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Role of AMPK Throughout Meiotic Maturation in the Mouse Oocyte: Evidence for Promotion of Polar Body Formation and Suppression of Premature Activation

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Role of AMPK Throughout Meiotic Maturation in the Mouse Oocyte: Evidence for Promotion of Polar Body Formation and Suppression of Premature Activation

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Abstract:

This study was conducted to assess the role of AMPK in regulating meiosis in mouse oocytes from the germinal vesicle stage to metaphase II. Exposure of mouse cumulus cell-enclosed oocytes (CEO) and denuded oocytes (DO) during spontaneous maturation in vitro to AMPK-activating agents resulted in augmentation of the rate and frequency of polar body formation. Inhibitors of

AMPK had an opposite, inhibitory effect. In addition, the AMPK inhibitor, compound C (Cmpd C) increased the frequency of oocyte activation. The stimulatory action of the AMPK-activating agent, AICAR, and the inhibitory action of Cmpd C were diminished if exposure was delayed, indicating an early action of AMPK on polar body formation. The frequency of spontaneous and Cmpd C-induced activation in CEO was reduced as the period of hormonal priming was increased, and AMPK stimulation eliminated the activation response. Immunostaining of oocytes with antibody to active AMPK revealed an association of active kinase with chromatin, spindle poles and midbody during maturation. Immunolocalization of the α1 catalytic subunit of AMPK showed an association with condensed chromatin and the meiotic spindle, but not in the spindle poles or midbody; α2 stained only diffusely throughout the oocyte. These data suggest that AMPK is involved in a regulatory capacity throughout maturation and helps promote the completion of meiosis while suppressing premature activation.

INTRODUCTION

AMP-activated protein kinase (AMPK) is an important cellular energy sensor that is activated in response to deficits in ATP and acts by shutting down energy consumption and turning on energy-generating pathways (Hardie, 2003; Carling, 2004). It is a trimeric protein comprised of β and γ regulatory subunits and an α catalytic subunit and is present in all tissues studied. Two isoforms of the catalytic subunit exist, α1 and α2 and both are present in mouse oocytes (Downs et al, 2002). Recent studies from our lab have demonstrated a role for AMPK in controlling the resumption of meiotic maturation in mouse oocytes. AMPK within the oocyte can be activated by hormones, stress and adenosine and AMP analogs, and such activation precedes, and is a causal force for, meiotic resumption; moreover, suppressing AMPK activity blocks meiotic induction brought about by these different stimuli (Chen et al, 2006; Downs and Chen, 2006; LaRosa and Downs, 2006, 2007; Chen and Downs, 2008).

While AMPK involvement in meiotic resumption is well established, much less is known about its participation in meiosis following germinal vesicle breakdown (GVB) in mouse oocytes. It was therefore the goal of the present study to examine how AMPK might contribute to mouse oocyte maturation from GVB to first polar body

extrusion. Through the use of stimulators and inhibitors of AMPK, and immunofluorescent localization of the kinase, evidence is presented that active AMPK associates with condensed chromatin and the meiotic spindle and exerts a positive influence on polar body formation, while suppressing activation.

RESULTS

Effects of AMPK Modulators on polar body formation

Activators Cumulus cell-enclosed oocytes (CEO) were cultured 16-17 h in medium containing increasing concentrations of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or AMP, and were assessed for polar body formation. The percentage of CEO progressing to MII in control cultures was 50-54%. AICAR stimulated a significant increase in PB formation at the lower two doses, but at the highest dose was inhibitory, while AMP was stimulatory at all doses tested (Fig. 1A). The degree of stimulation was similar (a 26-27% increase) within the two groups at the maximally effective concentration.
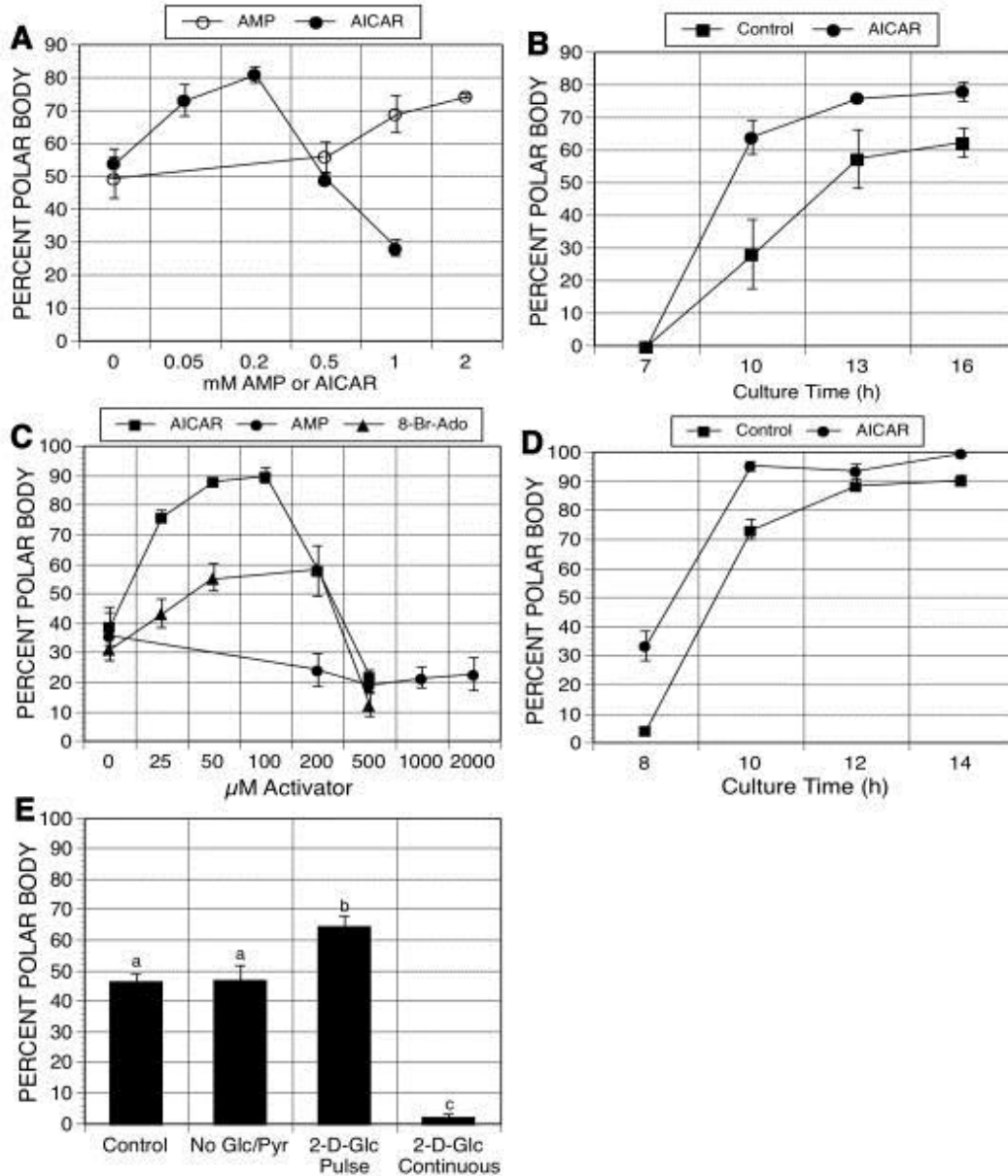
Figure 1 Effect of AMPK activation on polar body formation. (A) CEO were cultured 16-17 h in medium containing increasing concentrations of AICAR or AMP and assessed for polar body formation; (B) CEO were cultured for 7-16 h in control medium or medium containing 200 µM AICAR and checked every 3 h for polar body formation; (C) DO were cultured 9 h in medium containing increasing concentrations of AICAR, AMP or 8-Br-Ado and assessed for polar body formation; (D) DO were cultured 8-14 h in control medium or medium containing 100 µm AICAR and assessed every 2 h for polar body formation; (E) DO were pulsed 3 h in medium lacking pyruvate and glucose or in medium containing 10 mM 2-D-Glc, or they were continuously cultured in control medium or medium lacking pyruvate and glucose.

