The Role of PRKA-Mediated Stimulation of Fatty Acid Oxidation in the Regulation of Meiotic Maturation in Mouse Oocytes

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THE ROLE OF PRKA-MEDIATED STIMULATION OF FATTY ACID OXIDATION IN THE MEIOTIC MATURATION OF MOUSE OOCYTES

by

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ABSTRACT

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Meiosis is a defining aspect of sexual reproduction and its outcome often determines reproductive success. In mammalian females, oocytes initiate meiosis in early embryonic stages but arrest in the late prophase of meiosis-I around the time of birth. They remain arrested until hormonal induction during reproductive cycles after puberty, and then resume meiosis until a second arrest at metaphase-II. The process of oocyte meiosis from the release of prophase arrest till the metaphase-II arrest is known as oocyte maturation.

Meiotic arrest is a result of maintenance of elevated cAMP levels within the oocyte by its continuous production as well as by the inhibition of its breakdown. A mid-cycle surge of luteinizing hormone culminates in a dramatic increase in cAMP breakdown that eventually results in the activation of MPF (maturation-promoting factor), a cyclin-dependent kinase that orchestrates the downstream events of meiotic resumption. The product of cAMP breakdown is AMP. A high AMP: ATP ratio is known to activate a metabolic switch known as AMP-activated protein kinase (AMPK or PRKA) that turns off energy-consuming pathways while turning on energy-generating pathways. In a series of previous studies, our lab has established that activation of PRKA by pharmaceutical agents induces meiotic resumption, while hormone-induced meiotic resumption requires PRKA activation in mouse oocytes.

Since fatty acid oxidation (FAO) is a very important pathway stimulated by PRKA, our lab next investigated its involvement in PRKA-mediated meiotic resumption, and demonstrated that PRKA activator-induced meiotic resumption requires FAO and agents that stimulate FAO induce meiotic resumption in vitro. Therefore, it was hypothesized that PRKA induces meiotic resumption by the stimulation of FAO.

In my dissertation research, I demonstrated a requirement of FAO during hormone-induced meiotic resumption. By measuring FAO levels, I showed that hormone-induced meiotic resumption in vitro and in vivo involves a significant increase in FAO. Moreover, I examined the role of PRKA-mediated regulation of FAO by determining the inactivation of acetyl CoA carboxylase (ACC) during meiotic resumption, and showed that ACC inhibitors increase FAO and induce meiotic resumption. These data show that the PRKA-mediated inactivation of ACC and the resulting increase in FAO is required for and stimulatory to meiotic resumption.

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ABBREVIATIONS

ACC: acetyl coenzyme A carboxylase

AICAR: 5-aminoimidazaole-4-carboxamide-1-b-D-ribofuranoside

AMP: adenosine monophosphate

AMPK or PRKA: AMP-activated protein kinase

AR: amphiregulin

ATP: adenosine triphosphate

cAMP: cyclic AMP

CAT: carnitine-acylcarnitine translocase

CDK: cyclin-dependent kianse

CEO: cumulus cell-enclosed oocytes

cGMP: cyclic guanosine monophosphate

Cmpd C: compound C

CoA: coenzyme A

CPT1: carnitine palmitoyl transferase 1

dbcAMP: dibutyryl cAMP

DNA: deoxyribonucleic acid

DO: denuded oocytes

eCG: equine chorionic gonadotropin

EGF: epidermal growth factor

FA: fatty acid

FACS: fatty acyl CoA synthetase

FADH2: flavin adenine dinucleotide

FAO: fatty acid oxidation

FSH: follicle stimulating hormone

GV: germinal vesicle

GVB: GV breakdown

hCG: human chorionic gonadotropin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IBMX: 3-isobutyl-1-methylxanthine

LCFA: long-chain fatty acids

LH: luteinizing hormone

MAPK or ERK1/2: mitogen-activated protein kinase

MCD: malonyl CoA decarboxylase

MEM: minimum essential medium

MI: metaphase I

MII: metaphase II

MPF: maturation promoting factor

NADH: nicotinamide adenine dinucleotide

NPPC: natriuretic peptide precursor C

NPR2: natriuretic peptide receptor 2

pACC: phospho-ACC

PB: polar body

PKA: protein kinase A

ROS: reactive oxygen species

Chapter I

BACKGROUND

Meiosis is a defining feature of sexual reproduction and its accuracy often determines reproductive outcome. It is the mechanism by which haploid gametes containing recombined genetic material are produced and is thus important in creating variation in the population.

In most mammals, females differ from males in many aspects of meiosis. In males, production of sperms and thus meiosis in germ cells does not begin until puberty, and meiotic division of each male germ cell results in the formation of four haploid spermatids that all go on to form mature spermatozoa. On the other hand, meiosis begins in the early female embryo at day 13.5 post-coitum in mice (Speed, 1982; Bowles and Koopman, 2007). However, meiosis in female germ cells is paused at prophase of the first meiotic division (i.e. prophase-I) around the time of birth and is not resumed until puberty. Furthermore, after hormonal stimulation at puberty, female meiosis resumes until it undergoes another arrest at metaphase-II and is not completed unless fertilization or parthenogenesis occurs. Another difference between male and female gametes is that female meiotic division is asymmetrical: in the first meiotic division, a primary oocyte divides into a secondary oocyte (egg) and a much smaller first polar body. Upon fertilization or parthenogenesis, the second meiotic division, again asymmetrical, forms the fertilized egg and second polar body; while polar body-I may or may not divide to give another polar body. In this way, of the three or four daughter cells produced, only

the secondary oocyte (egg) gets fertilized and forms a zygote (Conti, 2013; Mehlmann, 2013).

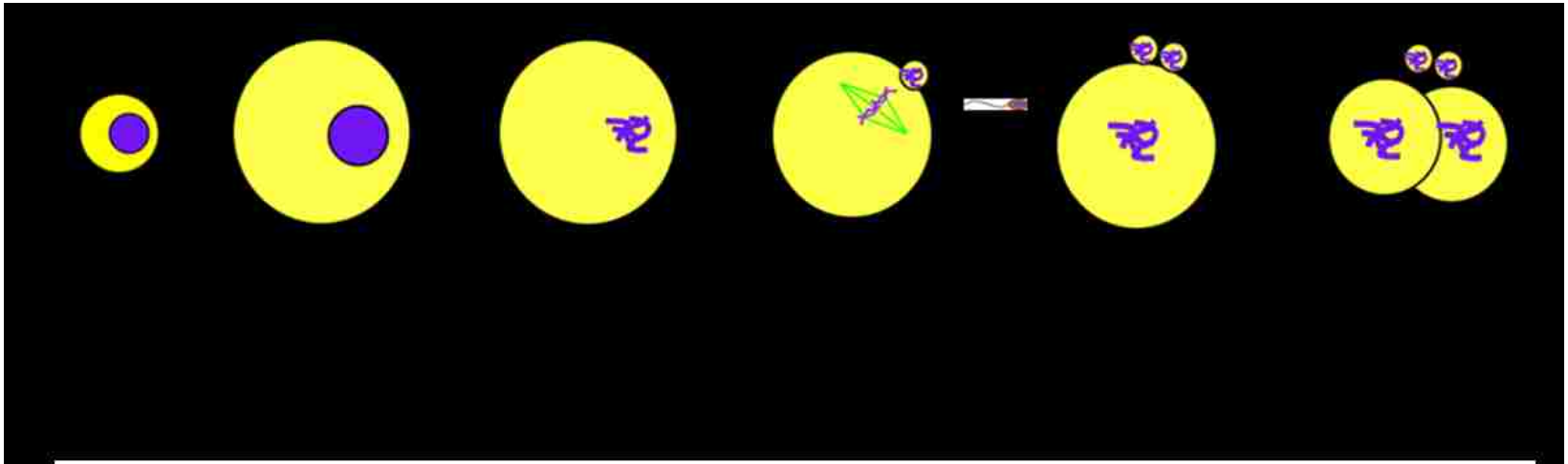


Figure 1.1. Oocyte Maturation.

Oocytes get arrested in prophase-I of meiosis around the time of birth. After puberty, in estrous cycles, the action of FSH stimulates oocyte and follicle growth, resulting in fully grown, meiotically competent oocytes, arrested in GV stage. A mid-cycle surge of LH acts on cells of the follicle and induces meiotic resumption, resulting in GV breakdown and progression to metaphase-II, at which point the oocyte faces a second arrest. This arrest is relieved at fertilization by sperm (or egg activation, not shown here), resulting in completion of meiosis, fusion of egg and sperm chromosomal material, formation of the zygote, and subsequent embryonic cleavages.

Ovarian follicles

Meiosis in mammalian females is a highly regulated process. However, it is important to note that oocytes do not exist in isolation; they are nestled in structures in the ovary called follicles. Follicles and the oocytes within undergo growth and development in response to gonadotropin stimulation in each ovarian cycle. Each follicle contains a single oocyte surrounded by specialized somatic cells, the number and kind of which depend on the growth stages of follicle. Each follicle contains a single oocyte.

Generally, most of the follicles in the ovary at any given time exist as primordial follicles containing an oocyte surrounded by a single layer of flattened, i.e. squamous, granulosa cells. In response to gonadotropins, some follicles start to develop to the next stage, viz. primary follicle stage. In primary follicles, the granulosa cells become cuboidal. Granulosa cells then divide to form more than one layer of cells in the secondary follicle stage. Next, the granulosa cells start secreting antral fluid and a cavity (antrum) begins to form in the follicle which is now called a tertiary follicle. A fully mature tertiary follicle that contains a large antrum and a fully grown oocyte is known as the Graafian follicle. At this point, the granulosa cells that are intimately attached to the oocyte via gap junctions are known as cumulus granulosa cells, or simply cumulus cells. The remaining outer granulosa cells that either line the antral cavity or attach to cumulus cells via gap junctions are known as mural granulosa cells, and will be referred to as 'mural cells' in this dissertation. These granulosa cells are surrounded by a layer of extracellular matrix- the basement membrane, outside of which lie two layers of ovarian cells: theca interna and theca externa. Theca cells produce androgen in response to

luteinizing hormone (LH), and androgen is then converted to estrogen by the enzyme aromatase in granulosa cells.

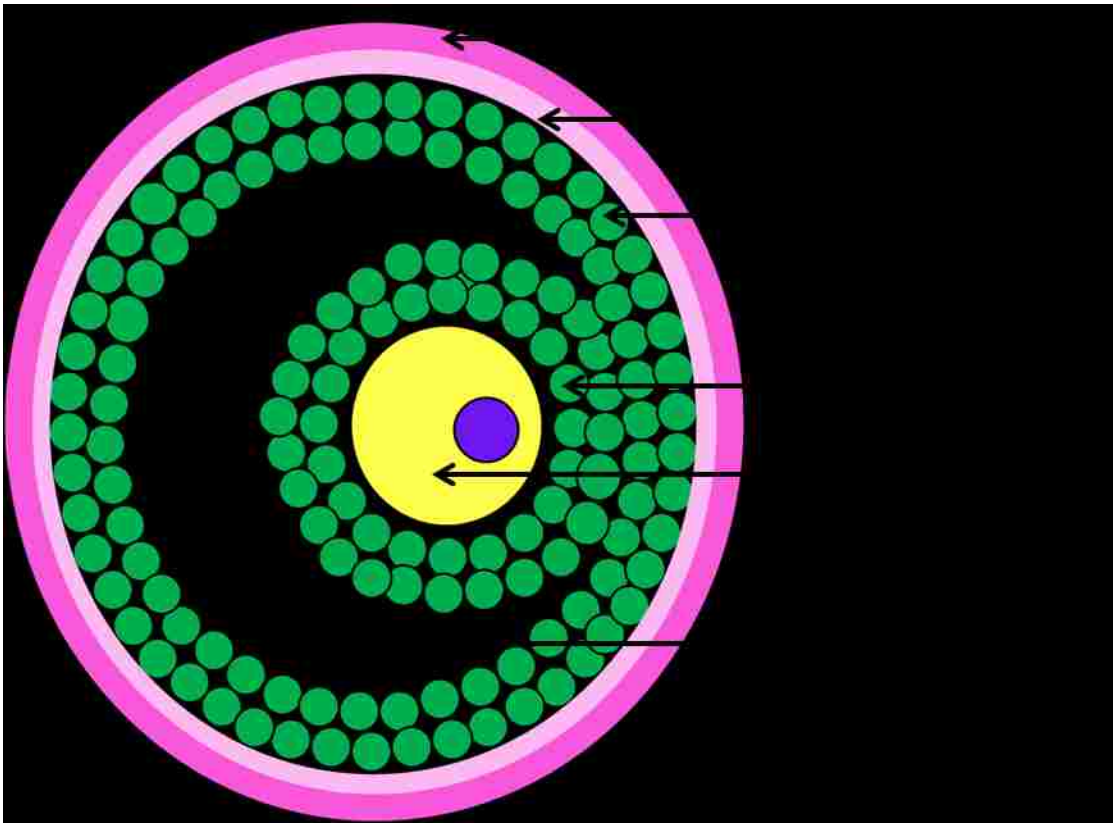


Figure 1.2. The Ovarian Follicle.

The oocyte is nestled in structures called ovarian follicle. Fully grown antral follicle is shown here, depicting the 2 outermost theca layers, mural and cumulus granulosa cells, the antral cavity, and the oocyte.

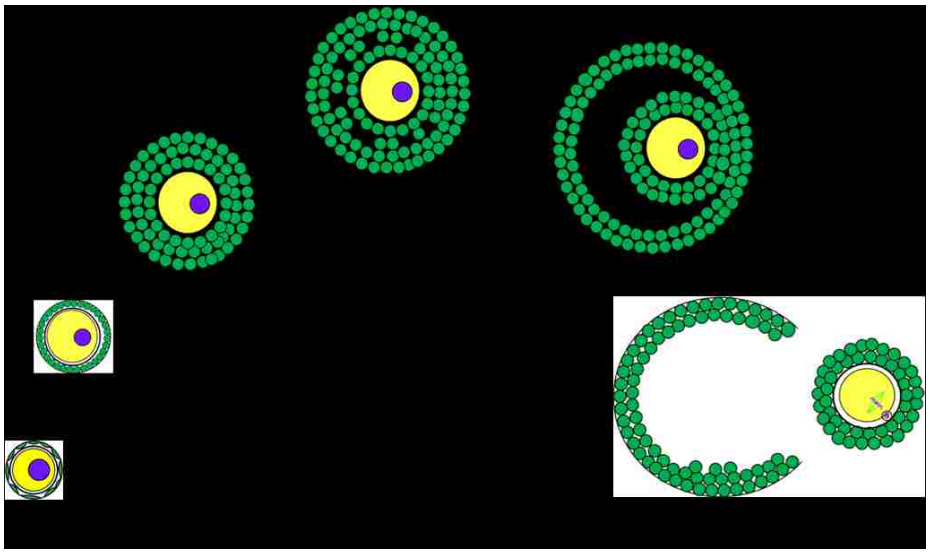


Figure 1.3. Stages of Follicle Development during Ovarian Cycles.

Most follicles in the ovary exist as primoridal follicles. In the first phase of ovarian cycle, FSH acts on the follicle and oocyte to stimulate their growth. Cells that surround the oocyte in the follicle change their shape from squamous to cuboidal, and multiply. Eventually, the fully grown follicle contains several layers of granulosa cells enclosing an antral cavity that contains an oocyte surrounded by several cumulus cell layers. A mid-cycle LH surge induces meiotic resumption and release of the metaphase-II-arrested oocyte (Green, 1966; Li and Albertini, 2013).

The ovarian and uterine cycles in the mouse

Each reproductive cycle (estrous cycle) of the laboratory mouse lasts about 4-5 days (Green, 1966). Follicle-stimulating hormone (FSH) is a gonadotropin secreted by the anterior pituitary that initiates growth of several primordial follicles in the ovary in the first phase (diestrus) of the cycle. The second phase, proestrus, involves rapid growth of some of these follicles culminating in the Graafian, pre-ovulatory stage. At estrus, a surge of LH stimulates the oocytes within some antral follicles to resume meiosis. In about 12 hours, the mature, metaphase-II-arrested oocytes (now called eggs) are released (ovulated) into the oviduct. The rest of the follicle that contains granulosa cells and theca cells remains in the ovary and becomes a secretory tissue called corpus luteum. The next phase (metestrus) of the cycle involves secretion of progesterone by this luteal tissue that is mainly responsible for thickening the uterine lining. In the absence of egg fertilization by sperm, the corpus luteum is degraded, uterine lining rearranged, and the next cycle starts.

Meiosis stages in the oocyte

Meiosis involves one round of DNA replication followed by two rounds of cell division named meiosis I and meiosis II, finally resulting in four haploid cells: one secondary oocyte and 2-3 polar bodies. Both meiotic divisions comprise four phases. In prophase, one of the most important processes is condensation of chromosomes. In meiosis-I the prophase is further separated into leptotene, zygotene, pachytene, diplotene,

and diakinesis. Prophase-I results not only in condensed chromosomes but is also accompanied by recombination of genetic material between homologous chromosomes. Diplotene is the phase where prophase arrest sets in, and the resulting prolonged stage is known as the dictyate stage. The nucleus of the prophase-arrested oocyte is clearly visible and is known as the germinal vesicle (GV). The resumption of meiosis is accompanied by breakdown of the nuclear envelope (GVB; germinal vesicle breakdown) and movement of condensed chromosomes towards the metaphase plate. In the metaphase plate of meiosis-I, closely associated homologous chromosomes are held by microtubules from opposite spindle poles. In the next phase (anaphase-I), homologous chromosomes are separated and migrate towards opposite spindle poles. Unlike mitosis, no distinct telophase is observed in meiosis. Chromosome separation is followed by cytokinesis i.e. separation into two daughter cells- the secondary oocyte (egg) and polar body-I. This is followed by meiosis-II that involves neither DNA replication nor recombination. In metaphase-II, the sister chromatids of each chromsome are held by microtubules from opposite spindle poles. Fertilization or activation of the egg results in separation of sister chromatids and formation of polar body-II and the zygote that contains male and female pronuclei that fuse together (Gilbert, 2000).

Regulation of prophase meiotic arrest

Once oocytes reach late prophase-I, they are maintained in meiotic arrest from the time of birth until at least puberty. However, fully grown meiotically competent oocytes, when removed from Graafian follicles and cultured in appropriate conditions,

spontaneously resume meiosis (Pincus and Enzmann, 1935; Edwards, 1965). Therefore it has been suggested that the somatic part of the follicle is important in maintaining the oocyte in meiotic arrest. Maintenance of a high cAMP level within the oocyte is important in keeping the oocyte GV-arrested (Cho et al., 1974; Magnusson and Hillensjö, 1977; Schultz et al., 1983) . This is achieved by the action of Gpr3, a constitutively active G-protein-coupled receptor (Mehlmann, 2005). Gpr3 ensures a high cAMP production by activating adenylyl cyclase (AC) in the oocyte. High cAMP levels result in the activation of protein kinase A (PKA) that prevents the activation of maturation promoting factor (MPF), a complex of a cyclin and a cyclin-dependent kinase, whose activity orchestrates the downstream events of meiosis, including GVB (Jones, 2004).

Cyclic AMP levels in the oocyte are regulated not only by its constitutive synthesis but also by the rate of its breakdown, mediated by an oocyte-specific phosphodiesterase 3A (PDE3A). It is now known that PDE3A is inhibited by cGMP (Shitsukawa et al., 2001), which is produced by a guanylyl cyclase called natriuretic peptide receptor 2 (Npr2) that is predominantly expressed in cumulus cells. This cGMP diffuses to the oocyte via gap junctions. Thus, when the oocyte (cumulus enclosed or not) is removed from the follicle, it loses meiotic arrest and undergoes GVB (Zhang et al., 2010). In addition, the antral fluid of the follicle has been found to contain hypoxanthine (HX) that inhibits meiotic resumption. The salvage pathway for purines converts hypoxanthine to inosine monophosphate (IMP), a precursor of GTP, whose breakdown by NPR2 produces cGMP. Inosine monophosphate dehydrogenase (IMPDH) is a key enzyme in the conversion of IMP to GTP, and its activity is controlled by oocyte-

derived paracrine factors (ODPFs). Therefore, the oocyte itself plays a major role in maintaining its own meiotic arrest (Wigglesworth et al., 2013).

