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Potential upstream regulators and downstream targets of AMP-activated kinase signaling during oocyte maturation in a marine worm

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Abstract

Unlike in mice, where the onset of oocyte maturation (germinal vesicle breakdown, GVBD) is blocked by cAMP and triggered by AMP-activated kinase (AMPK), oocytes of the marine nemertean worm *Cerebratulus* undergo GVBD in response to cAMP elevations and AMPK deactivation. Since the pathways underlying AMPK's effects on mammalian or nemertean GVBD have not been fully defined, follicle-free nemertean oocytes were treated with pharmacological modulators and subsequently analyzed via immunoblotting methods using phospho-specific antibodies to potential regulators and targets of AMPK. Based on such phosphorylation patterns, immature oocytes possessed an active LKB1-like kinase that phosphorylated AMPK's T172 site to activate AMPK, whereas during oocyte maturation, AMPK and LKB1-like activities declined. In addition, given that MAPK can deactivate AMPK in somatic cells, oocytes were treated with inhibitors of ERK1/2 MAPK activation. However, these assays indicated that T172 dephosphorylation during maturation-associated AMPK deactivation did not require MAPK and that an observed inhibition of GVBD elicited by the MAPK kinase blocker U0126 was actually due to ectopic AMPK activation rather than MAPK inactivation. Similarly, based on tests using an inhibitor of maturation-promoting factor (MPF), T172 dephosphorylation occurred upstream to, and independently of, MPF activation. Alternatively, active MPF and MAPK were necessary for fully phosphorylating a presumably inhibitory S485/491 site on AMPK. Furthermore, in assessing signals possibly linking AMPK deactivation to MPF activation, evidence was obtained for maturing oocytes upregulating target-of-rapamycin activity and downregulating the cyclin-dependent kinase inhibitor Kip1. Collectively, these findings are discussed relative to multiple pathways potentially mediating AMPK signaling during GVBD.
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Introduction

In response to proper stimuli, oocytes start to mature by undergoing a process of nuclear disassembly referred to as germinal vesicle breakdown (GVBD). Based on comparisons across the animal kingdom, GVBD is differentially regulated by intraoocytic concentrations of the secondary messenger cAMP. In mammals, other vertebrates, and starfish, GVBD is characteristically blocked by elevated levels of cAMP (Voronina & Wessel 2003, Mehlmann 2005, Richard 2007), whereas intraoocytic cAMP elevations can trigger, rather than inhibit, GVBD in several marine invertebrates, including worms of the phylum Nemertea (Stricker & Smythe 2001).

In addition to cAMP, various protein kinases are also integrally involved in oocyte maturation via their eventual downstream effects on a proximate inducer of GVBD called maturation-promoting factor (MPF; Kishimoto 2003). In investigations of kinase-mediated processes during MPF-induced GVBD, the evolutionarily conserved AMP-activated kinase (AMPK) pathway has recently been analyzed, since in addition to integrating intracellular energy states, AMPK can also regulate cell cycling (Carling 2005, Alessi *et al.* 2006, Motoshima *et al.* 2006, Hardie 2007). Moreover, given that cAMP hydrolysis not only reduces cAMP levels but also raises AMP levels that in turn help to activate AMPK, signaling pathways involving AMPK may play key roles both in normal oocytes (Downs *et al.* 2002) and in those affected by diseases such as diabetes and polycystic ovary syndrome (Ratchford *et al.* 2007, Dupont *et al.* 2008).

Accordingly, various analyses have shown that AMPK triggers GVBD in denuded or cumulus-enclosed oocytes of mice (Chen *et al.* 2006, LaRosa & Downs 2006, 2007, Chen & Downs 2008). Alternatively, AMPK agonists can block GVBD in other cumulus-enclosed mammalian oocytes (Bilodeau-Goeseels *et al.* 2007, Mayes *et al.* 2007, Tosca *et al.* 2007), although such blockage is apparently due to effects on follicle cells and/or to non-AMPK-mediated pathways (Mayes *et al.* 2007, Tosca *et al.* 2007, Bilodeau-Goeseels *et al.* 2010). However,

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oocytes of nemertean worms lack follicle cells (Stricker *et al*. 2001), and AMPK activations nevertheless inhibit nemertean GVBD (Stricker *et al*. 2010*a*, 2010*b*), indicating that intraoocytic AMPK can stimulate or block MPF activation in different animals.

To determine how AMPK modulates GVBD, signaling pathways have been examined in follicle cells (Tosca *et al*. 2005, 2006, 2010, Kayampilly & Menon 2009, Richardson *et al*. 2009) and within oocytes themselves. For example, recent studies of intraoocytic AMPK in mice have assessed the relationship between diabetes and AMPK-mediated targets in glucose metabolism (Ratchford *et al*. 2007), the activation of AMPK by FSH and amphiregulin (Chen & Downs 2008), and the interconnections between AMPK and fatty acid oxidative pathways (Downs *et al*. 2009). Moreover, how AMPK interacts with MAPK and other signals has been evaluated in oocytes of cows and pigs (Bilodeau-Goeseels *et al*. 2007, Mayes *et al*. 2007, Tosca *et al*. 2007).

However, in spite of these and other analyses, intraoocytic AMPK signaling is incompletely understood, particularly with regard to some of the key upstream regulators and downstream targets that are known to mediate AMPK's effects in somatic cells. Thus, this study aims to track activity-related phosphorylations of AMPK signaling proteins in nemertean oocytes that are stimulated to mature in the presence or absence of pharmacological modulators. Based on these methods, new findings are presented regarding such regulators of intraoocytic AMPK as the AMPK kinase (AMPKK) LKB1, MAPK, and MPF. Moreover, target-of-rapamycin (TOR) signaling and the cyclin-dependent kinase (CDK) inhibitor p27 Kip1 are evaluated for the first time as possible targets linking AMPK deactivation to MPF activation, and collectively such results are discussed in the context of multiple pathways potentially mediating AMPK's effects during GVBD.