After a total culture time of 9 h, oocytes were assessed for polar body formation. A different letter at the top of the bar denotes a significant difference.

The action of AICAR on the kinetics of PB formation was next tested. CEO were cultured 7, 10, 13 or 16 h in control medium or medium containing 200 µM AICAR. In control cultures, PB formation was initiated between 7 and 10 h of culture, with 28% of the oocytes reaching metaphase II (MII) at 10 h and 62.5% by 17 h (Fig. 1B). The frequency of PB formation in AICAR-treated oocytes was already 64% after 10 h and this number peaked 6 h later at 78%. It is clear from these data that AICAR stimulated both the rate and frequency of PB formation. When CEO were cultured in 2 mM AMP, a comparable increase in PB formation was observed at 10 h (average of 62% MII in AMP-treated oocytes compared to 27% in controls; n = 3).

To determine if these agents act directly on the oocyte, similar experiments were conducted with denuded oocytes (DO). An AICAR dose response experiment first determined the most effective concentration. Since preliminary experiments showed a higher PB percentage in DO than CEO when cultured in control medium for 16-17 h (≥ 90%), the culture period for this experiment was shortened to 9 h. As shown in Fig. 1C, the effective dose for AICAR peaked at 100 µM, with an increase in PB formation from 39% to 90%, and the highest dose tested (500 µM) proved to be detrimental to development (22% at MII). When 100 µM AICAR was then tested on the kinetics of PB formation, the frequency of PB formation in DO was increased by this agent at all time points (Fig. 1D), demonstrating a direct, stimulatory action on the oocyte.

To test whether other AMPK-activating agents can also promote PB formation in DO, a dose response experiment with AMP was carried out with DO cultured for 9 h. However, at the effective concentrations used for CEO, AMP had no significant effect on PB formation (Fig. 1C). Another AMPK activator, 8-Br-adenosine (8-Br-Ado), that has previously been shown to augment PB formation in CEO (Downs and Chen, 2006), was next tested. A response curve similar to that obtained with AICAR was observed, with the peak concentration (200 µM) increasing the PB rate by 27% (Fig. 1C).

Exposing mouse oocytes to different stresses stimulates meiotic resumption in an AMPK-dependent manner (Larosa and Downs, 2006,

2007). Initial experiments in which oocytes were heat stressed at 42°C for 30-60 min produced no beneficial effect on progression to MII. Thus, an alternative approach was utilized that stressed the oocyte at the level of energy metabolism. In a previous study, it was shown that the TCA cycle inhibitor, arsenite, promoted AMPK and GVB in arrested oocytes (LaRosa and Downs, 2007). However, it was important for the oocyte to recover from the stress and be capable of generating energy after the stress pulse, since maturation is an active, energy-requiring process (Downs et al, 2007). Thus, 2-deoxy-glucose (2-D-Glc) to achieve ATP depletion in DO. This agent depletes ATP by the action of hexokinase, since the product, 6-phospho-2-D-Glc, cannot be used in glycolysis to generate more ATP. Three test conditions were evaluated: (1) 3-h pulse with glucose- and pyruvate-free medium; (2) 3-h pulse with this same medium containing 10 mM 2-D-Glc; and (3) continuous exposure to 2-D-Glc in the absence of glucose and pyruvate. Total culture time for all groups was 9 h. As shown in Fig. 1E, pulsing in the presence, but not absence, of 2-D-Glc stimulated PB formation, whereas continuous exposure to 2-D-Glc had no effect on GVB but eliminated meiotic progression to MII.