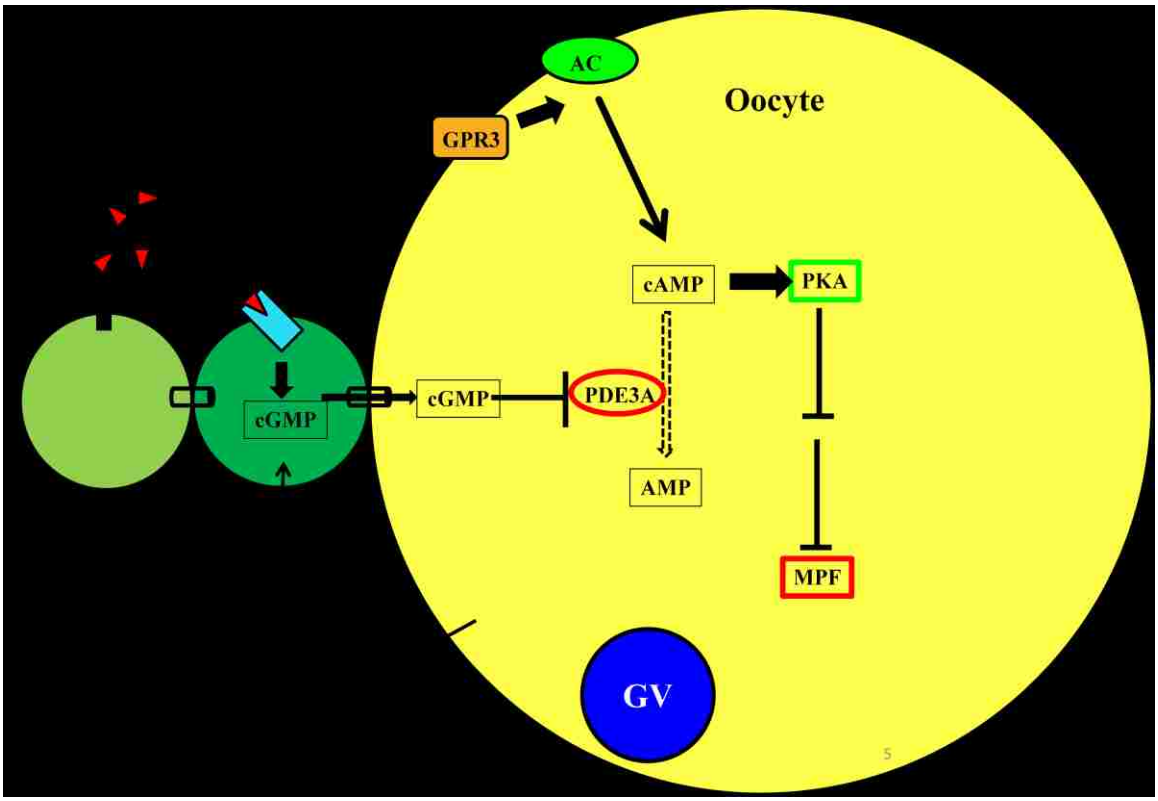


Figure 1.4. The maintenance of meiotic arrest in mouse oocytes.

In the oocyte, a high level of cAMP in the oocyte activates PKA (protein kinase A), that eventually prevents the activation of MPF (maturation promoting factor), the cyclin-dependent kinase required for meiotic resumption including germinal vesicle (GV) breakdown. The continuous production of cAMP is due to a constitutively active G-protein-coupled receptor, GPR3, which activates adenylyl cyclase (AC). However, cAMP levels are also affected by its breakdown, mediated by an oocyte-specific phosphodiesterase, PDE3A. PDE3A is inhibited by cGMP, whose source are the cumulus cells surrounding the oocyte. This cGMP is produced by a guanylyl cyclase called NPR2, which is a receptor for a peptide called NPPC (natriuretic peptide precursor C) that is produced by the mural granulosa cells surrounding the antral cavity of the ovarian follicle. Additionally, some oocyte-derived paracrine factors (ODPFs) aid in the maintenance of meiotic arrest.

Meiotic Resumption

In vivo, a surge of LH from the anterior pituitary at estrus is received by mural cells of the antral, preovulatory follicle that consequently transduce a signal to the meiotically competent arrested oocyte to resume meiosis. The exact mechanism is not completely clear, but LH binding to mural granulosa cells results in a transient increase of cAMP levels in these cells, which induces the expression of epidermal growth factor (EGF)-like peptides, such as amphiregulin and epiregulin (Downs and Chen 2008; Park et al. 2004; Panigone et al. 2008). This results, via an EGF receptor- and MAP kinase-mediated mechanism, in decreased expression of NPPC and NPR2; as well as a decrease in gap junction permeability (Su et al., 2003; Kalma et al., 2004; Norris et al., 2008; Kawamura et al., 2011). Thus results in the decrease in cGMP production by granulosa cells as well as a decrease in cGMP transfer to the oocyte and removal of PDE3A inhibition in the oocyte, reducing oocyte cAMP level, MPF activation and meiotic resumption. Also, it seems that LH may act via redundant pathways to stimulate meiotic resumption, as a highly specific dose of UO126, a MAP kinase inhibitor, fails to inhibit LH-induced meiotic resumption (Norris et al., 2008). Interestingly, oocyte-derived paracrine factors (ODPFs)- specifically, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15)- are pivotal in EGF receptor expression in cumulus cells (Su et al., 2010).

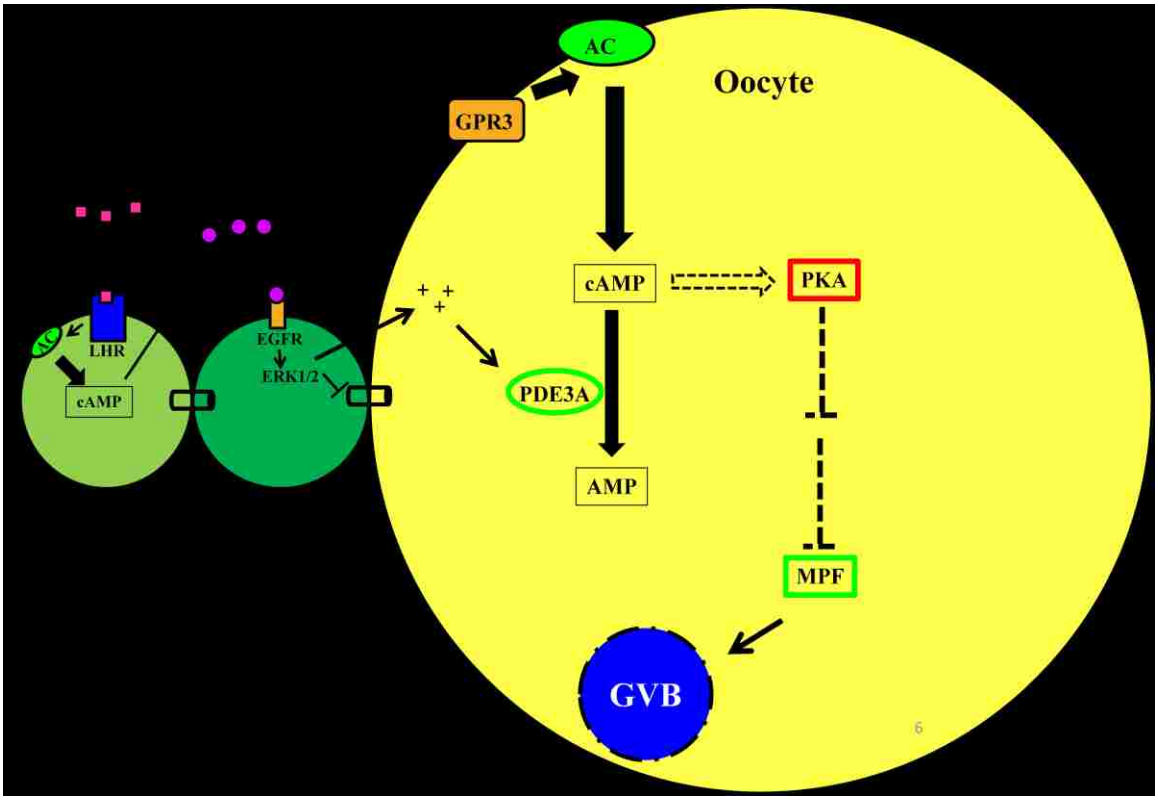


Figure 1.5. The regulation of meiotic resumption in mouse oocytes.

Luteinizing hormone (LH) activates LH receptors on mural granulosa cells, that causes elevated cAMP levels in these cells, leading to the production of EGF (epidermal growth factor)-like peptides (EGFp). These peptides then act on EGF receptors on cumulus cells and cause a closure of gap junctions via a MAP kinase (ERK1/2)-mediated pathway, thus preventing the passage of cGMP to the oocyte. This leads to cAMP breakdown in the oocyte, causing its levels to fall, and eventually results in the removal of MPF inhibition, causing the resumption of meiosis. Additionally, an unknown positive stimulus from cumulus cells aids in the activation of PDE3A.

AMP-activated protein kinase

Breakdown of cAMP by PDE3A produces 5'-AMP (usually referred to simply as AMP), a molecule thought for many years to be a byproduct with no specific function. However, a high AMP:ATP ratio in cells results in the activation of a kinase called AMP-activated protein kinase (formerly AMPK, now known as PRKA), an energy sensor that activates pathways that produce energy while inhibiting ones that spend energy (Hardie, 2011; Hardie et al., 2012; Gowans et al., 2013). One of the important pathways activated by PRKA is beta oxidation (referred to as fatty acid oxidation or FAO in this dissertation) (Hardie and Pan, 2002; Tong, 2005; Brownsey et al., 2006; Tong and Harwood, 2006). This pathway takes place in the mitochondria, where acyl CoA-linked fatty acids from the cytosol are imported inside by replacing CoA with L-carnitine (often referred to simply as carnitine), a small molecule derived from the amino acids L-lysine and L-methionine (Hoppel, 2003). The enzyme that produces fatty acyl carnitine from fatty acyl CoA is carnitine palmitoyl transferase-1 (CPT1), located on the mitochondrial outer membrane. The fatty acyl carnitine is then facilitated to the mitochondrial matrix by a carnitine/acylcarnitine translocase. Once in the matrix, the carnitine is then replaced with CoA by CPT-2, and the fatty acyl CoA undergoes beta oxidation (Bonnefont et al., Declercq et al., 1987; Weis et al., 1994). PRKA upregulates FAO by inactivating acetyl coenzyme A (CoA) carboxylase (ACC), an enzyme that indirectly inhibits FAO by producing malonyl CoA that is an inhibitor of CPT-1 (Goodwin and Taegtmeyer, 1999).

PRKA Involvement in Meiotic Resumption

Previous experiments in our lab have demonstrated the involvement of PRKA activation in meiotic resumption. Treatment of cumulus-enclosed or denuded oocytes with aminoimidazole carboxamide ribonucleiotide (AICAR), a pharmaceutical PRKA activator, induces meiotic resumption in the presence of one of several cAMP-elevating agents such as milrinone, dibutyryl cAMP, IBMX; and hypoxanthine. Furthermore, AICAR-induced and hormone-induced meiotic resumption in vitro is blocked by PRKA inhibitors compound C and araA (Downs et al., 2002b; Chen et al., 2006; Chen and Downs, 2008). PRKA is also a stress-response kinase, and subjecting oocytes to various kinds of stress induces meiotic resumption (LaRosa and Downs 2006; 2007).

Acetyl CoA carboxylase

Acetyl CoA carboxylase is an important substrate of PRKA. It is a biotin-binding protein that catalyzes the carboxylation of acetyl CoA to form malonyl CoA. In most eukaryotes, ACC is a single, multi-domain polypeptide chain. The three domains of ACC are biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyl transferase (CT) (see Fig. 3.1). Mammalian ACC exists in two isoforms: ACC1 or ACCα, and ACC2 or ACCβ, each with splice variants. The two isoforms, expressed from different genes (*Acaca* and *Acacb*), differ in tissue distribution and subcellular location and are believed to have different functions. While lipogenic tissues such as adipose and mammary glands predominantly express ACC1, ACC2 is the predominant

isoform expressed by lipolytic tissues such as the heart and skeletal muscle. In terms of protein length and molecular weight, ACC1 is 260 kDa and ACC2 is 280 kDa, ACC2 having 114 extra amino acids at the N-terminus. This N-terminal sequence in ACC2 begins with hydrophobic residues, suggesting membrane targeting (Brownsey et al., 1997, 2006; Shen et al., 2004; Zhang et al., 2004; Tong, 2005; Tong and Harwood, 2006). Using immunofluorescence as well as GFP fused to the ACC2 N-terminus, it was shown that ACC2 was indeed colocalized with CPT1 at the mitochondrial outer membrane in rat cardiomyocytes and HepG2 cells (a liver carcinoma cell line) (Abu-Elheiga et al., 2000). Considering tissue distribution and subcellular localization, it has been suggested that the malonyl CoA produced by the two isoforms has separate functions; malonyl CoA from ACC1 may be predominantly used as a precursor for long chain fatty acid CoA synthesis, whereas that produced by ACC2 may be used mainly for inhibiting CPT1 (Abu-Elheiga et al., 2000; Abu-Elheiga et al., 2001; Brownsey et al., 1997; Tong, 2005).

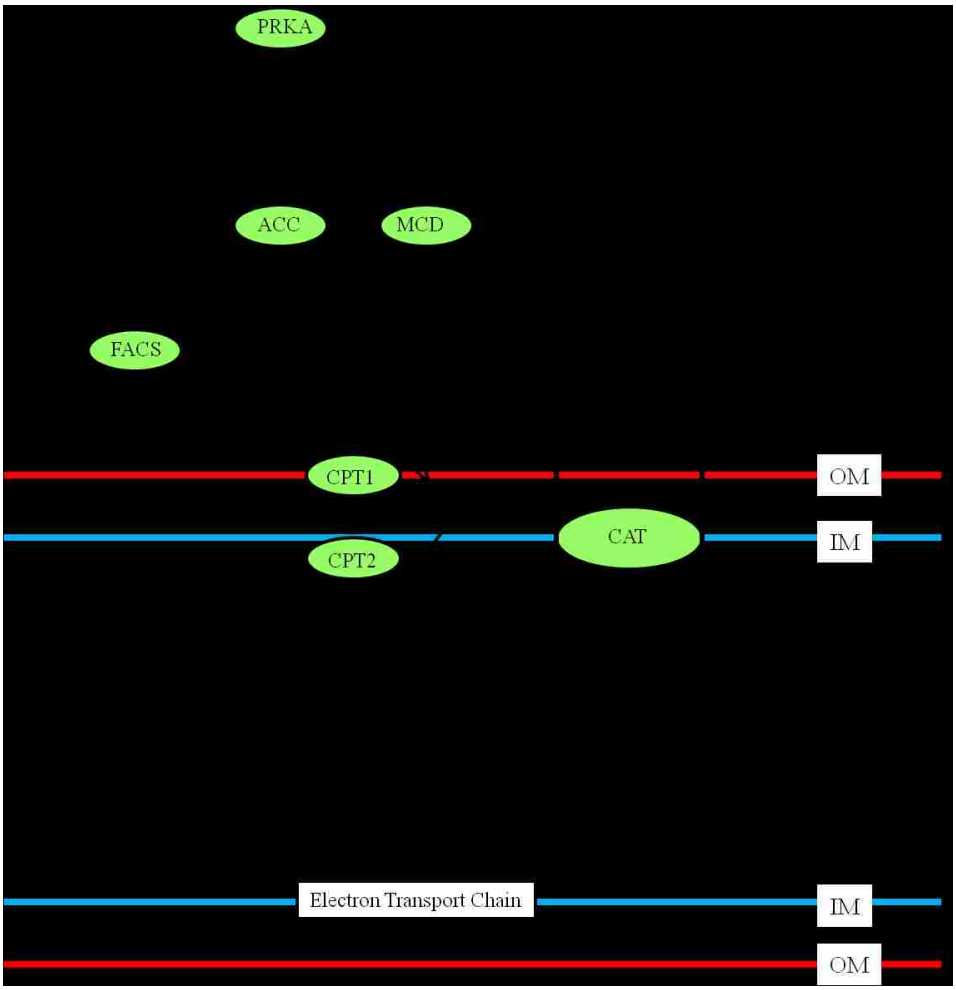


Figure 1.6. The role of PRKA in the regulation of fatty acid oxidation.

Coenzyme A is attached to fatty acids by fatty acyl CoA synthetase (FACS) in the cytosol. The CoA is then replaced with carnitine by carnitine palmitoyl synthetase 1 (CPT1), located on the outer mitochondrial membrane (OM). The fatty acyl carnitine then passes to the mitochondrial matrix by facilitated diffusion by the action of carnitine-acylcarnitine translocase (CAT). The carnitine is then again replaced with CoA by the action of CPT2 in the mitochondrial matrix. The fatty acyl CoA is then oxidized by beta-oxidation and the products acetyl CoA, NADH/FADH2, and water are formed. The NADH/FADH2 is then used to make ATP by the mitochondrial electron transport chain. The enzyme CPT1 is inhibited by malonyl CoA tht is produced from acetyl CoA by the enzyme acetyl CoA carboxylase (ACC) and converted to acetyl CoA by the enzyme malonyl CoA decarboxylase (MCD). AMPK-activated protein kinase (PRKA) plays a major role in FAO regulation by inactivating ACC and decreasing malonyl CoA levels, thereby removing CPT1 inhibition. Malonyl CoA is also a precursor for long-chain fatty acids (LCFA).

Regulation of acetyl CoA carboxylase

Mammalian ACC1, when active, forms filamentous polymers consisting of 10-20 protomers that comprise ACC dimers. The enzyme is regulated by feedforward regulation by citrate (precursor of acetyl CoA), as well as feedback regulation by long chain fatty acyl CoA (whose precursor is malonyl CoA). In addition, it is also regulated by covalent modifications. PRKA can phosphorylate both ACC isoforms, resulting in their inactivation. Although several serine residues are phosphorylated, Ser 79 (corresponding to Ser 218 in ACC2) phosphorylation appears to be sufficient and important for ACC inhibition. Protein kinase A also phosphorylates the ACCs at two serine residues, but the significance of this modification remains unknown (Brownsey et al., 1997, 2006; Shen et al., 2004; Zhang et al., 2004; Tong, 2005; Tong and Harwood, 2006). Importantly, assaying Ser 79 phosphorylation is a well-established method of determining PRKA activity (Munday and Hemingway, 1999; Chen et al., 2006; Chen and Downs, 2008).

Involvement of Fatty Acid Oxidation in Meiotic Resumption

In 2009, our lab, using different inhibitors of fatty acid oxidation showed its involvement in AICAR-induced meiotic resumption (Downs et al., 2009), whereas C75, a CPT1 activator, and carnitine-derivatives of long-chain fatty acids (e.g. palmitoyl carnitine) induce meiotic resumption. Also, C75-induced meiotic resumption was not blocked by the PRKA inhibitor Compound C, confirming that C75 was acting

downstream of PRKA. These important data led us to hypothesize that a major mechanism by which PRKA induces meiotic resumption is the stimulation of FAO. In this dissertation, I have investigated the effect of modulation of ACC, the important PRKA substrate that bridges PRKA with FAO regulation, and I have also studied the role of FAO during hormone-induced meiotic resumption in vitro and in vivo. Collectively, the data from our lab point to the model depicted below. This model proposes that FAO stimulation mediated by PRKA-mediated inactivation of ACC leads to the activation of MPF by a yet unknown mechanism, resulting in meiotic resumption.

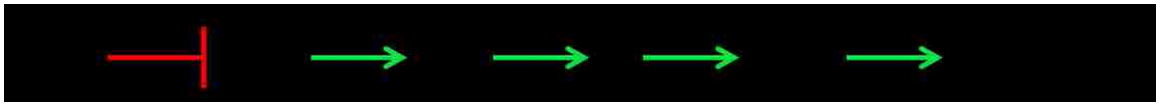


Figure 1.7. The Involvement of PRKA-mediated stimulation of FAO in meiotic resumption.

