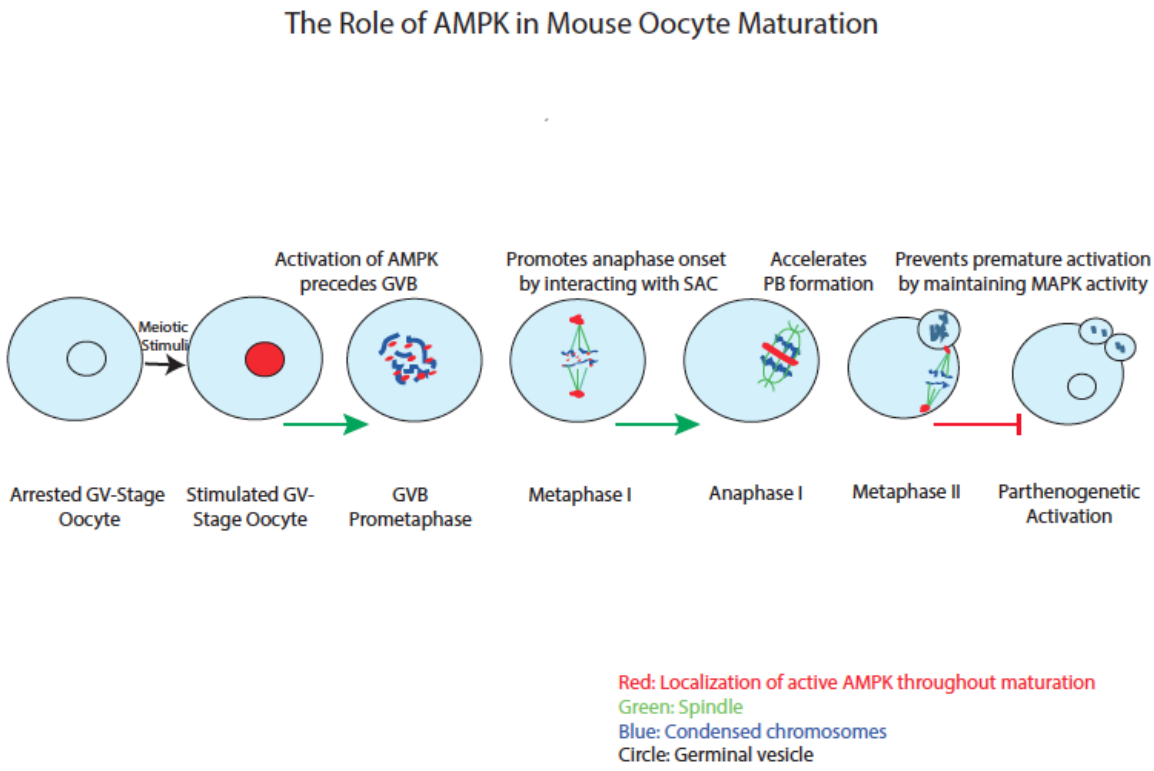

Figure 4. 1 The Role of AMPK in Mouse Oocyte Maturation

Figure summarizing the involvement of AMPK during mouse oocyte maturation.

The localization pattern of active AMPK is consistent with many other kinases that have been proven to regulate meiotic spindle. Although the reason why active AMPK adopts this localization pattern throughout maturation still remains elusive. Our evidence suggests that its localization of active AMPK has functional significance.

Firstly, the germinal vesicle localization of active AMPK seems to be good indication of oocytes stimulation and impending meiotic resumption. The likely impact of this accumulation would be increased phosphorylation of its nuclear substrates. The meiosis-inducing action of AMPK is dependent on the microtubule integrity. Perturbing microtubule dynamics using microtubule-targeted agents disrupts the normal nuclear localization of active AMPK, which is consistent with the inhibitory effects of microtubule perturbants on meiotic induction mediated by AMPK. In somatic cells or yeast, many conditions induce nuclear localization of AMPKα subunit, such as alkaline pH (Hong and Carlson, 2007), heat shock (Kodiha et al. 2007), and leptin stimulation (Suzuki et al., 2007). Even complicating, the upstream kinase, LKB1, was also found to shuttle between cytoplasm and nucleus (McGee et al., 2003). Compound C blocked both nuclear localization of active AMPK and GVB, indicating that such localization is vital for the induction process. However, induction can occur in its absence, since treatment with palmitoyl carnitine that directly activating downstream fatty acid oxidation pathway could significantly restore the GVB in nocodazole treated oocytes, though it should be noted that the extent of GVB after palmitoyl carnitine treatment did not reached the control level. Thus, we could speculate that activating other targets within the nucleus may also be involved that is required for stimulating MPF. An inhibitor of nuclear transport would be a valuable tool in further determining this mechanism.