Inhibitors If AMPK promotes PB formation, then suppressing its activity should have the opposite effect. Indeed, when CEO were cultured 16-17 h with the AMPK inhibitors, compound C (Zhou et al, 2001) and araA (Henin et al, 1996), suppression of meiotic progression was observed. Both inhibitors blocked PB formation in dose-dependent fashion, with Cmpd C the more effective agent (Fig. 2A). The highest dose of Cmpd C reduced PB formation by 44%, while this number was decreased 24% by araA treatment. Interestingly, Cmpd C, but not araA, stimulated oocyte activation, such that at a concentration of 2.5 µm, 19% of the oocytes were activated compared to 0% of controls. These oocytes displayed a prominent polar body and pronucleus and, upon overnight culture in control medium, most underwent some form of cleavage, though few normal two-cell stages were observed. Rather, most of the eggs exhibited unequal cleavage with differing degrees of cytoplasmic blebbing.
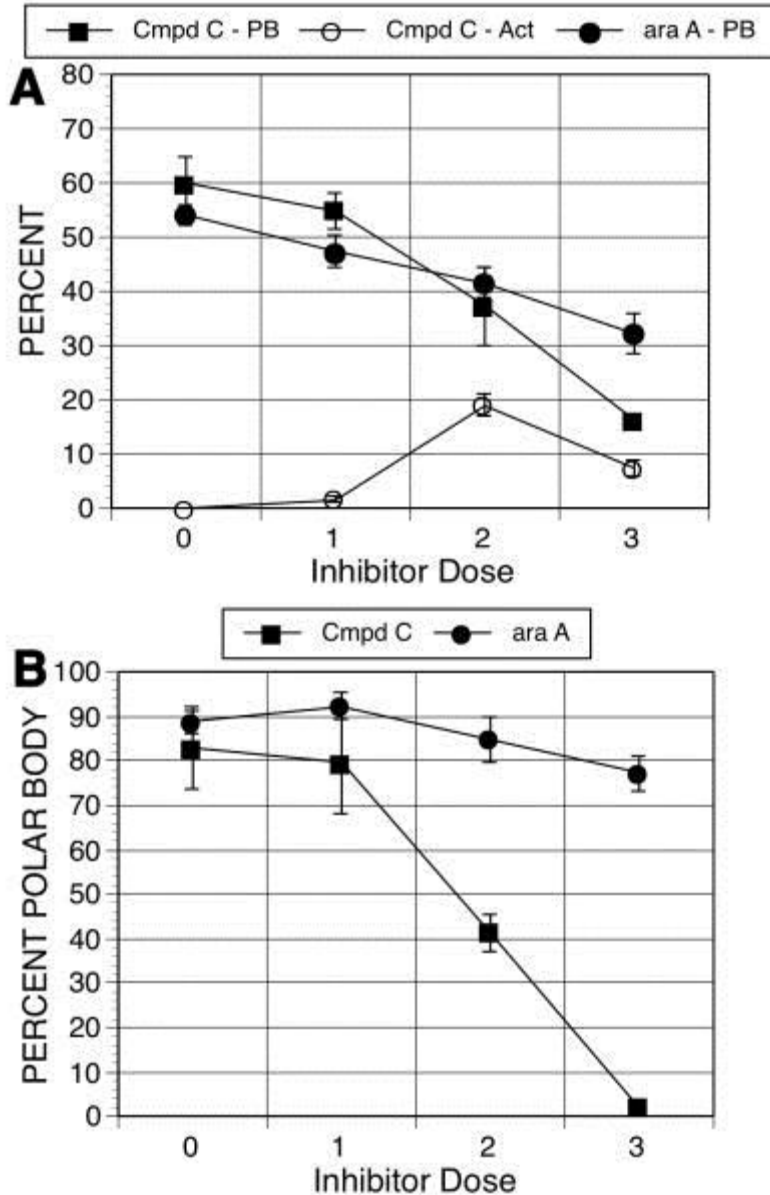
Figure 2 Effect of AMPK inhibition on polar body formation. CEO (A) or DO (B) were cultured for 16-17 h in medium containing increasing concentrations of Compound C or araA and assessed for polar body formation or activation. Inhibitor concentrations: 1, 2.5 and 5 µM Cmpd C; 1, 1.5 and 2 mM araA.

When the effect of these agents was tested on DO, Cmpd C demonstrated a similar potency in suppressing PB formation, while the effect of araA was less pronounced (Fig. 2B). Less activation was observed in Cmpd C-treated DO when compared to similarly treated

CEO, appearing only in the 2.5 µM group (9.8% activated), while no activation was observed in araA-treated DO.

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

To determine when in the culture period oocytes were sensitive to AICAR and Cmpd C treatment, CEO were cultured for 16-17 h and exposure to these agents was delayed for 0, 2 or 4 h. As shown in Fig. 3, delaying AICAR exposure reduced the stimulation of PB formation, suggesting an early effect of AMPK on this developmental endpoint. Consistent with this finding was the progressive loss of inhibition by Cmpd C of PB formation with later administration, such that when delayed 4 h, the percentage of MII oocytes was not significantly different than controls (43% versus 54%). This demonstrates that the action of Cmpd C is not due to a general toxic effect. However, the percentage of activated oocytes was similar (12-17%) regardless of when Cmpd C exposure took place, suggesting a later, protective action of AMPK on activation.
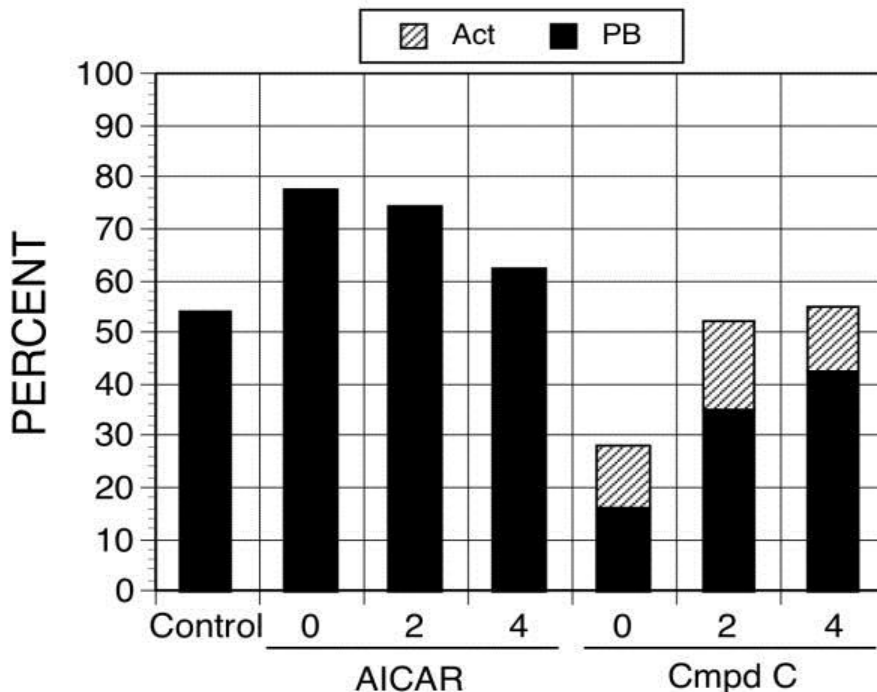

Figure 3 Effect of timing of exposure to AICAR and Cmpd C on polar body formation and activation. CEO were cultured 16-17 h and were exposed to 200 µM

AICAR or 2.5 µM Cmpd C at 0, 2 or 4 h after the onset of culture and then assessed for polar body formation and activation.