CHAPTER II

A REQUIREMENT FOR FATTY ACID OXIDATION IN THE HORMONE-INDUCED MEIOTIC RESUMPTION OF MOUSE OOCYTES

Summary

Our lab has previously shown that fatty acid oxidation (FAO) is required for AMP-activated protein kinase (PRKA)-induced maturation in vitro. In the present chapter, I have further investigated the role of this metabolic pathway in hormone-induced meiotic maturation. Incorporating an assay with ^{3}H-palmitic acid as substrate, I first examined the effect of PRKA activators on FAO levels. There was a significant stimulation of FAO in cumulus cell-enclosed oocytes (CEO) treated with AICAR and RSVA405. In denuded oocytes (DO), AICAR stimulated FAO only in the presence of carnitine, the molecule that facilitates fatty acyl CoA entry into mitochondria. The carnitine palmitoyltransferase 1 (CPT1) activator C75 successfully stimulated FAO in CEO. All three of these activators trigger germinal vesicle breakdown. Meiotic resumption induced by follicle-stimulating hormone (FSH) or amphiregulin was completely inhibited by the FAO inhibitors etomoxir, mercaptoacetate, and malonyl CoA. Importantly, FAO was increased in CEO stimulated by FSH and epidermal growth factor (EGF), and this increase was blocked by FAO inhibitors. Moreover, compound C, a PRKA inhibitor, prevented the FSH-induced increase in FAO. Both carnitine and palmitic acid augmented hormonal induction of maturation. In a more physiological setting, etomoxir eliminated hCG-induced maturation in follicle-enclosed oocytes. In

addition, CEO and DO from hCG-treated mice displayed an etomoxir-sensitive increase in FAO, indicating that this pathway was stimulated during in vivo meiotic resumption. Taken together, our data indicate that hormone-induced maturation in mice requires a PRKA-dependent increase in FAO.

Introduction

Meiotic resumption is regulated by a number of different molecules in the oocyte as well as the cumulus granulosa and mural granulosa cells. The granulosa compartment of the follicle plays a key role in maintaining the oocyte in prophase-I arrest. Thus, oocytes removed from the follicle and cultured in suitable medium resume meiosis spontaneously in the absence of gonadotropins, visually evident by the loss of the nuclear envelope, called germinal vesicle breakdown (GVB). Meiotic arrest is maintained by keeping cAMP levels high in the oocyte, which blocks the activation of maturation promoting factor, responsible for triggering the downstream events of meiosis, including GVB (Han and Conti, 2006). This cAMP-mediated arrest is overcome by gonadotropin stimulation of the somatic compartment that leads to phosphodiesterase-mediated degradation of cAMP. AMP, the end product of such degradation, is an activator of AMP-activated protein kinase (PRKA) that acts as a fuel gauge and responds to a high AMP:ATP ratio by turning off energy-consuming pathways and turning on energy-generating pathways (Hardie et al., 1998; Hardie, 2011).

Activation of PRKA is essential for the resumption of meiotic maturation in mouse oocytes (Chen et al. 2006; Chen and Downs 2008; LaRosa and Downs 2006; 2007). Treatment of meiotically arrested mouse oocytes with PRKA activators 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Corton et al., 1995) or the resveratrol analogue RSVA405 (Vingtdeux et al., 2011) stimulates meiotic resumption in vitro (Downs et al., 2002b; Chen et al., 2006; Ya and Downs, 2012). On the other hand, Compound C, a PRKA inhibitor (Zhou et al., 2001), blocks both the activation of PRKA

and meiotic induction brought about by treatment with AICAR (Chen et al., 2006), hormones (Chen and Downs, 2008) or stress (LaRosa and Downs, 2006, 2007), implicating PRKA as an important player in the mechanism(s) regulating meiotic induction.

Acetyl CoA carboxylase (ACC), an important substrate of PRKA, converts acetyl CoA to malonyl CoA, which plays two important roles in lipid metabolism: 1) it acts as precursor in the synthesis of long-chain fatty acids; and 2) it inhibits carnitine palmitoyltransferase 1 (CPT1), the mitochondrial outer membrane enzyme that links a carnitine moiety to fatty acyl to facilitate transport into mitochondria for beta-oxidation (Hardie and Pan, 2002). PRKA phosphorylates ACC at Serine 79 (mouse), resulting in its inactivation, and the resulting decrease in malonyl CoA removes the impediment to mitochondrial fatty acid transport and thereby promotes fatty acid oxidation (FAO)(Hardie and Pan, 2002). Thus, ACC provides an important link between PRKA activation and FAO.

Carbohydrates have attracted more attention than lipids as potential energy sources for the study of mammalian oocyte maturation, and their utilization has been taken into account for many culture medium formulations. Pyruvate is an important substrate utilized directly by the oocyte (Biggers et al., 1967; Downs et al., 2002a; Harris et al., 2009), and, although the oocyte is poorly equipped to metabolize glucose, this substrate is crucial for meiotic and developmental competence (Sutton-McDowall et al., 2010), since the somatic follicular cells readily metabolize glucose to pyruvate that is then utilized by the oocyte (Biggers et al., 1967; Donahue and Stern, 1968; Leese and

Barton, 1985). Relative carbohydrate levels in culture medium have immense effect on meiotic resumption (Downs & Mastropolo, 1994; Fagbohun & Downs, 1992).

With so much support for carbohydrate involvement in oocyte development, there has been less investigation to address lipid metabolism, particularly in rodent oocytes. Rodents have been a popular model system for oocyte maturation studies, but these oocytes have a very low level of lipid when compared to other mammals (e.g., 4 ng/oocyte in the mouse (Loewenstein and Cohen, 1964) versus 63 ng in the cow, 89 ng in the sheep and 161 ng in the pig (McEvoy et al., 2000)). It is not surprising that lipids represent an important energy source for oocytes from domestic species (reviewed by (Sturmey et al., 2009; Songsasen, 2012)). One untested speculation attempting to explain the disparity of lipid content between species is the different amounts of time taken by embryos to implant in the uterine wall and thus different needs of energy stores within the oocyte (Sturmey et al., 2009). In a study by Cetica et al (2002) (Cetica et al., 2002), the lipase activity in bovine oocytes was found to increase during meiotic maturation, whereas Ferguson and Leese (2006) (Ferguson and Leese, 2006) reported that methyl palmoxirate, a CPT1 inhibitor, decreased oxygen consumption in maturing bovine oocytes and affected subsequent blastocyst development.

Despite low levels of lipid in mouse oocytes, PRKA involvement in oocyte maturation and its well established regulation of FAO led us to examine a possible role for FAO in meiotic induction in mouse oocytes (Downs et al., 2009). The results of this earlier study provided compelling evidence that FAO plays an important role in PRKA-induced GVB in mouse oocytes. Among our findings, AICAR-induced maturation in vitro was blocked by inhibitors of fatty acid oxidation that include etomoxir, malonyl

CoA and mercaptoacetate. Etomoxir works in a similar fashion to that of malonyl CoA, by inhibiting CPT1-mediated fatty acid transport into mitochondria (Declercq et al., 1987), whereas mercaptoacetate inhibits long chain acyl CoA dehydrogenase, thereby blocking the first step in the beta-oxidation pathway (Bauché et al., 1981). On the other hand, stimulation of oocytes with C75, a CPT1 activator (Thupari et al., 2002), increased meiotic resumption in vitro, and this stimulatory effect was not blocked by inhibiting PRKA, suggesting that FAO stimulation can bypass PRKA inhibition.

Since our study, an important series of experiments has been presented by Dunning and associates (Dunning et al. 2010) that further implicated lipid metabolism as an integral component of oocyte maturation in mice. They demonstrated by radioisotopic assay, etomoxir-sensitive oxidation of fatty acids by CEO and DO as well as a direct relationship between the extent of FAO during oocyte maturation and developmental competence (similar findings have been reported for cow (Ferguson and Leese, 2006) and pig (Sturmey and Leese, 2003) oocytes); moreover, carnitine increased the cleavage percentage of one-cell embryos in the absence of external carbohydrate or protein sources, indicating utilization of internal lipid stores (Dunning et al. 2010). They extended these findings to show carnitine stimulation of FAO associated with increased developmental potential of oocytes grown in follicle culture (Dunning et al. 2011). Other groups have reported similar beneficial effects of carnitine on oocytes from a variety of species (e.g., (Abdelrazik et al., 2009; Somfai et al., 2011; Wu et al., 2011; You et al., 2012)), which is consistent with increased lipase activity during oocyte maturation (Cetica et al., 2002). Carnitine has beneficial effects beyond fatty acid utilization, also providing protection against reactive oxygen species (Ye et al., 2010); however, results of

experiments measuring FAO and effects of FAO inhibitors suggest a significant portion of its influence is through modulation of FAO. Manipulating FAO by providing media supplements such as carnitine holds promise for improving developmental competence in oocytes matured in vitro (Dunning & Robker, 2012).

In the present study, I have incorporated a metabolic assay for FAO to extend our work on meiotic induction, with special emphasis on more physiological hormonal stimulation. To this end, I(1) confirm the involvement of FAO in meiotic induction triggered by pharmacological PRKA activation and (2) demonstrate that hormone-induced maturation, whether occurring in vivo or in vitro, also requires FAO.

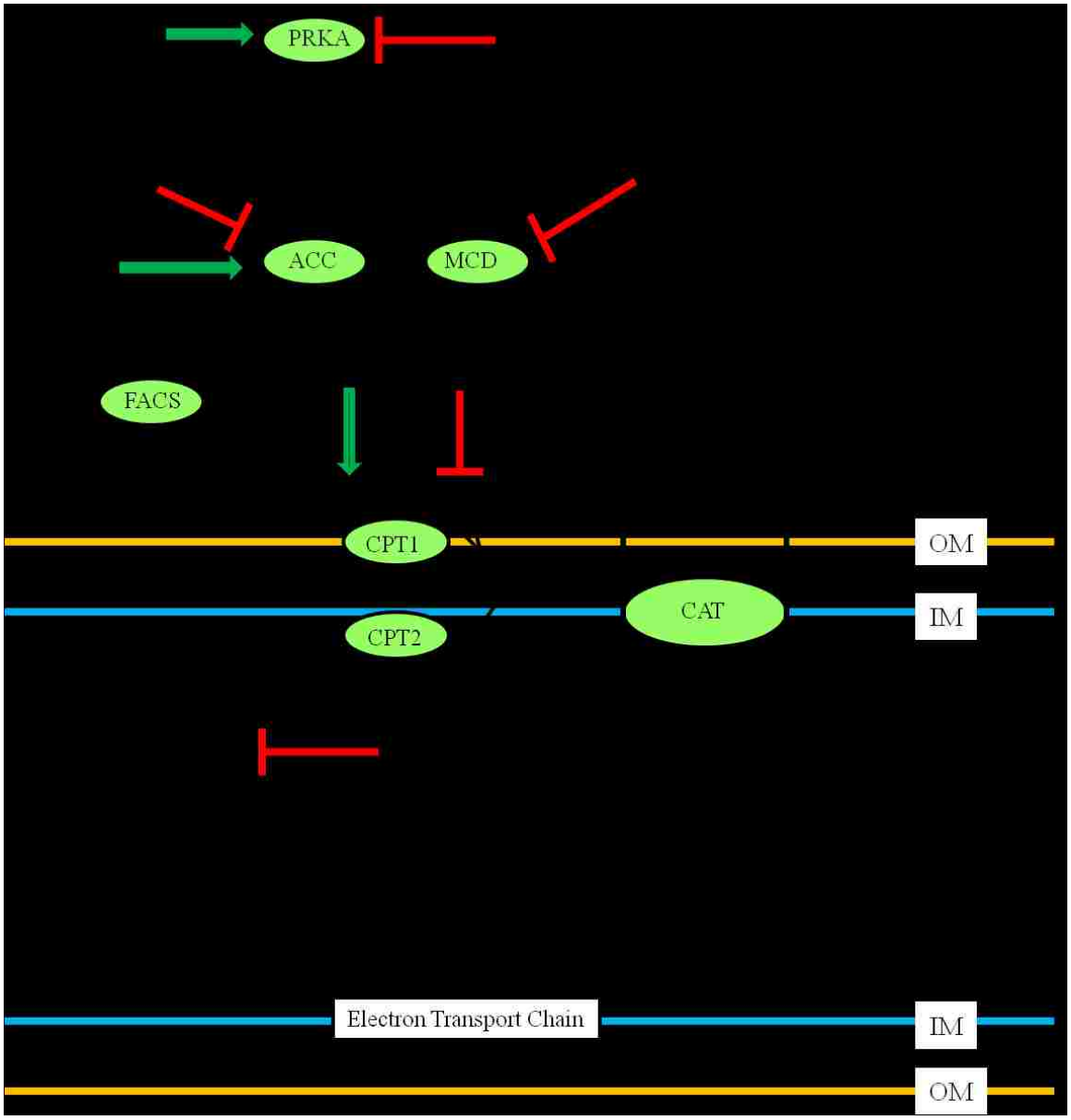


Figure 2.1. The sites of action of various activators and inhibitors that modulate fatty acid oxidation.

AICAR and RSVA405 are pharmacological activators or PRKA, whereas Compound C is a pharmacological inhibitor of PRKA. The synthetic compounds CP-640186 and Soraphen A inhibit both isoforms of ACC, whereas citrate, a physiological compound, activates ACC. The compound CBM-301106 is synthetic inhibitor of MCD, whereas C75 is a synthetic activator of CPT1. On the other hand, malonyl CoA is a physiological inhibitor of CPT1 and its action is mimicked by its synthetic analogue, etomoxir. Finally, mercaptoacetate is a pharmacological inhibitor of the first step in mitochondrial fatty acid oxidation.

Materials and Methods

Chemicals

Equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) and FSH were purchased from the National Hormone and Peptide Program and Dr. AF Parlow, whereas amphiregulin and epidermal growth factor (EGF) were obtained from R&D Systems. AICAR was from Toronto Reearch Chemicals, RSVA405 from ChemBridge and Compound C from Calbiochem (EMD Millipore). Tritiated palmitic acid and water were purchased from PerkinElmer. All other chemicals, including L-carnitine, were supplied by Sigma-Aldrich.

Oocyte Isolation and Culture

C57BL/6JxSJL/J F1 mice were used for all experiments. All work was carried out with the preapproval of the Marquette University Institutional Animal Care and Use Committee. 19-23-day-old females were primed with 5 IU eCG and killed 48 h later by cervical dislocation. Ovaries were dissected out and placed in a dish containing culture medium, and cumulus cell-enclosed oocytes (CEO) were isolated by puncturing large antral follicles with sterile 26 gauge needles. CEO were washed several times and distributed to tubes containing 1 ml of appropriate medium or microwells of a 96-well plate with a final medium volume of 100 μl, depending on the experiment involved. Denuded oocytes (DO) were obtained by repeated pipetting of CEO with a small bore

pipet to remove cumulus cells. For culture in tubes, Eagle's minimum essential medium (MEM) containing L-glutamine, and supplemented with 0.23 mM sodium pyruvate, 26 mM sodium bicarbonate, penicillin, streptomycin, and 3 mg/ml crystallized lyophilized bovine serum albumin (BSA) was used. Tubes were gassed with a humidified mixture of 5% CO_2/5% O_2/90% N_2, capped and sealed with parafilm, and incubated at 37^{0}C in a water bath. For fatty acid oxidation (FAO) assay cultures in 96-well plates, and for experiments testing the effect of palmitic acid or carnitine on FSH-induced maturation, fatty acid-free BSA was used. For cultures in 96-well plates (both FAO assays and maturation experiments), the above medium was buffered with 12.5 mM HEPES and 15.5 mM sodium bicarbonate.

Follicle culture

Ovaries from eCG-primed mice were transferred to a dish of Leibovitz's L-15 medium supplemented with 3 mg/ml BSA and antral follicles were dissected free using sterile 27 gauge needles. Follicles were then washed in MEM/5% fetal bovine serum (FBS) and transferred to 1ml of MEM/FBS in a 10 ml stoppered flask, gassed with 95% O_2/5% CO_2, capped, and incubated in a water-jacketed incubator at 37^{0}C with constant, gentle agitation for 3.5 hours .

Solubilization of palmitic acid

For experiments testing the effect of palmitic acid doses on FSH-induced

resumption of maturation, BSA-bound palmitic acid was used to enhance its solubility and reduce precipitation, and was prepared using a method described by Kane (1979). 500 mg of fatty acid-free BSA was dissolved in 30 ml sterile water to which was added 23 mg palmitic acid in 1 ml of absolute ethanol. This mixture was shaken well till clear, frozen overnight, and lyophilized.

Fatty Acid Oxidation Assay

Fatty acid oxidation (FAO) was measured using a modification of the protocol described by Dunning et al.(2010). All cultures were carried out in a volume of 100 µl in wells of a 96-well plate and contained 9 µCi 9,10-[^{3}H]palmitate, with cold palmitate added to bring the final concentration to 0.3 mM. Medium also contained 3 mg/ml fatty acid-free BSA, 12.5 mM HEPES, 15.5 mM sodium bicarbonate and, when appropriate, stimulators, or stimulators plus inhibitors, of FAO. A 2X solution of palmitate/BSA was first made by adding 250 µl 12 mg/ml fatty acid-free BSA in sterile water, 36 µl 9,10-[^{3}H]palmitate (specific activity 32.4 Ci/mmol, 5 mCi in 1 ml ethanol) and 25 µl 24 mM cold palmitate in ethanol to a plastic test tube and drying down at 55^{0}C under N_2 gas. It was then resuspended in 1 ml warm BSA-free MEM and maintained at 37^{0}C in a water bath until use. 50 µl of this mixture was placed in each microwell, to which was added 50 µl MEM/fatty acid-free BSA (3mg/ml) containing the appropriate number of CEO or DO and 2X concentration of milrinone, an inhibitor of oocyte-specific phosphodiesterase, and appropriate FAO modulators. The 96-well plates were transferred to a modular incubator gassed with a humidified mixture of 5% CO_2/5% O_2/90% N_2 and placed in a

37^0C water-jacketed incubator. Following culture, samples were processed as described by Dunning et al (Dunning et al., 2010), and 0.5 ml of the final supernatant was counted for each sample. 25 CEO or 40 DO per well were used for each experiment. Experiments were carried out at least 3 times, with duplicate groups per treatment. For each experiment, blanks were included that contained no tissue to determine background counts that were then subtracted from the radioactivity from each sample, and values were corrected for dilution during processing, percent recovery, and dilution with cold palmitate.

Statistical analysis

All experiments were repeated at least three times, and those involving oocyte maturation included at least 25 CEO or DO per group per experiment. Percent GVB values were subjected to arcsine transformation followed by ANOVA and Duncan's multiple range test to assess statistical significance, with a P value less than 0.05 considered significant. Maturation data are plotted as mean percent GVB ± SEM. FAO assay data were plotted as mean ratio of the treatment group compared to control ± SEM and were analyzed directly by ANOVA and Duncan's multiple range test.

Results

Pharmacological activators of PRKA induce maturation and stimulate FAO

I have previously shown that the PRKA activators, AICAR and RSVA405, stimulate meiotic maturation (Downs et al., 2002; Ya & Downs, 2013) and that AICAR-induced maturation is prevented by agents that block FAO (Downs et al., 2009). These results implicated FAO as an essential component of the meiotic induction process. It was therefore important to establish that FAO could indeed be demonstrated during meiotic induction triggered by pharmacological activation of PRKA. To this end, I used an assay reported by Dunning et al (Dunning et al., 2010) in which oxidation was determined by the production of tritiated water from radiolabeled palmitic acid. Before carrying out the FAO assays, I first established that meiotic induction was observed under the conditions of the assay; ie, when oocytes maintained in meiotic arrest with milrinone in 100 μl cultures within 96-well plates were stimulated with the appropriate meiotic inducer. Under these conditions, AICAR and RSVA405 triggered germinal vesicle breakdown (GVB) in CEO by 52% and 25%, respectively (Fig.2.3).

FAO assays were then carried out for CEO cultured in the presence of carnitine, PRKA activator, or the two agents together. Carnitine is a molecule that is bound to fatty acids at the outer mitochondrial membrane by the action of carnitine palmitoyltransferase 1 and helps facilitate transport into mitochondria, thereby promoting their oxidation (Hardie and Pan, 2002). In the presence of carnitine alone, FAO was stimulated in CEO by nearly 1.8-1.9-fold (Fig. 2.2 A, C). AICAR and RSVA405 also stimulated oxidation, by 1.6- and 1.4-fold, respectively. The addition of carnitine to AICAR- or RSVA405-

containing medium did not result in an additive effect (Fig. 2.2A, C). When DO were tested, AICAR stimulated GVB in the microwells (Fig. 2.3); however, neither carnitine nor AICAR alone stimulated FAO, although the two agents together increased FAO by 1.8-fold (Fig.2.2 B).

Our lab has shown that C75, a CPT1 activator (Thupari et al., 2002), induces meiotic resumption (Downs et al., 2009); herein, I confirmed that C75 stimulates meiotic resumption in 96-well plates (Fig. 2.3). As shown in Fig. 2.2D, C75 stimulated FAO in CEO by 1.6-fold. In this way, C75 stimulation of maturation was associated with an increase in FAO, and its effect in combination with carnitine was not additive.