The function of the spindle microtubule is well studied for later oocyte maturation stages. In contrast, less is known about its requirement during meiotic induction. I demonstrated that microtubule integrity is important for FSH-and AICAR-induced maturation. Perturbing microtubules blocked meiotic induction but not spontaneous maturation of oocytes. It is plausible to expect that during GV stage, the microtubule network is mainly required for protein transport (see p65 for details). One possible explanation would be that meiotic induction and spontaneous maturation are two different physiological processes in such that extra microtubule modulator-sensitive component is required for meiotic induction. Another explanation is mainly contributed by the different GVB kinetics of meiotic induction and spontaneous maturation of the mouse oocytes. Once released from the follicles, most mouse oocytes undergo GVB within an hour as opposed to hours in meiotic induction, which may be too soon for microtubule modulators to exert an effect.

Secondly, the correct spindle pole localization of active AMPK seems to be correlated with the oocyte's potential of completing maturation. Most LT oocytes arrest at metaphase I and have a high incidence of abnormal active AMPK localization at the spindle poles. AICAR stimulated meiotic progression of LT oocytes past metaphase I and significantly restored the normal spindle pole localization of active AMPK. This spindle pole localization of active AMPK is clearly depending on spindle microtubule integrity. Although we did not observe a reverse regulation of spindle microtubule by AMPK like in somatic cells, it is still possible that a residual AMPK activity is present after compound C treatment, which is sufficient to exert its effects on spindle dynamics. Recently, it has been found that blocking polo-like kinase 1 activity abolished activation

of AMPK and the correct spindle apparatus localization. Polo-like kinase 1 is an important protein that regulates spindle assembly, centrosome maturation, chromosome congression, separation and cytokinesis(Vazquez-Martin et al., 2011). It is likely that AMPK is also one of its down stream effectors in the oocytes.

Thirdly, active AMPK also concentrated in the kinetochore region at metaphase I in addition to the spindle poles, which may indicate its role as a chromosome passenger protein. Unfortunately, we were unable to perform colocalization staining with kinetochore because the CENP-A antibody we purchased did not stain. Regardless, this subcellular pattern is consistent with the data that AICAR accelerated MAD2 loss from the kinetochore. How this interaction occurs remains to be identified, but research has shown that AMPK phosphorylates microtubule associated proteins (MAPs) (Mihaylova and Shaw, 2011), which may relate to this.

Lastly, the midbody localization of the active AMPK during anaphase coincides with the future cleavage furrow that recruits MRLC. Cytokinesis is a highly coordinated process that can be divided into multiple stages; specification of the cleavage plane, ingression of the furrow, formation of the midbody, and abscission. Any defect of the above processes will lead to cytokinesis failure. Cytokinesis is also dependent on the actin filaments located outside of the furrow (Normand and King, 2010). Blocking AMPK with compound C inhibited meiotic progression past metaphase I, but did not suppress spindle migration and the excessive polymerization of the F actin filaments. Interestingly, polo-like kinase 1 that is involved in the later stage of cytokinesis also directs AMPK phosphorylation of MRLC (Vazquez-Martin et al., 2012). We are not sure whether there is any crosstalk between polo-like kinase and LKB1 regulation of AMPK,

especially on establishing cell polarity. Based on these observations, I propose that AMPK is more involved in the later stages of the cytokinesis, such as myosin recruitment and activation, which are crucial for ingression of the furrow.

The translocation of AMPK throughout maturation suggests a dynamic process at work, which also coincides with its potential functions at different stages. The germinal vesicle localization of AMPK is most likely dependent on microtubule meditated nuclear import. The poleward tanslocation of active AMPK might be dependent on the minus-end directed motor protein, dynein, which has been shown to be critical for spindle pole localization of the proteins such as NuMA and Eg5 (Merdes et al., 2000; Uteng et al., 2008). In contrast, kinesin family members may mediate the midbody translocation of active AMPK. For instance, citron kinase (CIK-K), a major component of the contractile ring, requires kinesin family member 14 (KIF-14) for proper localization to the cleavage site (Gruneberg et al., 2006). It is likely that active AMPK is transported from spindle poles to the cleavage furrow by plus-end directed kinesin. However, I could not rule out the possibility that more AMPK is activated and recruited during anaphase. A GFP-tagged fusion protein would be useful to track the movement of active AMPK during maturation.

AMPK restored the meiotic behavior of LT oocytes, which includes release of metaphase I arrest, suppression of spontaneous activation, and correction of normal spindle pole localization of the active AMPK. Early development of CSF activity is not associated with the meiotic defect of LT oocytes. According to these observations, there are two likely scenarios whereby AMPK may correct the meiotic defects of LT oocytes; one of these would be that activation of AMPK overrides high levels of CSF activity to

induce anaphase, yet leaving MAPK1/3 activity intact. The second scenario would be that activation of AMPK completely restores the normal CSF profile during meiosis in that premature development of CSF activity is prevented but later helps maintain normal levels of activity during metaphase II arrest.

Although Western blot analysis of AMPK activity and levels in CEOs were not different from the control F1 mouse CEOs (data not shown), the direct assaying of AMPK by western analysis of oocyte phospho-ACC level was hindered by the poor reproductive performance of LT mice (Westerns of DOs require many more oocytes than those of CEOs). Regardless, evidence collected so far indicates that the malfunctioning of AMPK may be an important contributing factor to the LT oocyte phenotypes.

In fact, in addition to LKB1, calcium/camodulin-dependent protein kinase kinase β (CaMKKβ) is another upstream activator of AMPK. Evidence suggests that CaMKKβ particularly activates AMPK in neurons and T cells (Mihaylova and Shaw, 2011). Although there has been report on the involvement of CaMKK on pig oocyte maturation, they did not distinguish CaMKKα and CaMKKβ (Xu et al., 2009). Furthermore, we are able to stain oocytes for active LKB1 but not CaMKK (Downs unpublished data). Thus, I favor the hypothesis that LKB1 pathway is predominantly active in the mouse oocytes.

To why there are opposite consequences for AMPK activation on G2/M transition between somatic cells and mouse oocytes, below are some possible explanations. 1). *Differential response to energy substrates*. High glucose level suppresses GVB (see p43 for details). 2) *The origin of the AMP*. In oocytes, elevated AMP mostly comes from degradation of cAMP, while the source is mostly ATP breakdown in somatic cells in

response to low energy and stress conditions. It is possible that a difference in AMP/ATP ratios between two types of cells may lead to the differential downstream effects.

It is important to note that our experiments are mainly based on the pharmacological manipulation of AMPK. An AMPK null mouse model is a valuable tool for testing the absolute requirement for AMPK in meiotic regulation; however, the double knockout of both catalytic alpha subunit is embryonic lethal. Thus, we are trying an alternative route to generate double knockout mice by using the Cre-loxP system in an α2 mutant mouse background, which should lead to a specific α1 subunit deletion in the oocytes that are already mutated for α2 allele (Lewandoski et al., 1997). Currently, we have produced the α1 oocyte-specific knockout animals, and the α1 mutant oocytes did not exhibit any meiotic defects, which maybe due to the functional redundancy of two catalytic alpha subunits. However, when the female mice carrying α1 single mutant oocytes were mated with males that carry two homozygous floxed alleles for α1, the isolated embryos showed a significantly reduced rate of 2-cell embryo and blastocyst formation. This is likely caused by the active Cre recombinase in the oocyte that is still able to excise the floxed α1 allele in the sperm, rendering some of the resulting embryos null for α1(De-Vries et al., 2000). This result is promising and suggests that α1 subunit is critical for the early embryonic development. The generation of double knockout mice is still in progress.

In conclusion, my study has shown a multifunctional role of the energy sensor, AMPK, in mouse oocyte maturation. It will be important to determine whether similar functions for AMPK exist in human oocytes.

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