Effects of PMSG Priming on Polar Body Formation and Oocyte Activation

In a previous study (Downs, 1990), oocytes from mice primed only 1 d had a significantly higher percentage of activation during spontaneous maturation than oocytes from mice primed 2 days. This finding was exploited to further analyze the effects of AMPK on PB formation and activation. CEO from unprimed mice and from mice primed for 1 d or 2 d were cultured for 21-22 h in control medium or medium containing either 200 µM AICAR or 2.5 µm Cmpd C and then assessed for PB formation and activation. The culture time was extended in these experiments, because longer culture times were shown to augment the effect on activation (Downs, 1990). These data are presented in Figure 4A. Control oocytes from 1-d- and 2-d-primed mice displayed identical percentages at MII (62%), with this value reduced in oocytes from unprimed mice (47%). In all three groups, AICAR treatment significantly stimulated PB formation (by 21-22% in oocytes from primed animals and 46% in oocytes from unprimed animals). As the period of priming increased, the degree of spontaneous activation decreased (10%, 6% and 1%, respectively, in 2-d-primed, 1-d-primed and unprimed mice) as well as the extent of Cmpd-C-stimulated activation (47%, 38% and 19%, respectively). Moreover, spontaneous activation was completely eliminated by AICAR treatment in all groups. These results support the idea that activation of AMPK promotes meiotic progression to MII while, at the same time, suppressing spontaneous activation of the oocyte. In addition, priming mice with eCG protects the oocytes from activation during spontaneous maturation in culture.
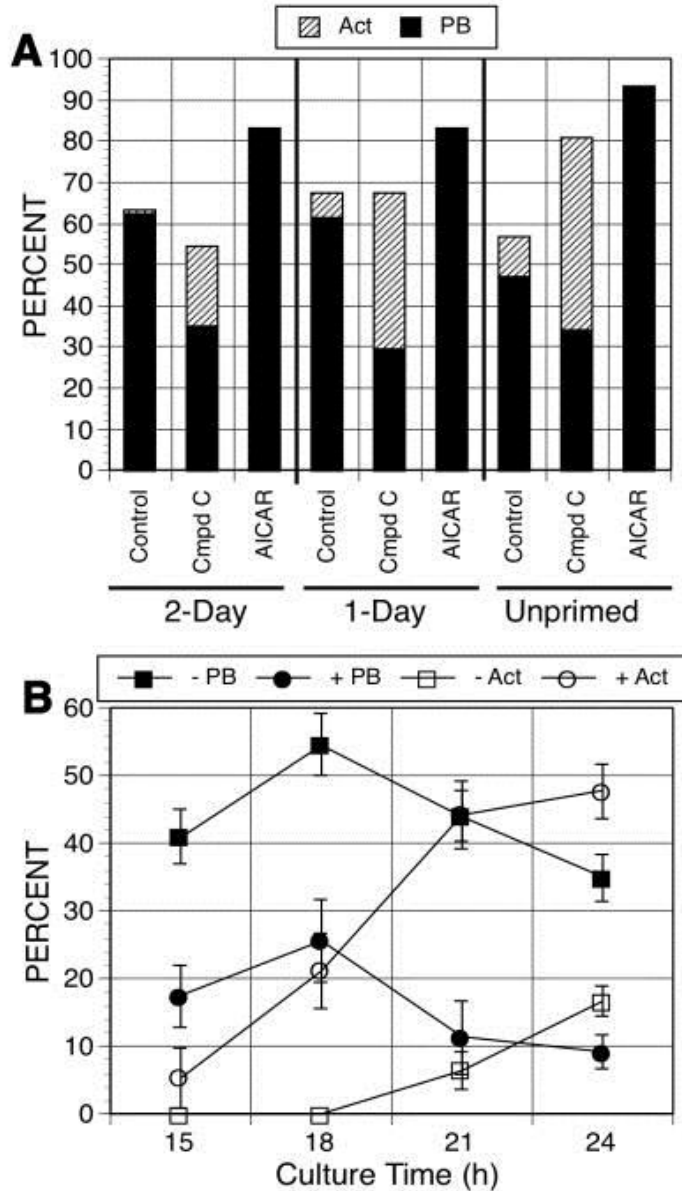
Figure 4 Effect of priming on polar body formation and oocyte activation. (A) CEO were derived from unprimed mice or mice primed for 1 or 2 d, cultured 21-22 h in control medium or medium containing 200 µM AICAR or 2.5 µM Cmpd C, and assessed for polar body formation and activation; (B) CEO from 1-d-primed mice were cultured 15-24 h in either the absence (−) or presence (+) of 2.5 µM Cmpd C and assessed every 3 h for polar body formation (PB) and activation (Act).

A kinetics experiment was carried out to determine the timing of oocyte activation as it relates to PB formation. CEO from 1-d-primed mice were cultured for varying periods of time from 15 h to 24 h in the

presence or absence of 2.5 µM Cmpd C and assessed for PB formation and activation. In control oocytes, the percentage of oocytes at MII peaked at 18 h (55%) and then declined to 35% at 24 h (Fig. 4B). A similar pattern was observed in Cmpd C-treated oocytes, but at reduced rates, with PB frequency peaking at 26% and decreasing 6 h later to 9%. In control oocytes, activation was initiated between 18 and 21 h of culture, at the same time PB frequency started to decrease. The percentage of activated oocytes at 24 h (17%) was similar to the 20% decrease in MII oocytes at this time. Activation in Cmpd C-treated oocytes was initiated as early as 15 h (5%) and increased rapidly thereafter, to 48% at 24 h. These data suggest that a significant portion of the activated oocytes derived from MII-stage oocytes.

In a separate experiment (n = 6), CEO from 1-d-primed mice were cultured 21-22 h in MEM/BSA ± 2 mM AMP to determine if this agent could also prevent spontaneous oocyte activation. In the absence of AMP, 15.2 ± 3.5% of the CEO (42 out of 273) underwent activation, as noted by the presence of a second PB and pronucleus. AMP significantly suppressed the activation response, as only 2.5 ± 1.2% of the CEO (8 total out of 273) had responded in this manner.

Immunostaining of Active AMPK Throughout Meiotic Maturation

Oocytes were fixed at different stages of meiotic maturation and then stained for active AMPK using an antibody that recognizes phospho-threonine 172 on the catalytic subunit of the kinase. Four different maturation conditions were utilized for these experiments: (1) in vivo maturation following hCG injection to primed animals: (2) spontaneous maturation of CEO; and meiotic induction in (3) dbcAMP-arrested DO stimulated with AICAR and (4) dbcAMP-arrested CEO stimulated with FSH. Oocytes were examined at the following meiotic stages: GVB, metaphase I (MI), anaphase and metaphase II (MII).