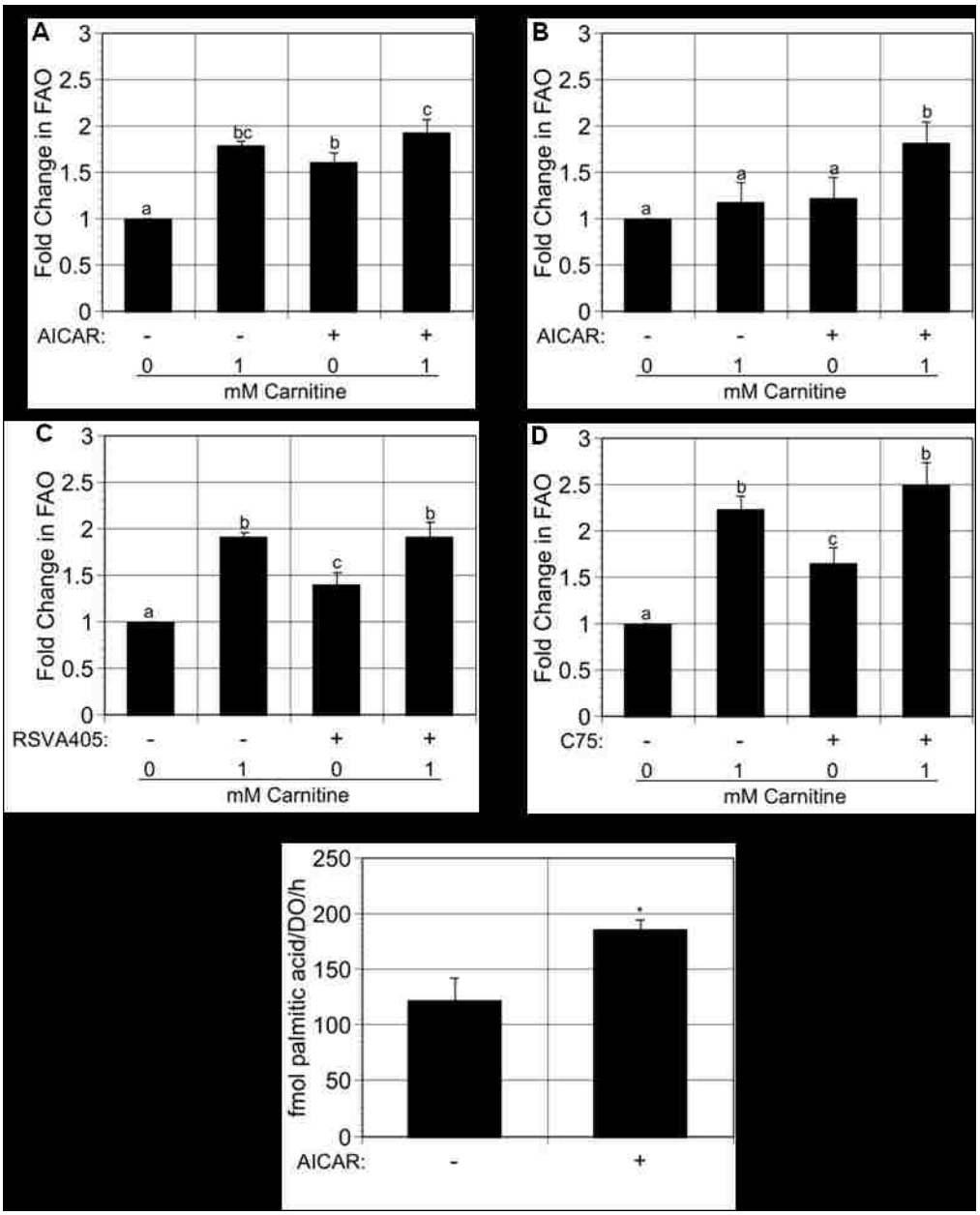


Figure 2.2. Pharmacological activators of PRKA, and C75, a CPT1 activator, stimulate FAO in mouse oocytes.

For FAO assay, CEO (A,C) were maintained in meiotic arrest with 2 μM milrinone and cultured with or without 1 mM carnitine and/or 250 μM AICAR (A) or 1.5 μM RSVA405 (C) for 18 h. For assays with DO (B), oocytes were cultured in 1 μM milrinone ± 1 mM carnitine ± 125 μM AICAR for 8 h. In (D), FAO levels were measured in CEO cultured in medium containing 2 μM milrinone ± 1 mM carnitine ± 25 μM C75. In (E), DO were pre-cultured in 1 μM milrinone ± 250 μM AICAR for 4 h followed by washing and well culture for FAO assay in 1 μM milrinone for 18 h. Groups with no common letter are significantly different. In (E), the asterisk indicates a significant difference by unpaired t-test (P value= 0.0381).

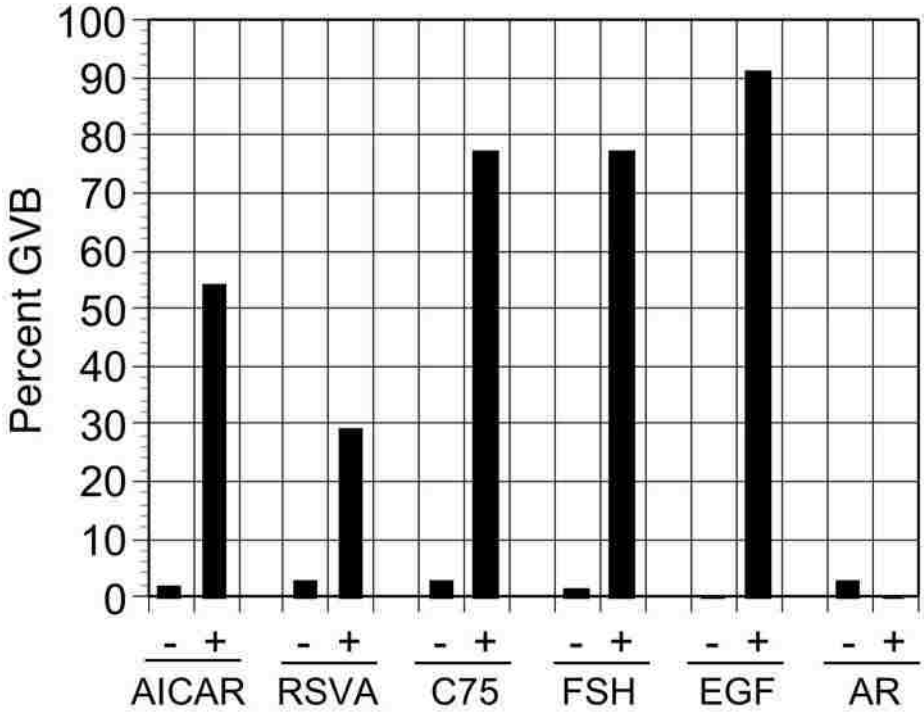


Figure 2.3. Effects of pharmacological and physiological stimulators on meiotic maturation in microwell culture system used for FAO assays.

CEO were cultured 17-18 h in 2 μM milrinone ± 250 μM AICAR, 1.5 μM RSVA405, 25 μM C75, 0.1 μg/ml FSH, 10 ng/ml EGF or 50 ng/ml amphiregulin and scored for GVB 18 h later.

Hormone-induced resumption of maturation is accompanied by an increase in FAO levels and blocked by inhibitors of CPT1 and FAO

I examined whether inhibitors of CPT1 and FAO affect FSH-induced maturation. CEO were cultured in tubes and maintained in meiotic arrest in medium containing dibutyryl cAMP (dbcAMP; a cAMP-mimicking agent, (Cho et al., 1974)), and FSH was added to induce maturation. To these FSH-treated cultures, I added increasing doses of etomoxir, a pharmacological inhibitor of CPT1 (Weis et al. 1994), malonyl CoA, a physiological inhibitor of CPT1 (McGarry, Leatherman, and Foster 1978), and mercaptoacetate, a direct inhibitor of FAO (Bauché et al., 1981). In control medium, only 13-20% of the oocytes underwent GVB whereas FSH stimulated maturation by 44-64 %, and this effect was completely blocked to control levels by all three inhibitors in a dose-dependent fashion (Fig.2.4A-C).

I also carried out assays to investigate the effect of FSH on FAO. As shown in Fig.2.4D, FSH alone stimulated FAO by 1.8-fold, whereas carnitine and FSH together had an additive effect (FAO increased 2.5-fold). On the other hand, the three FAO inhibitors blocked FAO to control levels (Fig. 2.4D). Also, FSH-stimulated FAO was blocked by Compound C (Fig.2.4 F), a PRKA inhibitor (Zhou et al., 2001) that blocks FSH-induced meiotic resumption (Chen and Downs, 2008).

Epidermal growth factor (EGF) has also been shown to induce GVB in cultured mouse CEO (Downs, Daniel, & Eppig, 1988), whereas the maturation-inducing effect of FSH on the oocyte via cumulus cells is mediated by EGF-like peptides such as Amphiregulin (AR) (Park et al. 2004; Shimada et al. 2006; Downs and Chen 2008).

Thus, I tested if the three FAO inhibitors also affected AR-induced GVB. CEO were again cultured in tubes, maintained in meiotic arrest with dbcAMP, and AR was added to stimulate maturation. As shown in Fig. 2.5A-C, all three inhibitors completely prevented AR-induced GVB. I also tested if AR stimulated meiotic resumption in microwells (Fig. 2.3), but I were unable to achieve meiotic induction even at a concentration 4 fold higher than those used in tubes (data not presented). Thus, for FAO assays in microwells, EGF was used in place of AR, since EGF successfully stimulated maturation in the wells (Fig. 2.3). EGF stimulation increased FAO by 1.6-fold whereas together with carnitine, it had a greater stimulatory effect (2.2-fold increase, Fig. 2.5D). The EGF-induced stimulatory effect was completely blocked by etomoxir (Fig.2.5E).

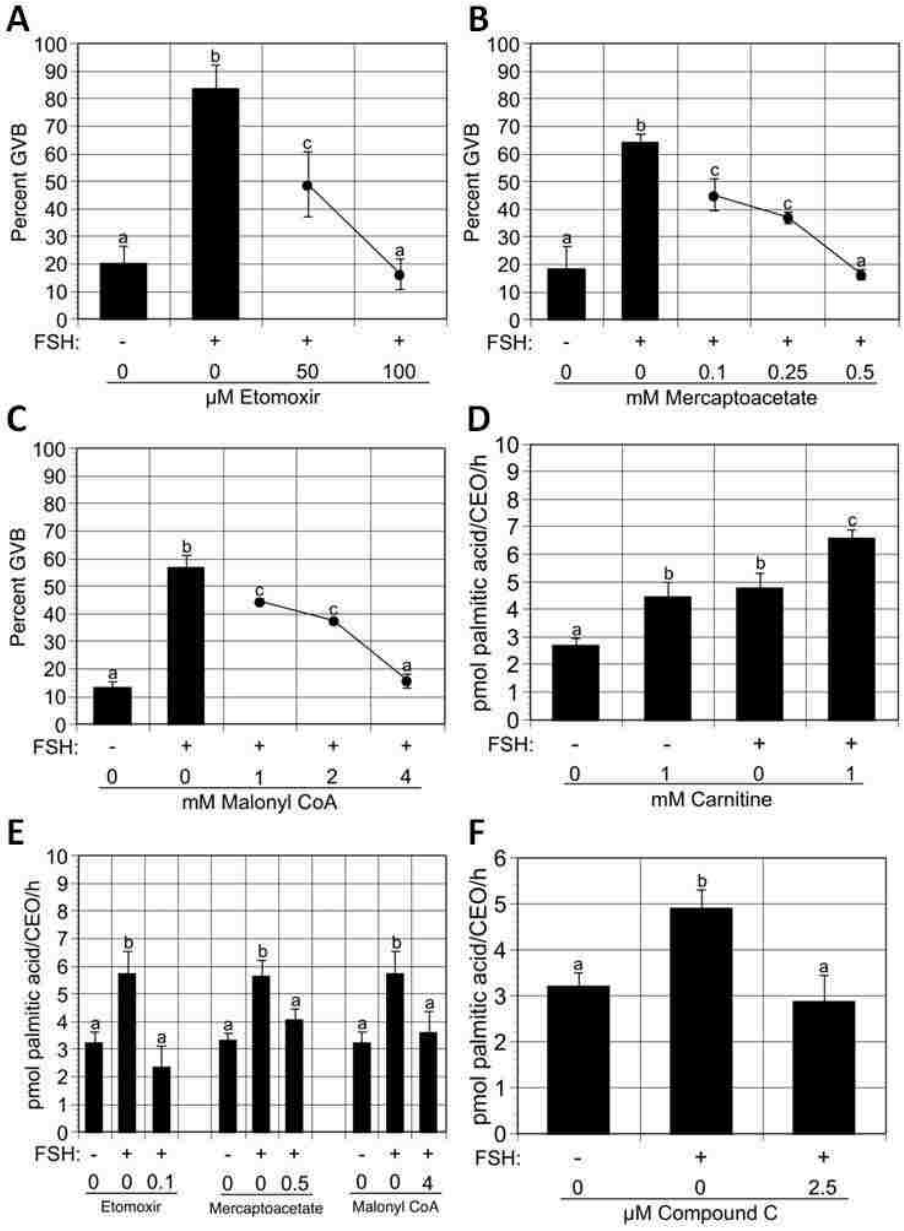


Figure 2.4. FSH-induced maturation is blocked by inhibitors of FAO and accompanied by increased FAO.

In (A-C), CEO were cultured in medium containing 300 µM dbcAMP ± 0.1 µg/ml FSH and exposed to different doses of etomoxir (A), mercaptoacetate (B) or malonyl CoA (C) for 17-18 h and assessed for GVB. FAO levels were measured in CEO cultured in 2 µM milrinone ± 0.1 µg/ml FSH for 18 h in the presence or absence of 1 mM carnitine (D), an FAO inhibitor (etomoxir, mercaptoacetate or malonyl CoA) (E), or 2.5 µM compound C, a PRKA inhibitor (F). In (E), concentrations of FAO inhibitors are in mM. Groups with no common letter are significantly different. The asterisk (*) indicates significant difference from control, obtained using a paired t-test with a p value of 0.0043.

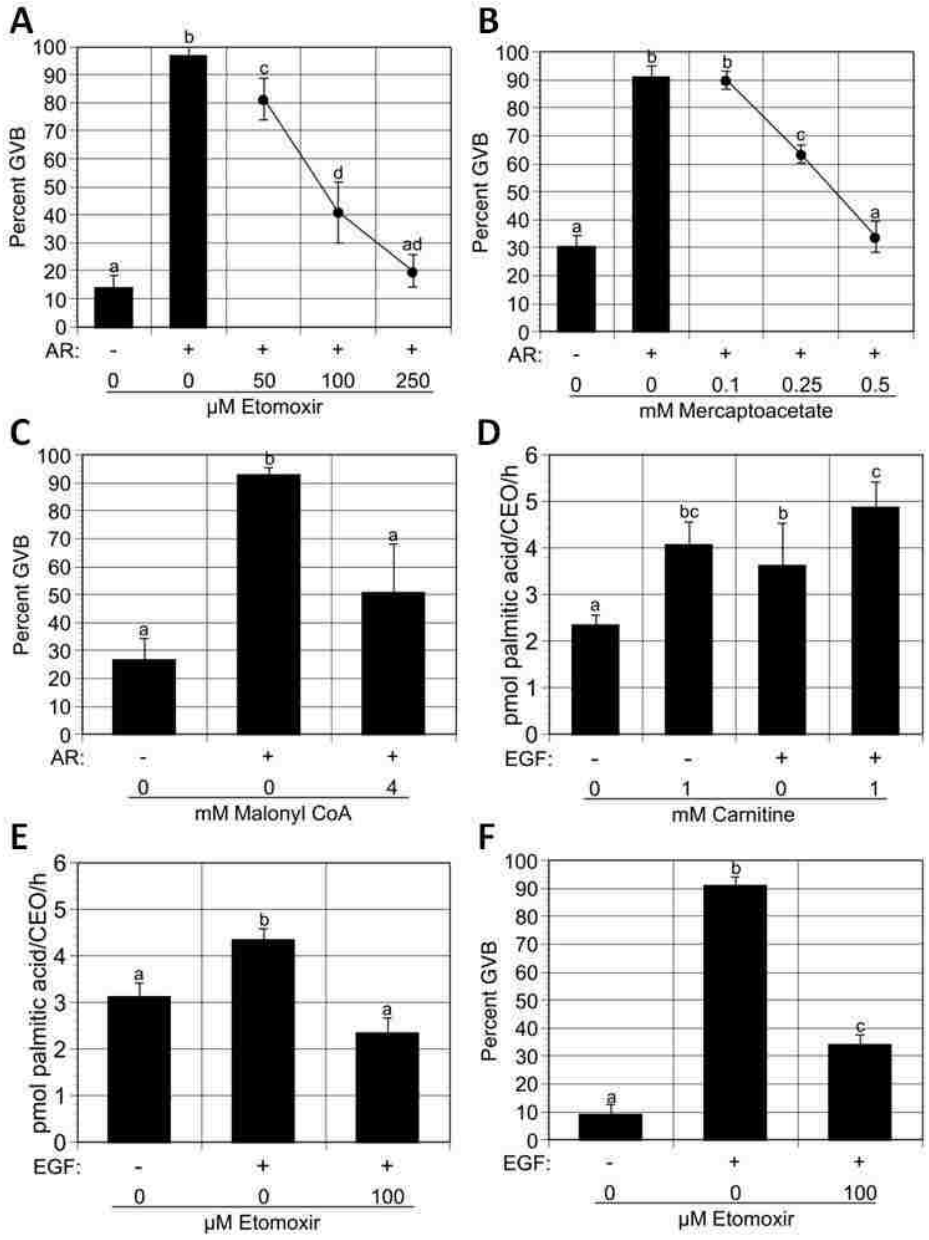


Figure 2.5. Amphiregulin-induced maturation is associated with an increase in FAO and blocked by inhibitors of FAO.

In (A-C), CEO were cultured in medium containing 300 μM dbcAMP ± 50 ng/ml amphiregulin (AR) and exposed to different doses of etomoxir (A), mercaptoacetate (B) or malonyl CoA (C), and assessed for GVB 17-18 h later. FAO was measured in CEO cultured in 2 μM milrinone ± 10 ng/ml EGF in the presence or absence of 1 mM carnitine (D) or 100 μM etomoxir (E) for 18 h. Groups with no common letter are significantly different.

Carnitine and Palmitic acid enhance hormone-induced meiotic resumption

Since PRKA activation-induced meiotic resumption was augmented by either carnitine or palmitic acid (Downs et al., 2009), I tested whether they also promoted maturation in hormone-stimulated CEO. These experiments were carried out in plastic tubes. CEO were maintained in meiotic arrest with dbcAMP and different doses of carnitine or palmitic acid were added in the presence or absence of FSH or AR. Both carnitine and palmitic acid significantly enhanced meiotic resumption at 1 mM and 0.5 mM, respectively, in the presence of FSH and AR, whereas they had no significant effect on GVB in the absence of hormone (Fig. 2.6A, B).

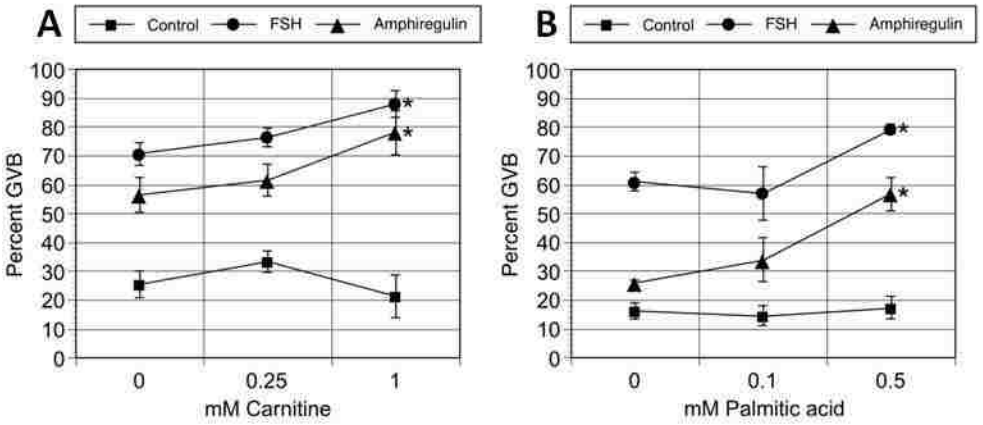


Figure 2.6. Carnitine and palmitic acid enhance hormone-induced maturation.