Results

Phosphorylation patterns of an LKB1-like AMPKK during oocyte maturation

Given that LKB1 is the most common AMPKK to activate AMPK via T172 phosphorylation in AMPK's activation loop (Shackelford & Shaw 2009, Ruderman *et al*. 2010), *Cerebratulus* oocytes were probed with a phospho-specific LKB1 antibody to track an S428 phosphorylation that can upregulate LKB1 (Liangpunsakul *et al*. 2008, Xie *et al*. 2008). Based on these tests, immature oocytes had relatively high T172 phosphorylation and likewise exhibited a putatively active LKB1 at the appropriate MW of ~52 kDa (Fig. 1A and C). Moreover, consistent with GVBD-related reductions in the phosphorylations of both AMPK's T172 site and S79 on the AMPK target acetyl-CoA carboxylase (ACC; Stricker *et al*. 2010*a*), such S428 phosphorylation decreased significantly

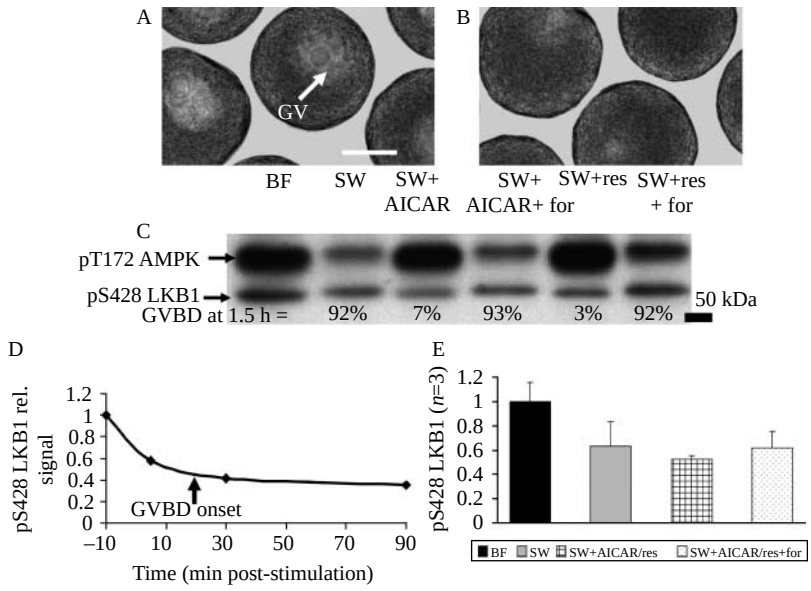

Figure 1 LKB1-like activity in nemertean oocytes. (A) Uncompressed living oocytes prior to maturation onset, showing intact germinal vesicles (GVs) in seawater (SW)+AICAR. (B) Mature oocytes after GVBD in SW+AICAR+the cAMP elevator IBMX; scale bar=50 μm. (C) Oocyte extracts dually probed with phospho-T172 and phosopho-S428 antibodies for tracking active forms of AMPK at 62 kDa and LKB1 at 52 kDa, showing a decrease in putative LKB1 activity during SW-induced GVBD (note: results were similar in separate blots using one antibody per blot). (D) Typical time-lapse sequence of decreasing phospho-S428 phosphorylations during SW-induced GVBD. (E) Even though T172 levels are elevated by SW solutions of the AMPK agonists AICAR and reseveratrol (res) (C), such treatments still allow as much LKB1 S428 dephosphorylation ($P>0.05$) as occurs in mature oocytes treated with either SW alone or with SW+AMPK agonists+cAMP elevators such as forskolin (for), suggesting that AMPK agonists may trigger an LKB1-independent activation of AMPK. BF=before treatment; other lanes on blots and graph in E=control and drug treatments at 1.5 h post-stimulation.

(44±22%; $N=23$; $P<0.05$) during maturation-associated AMPK deactivation (Fig. 1B and C). Alternatively, as potential evidence for LKB1-independent AMPK activation such as described elsewhere (Sakamoto *et al*. 2004, Sanders *et al*. 2007), seawater (SW) solutions of the AMPK agonists AICAR and resveratrol kept oocytes immature and AMPK activity high (Stricker *et al*. 2010*a*), and yet such agonist treatments in either SW or SW+cAMP elevators yielded phospho-S428 signals that were significantly lower ($P<0.05$) than in pre-treatment controls (Fig. 1B–E).

Effects of ERK1/2 MAPK on phosphorylations of AMPK's T172 and S485/491 sites

In somatic cells (Zheng *et al*. 2009), AMPKK/AMPK can be downregulated by ERK1/2 MAPK (MAPK3/1 or simply MAPK hereafter), and such MAPK activity normally increases during nemertean GVBD (Stricker and Smythe 2003, 2006*a*, Smythe & Stricker 2005). Thus, to determine if MAPK affects AMPK during GVBD, oocytes were treated with the MAPK kinase inhibitors CI1040, PD98059, and U0126. Based on such studies, CI1040- and PD98059-treated oocytes underwent GVBD in the absence of substantial MAPK activity and continued to deactivate AMPK, as evidenced by significant reductions ($P<0.05$) in both T172 and ACC phosphorylation, compared to immature controls (Fig. 2A and B).

Conversely, as noted previously (Smythe & Stricker 2005, Stricker 2009*b*), some oocyte batches remained immature in SW solutions of U0126. Accordingly, U0126-treated oocytes had significantly higher ($P<0.05$) AMPK activity than did mature controls in SW alone (Fig. 2A and B). Nevertheless, when stimulated by SW+cAMP-elevating drugs, U0126-treated specimens underwent GVBD (Stricker 2009*b*) and further dephosphorylated ($P<0.05$) AMPK's T172 site (Fig. 2C).

Oocytes treated with MAPKK inhibitors were also probed for S485/491 phosphorylation on AMPK, since such phosphorylation can inhibit AMPK in somatic cells (Horman *et al*. 2006, Hurley *et al*. 2006, Funahashi *et al*. 2009). However, as opposed to the marked S485/491 phosphorylation increase that occurs during normal GVBD (Stricker *et al*. 2010*a*), MAPKK blockers kept S485/491 phosphorylations in both immature and mature oocytes below ($P<0.05$) those exhibited by SW controls (Fig. 2D and E).

AMPK–MPF interactions during oocyte maturation

Given that positive feedback is apparently established between MPF and MAPK during nemertean GVBD (Stricker & Smythe 2006*a*), potential reciprocal interactions between AMPK and MPF were analyzed by treating immature oocytes with the MPF inhibitor roscovitine. In SW-stimulated oocytes kept immature by a roscovitine-mediated blockage of MPF activation, AMPK's T172 site continued to undergo control-like dephosphorylation, even in the absence of MAPK activity (Fig. 3A–C). Alternatively, since MPF blockers can more

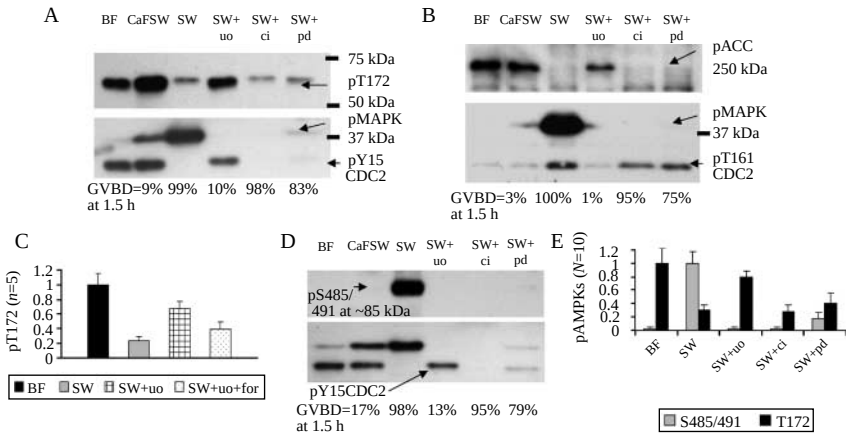

Figure 2 ERK1/2 mitogen-activated protein kinase (MAPK) and AMPK deactivation. (A and B) In spite of preventing MAPK activation, inhibitors of MAPK kinase (MAPKK; CI1040 (ci) or PD98059 (pd)) routinely allow GVBD as evidenced by reduced inhibitory Y15 phosphorylation on CDC2 or increased stimulatory phosphorylation at CDC2 T161. Similarly, based on control-like T172 dephosphorylation and decreased S79 phosphorylations on the AMPK target ACC, such oocytes lacking MAPK activity continue to undergo AMPK deactivation. (C) Alternatively, in some oocyte batches, SW solutions of the MAPKK blocker U0126 (uo) inhibit GVBD. However, T172 phosphorylations in these oocytes are significantly higher ($P<0.05$) than in SW controls, whereas co-treatments of uo plus cAMP elevators such as forskolin (for) further reduce T172 phosphorylation. Coupled with results for other MAPKK blockers and roscovitine (Fig. 3B–D), such findings suggest uo may not block GVBD via MAPK inhibition but rather by ectopic AMPK activation. (D and E) Unlike the MAPK-independence of the phospho-T172 signal, phosphorylating AMPK's putatively inhibitory S485/491 site apparently requires MAPK, since all MAPKK blockers reduce such phosphorylations to significantly below ($P<0.05$) control levels in SW alone. BF=before treatment; other lanes on blots/graphs=control and drug treatments at 1.5 h post-stimulation.

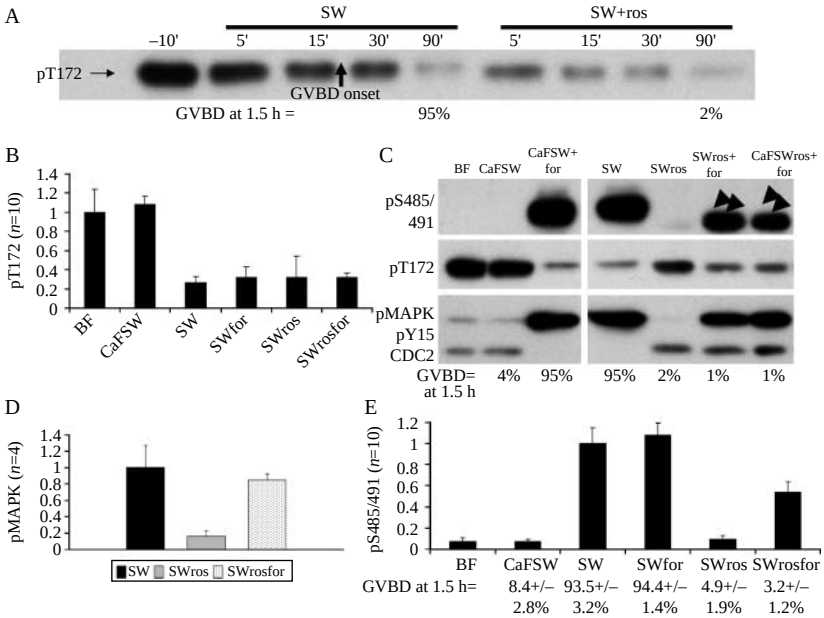

Figure 3 AMPK–MPF interactions. (A and B) T172 dephosphorylation continues in SW-stimulated oocytes that have low MPF activity owing to roscovitine (ros) treatments, as T172 phopsphorylations in such specimens are statistically the same ($P>0.05$) as in controls that matured in either SW or SW+forskolin. (C–E) As further evidence for the MAPK-independence of T172 dephosphorylations, T172 undergoes as much dephosphorylation ($P>0.05$) in roscovitine-treated oocytes as in SW controls (B) even though SW solutions of roscovitine cause significantly lower ($P<0.05$) MAPK phosphorylation than in SW alone (C and D). However, full phosphorylation of S485/491 apparently requires MPF in addition to MAPK, since in spite of control-like MAPK activities in forskolin (for)-stimulated oocytes (C and D), S485/491 phosphorylations in the presence of roscovitine are significantly lower ($P<0.05$; C and E) and downshifted (double arrowheads, C) compared to phospho-S485/491 bands of controls. BF=before treatment; other lanes on blots and graphs=control and drug treatments at 1.5 h post-stimulation; CaFSW=calcium-free seawater.

effectively deactivate MAPK in nemertean oocytes stimulated by SW alone versus by SW+cAMP elevators (Stricker & Smythe 2006*a*), roscovitine-mediated effects on S485/491 phosphorylations were also assayed. Accordingly, compared to roscovitine treatments in SW, elevated MAPK activations in SW solutions of roscovitine+cAMP elevators co-occurred with greater ($P<0.05$) S485/491 phosphorylation (Fig. 3C and D), further indicating the MAPK-dependence of such phosphorylation. However, phospho-S485/491 signals were downshifted nearer to the phospho-T172 signals after treatment with either roscovitine (Fig. 3C) or the less effective MPF inhibitor, olomoucine (data not shown). Moreover, in spite of relatively high MAPK activity (Fig. 3D), phospho-S485/491 signals remained weaker ($P<0.05$) than in SW controls lacking roscovitine (Fig. 3E), suggesting that along with MAPK-mediated phosphorylation, MPF may feed back on AMPK to facilitate S485/491 phosphorylation.

TOR signaling in maturing oocytes

Based on findings that AMPK can inhibit TOR signaling in somatic cells (Bolster *et al*. 2002, Kimura *et al*. 2003, Wullschleger *et al*. 2006), oocytes were probed with a phospho-S2448 TOR antibody that can track TOR upregulation (Rosner *et al*. 2010). In such blots, a putatively active TOR signal at the correct MW of ~285 kDa was significantly reduced ($P<0.05$) in immature versus mature controls (Fig. 4A–C). Moreover, SW solutions of the AMPK activators AICAR and resveratrol blocked GVBD while reducing ($P<0.05$) S2448 TOR phosphorylations below those of mature controls (Fig. 4B and C). In addition, as further evidence for differing modes of AMPK signaling during SW- versus cAMP-induced GVBD (Stricker *et al*. 2010*a*), adding cAMP elevators to SW solutions of AICAR or resveratrol restored GVBD and TOR phosphorylation to control-like levels in SW alone (Fig. 4B and C).

To provide added support for maturation-associated TOR activation during AMPK deactivation, oocytes were probed with a phospho-specific antibody to *Drosophila* p70/p85 S6 kinase (S6K), since S6K represents both a target and, via positive feedback, an activator of TOR complex 1 (Holz & Belnis 2006). For undetermined reasons, a weak and ephemeral phospho-p70 S6K signal was only occasionally observed during GVBD, whereas a strong phospho-S6K signal invariably occurred both at ~85 kDa and at upshifted sites (Fig. 4D), as noted for other phospho-S6Ks (Petritisch *et al*. 1995, Kraiss *et al*. 2000). Moreover, as shown for phospho-S2448 TOR, phospho-S6K signals increased ($P<0.05$) during

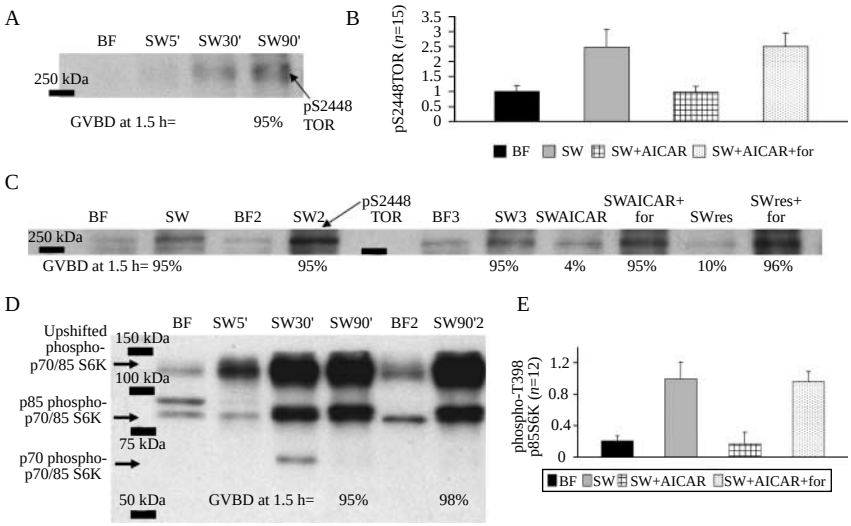

Figure 4 Target-of-rapamycin (TOR) signaling. (A) Typical time-lapse sequence, showing an increase in putatively active TOR during seawater (SW)-induced GVBD. (B and C) AMPK agonists AICAR and resveratrol (res) block GVBD and significantly reduce ($P<0.05$) SW-induced elevations in S2448 TOR phosphorylations, whereas cAMP elevators (e.g. forskolin (for)) restore GVBD/S4228 phosphorylations and allow AMPK deactivation (see Fig. 1C), collectively suggesting that maturation-associated AMPK deactivation might induce TOR activation. (D and E) As further evidence for maturation-associated TOR activation during AMPK deactivation, a target of active TOR, p70/85 S6K, undergoes a significant ($P<0.05$) increase in its phosphorylation during maturation. Moreover, phospho-p85 S6K signals are reduced ($P<0.05$) by AICAR but restored by forskolin (for). BF=before treatment; other lanes on blots C, G and graphs B, H=control and drug treatments at 1.5 h post-stimulation.

maturation, remained lower ($P<0.05$) in SW+AICAR than in SW controls, and re-elevated to control-like levels in SW solutions of AICAR+cAMP elevators (Fig. 4E).

Maturation-associated changes in the CDK inhibitor p27 Kip1

In somatic cells, AMPK-mediated activation of a TOR inhibitor, called tuberous sclerosis complex 2 (TSC2; Inoki *et al.* 2003, Coradetti *et al.* 2004, Abraham 2005), can block cell cycling by TSC2's stabilizing effects on p27 Kip1 (Rosner *et al.* 2006), since Kip1 is a CDK downregulator that typically targets CDK2 (Sherr & Roberts 1999) but can also inhibit the CDK1 (CDC2) of MPF (Font de Mora *et al.* 1997, Nakayama *et al.* 2004). Thus, oocytes were probed with a phospho-Kip1 antibody against a T187 site that, when phosphorylated, allows more effective Kip1 degradation (Vlach *et al.* 1997). In immature oocytes, such destabilizing Kip1 T187 phosphorylations at ~27 kDa were relatively weak, but during maturation, the phospho-T187 signal rose significantly ($P<0.05$) along with increased AMPK deactivation (Fig. 5A and B). Accordingly, signals

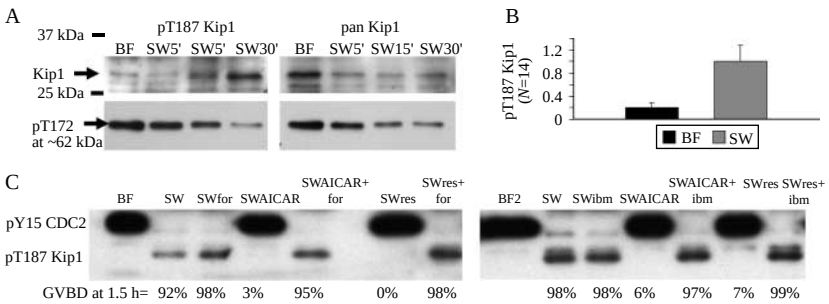

Figure 5 The cyclin-dependent kinase inhibitor p27 Kip1 during oocyte maturation. (A) Typical time-lapse sequence of a destabilizing (T187) phosphorylation on Kip1 (left) versus pan-Kip total protein levels (right), showing an increase and decrease, respectively, as oocytes mature and AMPK is deactivated by T172 dephosphorylation. (B) Quantification of the significant ($P<0.05$) increase in the destabilizing T187 phosphorylation on Kip1 during SW-induced maturation. (C) As with TOR signaling, AMPK agonists AICAR or resveratrol (res) prevent both SW-induced MPF activation (i.e. no decreases in inhibitory Y15-CDC2 phosphorylations) and phospho-187 Kip1 signals, whereas co-treatment with cAMP elevators (e.g. forskolin (for) or IBMX (ibm)) rescue such blockages. BF=before treatment; other lanes on blot C and graphs B, E=control and drug treatments at 1.5 h post-stimulation.

from a pan-Kip1 antibody for tracking total Kip1 levels declined during maturation (Fig. 5A). Moreover, the AMPK activator AICAR or resveratrol kept oocytes immature while preventing both T187-Kip1 phosphorylation and MPF activation (Fig. 5C). Conversely, as further evidence for different AMPK signaling pathways mediating SW- versus cAMP-induced GVBD, adding cAMP elevators to SW solutions of AICAR or resveratrol restored AMPK deactivation (Stricker *et al*. 2010*a*), T187 phosphorylation, and MPF activation to control levels (Fig. 5C).

Discussion

Phosphorylation changes during AMPK deactivation in maturing nemertean oocytes

Based on data reported here and elsewhere (Stricker *et al*. 2010*a*), AMPK activity declines during oocyte maturation in the nemertean worm *Cerebratulus*, and various AMPK agonists serve to block SW-induced GVBD, collectively indicating that AMPK deactivation is required for such GVBD. In this investigation, several proteins in the AMPK signaling pathway are shown to undergo phosphorylation changes during maturation. In general, such changes could involve either altered activities of upstream modulators acting upon already present proteins or changes in the overall amounts of proteins present in the oocyte. Currently, neither the levels of protein neosynthesis nor the proportions of phosphorylated pools relative to non-phosphorylated proteins are known for maturing oocytes, since total protein levels have yet to be analyzed, except for Kip1 where increased T187 phosphorylation is accompanied by decreased pan-Kip levels. However, given that high concentrations of protein synthesis inhibitors fail to keep nemertean oocytes from maturing (Stricker & Smythe 2001, Smythe & Stricker 2005; SA Stricker, unpublished observation), any new proteins added following maturation stimulation are presumably not required for GVBD. Moreover, regardless of whether AMPK signaling utilizes extant or newly synthesized proteins during GVBD, the activities of such proteins are nevertheless modulated by phosphorylations that can be tracked by phospho-specific antibodies. Accordingly, this study reveals highly repeatable differences in the overall amounts of several phosphorylations, based on multiple comparisons of pre- and post-treatment levels to control values in similar amounts of total cellular protein and oocytes loaded per lane. Furthermore, coupled with concordant data obtained using pharmacological modulators as well as verifications involving appropriate changes in the phosphorylations of downstream targets, such findings can collectively provide insight into maturation-associated alterations in the activities of possible upstream modulators and downstream targets of AMPK, as discussed further below.

Potential upstream regulators of AMPK activity in nemertean oocytes

In somatic cells, LKB1 upregulates AMPK by phosphorylating AMPK's T172 site (Alessi *et al*. 2006, Williams & Brenman 2008), and LKB1 homologues are also known to regulate polarity in oocytes (Pellettieri & Seydoux 2002). However, as novel evidence for LKB1 potentially blocking GVBD, immature nemertean oocytes display elevated T172 phosphorylation on active AMPK along with an apparently active LKB1-like kinase that seems to decrease its activity as oocytes mature and T172 undergoes dephosphorylation. Precisely which components of the intraovarian milieu serve to activate LKB1 in immature nemertean oocytes remain unknown. Moreover, whether these oocytes possess other AMPKKs such as CaMKKβ (Woods *et al*. 2005) or TAK1 (Xie *et al*. 2006) awaits additional testing.

In any case, it seems unlikely that upregulating LKB1 activity is the only way that AMPK agonists activate AMPK, since the AMPK stimulator AICAR maintains T172 phosphorylation in SW-stimulated oocytes and yet fails to keep LKB1's S428 site phosphorylated. It should be noted, however, that in some cells, S428 phosphorylation is either not required for LKB1 activity (Fogarty & Hardie 2009) or associated with LKB1 deactivation (Zheng *et al*. 2009), suggesting that reduced S428 phosphorylations in nemertean oocytes might not be indicative of decreased LKB1 activity. Alternatively, lowered S428 phosphorylation may indeed reflect LKB1 deactivation, and as reported for somatic cells, AICAR could stimulate AMPK in an AMPKK-independent manner by downregulating the phosphatase that normally dephosphorylates AMPK's T172 site (Sakamoto *et al*. 2004, Sanders *et al*. 2007).

In the case of ERK1/2 MAPK which can deactivate AMPK in somatic cells (Zheng *et al*. 2009), AMPK downregulation during nemertean GVBD occurs independently of such MAPK, given that MAPK inhibitions via CI1040 or PD98059 fail to prevent GVBD or AMPK deactivation. Alternatively, some oocyte batches treated with SW solutions of the MAPKK inhibitor U0126 do not undergo GVBD. Nevertheless, when co-stimulated with SW + cAMP elevators, nearly all U0126-treated oocytes mature in the absence of marked MAPK activity (Stricker 2009*b*), and as is the case with somatic cell extracts where U0126 has been shown to activate AMPK (Dokladda *et al*. 2005), U0126 reduces the amount of T172 dephosphorylation compared to controls in SW alone. Thus, U0126 does not seem to block GVBD by downregulating MAPK *per se* but rather by ectopically activating AMPK. Accordingly, although U0126-mediated AMPK activation is not universally reported (Kayampilly & Menon 2009), results presented here and elsewhere (LaRosa & Downs 2005) suggest that analyses relying exclusively on U0126 should be interpreted with caution.

Contrary to findings that T172 dephosphorylation proceeds independently of ERK1/2 MAPK, oocytes lacking such MAPK activity display reduced S485/491 phosphorylations on AMPK. These data suggest that MAPK is required for AMPK's S485/491 phosphorylations and also have implications for multiple pathways potentially regulating nemertean GVBD. For example, in previous tests of AMPK agonists (Stricker *et al.* 2010*a*), immature nemertean oocytes always exhibited high phospho-T172 and low phospho-S485/491 signals, whereas mature specimens invariably displayed the opposite pattern of these phosphorylations. Moreover, GVBD and S485/491 phosphorylation were significantly reduced in SW solutions of the protein kinase B (PKB) inhibitor API-2, whereas maturation and S485/491 phosphorylations were restored, when API-2 was dissolved in SW+cAMP elevators (Stricker *et al.* 2010*a*). Collectively, such findings indicated that S485/491 phosphorylation promoted AMPK deactivation and that such phosphorylation might be mediated by PKB in SW versus by cAMP-dependent protein kinase A (PKA) in the presence of cAMP elevators, based on the API-2 results and the fact that both PKB and PKA phosphorylate S485/491 in somatic cells (Horman *et al.* 2006, Hurley *et al.* 2006).

However, given the results obtained here with MAPKK inhibitors, S485/491 phosphorylation is evidently non-essential for T172 dephosphorylation. Nevertheless, S485/491 phosphorylation might downregulate AMPK during normal GVBD where MAPK is activated, whereas in the absence of S485/491 phosphorylation, a different mode of dephosphorylating T172 such as phosphorylation at AMPK's S173 site (Djouder *et al.* 2010) could compensate for such a loss. To test these hypotheses as well as to determine which signals downregulate AMPKK during AMPK deactivation requires additional analyses employing more precise means of altering and assessing AMPK phosphorylation status.

Potential downstream targets of AMPK deactivation during nemertean GVBD

Based on tests with SW solutions of the MPF inhibitor roscovitine, T172 dephosphorylation continues in SW-stimulated oocytes with low MPF levels, suggesting that T172 dephosphorylation occurs upstream to, and independently of, MPF activation. Conversely, roscovitine-mediated MPF deactivation causes a downward mobility shift and decrease in S485/491 phosphorylations that are not simply due to a lack of MAPK activity. Collectively, such findings suggest that, along with MAPK, MPF may facilitate full S485/491 phosphorylation via positive feedback between AMPK deactivation and MPF activation.

As for possible steps between AMPK deactivation and MPF activation, data presented here coincide with those reported for somatic cells (Bolster *et al.* 2002, Kimura *et al.* 2003) by indicating that active AMPK inhibits TOR. Accordingly, TOR upregulation as AMPK levels decline in maturing oocytes may trigger meiotic resumption via MPF activation in a manner analogous to mitosis induction by constitutively active TOR in cancerous cells (Wullschleger *et al.* 2006). However, exactly how TOR might activate MPF in nemertean oocytes remains unclear.

Alternatively, AMPK can inhibit TOR through TSC2 activation (Inoki *et al.* 2003, Coradetti *et al.* 2004, Abraham 2005), which in turn also stabilizes the CDK inhibitor Kip1 (Rosner *et al.* 2006). Accordingly, Kip1 levels appear to be elevated in immature oocytes and subsequently downregulated after AMPK deactivation in a manner resembling that described for rat follicle cells (Kayampilly & Menon 2009). Such findings suggest that, as an alternative to possible TOR-mediated signaling, MPF activation could be due to decreased TSC2 activity in the wake of AMPK deactivation that might not only activate TOR but also downregulate the MPF inhibitor Kip1. However, currently no commercially available antibody for monitoring TSC2 activity has been found to successfully cross-react with nemertean oocytes. Thus, testing

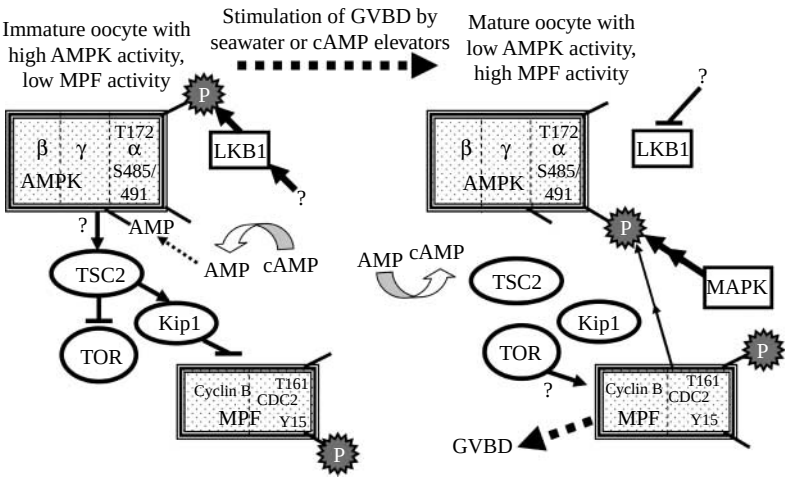

Figure 6 Summary of multiple pathways potentially mediating intraoocytic AMPK signaling during GVBD in a marine nemertean worm. AMPK, AMP-activated kinase composed of α, β, γ subunits; GVBD, germinal vesicle breakdown; Kip1, p27 Kip1 cyclin-dependent kinase; LKB1, AMPK kinase; MAPK, ERK1/2 mitogen-activated kinase; MPF, maturation-promoting factor composed of Cyclin B and CDC2 kinase; P, phosphorylation; TOR, target of rapamycin; TSC2, tuberous sclerosis complex 2.

these hypotheses awaits genomic sequencing as well as more specific means to manipulate and track the activities of such potential mediators of AMPK signaling.

Nevertheless, regardless of how AMPK regulates MPF activity, the question remains as to why AMPK blocks GVBD in nemerteans and cumulus-enclosed oocytes of some mammals, whereas AMPK stimulates GVBD in mice. One possible explanation involves the fact that AMPKs are heterotrimers that comprise varying combinations of α, β, and γ subunits (Hardie 2007). Accordingly, disparate effects of AMPK on GVBD could be due to alternative types of AMPKs, given that in somatic cells AMPKs can exhibit isotype-specific responses to modulating stimuli (Salt *et al*. 1998, Higaki *et al*. 2001, Zierath 2002). In any case, data reported here and summarized in Fig. 6 not only provide added support for the view that multiple pathways can mediate AMPK activity during stimulation of nemertean oocytes by SW versus cAMP elevators (Stricker *et al*. 2010*a*, 2010*b*) but also generate new findings as well as hypotheses to test regarding intraoocytic AMPK signaling in general.

Materials and Methods

Fully-grown immature oocytes lacking follicle cells but containing an intact germinal vesicle (Stricker & Schatten 1989) were obtained from gravid specimens of an undescribed species of the nemertean genus *Cerebratulus* that were collected during summers in an intertidal region on San Juan Island, WA, USA (Stricker 1987, Stricker *et al*. 2010*a*, 2010*b*, 2010*c*). Isolated oocytes were pre-incubated ∼2 h in ice-cold calcium-free seawater (CaFSW) to reduce spontaneous GVBD and subsequently dejellied via a 150-μm Nitex filter before being gently pelleted. Using such methods, spontaneous GVBD levels were typically 0–10% before the start of experiments, based on inverted microscopy of uncompressed living oocytes. Moreover, at 12–14 °C, GVBD and metaphase-I arrest began by ∼20 and ∼90 min respectively, in dejellied controls that had been stimulated to mature by SW or cAMP elevators (10 μM forskolin (LC Labs, Woburn, MA, USA) or 150 μM IBMX (EMD Chemicals, Gibbstown, NJ, USA) dissolved in SW or CaFSW). For most runs, a drop of ∼200 dejellied oocytes suspended in CaFSW was either directly pelleted and frozen in liquid nitrogen (LN) after fluid removal (the 'Before' sample) or added to 1 ml of test solution kept at 12–14 °C for 90–120 min. The incubated samples were then assessed for GVBD, pelleted, and frozen in LN. Alternatively, for time-lapse sequences, 3–5 drops of oocytes were added to 4 ml test solution, and pelleted aliquots were frozen in LN several times during the run.

For manipulations of AMPK signaling, drugs were routinely dissolved in solutions of SW or SW + cAMP elevators, and to reduce the likelihood of off-target drug effects (Davies *et al*. 2000), the following doses intermediate between half- and maximally effective concentrations were adopted: 300 μM AICAR, 20 μM resveratrol (Enzo Life Sciences, Plymouth Meeting, PA, USA); 30 μM roscovitine, 25 μM U0126 (LC Labs); 250 μM olomoucine, 50 μM PD98059 (Tocris Biosciences, Ellisville, MO, USA); and 25 μM CI1040 (a gift from Pfizer, Inc., Groton, CT, USA). In all experimental incubations, oocytes were continuously treated with a drug solution that had been pre-mixed ∼0.5–1 h before the onset of the run and equilibrated to 12–14 °C prior to addition of oocytes.

For immunoblotting analyses, LN-frozen samples were lysed in a protease- and phosphatase-containing buffer (Carroll *et al*. 2000), and based on Bradford assays of total-cell lysates, 25 μg cellular protein/lane was loaded on 4%/10% SDS-PAGE gels run at 100 kV for 2–2.5 h. Following transfer to PVDF membranes for 75 min at 100 mV and subsequent blocking for 1 h in 5% dry milk in Tween 20-containing Tris-buffered saline (TTBS), blots were incubated overnight at 4 °C in TTBS containing 1–5% BSA plus primary antibody that was obtained from suppliers and typically diluted as follows: from Cell Signaling Technology (Danvers, MA, USA) – 1:1000 phospho-T172 AMPK #2535; 1:2500 phospho-S485/491 AMPK #4185; 1:2000 phospho-Y15 CDC2 #9111; 1:1500 phospho-T161 CDC2 #9114; 1:2000 phospho-S428 LKB1 #3482; 1:1500 phospho-S2448 mTOR #2971; 1:2000 phospho-T398 *Drosophila* p70/85 S6K #9209; 1:2500 phospho-T202/T204 ERK1/2 MAPK#9101; from Genscript (Piscataway, NJ, USA) – 1:1000 pan p27 Kip1 #A00436, 1:2000 phospho-T187 p27 Kip1#A00333; or from Millipore (Billerca, MA, USA) – 1:4000 phospho-S79 ACC #07-303. Thereafter, blots were treated 1.5 h with 1:5000 HRP-conjugated secondary antibody (#sc-2054, Santa Cruz Biotechnology, Santa Cruz, CA, USA) dissolved in milk-containing TTBS, and bands were visualized by ECL before being recorded on X-ray film using exposures typically 0.5–5 min long.

To assay for bands at expected MWs and ectopic sites, whole PVDF membranes were initially incubated with a single primary antibody. However, after confirming the absence of confounding signals for the following three antibody pairs, subsequent blots were often dually probed with phospho-T172 + phospho-S485/491 AMPK, phospho-T172 AMPK + phospho-LKB1, or phospho-MAPK + a phospho-CDC2 antibody (Y15/T161). Alternatively, with MW markers on both sides of the membrane for guidance, each membrane was cut into 3 or 4 horizontal strips that were individually treated with either a single antibody or one of the three above-listed pairs, thereby allowing each composite figure presented here (i.e. stacked set of bands separated by a white line) to originate from a single gel transfer. Moreover, since each collection of strips was probed with antibodies whose signals have been shown to decrease (e.g. pT172 AMPK, pY15 MPF) versus increase (e.g. pS485/491 AMPK, pT161 MPF, pMAPK) during GVBD (Stricker *et al*. 2006*a*, 2010*a*), such parallel incubations with oppositely-trending antibodies helped to verify even gel loadings, since the occasional cases of markedly over- or under-loaded lanes tended to display inappropriately higher or lower signals across all strips and were thus excluded from analysis. In addition, to minimize female-specific variation and blot-to-blot differences in handling, densitometry of background-subtracted bands was quantified for at least three blots from two or more females, and band intensities were expressed relative to pre- and post-stimulation control levels on the same blot. For statistical significance, densitometric data were assessed by a Mann–Whitney *U* test (Smythe & Stricker 2005).

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Abraham RT 2005 TOR signaling: an odyssey from cellular stress to the cell-growth machinery. *Current Biology* **15** R139–R141. (doi:10.1016/j.cub.2005.02.015)

Alessi DR, Sakamoto K & Bayascas JR 2006 LKB1-dependent signaling pathways. *Annual Review of Biochemistry* **75** 137–163. (doi:10.1146/annurev.biochem.75.103004.142702)

Bilodeau-Goeseels S, Sasseville M, Guillemette C & Richard FJ 2007 Effects of adenosine monophosphate-activated kinase activators on bovine oocyte nuclear maturation *in vitro*. *Molecular Reproduction and Development* **74** 1021–1034. (doi:10.1002/mrd.20574)

Bilodeau-Goeseels S, Panich PL & Kastelic JP 2010 Activation of AMP-activated protein kinase may not be involved in AICAR- and metformin-mediated meiotic arrest in bovine denuded and cumulus-enclosed oocytes *in vitro*. *Zygote* **19** 97–106. (doi:10.1017/S096719940000195)

Bolster DR, Crozier SJ, Kimball SR & Jefferson LS 2002 AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *Journal of Biological Chemistry* **277** 23977–23980. (doi:10.1074/jbc.C200171200)

Carling D 2005 AMP-activated protein kinase: balancing the scales. *Biochimie* **87** 87–91. (doi:10.1016/j.biochi.2004.10.017)

Carroll DJ, Albay DT, Hoang KM, O'Neill FJ, Kumano M & Foltz KR 2000 The relationship between calcium, MAP kinase, and DNA synthesis in the sea urchin egg at fertilization. *Developmental Biology* **217** 179–191. (doi:10.1006/dbio.1999.9526)

Chen J & Downs SM 2008 AMP-activated protein kinase is involved in hormone-induced mouse oocyte meiotic maturation *in vitro*. *Developmental Biology* **313** 47–57. (doi:10.1016/j.ydbio.2007.09.043)

Chen J, Hudson E, Chi MM, Chang AS, Moley KH, Hardie DG & Downs SM 2006 AMPK regulation of mouse oocyte meiotic resumption *in vitro*. *Developmental Biology* **291** 227–238. (doi:10.1016/j.ydbio.2005.11.039)

Coradetti MN, Inoki K, Bardeesy N, DePinho RA & