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Acetyl CoA Carboxylase Inactivation and Meiotic Maturation in Mouse Oocytes

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SUMMARY

In mouse oocytes, meiotic induction by pharmacological activation of PRKA (adenosine monophosphate-activated protein kinase; formerly known as AMPK) or by hormones depends on stimulation of fatty acid oxidation (FAO). PRKA stimulates FAO by phosphorylating and inactivating acetyl CoA carboxylase (ACAC; formerly ACC), leading to decreased malonyl CoA levels and augmenting fatty-acid transport into mitochondria. We investigated a role for ACAC inactivation in meiotic resumption by testing the effect of two ACAC inhibitors, CP-640186 and Soraphen A, on mouse oocytes maintained in meiotic arrest in vitro. These inhibitors significantly stimulated the resumption of meiosis in arrested cumulus cell-enclosed oocytes, denuded oocytes, and follicle-enclosed oocytes. This stimulation was accompanied by an increase in FAO. Etomoxir, a malonyl CoA analogue, prevented meiotic resumption as well as the increase in FAO induced by ACAC inhibition. Citrate, an ACAC

activator, and CBM-301106, an inhibitor of malonyl CoA decarboxylase, which converts malonyl CoA to acetyl CoA, suppressed both meiotic induction and FAO induced by follicle-stimulating hormone, presumably by maintaining elevated malonyl CoA levels. Mouse oocyte-cumulus cell complexes contain both isoforms of ACAC (ACACA and ACACB); when wild-type and *Acacb*^{-/-} oocytes characteristics were compared, we found that these single-knockout oocytes showed a significantly higher FAO level and a reduced ability to maintain meiotic arrest, resulting in higher rates of germinal vesicle breakdown. Collectively, these data support the model that ACAC inactivation contributes to the maturation-promoting activity of PRKA through stimulation of FAO.

Abbreviations

ACAC acetyl CoA carboxylase
BC biotin carboxylase domain
CEO cumulus cell-enclosed oocytes
CPT1 carnitine palmitoyl transferase I
CT carboxyl transferase domain
DO denuded oocytes
FAO fatty acid oxidation
FSH follicle-stimulating hormone
GV[B] germinal vesicle [breakdown]
hCG human chorionic gonadotropin
IBMX 3-isobutyl-1-methylxanthine
MLYCD malonyl CoA decarboxylase
PRKA adenosine monophosphate-activated protein kinase

INTRODUCTION

Oocyte maturation is the developmental process whereby prophase I-arrested, pre-ovulatory oocytes resume nuclear maturation, complete the first meiotic division, and arrest at metaphase II while concurrent cytoplasmic changes help prepare the oocyte for fertilization and embryogenesis. Oocyte metabolism plays a major role in determining meiotic and developmental outcome. Carbohydrate metabolism has long been a popular research topic in germ-cell and embryo development, with a more-recent upsurge in studies focusing on lipid metabolism.

Our lab has investigated the importance of the energy-sensitive kinase PRKA (adenosine-monophosphate [AMP]-activated protein kinase; also known as AMPK) in meiotic regulation through its control of fatty acid oxidation (FAO). PRKA is a cellular energy sensor, activated by a high AMP/ATP ratio and by stress, that can turn off energy-consuming anabolic pathways while turning on energy-generating catabolic pathways (Hardie et al., 2012). Recent work, however, revealed that PRKA also has critical functions in the regulation of mitosis in somatic cells (Koh and Chung, 2007; Banko et al., 2011; Sanli et al., 2014). For example, active PRKA localizes to condensed chromosomes, spindle poles, and the midbody of dividing cells (Vazquez-Martin et al., 2009a, 2009b; Thaiparambil et al., 2012; Wei et al., 2012), specifically co-localizing with polo-like kinase-1 at spindle poles (Vazquez-Martin et al., 2011) and with myosin regulatory light chain at the cleavage furrow (Vazquez-Martin et al., 2012). Furthermore, depletion or disruption of PRKA suppresses cell-cycle progression (Thaiparambil et al., 2012; Wei et al., 2012).

Meiotic maturation shares many features with mitosis (Solc et al., 2010). In mouse oocytes, active PRKA co-localizes with condensed chromosomes, spindle poles, and the midbody; PRKA is activated prior to germinal vesicle breakdown (GVB) and throughout maturation; and blocking PRKA activity prevents meiotic induction

(Chen et al., 2006; Chen and Downs, 2008; Downs et al., 2010). One of the key substrates of PRKA is acetyl CoA carboxylase (ACAC; also known as ACC), whose product, malonyl CoA, plays a vital role in lipid metabolism by serving as precursor for long-chain fatty acid biosynthesis and by suppressing carnitine palmitoyltransferase (CPT1) activity (Thupari et al., 2002) and, thus, fatty acid transport into mitochondria (Hardie and Pan, 2002; Brownsey et al., 2006; Tong and Harwood, 2006) (Fig. 1). PRKA-dependent phosphorylation of ACAC inhibits its carboxylase activity, which switches from fatty-acid synthesis to FAO by decreasing the availability of malonyl CoA (Ruderman et al., 2003; Tong and Harwood, 2006). Past studies have shown that both pharmacological and physiological induction of GVB in mouse oocytes depends on FAO (Downs et al., 2009; Valsangkar and Downs, 2013), and promoting FAO during meiotic maturation augments developmental competence (Dunning et al., 2014).