CEO were cultured in medium containing 300 μM dbcAMP ± 10 ng/ml FSH or 1 ng/ml AR, and treated with increasing concentrations of carnitine (A) or palmitic acid (B) and scored for percent GVB 17-18 h later. An asterisk denotes a significant difference from controls.

hCG-induced meiotic resumption and stimulation of FAO is blocked by etomoxir

Meiotic arrest in follicle-enclosed oocytes in culture is maintained by the granulosa cells, and meiotic resumption requires gonadotropin stimulation (Downs, 2010). Therefore, follicle culture more closely mimics the physiological system of oocyte maturation than isolated oocyte-cumulus complexes, making it crucial to test the effect of FAO inhibitors on oocyte maturation in follicle-enclosed, hCG-stimulated oocytes. Follicles were cultured in inhibitor-free medium plus or minus hCG, and the effect of etomoxir was tested. After 3.5 h of culture, 13% of control oocytes had undergone GVB, and hCG increased this number to 90% (Fig. 2.7A). The addition of etomoxir completely blocked hCG-stimulated maturation (14% GVB).

In addition, I measured FAO in oocytes isolated 2 h after hCG injection to eCG-primed mice. Both CEO and DO retrieved from hCG-injected mice displayed higher levels of FAO compared to untreated control oocytes (increases of 1.8- and 1.9-fold) that were blocked by etomoxir (Fig.2.7 B,C).

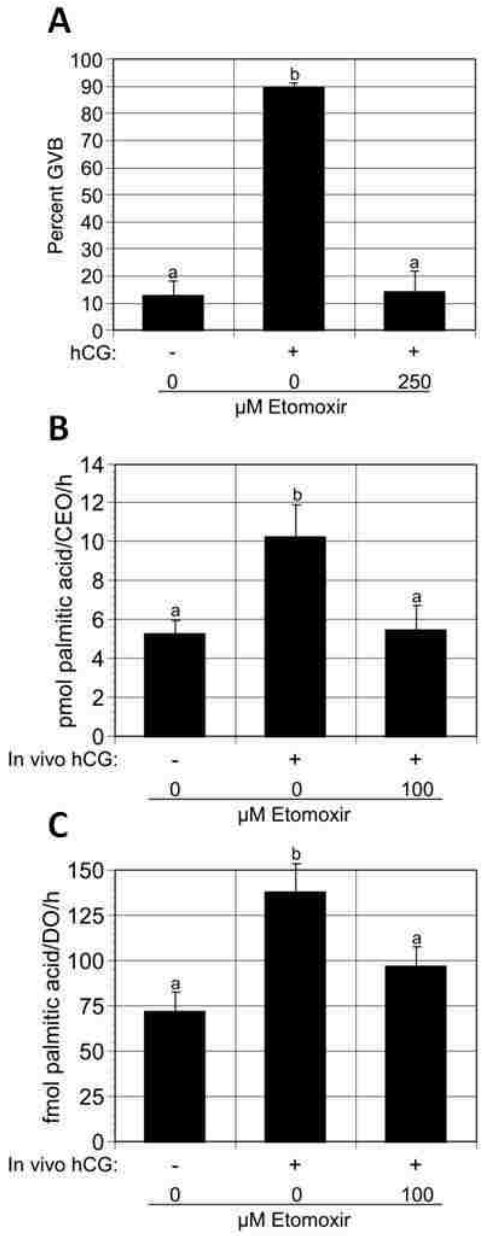


Figure 2.7. Human CG-induced meiotic resumption and stimulation of FAO are blocked by etomoxir.

In (A), antral follicles from eCG-primed mice were cultured in the presence or absence of 5 IU/ml hCG ± 250 μM etomoxir and GVB was assessed 3.5 h later. For (B,C), eCG-primed mice were injected with 5 IU hCG, and CEO (B) or DO (C) were isolated 2 h post-hCG injection. FAO was measured after 16 h culture in the presence of 2 μM (B) or 1 μM (C) milrinone ± 100 μM etomoxir. Oocytes from eCG-primed mice not injected with hCG were cultured as controls. Groups with no common letter are significantly different.

Discussion

In a previous study (Downs et al., 2009), the use of PRKA and FAO activators and inhibitors implicated FAO in the pharmacological stimulation of meiotic resumption in vitro. Herein, I have confirmed this finding by carrying out FAO assays under similar experimental conditions. In addition, I extended these experiments by examining the role of FAO in hormone-induced GVB in both cumulus cell- and follicle-enclosed oocytes. Whether maturation was stimulated by FSH, EGF-related peptides or hCG, two consistent findings were obtained: (1) meiotic induction was blocked by FAO inhibitors; and (2) the hormones stimulated FAO that was also sensitive to these inhibitors. Since the resumption of maturation also requires the actions of PRKA, these results provide compelling evidence that hormone-induced maturation in mouse oocytes requires a chain of events involving PRKA stimulation of FAO.

The PRKA activators AICAR and RSVA405 (Corton et al., 1995; Vingtdeux et al., 2011) and the CPT1 activator C75 (Thupari et al., 2002) all stimulate oocyte maturation. I have used a radioisotopic FAO assay to show that this meiotic resumption is associated with increased FAO, thereby confirming what was inferred by the use of FAO-modulating agents in our earlier study (Downs et al., 2009). Treatment of CEO with these activators led to stimulation of FAO; however, in DO, AICAR stimulated FAO only when carnitine was present. Carnitine, a crucial compound for shuttling long chain fatty acids into mitochondria (Hardie and Pan, 2002), also stimulated FAO by itself, but only in CEO. The lack of its effect in DO is not due to an inability of the

oocyte to take up this compound, since AICAR stimulation of FAO in DO was carnitine-dependent.

Baseline levels of palmitate oxidation in control CEO and DO maintained in meiotic arrest with milrinone were, on the average, 3 ± 0.2 pmol/CEO/hr and 0.06 ± 0.01 pmol/DO/hr. These results indicate that the majority of FAO in CEO resides within the cumulus oophorus and suggest that such cumulus cell activity may play a role in meiotic regulation. Nevertheless, the direct effects of FAO-modulating agents on beta-oxidation and meiotic maturation in DO (Downs, Mosey, and Klinger 2009; data herein) demonstrate that stimulation of FAO within the oocyte is essential for successful meiotic induction. It should be noted that our levels of beta-oxidation are considerably higher than those reported by Dunning *et al* (Dunning et al., 2010). The reason for this is not clear, but may be influenced by type of culture medium, strain of mouse, or the degree of palmitate solubilization.

To further investigate the role of FAO in meiotic resumption, I examined its participation in hormone-induced maturation. Etomoxir, malonyl CoA and mercaptoacetate were used to test the effect of FAO inhibition on FSH- or amphiregulin-induced GVB. All three inhibitors completely blocked induction of oocyte maturation by either hormone. Furthermore, FSH and EGF caused a 1.7- and 1.4-fold stimulation of FAO, respectively, that was reduced to control levels by FAO inhibitors. The addition of carnitine to FSH-treated, but not EGF-treated, CEO produced an additive effect on FAO. In this way, I demonstrated that hormonal induction of maturation, like that achieved with PRKA activators, is accompanied by increased FAO, whereas inhibition of FAO blocks meiotic resumption. That carnitine and palmitic acid each significantly enhanced

FSH- and AR-induced maturation provides further support for the participation of the FAO pathway in the mechanism(s) controlling hormone-induced GVB in isolated CEO in vitro. Moreover, since FSH-induced GVB requires activation of PRKA (Chen and Downs, 2008) and one of the major effects of PRKA activation in cells is an increase in FAO (Hardie et al., 2006), the suppression of FSH-induced FAO by compound C indicates that active PRKA was crucial for the increase in FAO by FSH.

While carnitine alone stimulates FAO in CEO, it does not induce meiotic resumption, though it augments the meiosis-inducing action of PRKA (Downs et al., 2009) and hormone stimulation. This suggests that FAO is required, but by itself not sufficient, to induce GVB without stimulation of PRKA or some other physiological response. Yet C75 alone stimulates FAO to a similar extent but also triggers meiotic resumption; furthermore, the fatty acid derivatives palmitoyl carnitine and stearoyl carnitine also stimulate maturation, presumably by feeding directly into the FAO pathway (Downs et al., 2009). The reason for these discrepancies is not clear, but possibilities include differing kinetics of FAO activation by various modulating agents or limitations in our assay in discriminating between subtle differences in FAO levels. Also, it should be noted that carnitine or palmitic acid are substrates of CPT1, not its activators.

To increase the physiological relevance of our findings, I tested follicle cultures and oocytes retrieved 2h after hCG administration to primed animals. Similar to results in hormone-stimulated CEO in vitro, the increase in maturation of follicle-enclosed oocytes brought about by hCG was completely abrogated by etomoxir. And, as predicted, DO and CEO from hCG-injected mice displayed 1.8-1.9-fold increases in FAO that were inhibited by etomoxir. This finding is important not only because the FAO

increase occurred in response to an in vivo hormonal stimulus, but also because it supports a role for FAO within the oocyte during intrafollicular meiosis reinitiation.

Dunning et al. (2010, 2011) demonstrated etomoxir-sensitive hormonal regulation of beta-oxidation in mouse CEO and DO and augmentation of this effect with carnitine. In addition, they reported that modulation of FAO during follicular development and oocyte maturation has a profound effect on developmental competence. Also, interestingly, stimulation of FAO by carnitine in the absence of exogenous energy sources provided sufficient energy to drive embryo development. I have shown that beta-oxidation is also an essential feature of meiotic induction in mouse oocytes, whether it occurs in response to pharmacological stimulation in vitro or is driven by hormones in vitro or in vivo. Taken together, these data show a role for FAO throughout oocyte maturation, beginning at the earliest stage of GVB and extending to its completion at metaphase II, and during embryogenesis. (Chen & Downs, 2008; Chen et al., 2006; Downs, 2010; Ya & Downs, 2013). Based on the well-established regulatory role for PRKA in beta-oxidation and our confirmation of this relationship in the mouse oocyte, it is reasonable to propose that a significant portion of PRKA influence on mouse oocyte physiology during this period is mediated by FAO.

In conclusion, I have demonstrated an essential role for FAO in the meiotic induction process in mouse oocytes. The fact that mouse oocytes have very limited lipid reserves does not discount their potential importance, since only a small amount of energy-rich lipid would be required to produce considerable energy via beta-oxidation to drive meiotic resumption (Dunning et al. 2010; Sturmey et al. 2009). Meiotic maturation in the mouse oocyte requires PRKA activation (Chen et al., 2006) that leads to increased

FAO (Downs, Mosey, and Klinger 2009 and data herein) through the inactivation of acetyl CoA carboxylase located at the mitochondrial outer membrane (Dunning et al. 2010; see Introduction). This would necessitate close proximity of lipid substrate to the mitochondria, and, consistent with this idea, mitochondria/lipid droplet colocalization has been demonstrated in the porcine oocyte (Sturmey et al., 2006). Beta oxidation may also remove fatty acids that would prove harmful to the oocyte or suppress maturation if allowed to reach threshold levels (eg, through long-chain acetyl CoA synthetase-mediated palmitoylation reactions (Wang et al., 2012)). Future studies will hopefully shed important light on the interplay between the different metabolic pathways involved in energy dynamics during gametogenesis.

CHAPTER III

THE INVOLVEMENT OF ACETYL COA CARBOXYLASE INACTIVATION DURING MEIOTIC RESUMPTION OF MOUSE OOCYTES

Summary

In previous studies, I have shown that various inhibitors of fatty acid oxidation prevent meiotic resumption, whether induced by pharmacological activation of PRKA (AMP-activated protein kinase), or by hormones in vitro or in vivo (Downs et al., 2009; Valsangkar and Downs, 2013). Moreover, I have also shown that this meiotic resumption is accompanied by a significant stimulation of fatty acid oxidation (FAO). PRKA stimulates FAO by phosphorylating and inactivating acetyl CoA carboxylase (ACC), an enzyme that converts acetyl CoA to malonyl CoA, an inhibitor of fatty acid transport into mitochondria where FAO takes place (Hardie and Pan, 2002; Tong and Harwood, 2006). To determine whether a majority of PRKA action on meiotic resumption is mediated via the inactivation of ACC, I investigated the role of ACC inactivation in meiotic resumption in this study. To this end, I first tested the effect of two ACC inhibitors that act on different sites in the ACC molecule on the meiotic status of mouse oocytes in vitro. Both ACC inhibitors significantly stimulated meiotic resumption in cumulus cell-enclosed oocytes (CEO), denuded oocytes (DO), as well as follicle enclosed oocytes. Moreover, this was also accompanied by an increase in FAO. Etomoxir, a malonyl CoA analogue, prevented meiotic resumption as well as FAO increase induced by the two ACC inhibitors. The physiological ACC activator citrate and CBM-301106, an inhibitor

of malonyl CoA decarboxylase (MCD, an enzyme that reduces malonyl CoA levels by converting it back to acetyl CoA, thereby being stimulatory to FAO) both prevented meiotic resumption and FAO increase induced by FSH (follicle stimulating hormone). I also determined that mouse oocytes contain both isoforms of ACC (ACC1 and ACC2). Finally, to examine the effect of in vivo ACC2 loss, I compared FAO and the rate of meiotic resumption in WT and ACC2 knockout oocytes and found that the KO showed a significantly higher FAO level and meiotic resumption. Collectively, these data support the idea that a majority of maturation-promoting action of PRKA may be through the stimulation of FAO by ACC inactivation.

Introduction

Oocyte metabolism is an important aspect of meiotic and developmental outcome. Carbohydrate metabolism has been a popular topic of research in mouse oocyte and cumulus cells, whereas relatively very little is known about fatty acid metabolism. In 2009, our lab showed that meiotic resumption induced by pharmacological activation of PRKA (Protein Kinase, AMP-activated; a protein previously shown to be important for meiotic resumption) requires fatty acid oxidation (FAO) (Downs et al., 2009). Induction with AICAR, a PRKA activator (Corton et al., 1995), is blocked by pharmacological and physiological inhibitors of fatty acid oxidation, whereas C75, a pharmacological activator of carnitine palmitoyl transferase 1 (CPT1; the enzyme that facilitates fatty acid entry into mitochondria for oxidation) (Thupari et al. 2002; see Fig. 2.1) induces meiotic resumption. Next, I showed that hormone-induced meiotic resumption in vitro also requires FAO and is accompanied by FAO increase in vitro as well as in vivo (Valsangkar and Downs, 2013). PRKA is a cellular energy sensor that is activated by a high AMP:ATP ratio as well as stress (Hardie et al., 2012). Active PRKA turns off metabolic pathways that cost energy while turning on pathways that generate energy. One of the catabolic pathways activated by PRKA is FAO. PRKA inactivates an enzyme (acetyl CoA carboxylase, ACC), whose product, malonyl CoA, blocks fatty acid entry into mitochondria by inhibiting the activity of CPT1 (Hardie and Pan, 2002; Brownsey et al., 2006; Tong and Harwood, 2006). Apart from inhibiting FAO, malonyl CoA also serves as a precursor for the synthesis of long-chain fatty acids. Thus, by preventing malonyl CoA production, PRKA stimulates FAO while blocking long chain fatty acid

synthesis. In most eukaryotes, ACC is a single, multi-domain polypeptide chain (Brownsey et al., 1997; Tong and Harwood, 2006; Kim et al., 2010). The three domains of ACC are biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyl transferase (CT) (Fig. 3.1). In the first enzymatic step, the BC domain carboxylates the biotin molecule covalently linked to the BCCP domain. In the second step, the CT domain transfers the carboxyl group from biotin to acetyl CoA that results in the formation of malonyl CoA. In mammals, ACC exists in two isoforms ACC1 and ACC2 that are transcribed from two different genes and differ in tissue distribution and molecular weights. Although transcribed from different genes, the two proteins have highly similar sequences, the only major difference being the first ~100 extra amino acids at the N-terminus of ACC2 that begins with a hydrophobic sequence that suggests membrane targeting (Abu-Elheiga et al., 2000). Indeed, ACC2 and its N-terminal sequence fused to GFP were shown to be co-localized with CPT1 that is located on the mitochondrial outer membrane. Furthermore, while lipogenic tissues such as adipose and mammary glands predominantly express ACC1, lipolytic tissues such as the heart and skeletal muscle mainly express ACC2. This differential tissue distribution and cellular locations suggested different functions for the two isoforms. It is believed that the malonyl CoA produced by ACC1 (the cytosolic isoform) is mainly channeled towards the synthesis of long-chain fatty acids, whereas that produced by ACC2 (the isoform thought to be located on the mitochondrial outer membrane in the vicinity of CPT1) is mainly used to block FAO by inhibiting CPT1 (Abu-Elheiga et al., 2000; Tong and Harwood, 2006). PRKA can phosphorylate and inactivate both isoforms (Tong, 2005; Tong and Harwood, 2006). The phosphorylation site involved in this inactivation is the same in

both isoforms: in ACC1, it is Ser 79 and in ACC2, it corresponds to Ser 218 due to the first extra amino acids.

Since our lab has previously demonstrated the involvement of PRKA and FAO in meiotic maturation, it was of interest to investigate if ACC inactivation plays a role in this process. Therefore, in this study, I have tested the effect of ACC inhibitors and activators on meiotic maturation of mouse oocytes. Furthermore, I also studied the effect of ACC2 knockout on meiotic maturation and FAO in mouse oocytes and oocyte-cumulus complexes.

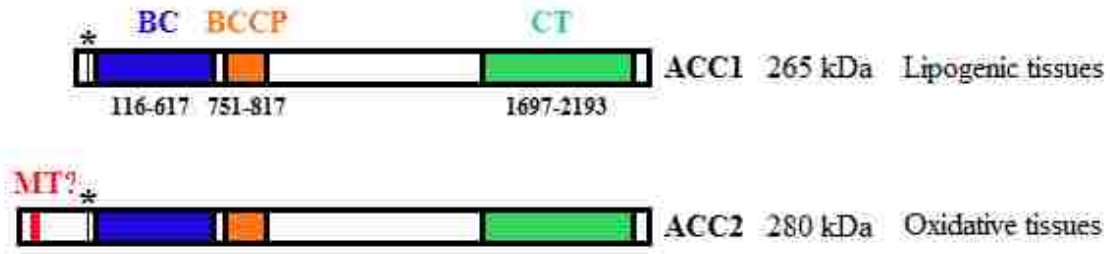


Figure 3.1. ACC isoforms.

Mouse ACC has 2 isoforms expressed from 2 different genes. While ACC1 predominates in lipogenic tissues, ACC2 is predominant in oxidative tissues. The two isoforms have a high sequence similarity, except ACC2 has extra amino acids at the N-terminus that includes a short hydrophobic sequence that has been suggested to be membrane-targeting (MT). Both isoforms have three domains: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyl transferase (CT). Length of each domain is shown with the beginning and ending amino acid number below each domain. PRKA phosphorylates ACC at several sites, but the only site for which a regulatory significance is known is the Ser 79 site in ACC1 that corresponds to Ser 218 in ACC2. This site is denoted with an asterisk and a vertical line in the two isoforms above. Note: diagrams are not to scale.

Materials and Methods

Chemicals

The hormones equine chorionic gonadotropin (eCG), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG) were purchased from the National Hormone and Peptide Program and Dr AF Parlow. AICAR was from Toronto Research Chemicals, CP-640186 was a gift from Pfizer, and Soraphen A, a gift from Dr Rolf Jansen. Compound C was from Calbiochem (EMD Millipore), and the MCD inhibitor CBM-301106 was a gift from Dr Gary Lopaschuk (University of Alberta). Tritiated palmitic acid and tritiated water were purchased from PerkinElmer. All other chemicals, including L-carnitine, were supplied by Sigma-Aldrich.

Oocyte Isolation and Culture

C57BL/6JxSJL/J F_1 mice or B6/129 F_2 mice (either WT or ACC2-/-) were used for all experiments. The ACC2-/- mice were kindly provided by Dr Wakil from the Baylor College of Medicine. All work was carried out with the preapproval of the Marquette University Institutional Animal Care and Use Committee. 19-23-day-old females were injected with 5 IU eCG and euthanized 48 h later by cervical dislocation. Ovaries were dissected out and placed in a dish containing culture medium, and large antral follicles were punctured with sterile 26 gauge needles to isolate cumulus cell-enclosed oocytes (CEO). CEO were washed 2 times and distributed to tubes containing 1

ml of appropriate medium or microwells of a 96-well plate with a final medium volume of 100 µl, depending on the experiment involved. Denuded oocytes (DO) were obtained by repeated pipetting of CEO with a small bore pipet to remove cumulus cells. For culture in tubes, Eagle minimum essential medium (MEM) containing L-glutamine, and supplemented with 0.23 mM sodium pyruvate, 26 mM sodium bicarbonate, penicillin, streptomycin, and 3 mg/ml crystallized lyophilized bovine serum albumin (BSA) was used. Tubes were gassed with a humidified mixture of 5% CO_2/5% O_2/90% N_2, capped and sealed with parafilm, and incubated at 37°C in a water bath. For fatty acid oxidation (FAO) assay cultures in 96-well plates, fatty acid-free BSA was used. For cultures in 96-well plates (both FAO assays and maturation experiments), MEM culture medium described above was buffered with 12.5 mM HEPES and 15.5 mM sodium bicarbonate.