Maturation in vivo In oocytes undergoing maturation in vivo in response to hCG stimulation, active AMPK became associated with the condensed chromosomes immediately after GVB (Fig. 5). Active AMPK then began to move to two regions at opposite ends of the chromatin mass, and, at MI, staining was prominently positioned at the spindle

poles. During chromosome segregation, however, staining at the poles disappeared and intense staining appeared in the midbody region. After polar body formation, intense staining was often seen throughout the polar body, but only in oocytes that had recently completed anaphase. As the MII spindle was established, staining in the PB disappeared and again became prominent at the spindle poles. Specificity for active AMPK was demonstrated in MI-stage oocytes by the disappearance of staining at the spindle poles when primary antibody was omitted or when primary antibody was preincubated with blocking peptide specific for the PT172 epitope (Fig. 5).
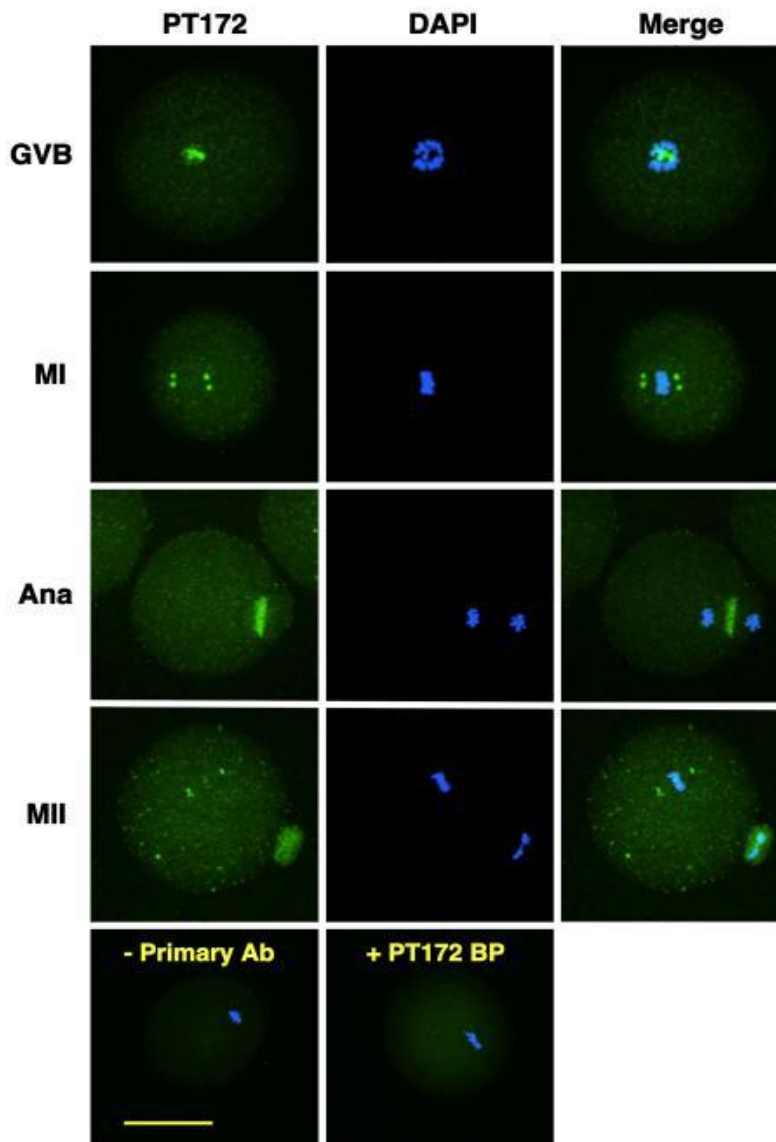
Figure 5 Immunolocalization of active AMPK throughout oocyte maturation. Oocytes were isolated at varying stages of meiosis after hCG administration to 2-d-primed mice, fixed and stained with anti-PT172 antibody for active AMPK (green) and DAPI for chromatin (blue). Controls in the bottom row were of MI-stage oocytes and were either not exposed to primary antibody or anti-PT172 antibody was first treated with blocking peptide. GVB, germinal vesicle breakdown; MI, metaphase I, Ana, anaphase, MII, metaphase II.

Maturation in vitro When immuofluorescence for active AMPK was examined throughout maturation in the different in vitro model systems, localization in all three groups was identical to that observed in oocytes maturing in vivo (Supplementary Fig. 1). Thus, the temporal pattern of active AMPK distribution was consistent at the different stages of maturation regardless of whether maturation occurred spontaneously, was induced in vivo by hCG, or was induced in vitro by AICAR or FSH.

Immunostaining of isoforms of the catalytic subunit of AMPK

Since there are two different isoforms of the catalytic alpha subunit of AMPK, we examined the distribution pattern of each isoform using isoform-specific antibodies. A light diffuse staining of alpha 2 was manifest throughout maturation with no discrete localization associated with chromosomes or meiotic apparatus (not shown). On the other hand, alpha 1 showed intense colocalization with condensed chromosomes following GVB, was associated with MI and MII spindle MTs, and colocalized with migrating chromosomes during anaphase (Fig. 6). This stain was eliminated when primary antibody was omitted or when primary antibody was pretreated with blocking peptide for alpha 1. Blocking peptide for alpha 2 had no effect on alpha 1 staining. These results are interesting in that, similar to that for active AMPK, alpha 1 staining was associated with chromosomes and the meiotic spindle, but the precise localization after spindle formation did not coincide with that of the active kinase.
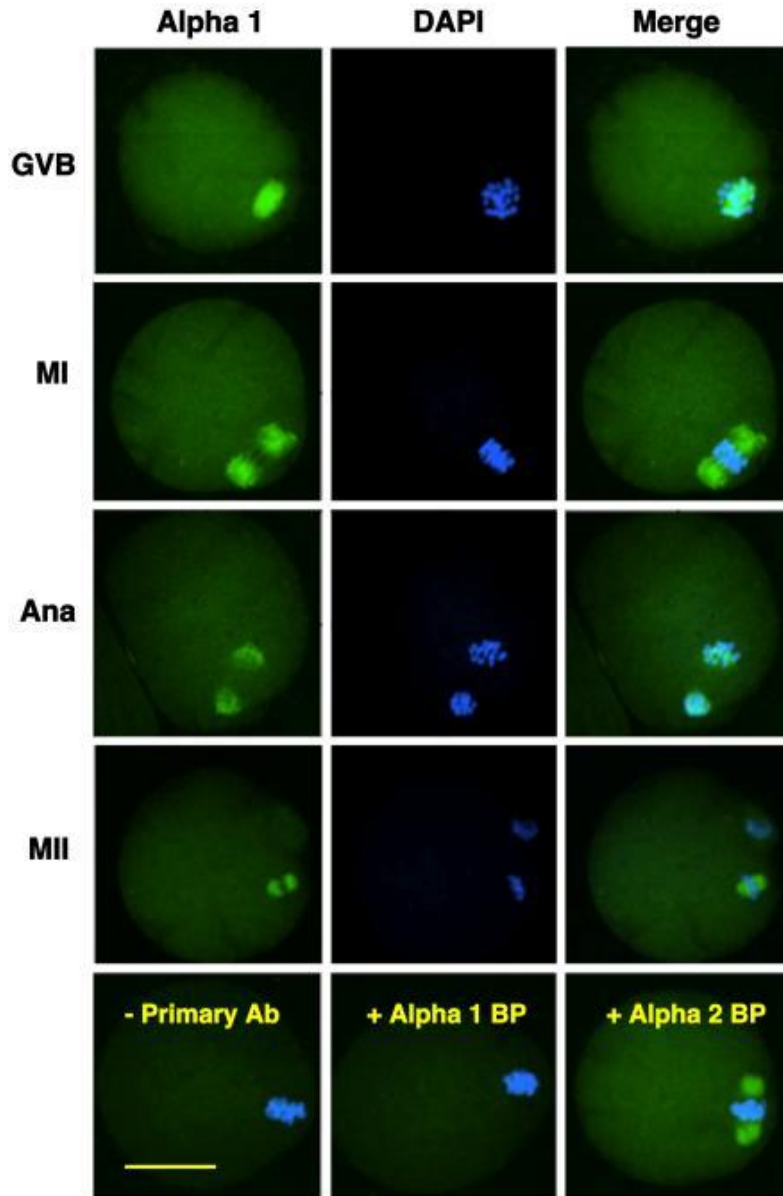
Figure 6 Immunolocalization of the α1 catalytic subunit of AMPK. Oocytes were fixed at varying stages of meiosis following hCG injection to 2-d-primed mice and stained for with anti-α1 antibody (green) and with DAPI for chromatin (blue). Controls in the bottom row were of MI-stage oocytes and were either not exposed to primary antibody or anti-α1 antibody was first treated with α1 blocking peptide or α2 blocking peptide. GVB, germinal vesicle breakdown; MI, metaphase I; Ana, anaphase; MII, metaphase II.