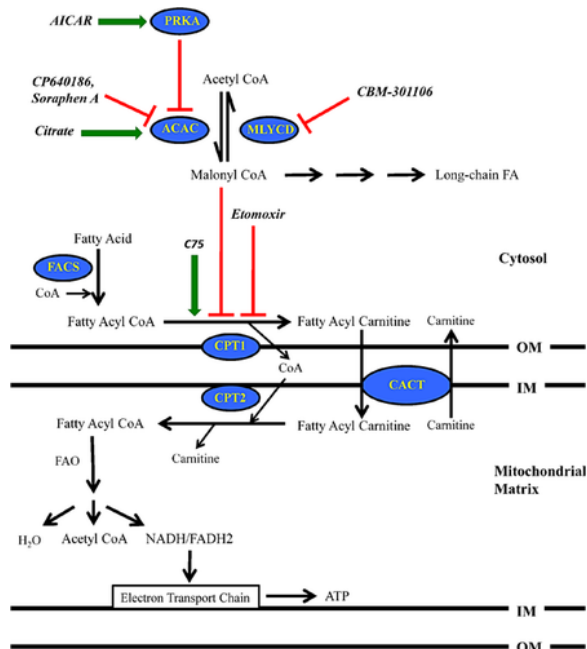


Figure 1 FAO and its modulation by various compounds used in this study. Coenzyme A is attached to fatty acids by fatty acyl CoA (CoA) synthetase (FACS) in the cytosol. CoA is then replaced with carnitine by carnitine palmitoyltransferase 1 (CPT1), located on the outer mitochondrial membrane (OM). The fatty acyl carnitine passes to the mitochondrial matrix by facilitated diffusion via the action of carnitine-acylcarnitine translocase (CACT). Carnitine is replaced with CoA by the action of carnitine palmitoyltransferase 2 (CPT2) in the mitochondrial matrix. The resulting fatty acyl CoA is oxidized, and the products acetyl CoA, NADH/FADH₂, and water are formed; NADH/FADH₂ is used to make ATP by the mitochondrial electron transport chain. CPT1 is inhibited by malonyl CoA produced from acetyl CoA by the enzyme acetyl CoA carboxylase (ACAC); malonyl CoA is converted to acetyl CoA by the enzyme malonyl CoA decarboxylase (MLYCD). Adenosine monophosphate (AMP)-activated protein kinase (PRKA) plays a major role in FAO regulation by inactivating ACAC and decreasing malonyl CoA levels, thereby removing the inhibition on CPT1. Malonyl CoA is also a precursor for long-chain fatty acids. AICAR is a pharmacological activator of PRKA. The compounds CP-640186 and Sorafen A inhibit both isoforms of ACAC, whereas citrate, a physiological compound, activates ACAC. CBM-301106 is synthetic inhibitor of MLYCD; malonyl CoA is a physiological inhibitor of CPT1, and its action is mimicked by its synthetic analogue, etomoxir. C75 is an activator of CPT1.

In most eukaryotes, ACAC is a single, multi-domain polypeptide chain (Brownsey et al., 1997; Tong and Harwood, 2006; Kim et al., 2010). The three domains of ACAC are biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyl transferase (CT). The BC domain first carboxylates the biotin molecule covalently linked to the BCCP domain, followed by CT domain-catalyzed transfer of the carboxyl group from biotin to acetyl

CoA, resulting in the formation of malonyl CoA (Brownsey et al., **2006, 1997**; Tong, **2005**). In mammals, ACAC exists in two isoforms—ACACA (ACC1) and ACACB (ACC2)—that differ in tissue distribution and molecular weight. Although transcribed from different genes, the two proteins have high sequence homology; the primary difference lies in the first ~100 extra amino acids at the amino-terminus of ACACB, which encode a hydrophobic, membrane-targeting sequence (Abu-Elheiga et al., **2000**) that results in its co-localization with CPT1 at the mitochondrial outer membrane. While lipogenic tissues such as adipose and mammary glands predominantly express ACACA, lipolytic tissues such as the heart and skeletal muscle mainly express ACACB. These differential tissue distribution and cellular localization patterns suggest that each isoform has a distinct function: the malonyl CoA produced by ACACA (the cytosolic isoform) is thought to be channeled towards the synthesis of long-chain fatty acids, whereas that produced by ACACB on the mitochondrial outer membrane blocks 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**), utilizing an analogous phosphorylation site: ACACA Ser 79, which corresponds to Ser 218 in ACACB due to the extra amino-terminal localization sequence.

Mouse knockouts for both ACAC isoforms have been generated. Whereas *Acaca*^{-/-} mice are embryonically lethal (Abu-Elheiga et al., **2005**), *Acacb*^{-/-} mice are fertile and have a normal life span (Abu-Elheiga et al., **2001**). Several lines of *Acacb*^{-/-} mice have been created, and all exhibit increased levels of FAO. In one knockout line, the loss of the *Acacb* protects against obesity and diabetes (Abu-Elheiga et al., **2001,2012**; Choi et al., 2007); however, these benefits were not observed in the other knockout lines (Hoehn et al., **2010**; Olson et al., **2010**). Such inconsistencies have not been sufficiently reconciled, and cast doubt on the effectiveness of targeting *Acacb* to treat these pathologies.

Considering the importance of PRKA and FAO in the meiotic maturation of mouse oocytes, we were interested in examining the contribution of ACAC in this process. We previously showed that phosphorylation of oocyte ACAC precedes meiotic induction by stress (LaRosa and Downs, **2006**) and by pharmacological (Chen et al., **2006**) and physiological (Chen and Downs, **2008**) stimuli, and is associated with stimulation of FAO. In the present study, we tested the effect of ACAC inhibitors and activators, as well as the knockout of *Acacb*, on meiotic maturation and FAO in mouse oocytes.

RESULTS

ACAC Inhibitors Induce Meiotic Resumption in Denuded, Cumulus-Enclosed, and Follicle-Enclosed Oocytes

Denuded oocytes (DO) and cumulus-enclosed oocytes (CEO) were maintained in meiotic arrest with either hypoxanthine or 3-isobutyl-1-methylxanthine (IBMX), a non-selective phosphodiesterase inhibitor. Increasing doses of either CP-640186 or Soraphen A, both non-selective ACAC inhibitors (Shen et al., **2004**; Zhang et al., **2004**), were added, and then oocytes were scored for GVB 16–17 hr later. Both ACAC inhibitors induced GVB in DO and CEO arrested with IBMX (Fig. **2A** and **B**) or hypoxanthine (Fig. **2C** and **D**) in a dose-dependent fashion.

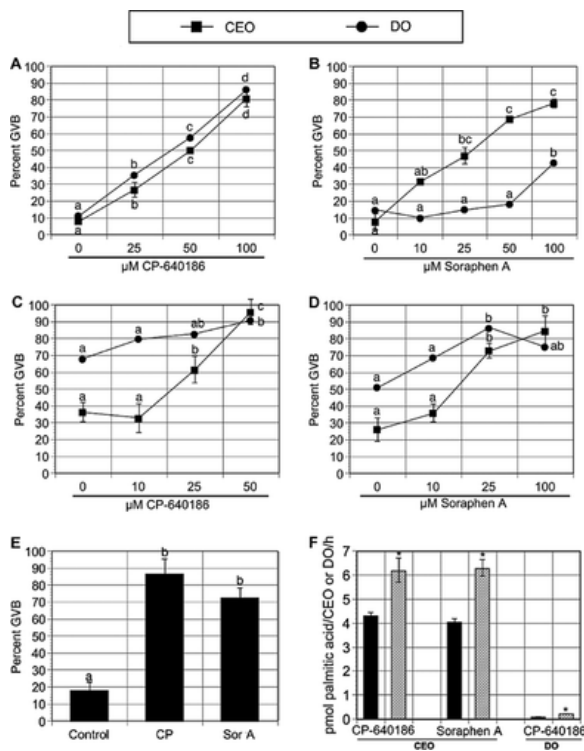


Figure 2 Pharmacological inhibitors of ACAC stimulate meiotic resumption in mouse oocytes by increasing FAO. A–D: CEO or DO, maintained in meiotic arrest with either (A, B) IBMX or (C, D) hypoxanthine, were treated with different doses of CP-640186 or Soraphen A. Percent GVB was scored 18 hr later. E: Ovarian follicles were dissected from ovaries and cultured in either control medium or medium containing 100 μ M CP-640186 [CP] or Soraphen A [Sor A]. After 3.5 hr in culture, oocytes were isolated from follicles and scored for percent GVB. F: Milrinone-arrested CEO were treated with CP-640186 or Soraphen A, or DO were treated with CP-640186 and FAO was assayed after 18 hr in culture. Groups with no common letter are significantly different in “A” through “E”; an asterisk indicates a significant difference by Student's t-test in “F.”

Dissected, pre-ovulatory follicles were also treated with CP-640186 or Soraphen A in vitro for 3.5 hr, and then scored for GVB. The ACAC inhibitors induced GVB in these follicle-enclosed oocytes, increasing the GVB percentage from 18% to 72–86% (Fig. 2E).

Meiotic Resumption by ACAC Inhibitors Is Accompanied by FAO Stimulation

FAO was measured in oocytes arrested with 2 μ M milrinone, a phosphodiesterase 3A (PDE3A) inhibitor, that were induced to resume meiosis with 50 μ M CP-640186 or 100 μ M Soraphen A. Milrinone was chosen because it selectively inhibits oocyte PDE3A, thereby averting non-specific effects on cumulus cells, and because it has been used as a meiotic-arresting agent in all of our FAO assays. Both ACAC inhibitors increased FAO in CEO about 1.5-fold (Fig. 2F) while CP-640186 increased FAO in DO by nearly 2.5-fold; however, the basal level of FAO in DO was only ~2% of that in CEO complexes. Furthermore, at the concentrations used for the 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.

Inhibition of FAO Prevents ACAC Inhibitor-Induced Meiotic Resumption

Etomoxir, a CPT1 inhibitor (Weis et al., 1994; Ishida, 1997), was used to determine if the effect of ACAC inhibitors on oocytes requires FAO. CEO were maintained in meiotic arrest with hypoxanthine and induced to resume meiosis with CP-640186 or Soraphen A. Etomoxir (100 and 250 μ M) was added, and then GVB was scored after 16–17 hr of culture. Etomoxir reduced GVB from 96% to 65% in CP-640186-treated CEO (Fig. 3A),

and from 86% to 36% in Soraphen A-stimulated CEO (Fig. 3B). Assessment of FAO in CP-640186-treated complexes showed that stimulation of FAO by the inhibitor was also suppressed by etomoxir (Fig. 3C). These results indicate that FAO is required for ACAC inhibitor-induced meiotic resumption.

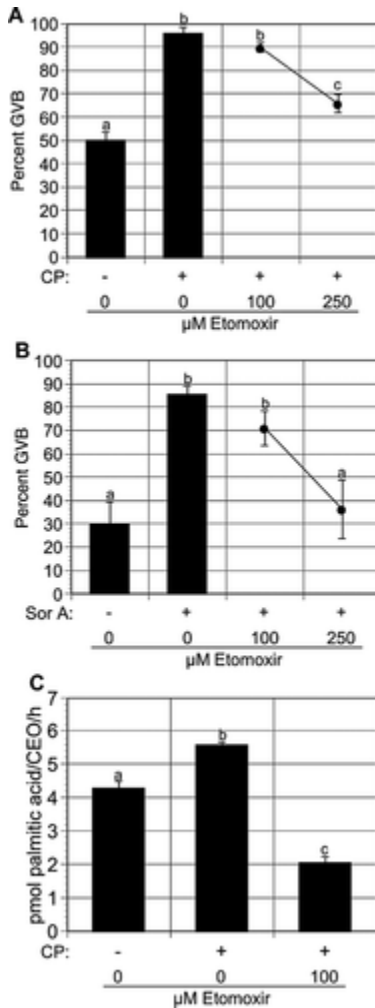


Figure 3 Meiotic resumption and FAO induced by ACAC inhibitors is prevented by etomoxir. **A, B:** CEO arrested in meiosis using hypoxanthine were induced to resume meiosis with (A) CP-640186 [CP] or (B) Soraphen A [Sor A], exposed to etomoxir, and then scored for GVB 18 hr later. **C:** An 18-hr FAO assay was carried out 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 ACAC and Inhibition of Malonyl CoA Decarboxylase Blocks Follicle-Stimulating Hormone-Induced GVB and FAO

Follicle-stimulating hormone (FSH)-induced meiotic resumption is accompanied by a PRKA-dependent increase in FAO (Valsangkar and Downs, 2013). Therefore, we tested the effect of citrate, a physiological activator of ACAC (Tong, 2005), on the FSH-induced increase in FAO to determine if ACAC activation would antagonize meiotic induction and elevate FAO by promoting malonyl CoA synthesis. Citrate reduced FSH-induced GVB from 89% to 67% (Fig 4A), which was accompanied by a decrease in FSH-induced FAO in CEO (Fig. 4B).

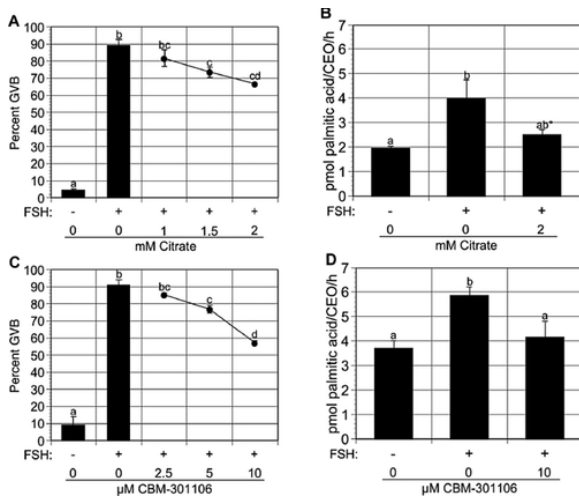


Figure 4 Activation of ACAC and inhibition of MLYCD prevent FSH-induced meiotic resumption as well as a FSH-induced increase in FAO. **A, C:** Milrinone-arrested CEO stimulated with FSH in the presence or absence of the physiological ACAC activator, citrate, or the MLYCD inhibitor, CBM-301106, were assessed for GVB 17–18 hr after culture. **B, D:** Milrinone-arrested CEO stimulated with FSH were exposed to citrate or CBM-301106, and assayed for FAO 18 hr later. Groups with no common letter are significantly different. An asterisk in “B” indicates a significant difference from the FSH-treated group when the fold changes in FAO were compared using ANOVA.