Follicle culture

Ovaries dissected from eCG-primed mice were transferred to a dish of Leibovitz L-15 medium supplemented with 3 mg/ml BSA. Using sterile 27 gauge needles, antral follicles were isolated carefully. Follicles were then washed in MEM/5% fetal bovine serum (FBS) and transferred to 1ml of MEM/FBS in a 10 ml stoppered flask, gassed with 95% O_2/5% CO_2, capped, and incubated in a water-jacketed incubator at 37°C with constant, gentle agitation for 3.5 hours . GVB was assessed after culture by puncturing the follicles with needles and denuding the oocytes within.

Fatty Acid Oxidation Assay

Fatty acid oxidation (FAO) was measured using a modification of the protocol described by Dunning et al (Dunning et al., 2010). All cultures were carried out in a volume of 100 µl in wells of a 96-well plate and contained 9 µCi 9,10-[^{3}H]palmitate, with cold palmitate added to bring the final concentration to 0.3 mM. Medium also contained 3 mg/ml fatty acid-free BSA, 12.5 mM HEPES, 15.5 mM sodium bicarbonate and, when appropriate, stimulators, or stimulators plus inhibitors, of FAO. A 2X solution of palmitate/BSA was first made by adding 250 µl 12 mg/ml fatty acid-free BSA in sterile water, 36 µl 9,10-[^{3}H]palmitate (specific activity 32.4 Ci/mmol, 5 mCi in 1 ml ethanol) and 25 µl 24 mM cold palmitate in ethanol to a plastic test tube and drying down at 55^{0}C under N_2 gas. It was then resuspended in 1 ml warm BSA-free MEM and maintained at 37^{0}C in a water bath until use. 50 µl of this mixture was placed in each microwell, to which was added 50 µl MEM/fatty acid-free BSA (3mg/ml) containing the appropriate number of CEO or DO and 2X concentration of milrinone, an inhibitor of oocyte-specific phosphodiesterase, and appropriate FAO modulators. The 96-well plates were transferred to a modular incubator, gassed with a humidified mixture of 5% CO_2/5% O_2/90% N_2 and placed in a 37^{0}C water-jacketed incubator. Following culture, samples were processed as described by Dunning et al (Dunning et al., 2010), and 0.5 ml of the final supernatant was counted for each sample. 25 CEO or 40 DO per well were used for each experiment. Experiments were carried out at least 3 times, with duplicate groups per treatment. For each experiment, blanks were included that contained no tissue to determine background counts that were then subtracted from the radioactivity from each

sample, and values were corrected for dilution during processing, percent recovery, and dilution with cold palmitate.

Western blots

CEO or DO were isolated from WT or ACC2-/- mice, washed in PBS/PVP containing a protease-inhibitor cocktail, and appropriate volume of 2X Laemmli buffer and 2-mercaptoethanol were added. The samples were then boiled for 5 min, vortexed briefly, immediately stored at -20°C, and used within 2-3 weeks. Samples were resolved using SDS-PAGE on a 1.0 mm thick 3-8% Tris-acetate gel from Life Technologies and NuPAGE Tris-acetate SDS-PAGE buffer. The gel was run at 150 V for the initial 30 min and then at 70 V for 3 h to get desired separation of the two ACC isoforms. After electrophoresis, the gel was cut out and proteins were blotted by wet transfer method using NuPAGE Transfer Buffer containing 2% SDS and 10% methanol at 400 mA for 1h 15min with an ice tray inserted in the buffer tank. Protein transfer was confirmed by staining the blot in Ponceau S solution. The blot was blocked in 5% milk for 2 h, washed briefly and incubated overnight at 4 °C with 1:1000 dilution of anti-ACC rabbit monoclonal antibody (#3676 from Cell Signaling) in 5% BSA, washed with TBS and 0.5% Tween-20, and incubated for 1 h at RT with 1:4000 goat-anti-rabbit-HRP-conjugated secondary antibody in 5% milk, and washed in TBS. Finally, the ACC protein bands were visualized on film after incubating with SuperSignal West Pico signal detection system from Pierce.

Immunofluorescence

Denuded oocytes were fixed in 4% formaldehyde/PBS/PVP in the refrigerator overnight in Petri dishes. They were then brought to RT and transferred to PBS/PVP at RT, with 2 changes of solution, and then stored in the refrigerator and used for immunostaining within 1 week. For immunostaining, the oocytes were collected from PBS/PVP and placed in a well of a 96-well-plate containing permeabilization solution containing 1% Triton-X-100 in blocking solution for 30 min. Next, they were transferred in the next well containing blocking solution that consisted of 10% sheep serum and 0.005% Saponin in PBS/PVP and blocked for 1h 30min. They were then incubated with a 1:50 dilution of anti-Ser79-phospho ACC (#11818 from Cell Signaling) overnight in the cold room. They were then warmed to RT for 30 min and transferred through 4 washes of blocking solution to remove excess antibody. Then the oocytes were incubated in 1:1000 dilution of sheep-anti-rabbit FITC antibody for 1 h and washed 4 times as described previously. Washed oocytes were transferred to a glass slide, let dry almost completely, and then mounted in DAPI. Oocytes were imaged at 63X using a Zeiss LASER scanning confocal immunofluorescence microscope. For each experiment, the scanning parameters were kept identical between treatment groups.

Statistical analysis

All experiments were repeated at least three times, and those involving oocyte maturation included at least 25 CEO or DO per group per experiment. Percent GVB

values were subjected to arcsine transformation followed by ANOVA and Duncan multiple range test to assess statistical significance, with a P- value less than 0.05 considered significant. Maturation data are plotted as mean percent GVB ± SEM. FAO assay data were plotted as mean ratio of the treatment group compared to control ± SEM and were analyzed directly by ANOVA and Duncan multiple range test.

Results

ACC inhibitors induce meiotic resumption in DO, CEO and follicle-enclosed oocytes

Since PRKA activation leads to meiotic resumption, and since ACC is an important substrate of PRKA, the effect of ACC inhibitors was tested on meiotic resumption. To this end, DO and CEO were maintained in meiotic arrest with either hypoxanthine (Hx) or 3-isobutyl-1-methylxanthine (IBMX, a non-selective PDE inhibitor), and increasing doses of either CP-640186 or Soraphen A, both non-selective ACC inhibitors, were added. Oocytes were scored for GVB 16-17 h later. The ACC inhibitor CP-640186 increased GVB from 8% to 81% for CEO, and from 11% to 86% for DO in the presence of IBMX (Fig.3.2 A). In the presence of Hx, CP-640186 increased GVB from 36% to 96% for CEO and from 68% to 91% for DO (Fig.3.2 B). Similarly, GVB increased from 8% to 78% for CEO and from 15% to 43% for DO in the presence of IBMX (Fig.3.2 C), and from 26% to 85% for CEO and 51% to 75% for DO in the presence of Hx (Fig.3.2 D). In this way, both ACC inhibitors significantly induced GVB in DO as well as CEO arrested with either Hx or IBMX. To investigate if ACC inhibitor could induce meiotic resumption in a more physiological culture system, follicle-enclosed oocytes were treated with CP-640186 for 3.5 h and then scored for GVB. The ACC inhibitor successfully induced GVB in follicle-enclosed oocytes (Fig.3.3).

Meiotic resumption by both ACC inhibitors is accompanied by FAO stimulation

To see if ACC inhibition indeed results in a change in FAO, FAO was measured in CEO arrested with 2 μM milrinone (a selective PDE3A inhibitor) induced to resume meiosis with 50 μM CP-640186 or 100 μM Soraphen A. Both ACC inhibitors increased FAO in CEO about 1.5-fold (Fig.3.2 E, F). In DO, CP-640186 stimulated FAO 2.41-fold (2.41 ± 0.41) (data not illustrated). I also confirmed meiotic resumption by the two ACC inhibitors in the presence of milrinone in the 96-well plates that were used to assay FAO. At the concentrations used for FAO assay, CP-640186 increased meiotic resumption from 4% to 50% in CEO and from 2% to 58% in DO, whereas Soraphen A increased meiotic resumption from 0% to 54% in CEO.

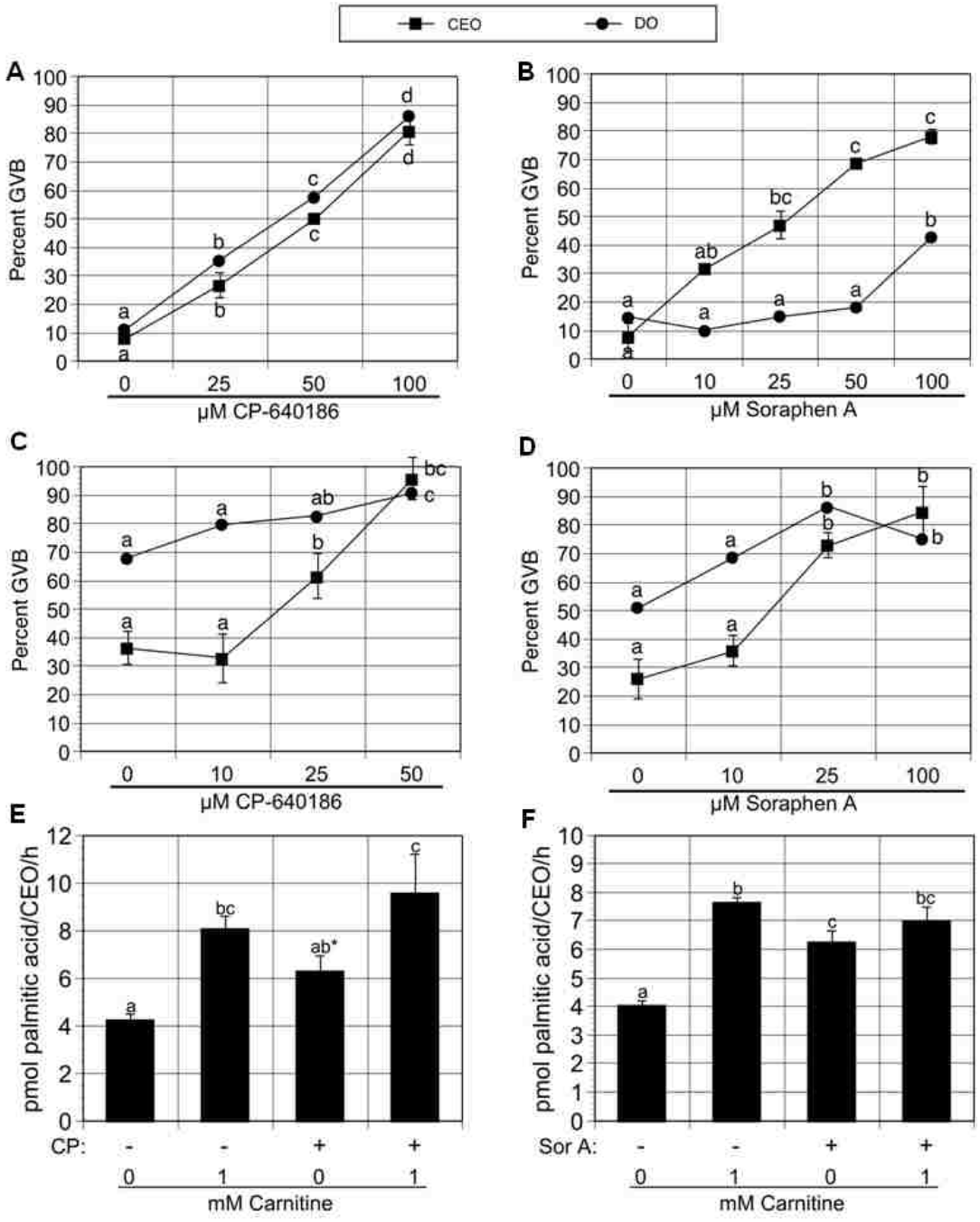


Figure 3.2. Pharmacological inhibitors of ACC stimulate meiotic resumption in mouse oocytes and this is accompanied by an increase in FAO.

CEO or DO maintained in arrest with either IBMX (A,B) or Hx (C,D) were treated with different doses of CP-640186 (A,C) or Soraphen A (B,D) and percent GVB was scored 18 h later. In (E,F), CEO were arrested with milrinone and treated with CP-640186 (E) or Soraphen A (F), and FAO was assayed for 18h. Groups with no common letter are significantly different. An asterisk (*) indicates significant difference by Student's t-test.

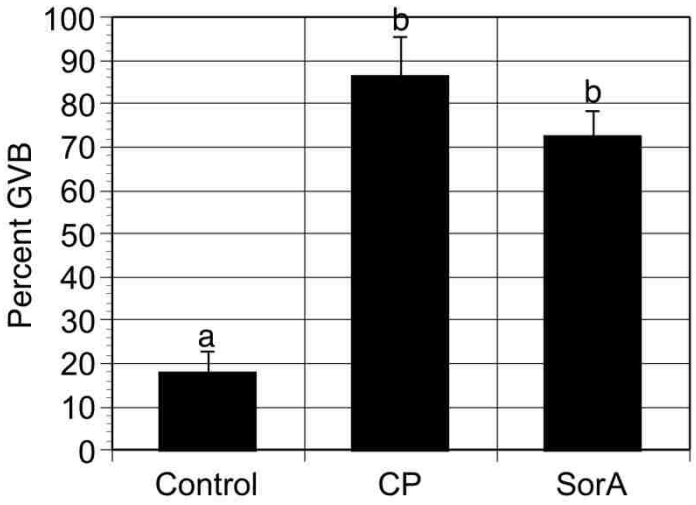


Figure 3.3. ACC inhibitors induce meiotic resumption in follicle-enclosed oocytes.

Follicles were isolated from ovaries and cultured in either control medium or medium containing 100 µM CP-640186 or Soraphen A. After 3.5h culture, oocytes were isolated from follicles and scored for percent GVB. Groups with no common letter are significantly different.

Inhibition of FAO prevents ACC inhibitor-induced meiotic resumption

To determine if the effect of ACC inhibitors on oocytes requires FAO, etomoxir, a CPT1 inhibitor (Weis BC et al., 1994; Ishida, 1997), was added to CEO induced to resume meiosis with CP-640186 or Soraphen A in the presence of Hx, and GVB was scored as described above. Blockage of fatty acid oxidation with etomoxir (as demonstrated in Fig. 3.4 C by a 2.7-fold decrease) resulted in inhibition of meiotic resumption in CEO. Etomoxir brought down GVB from 96% to 65% for CEO induced to mature with CP-640186, whereas for CEO stimulated with Soraphen A, it decreased GVB from 86% to 36% (Fig.3.4 A, B). These results indicate that FAO is required for ACC inhibitor-induced meiotic resumption.

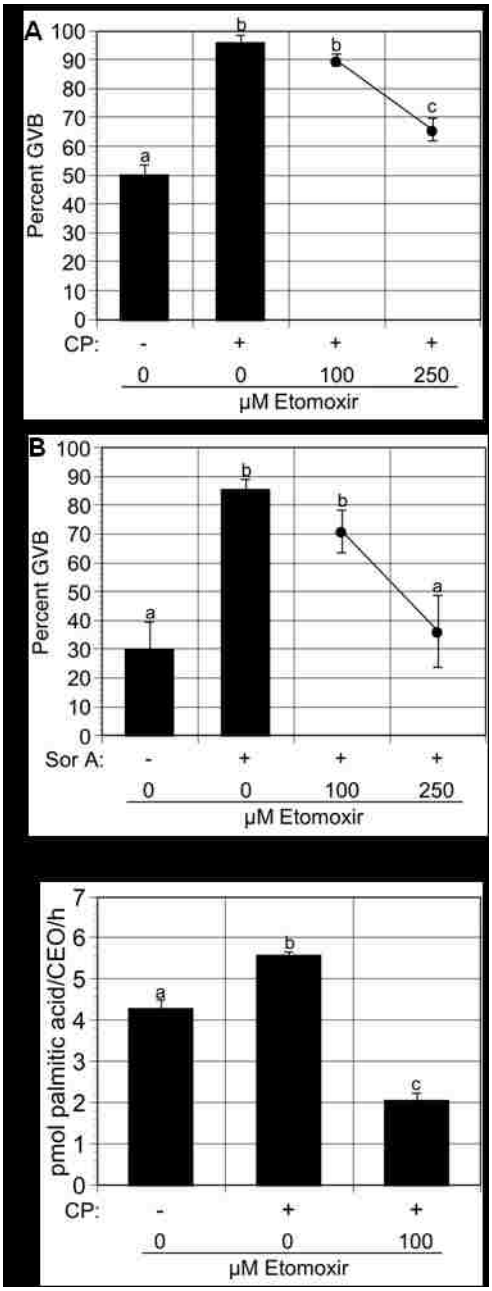


Figure 3.4. Meiotic resumption induced by ACC inhibitors is prevented by etomoxir, a CPT1 inhibitor that blocks FAO.

CEO arrested in hypoxanthine were induced to resume meiosis with CP-640186 (A) or Soraphen A (B), and exposed to two doses of etomoxir and GVB was scored 18 h later. In (C), an 18 h FAO assay was carried with CEO arrested in milrinone and treated with CP640186 in the absence or presence of etomoxir. Groups with no common letter are significantly different.

Activation of ACC and Inhibition of MCD blocks FSH-induced GVB and FAO

I have previously shown that FSH-induced meiotic resumption is accompanied by a PRKA-dependent increase in FAO (Valsangkar and Downs, 2013). To determine if ACC inactivation was necessary for this FAO increase, I tested the effect of citrate, a physiological activator of ACC (Tong, 2005), on FSH-induced increase in FAO. As shown in Fig. 3.5 B, citrate prevented the FSH-induced FAO increase in CEO. Importantly, this was accompanied by a decrease in FSH-induced GVB from 89% to 67% (Fig. 3.5 A). The malonyl CoA produced by ACC is responsible for the inhibitory effect of ACC on FAO via the inhibition of CPT1, and the level of malonyl CoA is also regulated by another enzyme known as malonyl CoA decarboxylase (MCD) that converts malonyl CoA back to acetyl CoA (Goodwin and Taegtmeyer, 1999; Samokhvalov et al., 2012). Thus, ACC and MCD have opposite effects on malonyl CoA level. Therefore, I investigated the effect of the MCD inhibitor CBM-301106 (Samokhvalov et al., 2012)on FSH-induced FAO as well as FSH-induced GVB. The MCD inhibitor is expected to increase malonyl CoA by blocking the conversion of malonyl CoA to acetyl CoA, thereby inhibiting CPT1 and decreasing FAO. As expected, the MCD inhibitor CBM-301106 reduced FSH-induced GVB from 92% to 57% at the highest dose, and decreased the FSH-induced FAO increase by 1.5-fold (Fig. 3.5 C, D). Taken together, these data indicate that ACC activators and MCD inhibitors block hormone-induced meiotic resumption.