DISCUSSION

Evidence is presented that activation of AMPK in the mouse oocyte promotes the completion of meiotic maturation and suppresses activation, while inhibition of this kinase has the opposite results. AMP and AICAR each stimulated an increase in both the rate and frequency of polar body formation in spontaneously maturing oocytes. On the other hand, Cmpd C and araA both blocked polar body formation, and Cmpd C promoted activation. Susceptibility of oocytes to activation was indirectly related to the extent of eCG priming; moreover, AICAR and AMP prevented activation, while Cmpd C augmented activation. Immunolocalization of active AMPK revealed an initial association with condensed chromosomes after GVB and with the meiotic apparatus thereafter, supporting a meiotic regulatory role throughout the entire period of oocyte maturation.

Previous work has shown that AMPK activation precedes GVB in mouse oocytes and is a causal force behind meiotic resumption (Chen et al, 2006). The present study was conducted to determine if, after GVB, AMPK continues to participate in the mechanisms regulating the progression of meiosis to MII. Several lines of evidence presented herein indicate an important role for AMPK throughout the maturation period. Both AICAR and AMP dose-dependently increased the extent, and accelerated the kinetics of, PB formation in spontaneously maturing CEO, while treatment with AMPK inhibitors produced the opposite effect. Furthermore, stressing the oocyte by depleting ATP with 2-deoxyglucose treatment also promoted the completion of maturation. Such treatment increases the AMP/ATP ratio and stimulates AMPK and is often used in somatic cells to activate the kinase (eg, Hurley et al, 2005; Zhu et al, 2005; Park et al, 2009; Fujimoto et al, 2010; Pradelli et al, 2010). The response to 2-DG pulsing is therefore consistent with the effects of AICAR and AMP. It should be noted that the frequency of MII-stage control oocytes, consistently maintained throughout the study at ~50-62%, was lower than that reported in an earlier study utilizing similar culture conditions (Downs and Hudson, 2000). While the reason for this difference is not known, it is not due simply to suboptimal culture conditions, since CEO from unprimed mice and DO from primed mice reached MII at very high levels (~90%) and AMPK activation increased both the extent and kinetics of PB formation.

Because a higher number of DO than CEO progressed to the MII stage after 16-17 h of culture, shorter time points were utilized to assess the effects of AMPK modulators on PB formation in DO. While AICAR increased the incidence of PB formation in DO after 9 h of culture by over 50%, AMP curiously had no effect. An additional activator of AMPK, 8-Br-Ado (Downs and Chen, 2008), produced a 29% increase in PB frequency. The reason for the failure of AMP to affect maturation in DO is not known, but it is possible that uptake by the oocyte may be hampered by removal of the cumulus cells. A similar discrepancy in efficacy between DO and CEO was observed with araA. Like Cmpd C, this inhibitor effectively suppressed PB formation in CEO; however, while Cmpd C was similarly effective in DO, araA displayed a much reduced inhibitory potency. Nevertheless, taken together these data strongly suggest that AMPK plays an important maturation-promoting role throughout the period from GVB to MII. It will be important to determine how the somatic compartment contributes to this regulation.

Additional support for AMPK involvement in the completion of meiotic maturation was provided by the stimulation of oocyte activation by Cmpd C during overnight culture. This is not unprecedented, since other protein kinase inhibitors have been shown to promote this developmental response. For example, a wide range of studies have shown that the general kinase inhibitor, 6-dimethylaminopurine, is an effective inducer of parthenogenesis in oocytes of many mammalian species, including mouse (Szllosi et al, 1993; Liu et al, 1997; Nakasaka et al, 2000), rat (Jiang et al, 2002; Sano et al, 2009), cat (Yin et al, 2007), rabbit (Liu et al, 2002), pig (Leal and Liu, 1998; Jilek et al, 2001), bovine (Winger et al, 1997; Liu et al, 1998), sheep (Loi et al, 1998; Choi et al, 2004; Alexander et al, 2006), goat (Ongeri and Krisher, 2001), camel (Wani, 2008), rhesus monkey (Mitalipov et al, 2001) and human (Nakagawa et al, 2001). This is due in large measure to interruption of maturation promoting factor (MPF) activity, which is required to maintain MII arrest and is regulated by mitogen-activated protein kinase (Fan and Sun, 2004). Accordingly, inhibitors of the upstream kinase, MEK, also trigger activation (Tong et al, 2003). Data herein suggest that blocking AMPK activity in mouse oocytes interferes with this MII meiotic control point and leads to activation, perhaps through suppression of ERK1/2 activity, though it should be noted that Cmpd C had no effect on

ERK1/2 phosphorylation in bovine oocytes (Tosca et al, 2007). The fact that CEO were more susceptible to activation than DO is consistent with studies showing that cumulus cells promote activation in aging mouse oocytes (Miao et al, 2005)

When the administration of AICAR was delayed after the onset of culture by 2 or 4 h, there was a progressive reduction in the stimulation of PB formation in CEO, suggesting that the increase in PB number requires early exposure to active AMPK. This is consistent with the finding that a similar delay in exposure to Cmpd C significantly reduces its inhibitory activity on PB formation. However, a more protracted period of susceptibility exists for Cmpd C-induced activation, since comparable levels of activation were observed whether exposure to this inhibitor occurred 0, 2 or 4 h after the onset of culture. It therefore appears that sustained AMPK activity during a substantial portion of the maturation period is important to maintain a normal course of maturation and suppress oocyte activation.

In an earlier study, oocytes from 1-d-primed mice were more susceptible to activation during spontaneous maturation than those from mice primed 2 d (Downs, 1990). This observation was expanded upon to further evaluate the role of AMPK in oocyte activation by comparing oocytes from unprimed mice with those from mice primed 1 or 2 d. While less than 1% of spontaneously maturing CEO from 2-d-primed mice became activated during 17-18 h of culture, 6 or 10% were activated in CEO from 1-d-primed or unprimed mice, respectively. That this phenomenon was sensitive to regulation by AMPK was supported by the observation that treatment with AICAR or AMP suppressed oocyte activation, while Cmpd C appreciably augmented the activation rate, most notably in the unprimed group, where an increase from 10% to 47% was observed.

The dynamics of oocyte activation received further scrutiny in the kinetics experiment that utilized oocytes from 1-d-primed mice, where extending the culture time to 24 h revealed an even greater activation response in both the absence and presence of Cmpd C. This stems from the fact that prolonged culture lessens the ability of oocytes to maintain MPF activity, making them more susceptible to spontaneous and induced activation (Fissore et al, 2002). PB formation peaked at 18 h and declined thereafter, in concert with a

corresponding increase in activation frequency, suggesting that a significant portion of the activated eggs originated from MII-stage oocytes. Indeed, the 20% decrease in MII-stage oocytes in the control group between 18 h and 24 h coincides closely with the 17% increase in activation. However, we cannot discount the possibility that some activated oocytes derive from MI-stage oocytes without transit through MII, as has been reported in LT/Sv and LTXBO mouse strains whose oocytes display high spontaneous activation rates (Kaufman and Howlett, 1986; Maleszewski and Yanagimachi, 1995; Eppig et al, 1996, 2000). It will be interesting to determine whether AMPK activity influences parthenogenetic activation brought about by a number of different chemical treatments (cf, Kaufman, 1983).

In previous double immunofluorescent staining experiments using an anti-phospho-threonine 172 antibody that targets the phosphorylated, active α subunit of AMPK, positive staining was shown in the GV of mouse oocytes prior to meiotic resumption and later colocalized with condensed chromatin following GVB (Chen et al, 2008). This was consistent with a causal role for AMPK in meiotic resumption and suggested further involvement in subsequent stages of meiosis. Regardless of the conditions of meiotic maturation--whether spontaneous, in situ induction via hCG injection, or in vitro induction by AICAR or FSH treatment--immunofluorescent staining with the anti-PT172 antibody revealed a reproducible pattern of localization after GVB which further supported the participation of AMPK throughout the entire maturation period. Active kinase appeared initially in condensed chromosomes, became focused at the spindle poles at MI, shifted to the midbody/cleavage furrow region during anaphase I, and then reappeared at the spindle poles at MII.

This pattern of localization for active AMPK throughout maturation, notably its association with the meiotic spindle, closely resembles that found in oocytes for a number of other kinases, including MEK1/2 (Sun et al, 2008), phospho-protein kinase C δ (Ma et al, 2008), aurora kinase (Yao and Sun, 2005; Shuda et al, 2009) and polo-like kinase (Wianny et al, 1998; Pahlavan et al, 2000; Tong et al, 2002; Fan et al, 2003). These kinases have all been shown to play a vital role in meiotic maturation, particularly in relation to spindle dynamics. The localization pattern for active AMPK in the mouse oocyte is also identical to that recently described in dividing human

cancer cells, mouse NIH-3T3 fibroblasts and 3T3-L1 mouse pre-adipocytes (Vazquez-Martin et al, 2009). These observations suggest an important role for AMPK in cell cycle regulation.

Indeed, results of a recent genome-wide survey of protein kinases revealed a direct link between energy sensing by AMPK and cell cycle progression (Bettencourt-Dias et al, 2004). This relationship was demonstrated in a study by Jones et al (2005) in which restricting glucose availability to mouse primary mouse embryonic fibroblasts activated AMPK and, subsequently, a p53-dependent metabolic checkpoint; similarly, cells expressing a constitutively active α2 AMPK catalytic subunit had impaired proliferative ability. This finding is consistent with a recent series of reports demonstrating cessation of cell growth and proliferation by AMPK stimulation in a variety of cell types (eg, Imamura et al, 2001; Igata et al, 2005; Motoshima et al, 2006; Baumann et al, 2007; Gwinn et al, 2008; Fogarty and Hardie, 2009; Ishii et al, 2009; Zang et al, 2009). Further evidence stems from the finding that mutations in the upstream AMPK kinase, LKB1 (Kyriakis, 2003), are responsible for Peutz-Jeghers syndrome, an autosomal dominantly inherited cancer in humans (Hemminki et al, 1998; Jenne et al, 1998). LKB1 is also involved in regulation of cell polarity through its action on AMPK (Alessi et al, 2006; Lee et al, 2007; Mirouse et al, 2007), and in mouse oocytes is present in the germinal vesicle and is associated with the meiotic spindle, though it is excluded from the polar microtubules and midbody (Szczepanska and Maleszewski, 2005).

Consistent with above observations in somatic cells are the findings that AMPK activation suppresses GVB in pigs (Mayes et al, 2007), cows (Tosca et al, 2007; Bilodeau-Goeseels et al, 2007)) and nemertean worms (Stricker et al, 2010). However, mouse oocytes are unique in that AMPK activation does not suppress, but, rather, promotes cell cycle progression such that arrested GV-stage oocytes are stimulated to reinitiate meiotic maturation (Chen et al, 2006), and polar body formation in maturing oocytes is increased (data herein). The effect of glucose concentrations on cell cycle regulation in mouse oocytes likewise differs substantially from that in somatic cells. Whereas in somatic cells lower glucose levels block cell proliferation, in mouse oocytes it has the opposite effect. In the presence of meiotic inhibitors, the G2/M transition is suppressed by increased glucose

concentration, while lower glucose levels promote maturation (Downs and Mastropolo, 1994; Downs, 1995). This may be related to the fact that, unlike in somatic cells, glucose is not a primary energy source for oocytes; rather, this role is taken by downstream metabolites of glucose, such as pyruvate, that are supplied by the somatic compartment of the follicle. Thus, meiotic inhibitors have a poor ability to maintain meiotic arrest in mouse oocytes when pyruvate levels are high and glucose levels are low, whereas the opposite is true when pyruvate is low and glucose is high. Though not yet specifically investigated, it is likely that the meiotic response of mouse oocytes to these differing energy substrate conditions is closely related to the activation status of AMPK. For example, glucose-derived ATP in the cumulus cells could transit gap junctions to reach the oocyte (Downs, 1995), lower the AMP/ATP ratio and maintain AMPK in an inactive state.

Since both type 1 and 2 α subunits for AMPK are present in the mouse oocyte (Downs et al, 2002), it was of interest to determine the localization pattern for each throughout maturation. The pattern for α2 was unremarkable, exhibiting a faint diffuse staining throughout the maturation period without any localized accumulation. On the other hand, staining for α1 showed an intense localization pattern associated initially with the condensed chromosomes and later with the meiotic spindle. Staining for this subunit also increased in the germinal vesicle prior to GVB, which was surprising based on reports that α2, but not α1, localizes to the nucleus in somatic cells (Salt et al, 1998). Curiously, α1 stained intensely within the spindle proper at both metaphase stages and colocalized with migrating chromosomes at anaphase, a pattern inconsistent with that observed for the active kinase. However, previous studies in somatic cells have failed to demonstrate a link between the phosphorylation state of threonine 172 of AMPK and subcellular localization of the α subunit of AMPK (Kohida et al, 2007; Vazquez-Martin et al, 2009). These results suggest that α1, and not α2, is the catalytic subunit most important for regulating the maturation of mouse oocytes.

In conclusion, results indicate that AMPK plays an important regulatory role in the completion of meiotic maturation in mouse oocytes. They also suggest that compromising AMPK activity during meiosis has detrimental downstream effects on developmental

potential, since although most activated oocytes from Cmpd C-treated CEO exhibited some form of mitotic division, cleavages were clearly abnormal, often accompanied by extensive blebbing. It will be of interest to determine how AMPK affects preimplantation embryonic development to see whether AMPK reverts to a suppressive influence on cell cycle progression, as has been previously demonstrated in mitotic cells.

MATERIALS AND METHODS

Oocyte Isolation and Culture Conditions

Animals were raised in the research colony of the principal investigator (SMD). All experiments were carried out with prior approval of the Marquette University Institutional Animal Care and Use Committee.

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

Immunofluorescent Staining

Oocytes were fixed with 4% formaldehyde at 4°C and permeabilized with 0.1% triton in blocking buffer (0.05% saponin in PBS, pH 7.4, plus 10% sheep serum) for 30 min. Oocytes were then washed free of triton and continuously blocked for another 90 min at room temperature. Oocytes were incubated with rabbit primary

antibodies (1:100) overnight at 4°C, washed in blocking buffer at room temperature, and incubated with FITC-conjugated sheep anti-rabbit antibody (1:1000) at room temperature for 1 h. For experiments involving blocking peptide, primary antibodies were incubated for 1 h at room temp with blocking peptide before adding oocytes for overnight incubation. After washing, oocytes were mounted on pre-washed slides with vectashield mounting medium containing DAPI (Vector laboratories, Inc., Burlingame, CA), and cover slips were sealed with nail polish.

Confocal Microscopy

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

Chemicals

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

## Statistical Analysis

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

Supplementary Material

Suppl Figure 1

Supplemental Figure 1. Immunolocalization of active AMPK throughout maturation. Oocytes were fixed at varying stages of meiosis using three different in vitro model systems: (1) spontaneous maturation of CEO; (2) dbcAMP-arrested CEO stimulated with FSH; and (3) dbcAMP-arrested DO stimulated with AICAR. Oocytes were fixed and stained with anti-PT172 antibody for active AMPK (green) and with DAPI for chromatin (blue). GVB, germinal vesicle breakdown; MI, metaphase I; Ana, anaphase: MII, metaphase II.
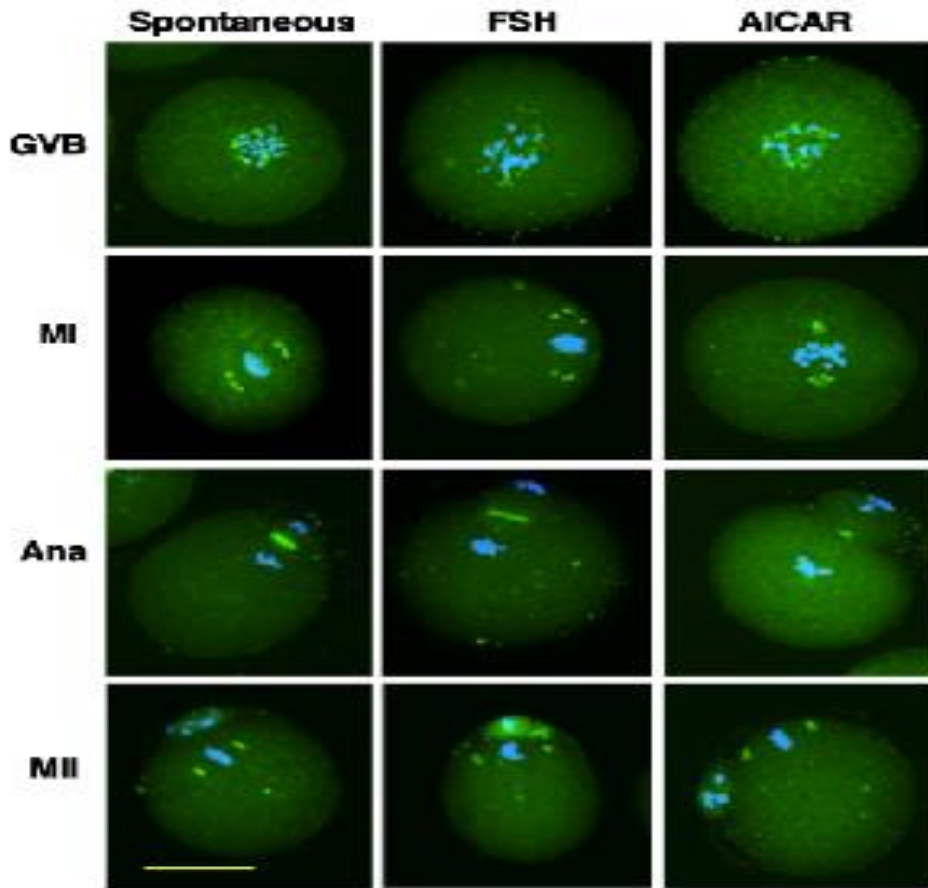
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