Elevated malonyl CoA levels can also be maintained by preventing its conversion to acetyl CoA using an inhibitor of malonyl CoA decarboxylase (MLYCD) (Goodwin and Taegtmeier, **1999**; Samokhvalov et al., **2012**) (Fig 1). We thus investigated the effect of CBM-301106, a MLYCD inhibitor (Samokhvalov et al., **2012**), on FSH-induced GVB and FAO under milrinone-arresting conditions. Consistent with the citrate results, CBM-301106 reduced FSH-induced GVB from 92% to 57% at the highest dose, and decreased FSH-induced FAO by 1.5-fold (Fig. 4C and D). Taken together, these data further support a role for malonyl CoA as a negative regulator of meiotic resumption.

Comparison of FAO and Meiotic Resumption in Wild-Type and *Acacb*^{-/-} Mouse Oocytes

Our studies with pharmacological inhibitors indicate that ACAC is important in the regulation of FAO and meiotic resumption in mouse oocytes. To further study the role of this enzyme, we utilized *Acacb*^{-/-} mice generated in a B6/129 background (Abu-Elheiga et al., **2001**; Abu-Elheiga et al., **2012**). We specifically compared the percent of meiotic resumption and FAO levels in CEO of B6/SJL F₁, B6/129 F₂ (control for *Acacb*^{-/-}), and *Acacb*^{-/-} strains in the presence of cyclic AMP (cAMP)-elevating agents during overnight culture. In the presence of 2 μM milrinone, the three strains showed no difference in percent of GVB (data not shown); however, *Acacb*^{-/-} CEO exhibited a higher percentage of GVB oocytes in the presence of hypoxanthine, a weak inhibitor of spontaneous meiotic resumption (Fig. 5A). This correlated with increased FAO in the *Acacb*^{-/-} compared to both B6/SJL and B6/129 control CEO populations (Fig. 5B).

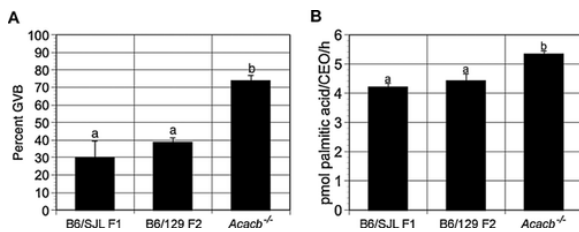


Figure 5 Reduced meiotic arrest and increased FAO in oocytes from *Acacb*-knockout oocytes. **A:** CEO from the three strains were cultured in the presence of 4 mM hypoxanthine and assessed for GVB 17 hr later. **B:** Milrinone-arrested CEO were cultured for 18 hr, and then assayed for FAO. Groups with no common letter are significantly different.

As loss of ACAC in situ could lower malonyl CoA levels to the extent that intrafollicular meiotic arrest became compromised, pre-ovulatory oocytes were isolated from 48-hr primed mice and scored for meiotic status. GVB percentages in the three genotypes were as follows: 0% in B6SJL (n = 180; 5 mice); 0.5% in B6/129 (n = 200; 5 mice); and 0% in *Acacb*^{-/-} (n = 119; 4 mice). Thus, loss of ACACB did not impair the ability of the follicle to maintain meiotic arrest.

Localization of Phosphorylated ACAC in Mouse Oocytes

Our lab has previously shown that active PRKA localizes to the germinal vesicle (GV); at condensed chromosomes after GVB; at spindle poles in metaphase I and II; and at the spindle midbody in anaphase I (Chen et al., 2006; Chen and Downs, 2008). Since both ACAC isoforms are substrates of PRKA, it was important to compare the localization of phosphorylated ACAC (pACAC) during meiotic resumption with that of active PRKA. Although PRKA phosphorylates ACAC at several residues, Ser79 of ACACA has been shown to be necessary and sufficient for its inactivation (Brownsey et al., 2006, 1997; Tong, 2005). Therefore, we first determined the localization of pACACA using a monoclonal rabbit antibody that recognizes phosphorylated Ser79. Staining of pACAC increased within the GV after in vivo human chorionic gonadotropin (hCG) stimulation, followed by intense staining near condensed chromosomes after GVB and at spindle poles during metaphase I and II (Fig. 6A), which is similar to the pattern of PRKA. By anaphase, however, no specific pACAC Ser79 localization was seen—in contrast to the persistent PRKA staining.

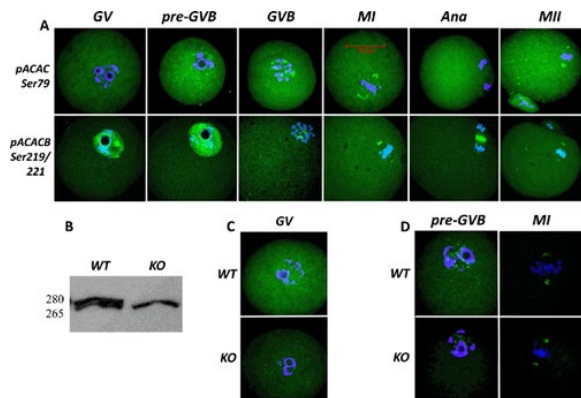


Figure 6 Localization of ACAC and determination of ACAC isoforms in mouse oocytes. **A:** Mice primed with equine chorionic gonadotropin (eCG) were injected with 5 IU hCG, and preovulatory oocytes were isolated from follicles at 0, 2, 8.5, and 10.5 hr post-hCG, while ovulated ova were isolated 13 hr post-hCG from oviductal ampullae to obtain oocytes at the following meiotic stages: GV, pre-GVB, GVB, metaphase I (MI), anaphase (Ana), and metaphase II (MII). Oocytes were stained with primary anti-phospho-Ser79 ACAC monoclonal rabbit antibody or primary anti-pACACB Ser218/221 rabbit polyclonal antibody. **B:** Western blots from wild-type and *Acacb*^{-/-} (KO) CEO (250 each) were probed with a primary, non-isoform specific anti-ACAC monoclonal antibody. Numbers to the left of the blot indicate molecular weights of the two isoforms, in kDa. **C:** GV-stage oocytes from 48-hr, eCG-primed wild-type and *Acacb*^{-/-} (KO) mice immunostained with the same antibody as in “B”. **D:** Oocytes obtained from wild-type and *Acacb*^{-/-} (KO) mice 2.5 (pre-GVB) and 8 hr post-hCG (MI) were stained with primary monoclonal anti-pACAC Ser79 antibody. Chromatin shown in blue, ACAC shown in green. Scale bar, 50 μ m.