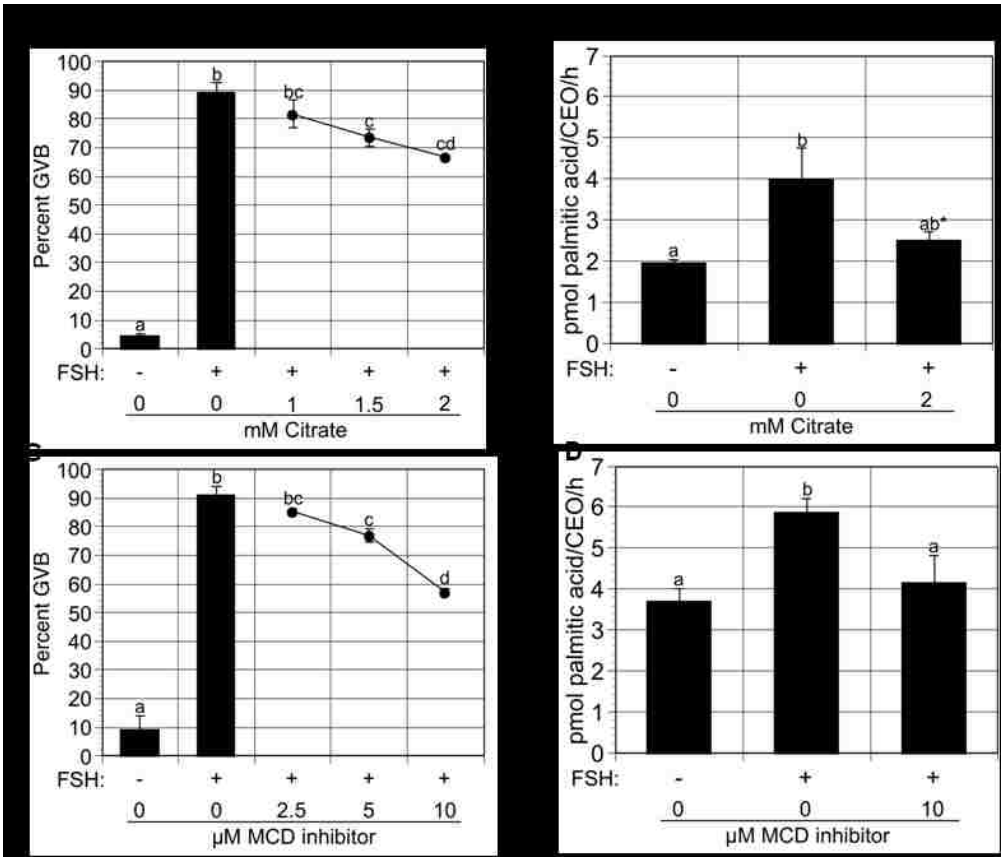


Figure 3.5. Activation of ACC and inhibition of MCD prevents FSH-induced meiotic resumption as well as FSH-induced increase in FAO.

In (A,C), CEO were cultured in the presence of milrinone and stimulated to mature with FSH with and without the physiological ACC activator, citrate (A) or the MCD inhibitor CBM-301106 (C) and GVB was assessed 17-18 h later. In (B,D), CEO arrested with milrinone and stimulated with FSH were exposed to citrate (B) or CBM-301106 (D), and assayed for FAO 18 h. Groups with no common letter are significantly different. In (B), an asterisk indicates that the citrate-treated group was significantly different from the group treated with FSH (but without citrate) when the fold changes in FAO were compared with ANOVA.

Comparison of FAO and Meiotic Resumption between WT and ACC2 -/- Mouse Oocytes

Our studies with pharmacological inhibitors of ACC indicate that ACC is important in the regulation of FAO as well as meiotic resumption in mouse oocytes. To further study the role of this enzyme, I procured ACC2-/- mice that have a B6/129 background and control mice for this strain (B6/129 F_2) (Abu-Elheiga et al., 2001; Abu-Elheiga et al., 2012). To see the effect of a physiological loss of the ACC2 gene, I compared the percent meiotic resumption and FAO levels in CEO of B6/SJl F_1, B6/129 F_2 and ACC2-/- strains in the presence of cAMP-elevating agents. As shown in Fig. 3.6, the ACC2 -/- CEO had a significantly higher rate of GVB in the presence of hypoxanthine (Fig. 3.6 A) as well as FAO in the presence of milrinone (Fig. 3.6 B) compared to both B6/SJL and B6/129 control CEO. In the presence of 2 μM milrinone, all three strains showed no difference in percent GVB (data not shown). This could be because milrinone is a more potent inhibitor of GVB, while Hx is a weaker inhibitor.

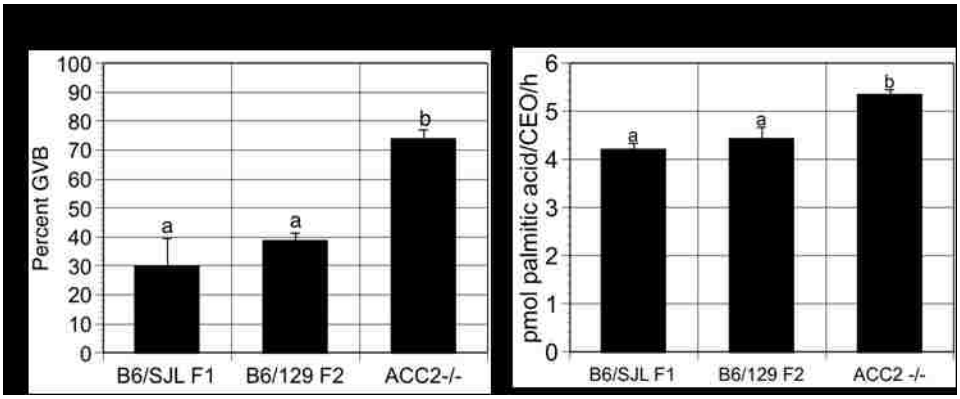
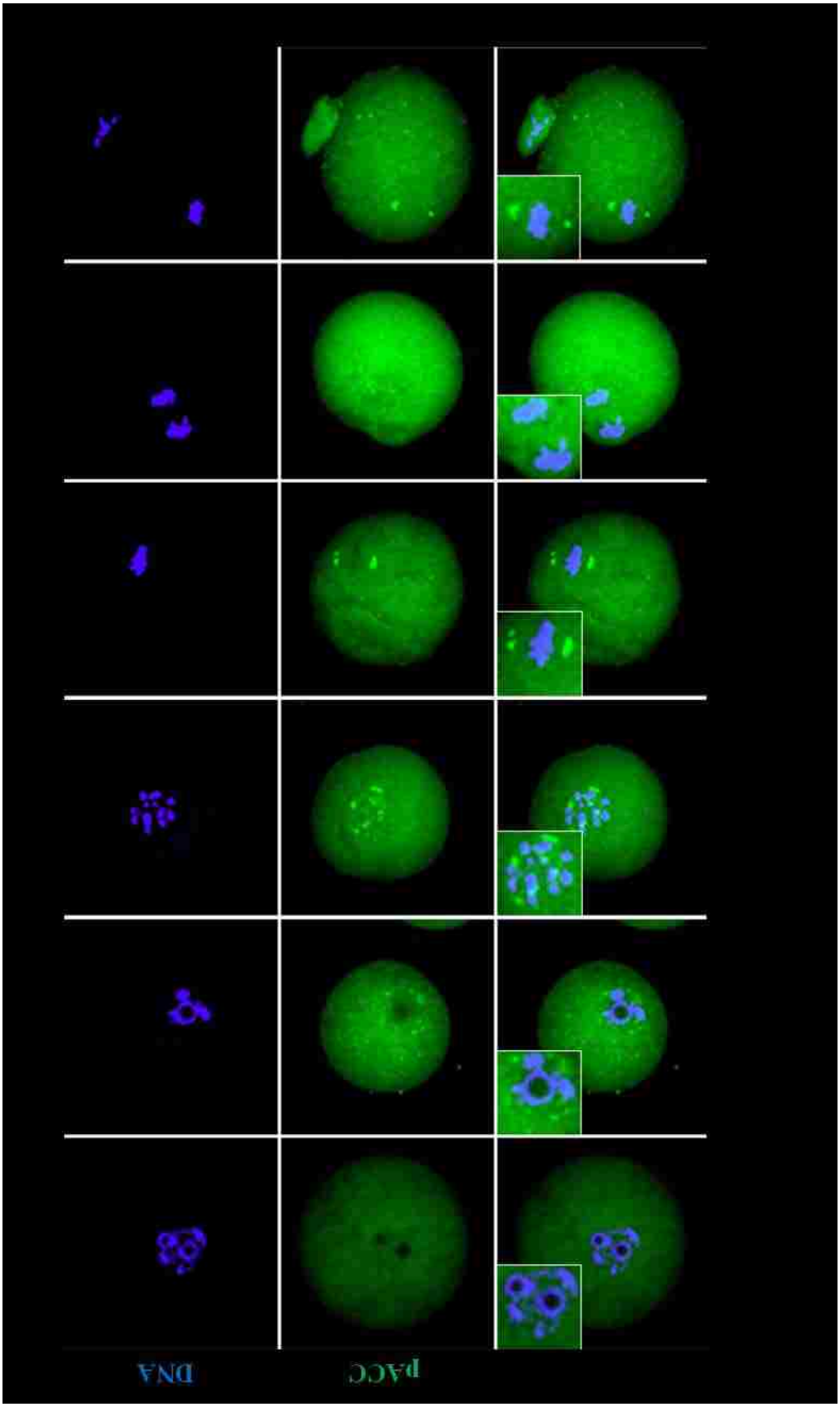


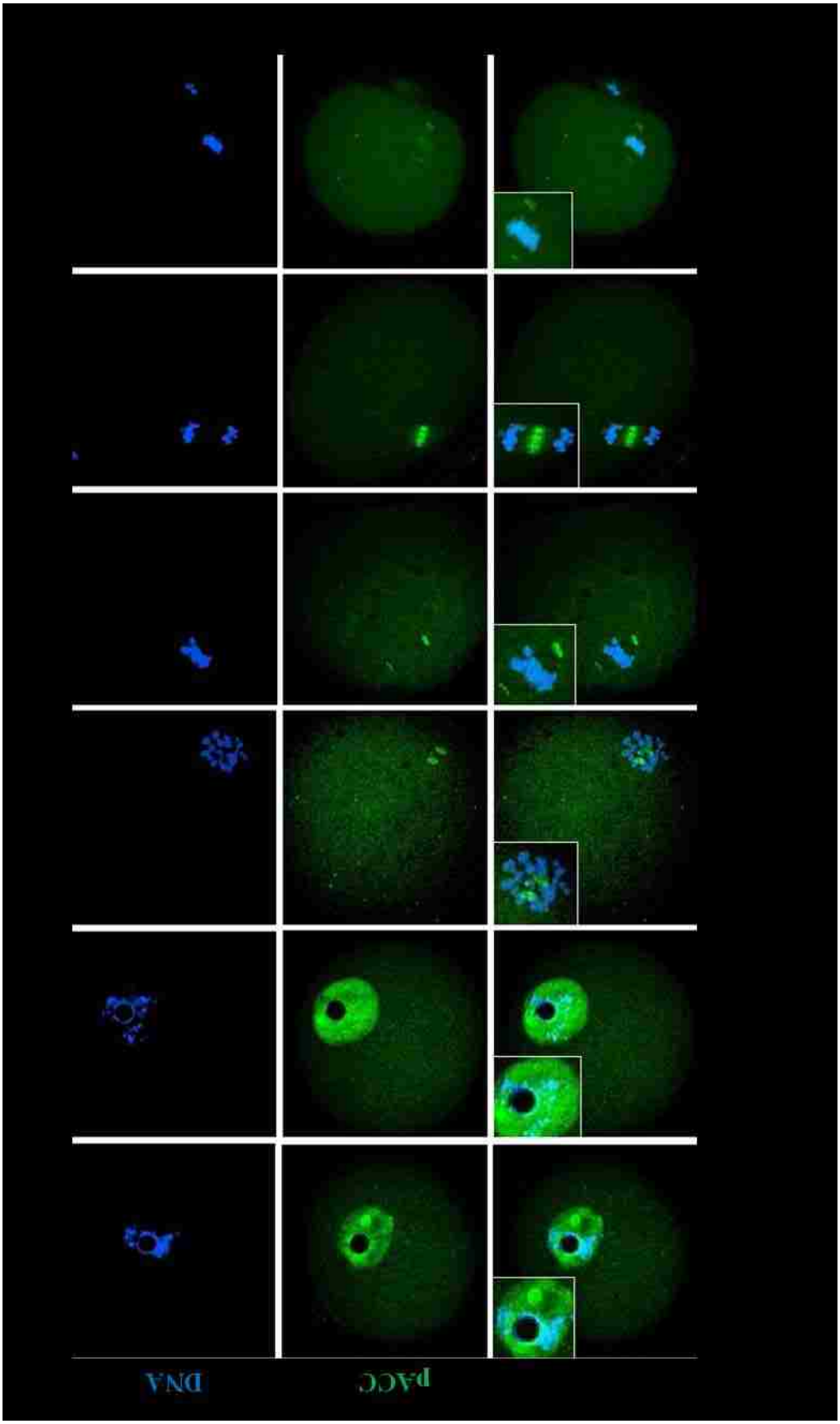
Figure 3.6. The effect of ACC knockout on the meiotic resumption and FAO in CEO.

In (A), CEO from the three strains were cultured in the presence of 4 mM Hx and assessed for percent GVB 17 h later. In (B), CEO were cultured in wells of a 96-well plate in the presence of 2 μM milrinone and assayed for FAO 18 h later. Groups with no common letter are significantly different.

Localization of PRKA-phosphorylated ACC in mouse oocytes

In previous studies (Chen et al., 2006; Chen and Downs, 2008), our lab, using immunofluorescence, has shown that active PRKA (phosphorylated at Tyr 172 at the alpha PRKA isoform) localizes to GV before GVB, at condensed chromosomes after GVB, at spindle poles in M-I and M-II, and at the spindle midbody in anaphase-I. Since ACC is a substrate of PRKA, it was important to determine the localization pattern of PRKA-phosphorylated ACC (pACC, ACC phosphorylated at Ser 79) during meiotic resumption. Phospho-ACC localization was very similar to that of active PRKA, except pACC was not seen to be localized at spindle midbody during anaphase-I in oocytes stained with a monoclonal anti-pACC antibody (Fig. 3.7). However, using another antibody that is produced against the pACC-2 isoform (but that cannot distinguish between ACC1 or ACC2 isoforms due to high identity between the Ser 79 and Ser 218 sites), pACC was found to be localized to the spindle midbody as well (Fig. 3.8), indicating that pACC co-localizes with active PRKA. A similar pattern of staining for pACC in somatic cells has been recently reported (Vazquez-Martin et al., 2013).

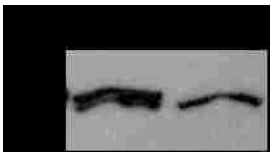

DNA
pACC


DNA
pACC

Identification of ACC isoforms present in mouse CEO and DO

To determine the type of isoform(s) present in mouse DO and CEO, I carried out Western blot assays using a monoclonal anti-ACC antibody and identified ACC isoforms based on their molecular weights. Both isoforms were present in WT CEO (Fig. 3.9A) while ACC2, as expected, was missing in ACC2 KO CEO. Data from a previous paper (Chen et al., 2006) show that DO contain both ACC isoforms as well. Immunofluorescence staining of meiotically arrested (non-stimulated) WT and KO DO with anti-ACC (isoform non-selective) revealed that although both strains contain ACC, KO DO stain much less intensely than WT DO, as expected (Fig. 3.9B). In addition, ACC appears to be present not only in the cytosol, but also in GV.

A



B

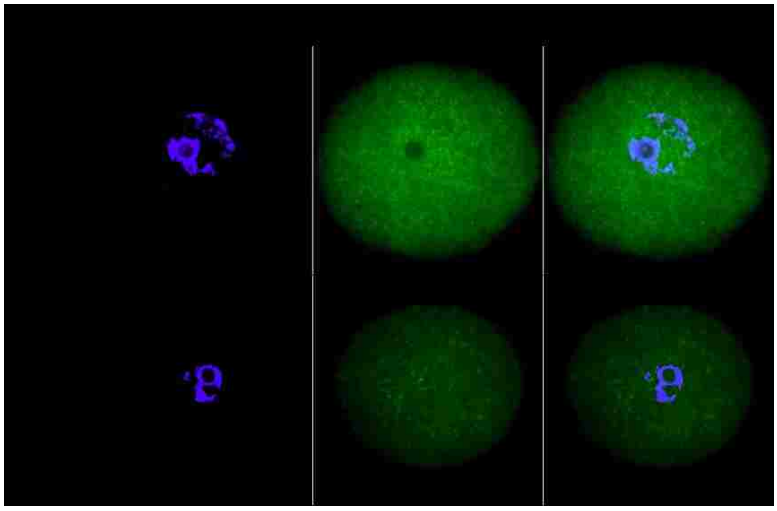


Figure 3.9. Determination of ACC isoforms in mouse CEO and DO.

In (A), Western blots from WT and ACC2 KO CEO (250 CEO each) were probed with anti-ACC antibody. Numbers to the left of the blot indicate molecular weights of the two isoforms in kDa. In (B), DO from WT and ACC2 KO mice were immunostained with the same antibody.

Localization of mitochondria during meiotic resumption

The majority of beta oxidation in cells takes place in the mitochondria, and a dramatic change in the localization (distribution) of mitochondria in oocytes of various species in both in vitro (including spontaneousmaturation) and in vivo (hormone-induced) conditions, including mouse has been described and in several cases, is associated with dramatic increases in ATP production (Van Blerkom and Runner, 1984; Calarco, 1995; Bavister and Squirrell, 2000; Stojkovic et al., 2001; Sun et al., 2001; Suzuki et al., 2005; Sturmey et al., 2006; Yu et al., 2010; Dalton, 2011; Wakai, 2012). I sought to investigate if a similar redistribution of mitochondria occurred during PRKA activator (AICAR)- induced maturation in vitro. To this end, CEO were cultured in the presence of 300 µM dbcAMP with and without 500 µM AICAR and DO isolated at 4 h and 10 h to get various stages of meiotic maturation. As seen in fig. 3.10, COX-IV staining indicated mitochondrial aggregation around the GV after meiotic induction and around condensed chromosomes and the M-I spindle, as reported previously using mitochondria-specific dyes and GFP-tagging of mitochondria.

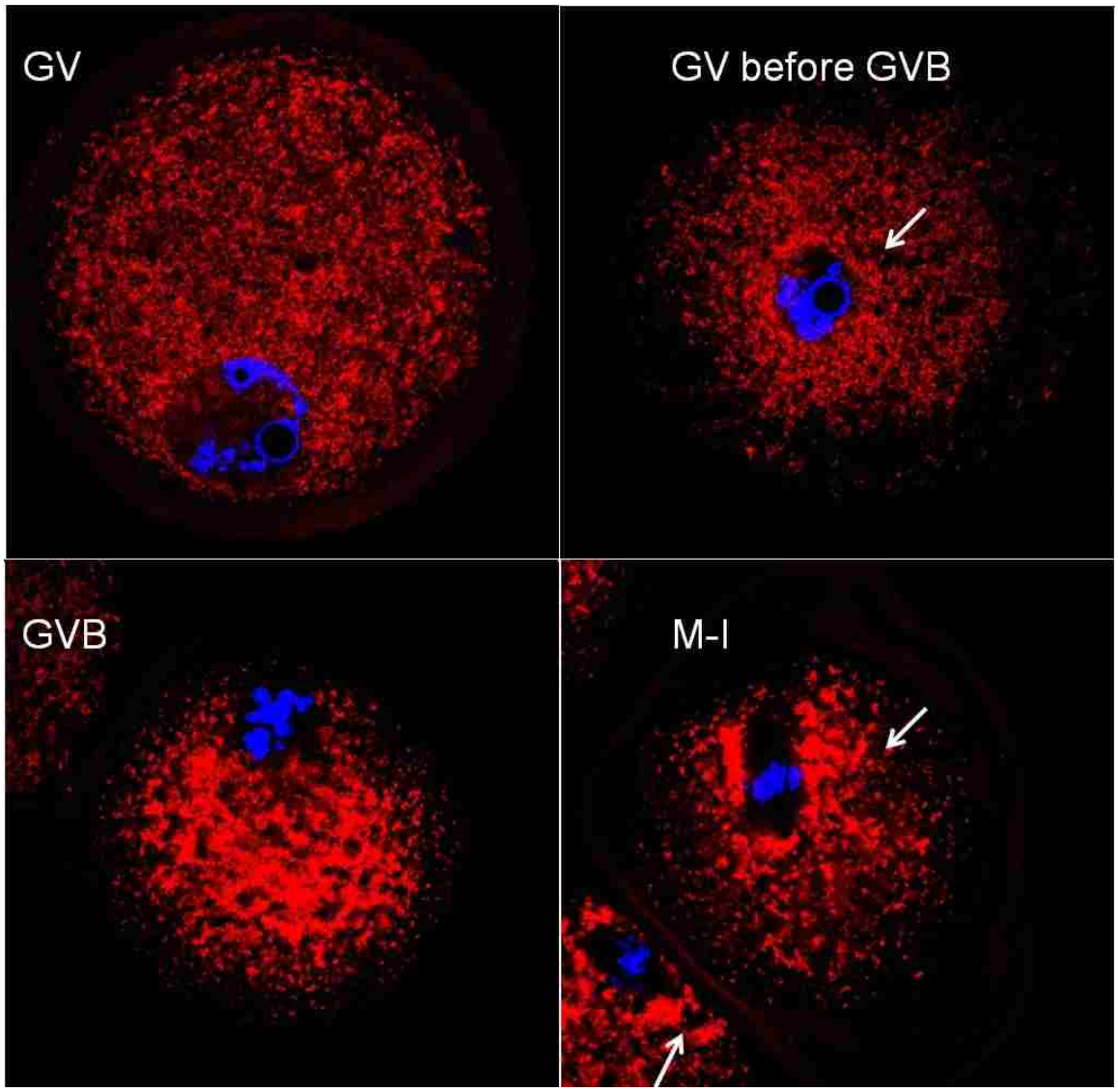


Figure 3.10. Localization of mitochondria in mouse oocytes during meiotic resumption.

CEO were induced to mature with 300 µM dbcAMP ± 500 µM AICAR and DO were isolated at 4 h (for GV and GVB stages) and 10 h (for M-I) followed by fixation and staining with anti-COX IV antibody that recognizes mitochondrial respiratory complex IV.

Discussion

I previously demonstrated the importance of PRKA activation and FAO stimulation in the pharmacological and physiological induction of meiotic resumption (Downs et al., 2002b, 2009; Chen et al., 2006; LaRosa and Downs, 2006; Chen and Downs, 2008; Valsangkar and Downs, 2013). Furthermore, I suggested that stimulation of FAO via the inactivation of ACC may be a significant portion of the mechanism of PRKA-induced meiotic resumption. In this study, I further tested the role of ACC inactivation and its effect on FAO and meiotic resumption in mouse oocyte maturation. The most important findings of this study were: (1) pharmaceutical inhibitors of ACC bring about meiotic resumption that is sensitive to FAO inhibition, and (2) physiological meiotic resumption was blocked by ACC activation and MCD inhibition. These data further establish not only a requirement for PRKA-mediated stimulation of FAO, but furthermore a regulatory role for this event in the meiotic resumption of mouse oocytes.

In this study, I have used two different ACC inhibitors CP-640186 and Soraphen A that act on two different sites on the ACC molecule. While CP-640186 binds to the active site at the carboxyltransferase (CT) domain and has been suggested to prevent binding of biotin to the CT domain (Zhang et al., 2004), Soraphen A binds to the biotin carboxylase (BC) domain and has been suggested to prevent oligomerization of this domain (Shen et al., 2004) that is important for enzyme activity (Tong, 2005). I demonstrated that both inhibitors induce meiotic resumption in mouse oocytes maintained in arrest with hypoxanthine, IBMX, or milrinone. The fact that these two compounds act on ACC by different mechanisms but both suppress its activity provides

compelling evidence that this enzyme has a critical regulatory role in mouse oocyte maturation. The ACC inhibitor-directed meiotic resumption is also associated with an increase in FAO that was measured using an assay first described in mouse oocytes by Dunning et al. (2010) and later extensively used by us (Valsangkar and Downs, 2013). This meiotic resumption is blocked by FAO inhibition, indicating that increased FAO mediated by ACC inhibition is required for meiotic resumption by these inhibitors. Furthermore, these experiments suggest that ACC inactivation and thereby FAO stimulation is sufficient for induction of meiotic resumption in vitro.

Since malonyl CoA is a crucial regulator of CPT1 and thereby of fatty acid oxidation, I tested the effect of citrate, a physiological activator of ACC (the malonyl CoA producer) and CBM-301106, a pharmacological inhibitor of MCD (the enzyme that converts malonyl CoA back to acetyl CoA), on the FSH-induced meiotic resumption of mouse oocytes (Bonnefont et al., 2004; Tong, 2005; Brownsey et al., 2006; Lopaschuk and Stanley, 2006; Samokhvalov et al., 2012). Both citrate and CBM-301106 blocked FSH-induced meiotic induction as well as FAO, confirming that malonyl CoA is an important regulator of FAO and in effect of meiotic resumption.

Using western blotting, I determined the types of isoforms expressed in the oocyte-cumulus complex and the oocyte alone. Both isoforms, viz. ACC1 and ACC2, were present in oocyte-cumulus complexes as well as denuded oocytes.

The ACC2 isoform has been suggested to be predominantly used for FAO inhibition, whereas the ACC1 isoform has been implicated mainly in long-chain fatty acid synthesis (Abu-Elheiga et al., 2000, 2001; Tong, 2005; Brownsey et al., 2006). To determine if an in vivo loss of these isoforms affects meiotic resumption, I sought to

obtain knockout mice lacking these isoforms. However, ACC1 knockout mice die in early embryonic stages (Abu-Elheiga et al., 2005), therefore it was not possible to study ACC1-/- oocytes. However, I procured ACC2 KO mice with a B6/129 background (Abu-Elheiga et al., 2001; Abu-Elheigaet al., 2012), and studied their oocytes for FAO level and meiotic maturation. The ACC2-/- CEO showed an increased level of FAO compared to both B6/SJL F1 and B6/129 F_2 CEO. This correlated with the differences in the basal level of meiotic resumption in these oocytes. In the presence of hypoxanthine, the knockout CEO showed significantly higher meiotic resumption than both the WT B6/129 F_2 mice and the B6/SJL F_1 mice, indicating that loss of ACC2 reduces the ability of the oocyte to maintain meiotic arrest, though a significant number of oocytes were still maintained in the GV stage. Also, ACC2 KO mice are not sterile. These data suggest that there may be some redundancy of functions in the two ACC isoforms. On the other hand, it is not clear if ACC inhibition or stimulation of FAO is sufficient to induce meiotic resumption. Two sets of data point to two contradictory conclusions. The fact that the two ACC inhibitors CP-640186 and Soraphen A, the CPT1 activator C75, and carnitine derivatives of long chain fatty acids (e.g. palmitoyl carnitine) induce meiotic resumption (Downs et al., 2009) suggest that increase in FAO via the inactivation of ACC is sufficient to induce meiotic resumption in vitro. However, palmitic acid and carnitine fail to induce meiotic resumption. There could be at least two reasons for this. First, these two compounds are substrates of CPT1, not activators (Bonnefont et al., 2004); thus, in the absence of CPT1 activity, the addition of either of these two compounds fails to stimulate FAO. However, previously our lab has shown that fatty acyl carnitine derivatives of fatty acids, including palmitoyl carnitine, induced meiotic

resumption. Thus, pre-made palmitoyl carnitine can directly enter mitochondria and presumably undergo fatty acid oxidation, thereby leading to meiotic resumption. However, in the presence of CPT1 activity, such as in PRKA-activator-induced meiotic resumption or hormone-induced meiotic resumption, carnitine and palmitic acid are able to enhance percent GVB (Downs et al., 2009; Valsangkar & Downs, 2013). A second possibility is that they have a slower kinetics of FAO stimulation than that needed for meiotic resumption.

The localization of active PRKA and inactive ACC in the GV, at condensed chromosomes, spindle poles and spindle midbody supports the evidence that these molecules play a role in the mechanism of meiosis. A similar localization of phospho-ACC has been reported in somatic cells during mitosis (Vazquez-Martin et al., 2013). However, further studies are needed to determine the significance of this protein localization.

In summary, our data indicate that ACC inactivation plays an important role in the stimulation of FAO and meiotic resumption in mouse oocytes. It is possible that the products of the FAO pathway such as ATP or NADH/$FADH_2$ may feed into reaction leading to the activation of MPF (Maturation promoting factor). The most important message from this data, however, is how fat metabolism can dramatically affect meiotic resumption. This should be an important consideration in designing culture media for in vitro maturation and fertilization, as well as in the assessment of reproductive repercussions of drugs that target metabolism.

Chapter IV

SUMMARY AND CONCLUSIONS

Previous findings from our lab have established a role for PRKA activation throughout mouse oocyte meiotic maturation, starting from meiotic resumption until polar body formation and prevention of premature activation. In addition, previous studies by our lab and by Dunning et al. have collectively shown that fatty acid oxidation is crucial in meiotic resumption as well as developmental competence. Specifically, our lab showed that meiotic resumption stimulated by PRKA activators in vitro requires fatty acid oxidation (Downs et al., 2009). In my studies, I have further extended this research to include investigation of the FAO requirement in physiological meiotic resumption, as well as exploring whether PRKA action is mediated via the stimulation of FAO by ACC inactivation.

Using an assay that measures fatty acid oxidation levels, I showed that during meiotic resumption, fatty acid oxidation is stimulated, whether induced by PRKA activators, FSH or EGF in vitro, or by hCG in vivo and these changes in maturation and FAO were blocked by three different FAO inhibitors. The FAO inhibitor etomoxir also blocked hCG-induced meiotic resumption in follicle-enclosed oocytes. Thus, it appears that however stimulated, meiotic induction requires fatty acid oxidation. Since PRKA stimulates FAO through the inactivation of ACC to reduce malonyl CoA levels, it was hypothesized that modulating the activities of ACC (that produces malonyl CoA) or MCD (that converts malonyl CoA back to acetyl CoA) would affect meiotic resumption

by changing FAO levels. Indeed, ACC inhibitors increased FAO and induced meiotic resumption significantly, and this was prevented by the FAO inhibitor etomoxir. The ACC inhibitors also induced GVB in follicle-enclosed oocytes. Moreover, hormone-induced meiotic resumption and FAO increase in vitro was blocked by the physiological ACC activator, citrate, and the pharmacological MCD inhibitor, CBM-301106, both treatments presumably leading to an increase in malonyl CoA levels. These data lend support to the importance of malonyl CoA in the regulation of FAO and meiotic resumption in mouse oocytes, bolstering our hypothesis that FAO plays an essential role during meiotic resumption.

The localization of PRKA-phosphorylated ACC was interestingly very similar to that of active PRKA during meiotic resumption. The most curious and unexpected localization of phospho-ACC is the germinal vesicle, because PRKA is known to have nuclear substrates, but no such function is known of ACC in mammals. Moreover, since PRKA-phosphorylated ACC is inactive, it is unclear what function it could serve there, although it is possible that ACC was already present in the nucleus and once PRKA was active, the ACC was phosphorylated. If ACC2 is indeed restricted to the mitochondrial outer membrane as widely perceived, it must be ACC1 staining in the GV; since mitochondria are not present in the nucleus, however, a nonspecific binding cannot be discounted. The localization of phospho-ACC and active PRKA at condensed chromosomes and spindle poles suggests a functional role for these proteins at these locations and further studies are needed to determine whether ACC knockdown or ACC with a mutation at the Ser79 site affects spindle assembly, stability or movement.

The changes in mitochondrial distribution during meiotic maturation reported in numerous mammalian species indicate a conserved function for this this process during meiotic maturation. The redistribution of mitochondria in the mouse has been reported by Van Blerkon and Runner (1984), Calarco (1995), Yu et al (2010), Wakai, (2012), and data presented in this paper. These changes in mitochondrial distribution in the maturing oocyte have been shown to be correlated with dramatic increases in ATP production as well as normal embryonic development in the cow (Stojkovic et al., 2001) and mouse (Nagai et al., 2006; Yu et al., 2010). These findings buttress the suggestion that mitochondrial aggregation in the vicinity of M-I spindle may serve to provide large amounts of energy where it is needed because in a very large cell like the oocyte, the rate of ATP diffusion to the sites of its need may not be as efficient (Van Blerkom and Runner, 1984). It is possible that PRKA-mediated ACC inactivation at the condensed chromosomes and spindle poles could additionally enhance mitochondrial FAO near these locations. In addition, mitochondrial FAO increase specifically at the sites of requirement may also serve to prevent ROS (reactive oxygen species) production in the rest of the oocyte.

In the yeast *Saccharomyces cerevisiae*, the activities of ACC and SNF1 (the yeast homologue of mammalian PRKA) have been found to regulate global histone acetylation by affecting the levels of acetyl CoA that is required for histone acetylation (Galdieri & Vancura, 2012; Zhang et al., 2013). Thus, in yeast, PRKA and ACC appear to be at the intersection of metabolism and chromatin structure. In mammals, the majority of the acetyl CoA required for histone acetylation is produced from citrate by another enzyme, ATP- citrate lyase, that is localized in both the cytosol and the nucleus. Thus, at least in

the cytosol, there could be a competition to utilize the acetyl CoA produced for long-chain fatty acyl synthesis and for diffusion to the nucleus for histone acetylation. If ACC indeed is present in the nucleus in addition to the cytosol as my data suggest, the nuclear pool of acetyl CoA would also be subject to depletion by ACC. Thus, it is plausible that ACC may play a role in the regulation of histone acetylation in the mouse oocyte as well. In fact, proteins other than histones are also subject to acetylation and perhaps ACC activity may additionally affect this process.

I hypothesized that PRKA mediates a majority of its meiosis-inducing action via the stimulation of FAO. Our data linking GVB with ACC inactivation by CP-640186 and Soraphen A, CPT1 activation by C75, and the direct FAO increase by the addition of fatty acyl carnitine derivatives (Downs et al., 2009; Valsangkar & Downs, 2013; data herein) firmly support this hypothesis. It is also buttressed by the fact that hormonal induction of maturation (that has been shown to require PRKA activation) is accompanied by FAO stimulation in vitro and in vivo. On the other hand, palmitic acid or carnitine alone do not induce meiotic resumption, but significantly enhance AICAR-induced or hormone-induced maturation. It is possible that since neither of these two compounds is a CPT1 activator; there may not be sufficient CPT1 activity to have a stimulatory effect. However, in the case when CPT1 is activated, presumably in AICAR-treated or hormone-treated oocytes, FAO would increase when carnitine, a direct substrate of CPT1, and palmitic acid, a precursor of palmitoyl CoA, another substrate of CPT1; are added. On the other hand, carnitine alone significantly stimulated FAO in CEO. It is possible that most of this stimulation may be occurring in cumulus cells that perhaps have an active CPT1.

Apart from our studies, there have been numerous reports concerning fatty acid metabolism during meiotic maturation and early embryonic development in the mouse and other domestic species. Dunning et al (2010) and data herein showed that in the mouse, a major portion of the fatty acid oxidation in the cumulus-oocyte complex comes from the cumulus cells. These authors also showed that the expression of *cpt1b* mRNA in the cumulus cells is increased during meiotic maturation. Also, recently it was shown that expression of *trib1* and *trib3* (Ser-Thr kinases implicated in increasing FAO) was increased in mouse cumulus cells during in vitro maturation whereas that of *acaca* (ACC1) decreased in these conditions (Brisard et al., 2014). Indeed, it is highly plausible that cumulus cells metabolize fatty acids to provide energy to the oocyte during meiotic resumption. However, data from my studies and previous studies from our lab show that (1) after hCG induction of maturation in vivo, FAO within cumulus-oocyte complexes as well as within isolated oocytes is significantly stimulated, (2) the ACC inhibitor CP-640186 significantly stimulated FAO and meiotic resumption in DO as well as CEO, (3) the CPT1 activator C75 stimulated GVB in both DO and CEO, (4) palmitoyl carnitine induced meiotic resumption in both DO and CEO, and (5) carnitine stimulated FAO in CEO but not in DO, and failed to induce meiotic resumption in CEO and DO. Thus, it seems that FAO increase in DO is sufficient for meiotic resumption and that in cumulus cells may not always lead to meiotic resumption.

There are at least two possibilities by which FAO increase may induce meiotic resumption (Fig. 4.1). First, FAO may simply provide a metabolite, for example, ATP or redox substrates required for meiotic resumption. It would be interesting to test if blocking FAO and providing energy by another mechanism at a similar rate drives

meiotic resumption. It is possible that the ATP provided by FAO may be feeding into phosphorylation reactions leading to the activation of MPF. In this case, PRKA activation would be a requirement for MPF activation, and in cases where PRKA is activated above a certain threshold rate (that would lead to a high rate of FAO), it would overcome cAMP-mediated arrest (as demonstrated in all of our experiments in vitro and in vivo), resulting in meiotic resumption via the activation of MPF.

Secondly, perhaps FAO depletes the oocyte of substances that may either be harmful or inhibitory to meiotic resumption. For example, high levels of certain fatty acids are toxic to cells. Also, it has been found that pharmacological inhibition of ACSL (Long-chain acyl CoA synthetase) that links CoA to long chain fatty acids to produce, for example, palmitoyl CoA, leads to a significant loss of meiotic arrest in mouse oocytes (Wang et al., 2012). Interestingly, the $G_{s\alpha}$ subunit of the constitutively active GPR3 protein undergoes palmitoylation, and depalmitoylation causes translocation of this subunit from plasma membrane to cytosol. It would be of interest to see if depletion of palmitic acid by FAO stimulation in mouse oocytes causes translocation of this subunit. However, the addition of external palmitoyl carnitine also causes meiotic resumption, and this cannot be explained by this proposed mechanism unless palmitoyl carnitine oxidation causes an overall utilization of internal fatty acid stores as well. It should be noted that the above study with ACSL inhibitor is an isolated experiment with no other supporting data in the mouse.

Taken together, all of these data establish that fatty acid oxidation plays a crucial role in the regulation of meiotic maturation in the mouse oocyte and stress the complexity of the role of metabolism in cellular processes.

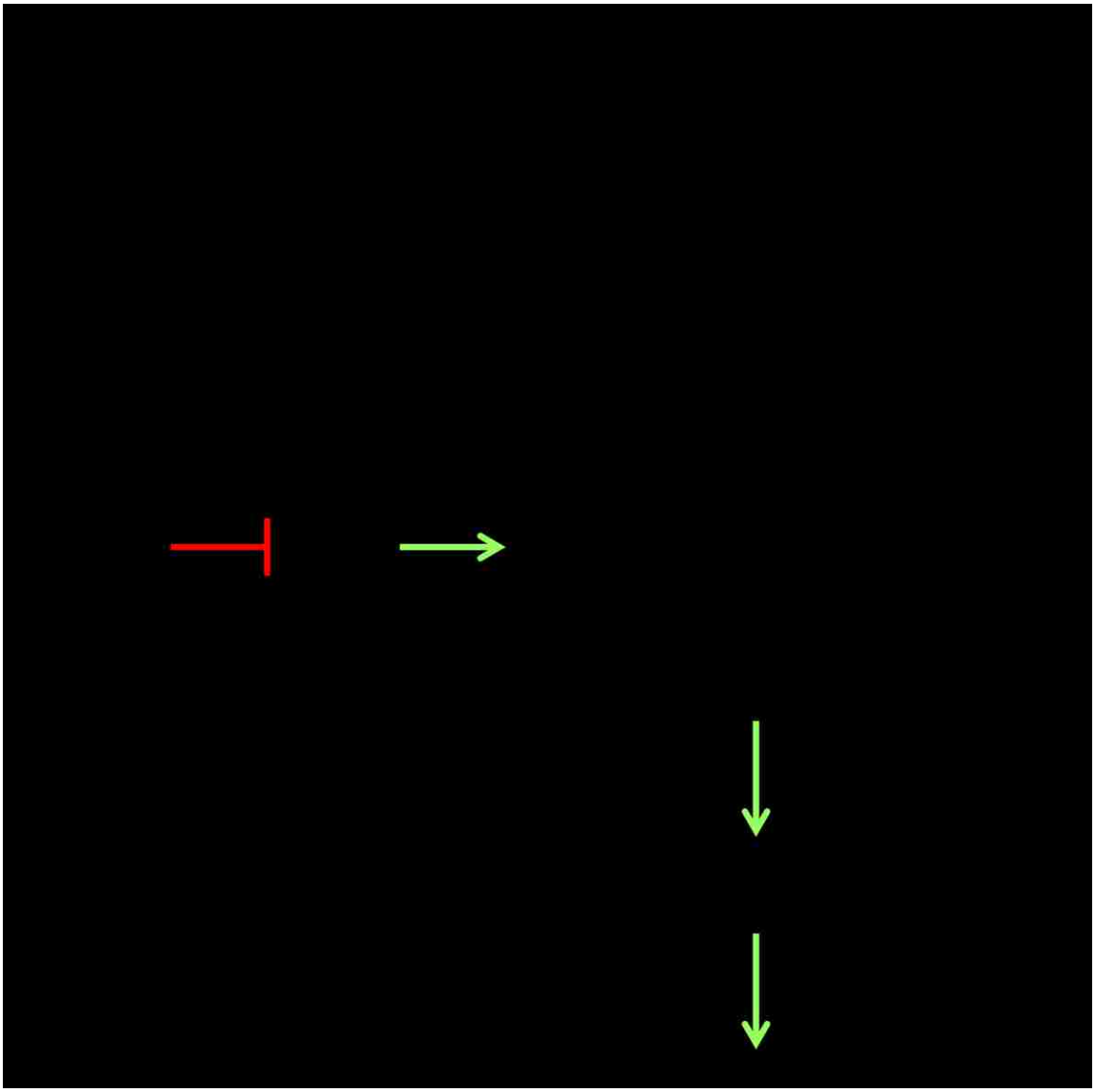


Figure 4.1. The various possible mechanisms that may be involved in PRKA-mediated induction of meiotic maturation.

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