When we used a polyclonal rabbit antibody produced against a dual-phosphorylated ACACB isoform (at Ser 218 and Ser 221 in human ACACB) that cannot distinguish between ACACA and ACACB due to the high identity between the relevant phosphorylation sites, pACAC was found to be additionally localized to the spindle midbody (Fig. 6A), where active PRKA resides. Staining was also evident within the GV in freshly isolated

oocytes, which intensified following hCG stimulation. The less-intense cytoplasmic staining for pACACB compared to that of pACACA Ser79 is likely a consequence of the different antibodies and exposure settings used to prevent overexposure of signal across the meiotic stages. It is important to note that similar pACAC localization was reported for dividing somatic cells from prophase to metaphase (Vazquez-Martin et al., 2013). In addition, when either of the primary antibodies for pACAC was omitted, no pACAC staining appeared in the oocyte, and when blocking peptide for the anti-phospho-Ser79 antibody was used to pre-absorb the active antibody, nuclear and cytoplasmic staining was eliminated.

Identification of ACAC Isoforms Present in Mouse CEO and DO

We next carried out Western blot assays using a monoclonal anti-ACAC antibody to determine which ACAC isoforms are present in mouse oocyte-cumulus cell complexes based on their molecular weights. As reported previously, both isoforms were present in wild-type complexes (Chen et al., 2006), whereas ACACB, as expected, was missing in *Acacb*^{-/-} complexes (Fig. 6B). When immunofluorescence staining of freshly isolated, non-stimulated DO was performed with the same monoclonal antibody, wild-type oocytes displayed a bright, diffuse cytoplasmic staining with a slightly increased punctate staining in the GV whereas *Acacb*^{-/-} DO staining was greatly reduced, with a few isolated punctate aggregations (Fig. 6C). Staining of 2.5-hr post-hCG oocytes with anti-pACAC Ser79 antibody revealed cytoplasmic and particularly intense nuclear puncta in controls, with barely discernible cytoplasmic staining and a modest number of punctate nuclear aggregates in knockout oocytes (Fig. 6D). Oocytes from both wild-type and *Acacb*-knockout mice at 9-hr post-hCG showed prominent pACAC staining at the metaphase-I spindle poles (Fig. 6D).

Localization of Mitochondria During Meiotic Resumption

A dramatic change in the distribution of mitochondria in oocytes of various species, including mouse, has been described during spontaneous in vitro maturation as well as hormone-induced in vivo maturation (Van Blerkom and Runner, 1984; Calarco, 1995; Bavister and Squirrell, 2000; Stojkovic et al., 2001; Sun et al., 2001; Suzuki et al., 2005; Sturmev et al., 2006; Yu et al., 2010; Wakai, 2012). In general, mitochondria aggregate around the GV as meiotic resumption is induced, and, in metaphase, surround the spindle. This conserved localization pattern is associated with dramatic increases in ATP production in the bovine oocyte (Stojkovic et al., 2001), and correlates with an increase in mitochondrial activity in the pig oocyte (Sun et al., 2001; Brevini et al., 2005). We examined whether a similar mitochondrial relocation pattern occurs during 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-induced meiotic resumption. To this end, CEO were cultured in the presence of 300 μM dibutyryl-cAMP with and without 500 μM AICAR, and DO were isolated at 4 and 10 hr to obtain various stages of meiotic maturation. Staining oocytes with an anti-mitochondrial oxidative phosphorylation complex IV (COX4) antibody showed increased mitochondrial aggregation near the GV before GVB in AICAR-stimulated oocytes. COX4 staining later surrounded condensed chromosomes and the metaphase-I spindle, similar to previous reports using mitochondria-specific dyes or GFP fusion proteins (Fig. 7).

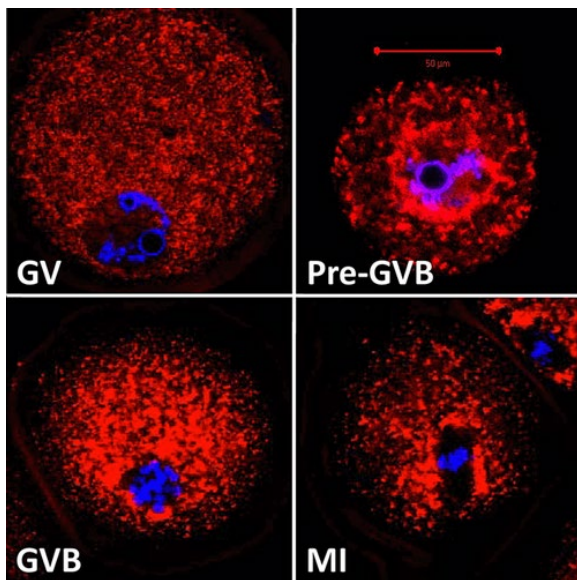


Figure 7 Mitochondria distribution in oocytes following stimulation by AICAR. Denuded oocytes were cultured in medium containing 300 μ M dbcAMP with or without 500 μ M AICAR, and isolated 4 and 10 hr later to obtain different meiotic stages. Oocytes were stained with anti-mitochondrial oxidative complex IV (COX4) antibody to localize mitochondria. Shown are freshly isolated, unstimulated oocytes (GV) and AICAR-treated oocytes at pre-GVB, GVB, and metaphase-I (MI) stages. Chromatin shown in blue; COX4 shown in red. Scale bar, 50 μ m.

DISCUSSION

We previously demonstrated the importance of PRKA activation and FAO stimulation in the pharmacological and physiological induction of meiotic resumption, and suggested that stimulation of FAO via the inactivation of ACAC may be how PRKA induces meiotic resumption (Downs et al., **2009, 2002**; Chen et al., **2006**; LaRosa and Downs, **2006**; Chen and Downs, **2008**; Valsangkar and Downs, **2013**). In the present study, we examined how altering ACAC activity affects these aspects of oocyte physiology. We report that (i) pharmaceutical inhibitors of ACAC bring about meiotic resumption and increased FAO through pathways that are sensitive to FAO inhibitors; (ii) physiological meiotic resumption was blocked by ACAC activation and MLYCD inhibition; and (iii) oocytes from *Acacb^{-/-}* mice show increased FAO and partial loss of meiotic arrest. These results support our contention that ACAC plays an important regulatory role in meiotic resumption, and help clarify how PRKA stimulation of FAO in mouse oocytes is mediated.

We used two different inhibitors, CP-640186 and Soraphen A, which act at two different sites on the ACAC molecule. CP-640186 binds to the active site at the CT domain and has been suggested to prevent biotin binding (Zhang et al., **2004**), whereas Soraphen A binds to the BC domain and has been suggested to prevent its oligomerization (Shen et al., **2004**), which is important for enzyme activity (Tong, **2005**). Both inhibitors induced meiotic resumption in mouse oocytes maintained in an arrested state with hypoxanthine, IBMX, or milrinone. A third inhibitor of ACAC, TOFA (McCune and Harris, **1979**), was equally effective as an inducer of meiotic resumption (data not presented). The fact that CP-640186 and Soraphen A suppress ACAC by different mechanisms and that both are effective in reversing meiotic arrest provide compelling evidence that this enzyme has a critical regulatory role in mouse oocyte maturation.

ACAC inhibitor-directed meiotic resumption in mouse oocytes was previously linked to an increase in FAO using an assay first described by Dunning et al. (**2010**) and later used by us (Valsangkar and Downs, **2013**). Suppression of meiotic resumption by FAO inhibition indicates that FAO mediates a meiotic response, and that ACAC inactivation—and thereby FAO stimulation—may be sufficient to induce meiotic resumption in mouse oocytes.

Malonyl CoA is a product of ACAC that suppresses CPT1, making it a critical regulator of FAO. Citrate, a physiological activator of ACAC, and CBM-301106, a pharmacological inhibitor of MLYCD, the enzyme that converts malonyl CoA to acetyl CoA, were therefore tested for their effects on FSH-induced meiotic resumption of CEO (Bonfont et al., **2004**; Tong, **2005**; Brownsey et al., **2006**; Lopaschuk and Stanley, **2006**; Samokhvalov et al., **2012**); use of these two complementary approaches should both result in elevated malonyl CoA levels, and we thus predicted that meiosis would be suppressed. Indeed, both citrate and CBM-301106 blocked FSH-induced meiotic resumption and FAO in mouse oocytes, which implicate the importance of malonyl CoA levels in the regulation of meiosis.

Mouse oocyte-cumulus complexes contain both isoforms of ACAC (Chen et al., **2006**), whereas only ACACA was present in *Acacb*^{-/-} complexes. ACACB is thought to be predominantly involved in FAO inhibition, whereas ACACA is mainly involved with long-chain fatty acid synthesis (Abu-Elheiga et al., **2000**, **2001**; Tong, **2005**; Brownsey et al., **2006**). We therefore utilized *Acacb*^{-/-} mice on a B6/129 background, kindly provided by Dr. Abu-Elheiga (Abu-Elheiga et al., **2001**, **2012**), to determine if the in vivo loss of this isoform affects meiotic resumption. The *Acacb*^{-/-} CEO showed increased FAO compared to both B6SJL F₁ and B6129 F₂ CEO. In the presence of hypoxanthine, but not milrinone, the knockout CEO showed significantly higher meiotic resumption than either the wild-type B6/129 F₂ or the B6/SJL F₁ oocytes, indicating that loss of *Acacb* can reduce the ability of the oocyte to maintain meiotic arrest. Yet this effect may only manifest when the inhibitory influence is weak since the much more potent inhibitor of spontaneous GVB, milrinone, effectively maintained meiotic arrest in knockout oocytes compared to hypoxanthine. Moreover, *Acacb*^{-/-} mice show normal fertility, suggesting that there is some redundancy of function wherein ACACA produces enough malonyl CoA to dampen FAO and premature meiotic resumption. The line of *Acacb*^{-/-} mice used in this study overexpress hypoxanthine-guanine phosphoribosyltransferase (Abu-Elheiga et al., **2001**), but this cannot account for the effects seen on meiosis and FAO because increased levels of this enzyme would be expected to produce effects opposite to those observed (Downs, **1997**).

The fact that the two ACAC 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**; data herein) suggests that the increase in FAO via the inactivation of ACAC is sufficient to induce meiotic resumption in vitro. Although they augment AICAR- and hormone-induced maturation, palmitic acid and carnitine fail to induce meiotic resumption by themselves. It is possible that palmitic acid and carnitine trigger slower FAO kinetics than are required for meiotic induction; however, palmitoyl carnitine is believed to directly enter mitochondria, thus bypassing the activity of CPT1 and reducing the time taken to initiate FAO.

The localization of pACAC in the GV and at condensed chromosomes, spindle poles, and the spindle midbody resembles that of phosphorylated PRKA. Such co-localization suggests a functional significance for PRKA-mediated inactivation of ACAC at these sites. Similar localization of pACAC has been reported in somatic cells during mitosis (Vazquez-Martin et al., **2013**), which is consistent with other data indicating a role for ACAC in somatic-cell division (Saitoh et al., **1996**; Al-Feel et al., **2003**; Mao et al., **2013**). On the other hand, residence of ACAC in the GV was unexpected since no such function for ACAC in mammals has been reported, although this localization pattern is somewhat consistent with reports that PRKA has nuclear substrates. Though the possibility of non-specific nuclear staining cannot be discounted, four lines of evidence support the specificity: (i) both anti-pACAC antibodies stained the GV; (ii) omitting primary antibody eliminated nuclear staining; (iii) blocking anti-phospho-Ser79 CACA antibody with the epitope-blocking peptide eliminated oocyte staining; and (iv) nuclear staining was greatly reduced in oocytes from *Acacb*-knockout animals. Indeed, a role for ACAC in the nucleus is plausible. In the yeast *Saccharomyces cerevisiae*, the activities of ACAC and SNF1 (the yeast homologue of mammalian PRKA) regulate global histone acetylation by affecting the levels of acetyl CoA that are required for this process (Galdieri and Vancura, **2012**; Zhang et al., **2013**). Therefore, PRKA and ACAC appear

to be at the intersection of metabolism and chromatin structure in yeast. In mammals, the majority of the acetyl CoA required for histone acetylation is produced from citrate by the enzyme ATP-citrate lyase found in both the cytosol and the nucleus; thus, there could be competition for acetyl CoA between long-chain fatty acyl synthesis in the cytosol and diffusion into the nucleus for histone acetylation. If ACAC is present in both the nucleus and cytosol, as our data suggest, the nuclear pool of acetyl CoA would also be susceptible to depletion by ACAC. It is therefore plausible that ACAC contributes to the regulation of histone or protein acetylation in the mouse oocyte. ACAC activity may also contribute to the acetylation of proteins other than histones, as in vivo liver-specific loss of both ACAC isoforms (a dual knockout) resulted in altered protein acetylation (Chow et al., **2014**). If ACACB is restricted to the mitochondrial outer membrane, as widely believed, ACACA must be the isoform present in the GV since mitochondria do not appear in the GV; indeed, nuclear staining is seen in *Acacb*^{-/-} oocytes probed for ACAC.

Changes in mitochondrial distribution during meiotic maturation have been reported in numerous mammalian species, suggesting that this dynamic rearrangement process is conserved. The redistribution of mitochondria in the mouse has been reported by Van Blerkom and Runner (**1984**), Calarco (**1995**), Yu et al. (**2010**), and Wakai, (**2012**). Such changes correlate 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**), which support the model that mitochondria aggregated near the chromosomes and meiotic spindle provide energy where it is needed because the rate of ATP diffusion may be limiting in a very large cells like an oocyte (Van Blerkom and Runner, **1984**), thereby necessitating functional compartmentalization (Van Blerkom, **2011**). This compartmentalization model is supported by the close association of mitochondria, and hence FAO, with lipid storage. Sturmey et al (**2006**) showed that mitochondria exhibit molecular-level co-localization with lipid droplets (within 10 nm) in porcine oocytes, thus supporting the concept of “metabolic units” proposed by Kruijff et al (**1983**). Additionally, consolidating mitochondrial FAO where it is required may serve to reduce the production of reactive oxygen species in the rest of the oocyte, thereby limiting potential oxidative damage.

Dunning et al. (**2010**) plus data herein showed that a major portion of FAO in the mouse cumulus-oocyte complex derives from the cumulus cells. Expression of *Cpt1b* mRNA in the cumulus cells increases during meiotic maturation and *Trib1* and *Trib3* (Ser-Thr kinases involved in FAO regulation) mRNA abundance increased in mouse cumulus cells during in vitro maturation, whereas *Acaca* transcript levels decreased under these conditions (Brisard et al., **2014**). High FAO activity in cumulus cells suggests that these somatic cells are an important source of fatty acid-derived energy during oocyte maturation. Yet data from this and previous studies have shown that (i) after hCG induction of maturation in vivo, FAO within oocytes is significantly stimulated (Valsangkar and Downs, **2013**); (ii) the ACAC inhibitor, CP-640186, stimulated FAO and meiotic resumption in DO; (iii) the CPT1 activator C75 stimulated GVB in DO (Downs et al., **2009**); and (iv) palmitoyl carnitine induced meiotic resumption in DO (Downs et al., **2009**). Thus, the increase in oocyte FAO occurs during meiosis is sufficient to drive GVB, though cumulus cells greatly accentuate the physiological response and their contribution may be required in situ.

It is important to consider potential mechanisms underlying how FAO induces meiotic resumption. One is through specific provisioning of energy for reactions critical for maturation-promoting factor (MPF), such as those involving phosphorylation of kinases. Alternatively, FAO could deplete the oocyte of substances that may be either harmful to the oocyte or inhibitory to meiotic resumption. For example, high levels of certain free fatty acids are toxic to somatic cells (Cnop et al., **2001**; Listenberger et al., **2003**, **2001**; Andrade et al., **2005**; Martins de Lima et al., **2006**) and oocytes (Wu et al., **2010**, **2011**; Aardema et al., **2013**; Luderer, **2014**). Pharmacological inhibition of long-chain acyl CoA synthetase, which links CoA to long-chain fatty acids, leads to a significant loss of meiotic arrest in mouse oocytes (Wang et al., **2012**). Interestingly, the G_{sα} subunit of the constitutively active G protein-coupled receptor 3 (GPR3), which maintains the production of cAMP by adenylyl cyclase in the oocyte

(Mehlmann, **2005**), undergoes palmitoylation. Depalmitoylation of $G_{s\alpha}$ causes its translocation from the plasma membrane to the cytosol, thereby reducing GPR3 signaling activity (Wedegaertner, **1998**; Chen and Manning, **2001**). It is thus reasonable to speculate that consumption of the long-chain fatty acid palmitoyl CoA via FAO would reduce its availability for $G_{s\alpha}$ palmitoylation and conceivably lead to its translocation, thereby reducing its inhibitory influence and contributing to GVB.

In summary, our data indicate that ACAC inactivation stimulates FAO and meiotic resumption in mouse oocytes. Studies carried out in other species provide evidence supporting the potential importance of ACAC in oocyte maturation. For example, *acac*-deficient mosquitos produce defective, non-viable eggs (Alabaster et al., **2011**); the phosphorylation state of ACAC from sea star (Pelech et al., **1991**), nemertean (Stricker et al., **2010**), and bovine (Bilodeau-Goeseels et al., **2010**) oocytes is altered during maturation; decreased ACAC phosphorylation in oocytes from diabetic mice is associated with reduced meiotic resumption (Ratchford et al., **2007**); and the ACAC inhibitor, CP640186, improves the in vitro maturation rate of canine oocytes (McGill et al., **2013**). Since biotin is required for carboxylase function, including ACAC, abnormal ACAC function could conceivably contribute to reproductive (Baez-Saldana et al., **2004**) and oocyte (Tsuji et al., **2015**) anomalies resulting from biotin deficiency, though non-carboxylase roles for biotin also exist (Zempleni, **2005**).

The increasing evidence linking fat metabolism to oocyte developmental competence should make fatty acid composition an important criterion when formulating culture media for in vitro maturation of oocytes—especially those destined for embryo production. These findings argue for a better understanding of how different metabolic pathways, particularly those involving energy generation, interact to successfully produce a developmentally competent ovum.

MATERIALS AND METHODS

Chemicals

The hormones equine chorionic gonadotropin (eCG), FSH, and hCG were purchased from the National Hormone and Peptide Program and Dr. AF Parlow. AICAR was obtained from Toronto Research Chemicals. Tritiated palmitic acid and tritiated water were purchased from PerkinElmer. All other chemicals, including L-carnitine, were sourced from Sigma–Aldrich (St. Louis, MO). We are indebted to Pfizer Pharmaceutical Co. for the gift of CP-640186; Dr. Rolf Jansen for the gift of soraphen A; and Dr. Gary Lopaschuk for the gift of CBM-301106.

Oocyte Isolation and Culture

C57BL/6JxSJL/J F_1 mice or B6/129 F_2 mice (either wild-type or *Acacb*^{-/-}) were used for all experiments. The *Acacb*^{-/-} mice were kindly provided by Dr. Wakil from the Baylor College of Medicine. All work was carried out with the prior approval of the Marquette University Institutional Animal Care and Use Committee. Females (19–23 days old) were injected with 5 IU eCG, and euthanized 48 hr later by cervical dislocation. Ovaries were dissected out and placed in a dish containing culture medium. Large antral follicles were punctured with sterile 26-gauge needles to isolate CEO, which were washed two times and distributed to tubes containing 1 ml of appropriate medium or to microwells of a 96-well plate with a final medium volume of 100 μ l, according to the experiment involved. DO were obtained by repeated pipetting of CEO with a small-bore pipet to remove cumulus cells.

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 bovine serum albumin (BSA), was used for oocyte culture in tubes. Following the addition of oocytes, culture medium was gassed with a humidified mixture of 5% CO₂/5% O₂/90% N₂; tubes were capped and sealed with parafilm, and then incubated at 37°C in a water bath. For cultures in 96-well plates (both FAO assays and maturation experiments), MEM

culture medium described above was additionally buffered with 12.5 mM HEPES and 15.5 mM sodium bicarbonate, and supplemented with fatty acid-free BSA.

Follicle Culture

Ovaries dissected from eCG-primed mice were transferred to a dish of Leibovitz L-15 medium supplemented with 3 mg/ml BSA. Antral follicles were carefully isolated using sterile 27-gauge needles. Follicles were then washed in MEM/5% fetal bovine serum (FBS), and transferred to 1 ml of MEM/FBS in a 10-ml stoppered flask, gassed with 95% O₂/5% CO₂, capped, and incubated in a water-jacketed incubator at 37°C with constant, gentle agitation for 3.5 hr. GVB was assessed after culture by puncturing the follicles with needles and denuding the oocytes within.

FAO Assay

FAO was measured using a modification of the protocol described previously (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-[³H]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. The FAO assay was carried out as described in our previous paper (Valsangkar and Downs, **2013**). For each experimental group, 25 CEO or 40 DO were used per well. Experiments were carried out at least three times, with two replicates per treatment.

Western Blots

CEO were isolated from wild-type or *Acacb*^{-/-} mice, washed in phosphate-buffered saline/polyvinylpyrrolidone (PBS/PVP) containing a protease-inhibitor cocktail. An appropriate volume of 2× Laemmli buffer and 2-mercaptoethanol were then added, and the samples were 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 using NuPAGE Tris-acetate SDS–PAGE buffer (Life Technologies, Grand Island, NY). The gel was run at 150 V for the initial 30 min, and then at 70 V for 3 hr to get desired separation of the two ACAC isoforms. After electrophoresis, the proteins were blotted by wet transfer using NuPAGE Transfer Buffer containing 2% SDS and 10% methanol at 400 mA for 75 min, with an ice tray inserted in the buffer tank; protein transfer was confirmed by staining the blot with Ponceau S. The blot was blocked in 5% milk for 2 hr, washed briefly, and incubated overnight at 4°C with a 1:1,000 dilution of anti-ACAC rabbit monoclonal antibody (#3676 from Cell Signaling, Danvers, MA) in 5% BSA. The blot was then washed with Tris-buffered saline containing 0.5% Tween-20 (TBS), incubated for 1 hr at room temperature with 1:4,000 goat-anti-rabbit-HRP-conjugated secondary antibody in 5% milk, and finally washed in TBS. Immunoreactive ACAC bands were visualized on film after incubating with SuperSignal West Pico signal detection system (Pierce).

Immunofluorescence

Denuded oocytes were fixed in 4% formaldehyde/PBS/PVP at 4°C overnight in Petri dishes, then equilibrated to room temperature PBS/PVP with two changes of solution, and stored at 4°C until used for immunostaining (within 1 week). Oocytes were collected from PBS/PVP and placed in a well of a 96-well plate containing permeabilization solution of 1% Triton X-100 in blocking solution. After 30 min of permeabilization, oocytes were transferred to a well containing blocking solution that consisted of 10% sheep serum and 0.005% saponin in PBS/PVP. Samples were blocked for 90 min, and then incubated overnight at 4°C with anti-phospho-Ser79 ACAC (1:50 dilution; #11818 from Cell Signaling, Danvers, MA); anti-ACAC (1:50 dilution; #3676 from Cell Signaling, Danvers, MA); anti-pACACB dual phosphorylated (Ser219/221) antibody (1:100 dilution; sc-30446-R from Santa Cruz Biotechnology, Dallas, TX); or anti-oxidative phosphorylation complex IV antibody (1:100 dilution; A21348 from Life Technologies, Grand Island, NY). Samples were equilibrated to room temperature for

30 min, and then washed four times, for 15 min each, with blocking solution to remove excess antibody. The specificity of anti-phospho-Ser79 ACAC antibody was tested by first pre-incubating the antibody dilution with blocking peptide (Cell Signaling) before adding the diluted solution to the oocytes. Oocytes were incubated in appropriate secondary antibody for 60 min, and then washed four times as described above. Washed oocytes were transferred to a glass slide, allowed to dry almost completely, and then mounted in DAPI. Oocytes were imaged at 63× using a Zeiss laser-scanning confocal immunofluorescence microscope. Scan parameters were maintained constant within groups for each experiment.

Statistical Analysis

Each experiment was repeated at least three times. For experiments investigating oocyte maturation in tubes, at least 25 CEO or DO were included per group per experiment. Percent GVB data were converted to arcsine values, and analyzed by ANOVA and Duncan multiple-range test to assess statistical significance; $P < 0.05$ was considered significant. Maturation data were graphed as mean percent GVB \pm standard error, and FAO assay data as mean picomoles of palmitic acid oxidized per CEO per hour or mean femtomoles of palmitic acid oxidized per DO per hour \pm standard error of the mean. These data were analyzed directly by ANOVA and Duncan multiple-range test.

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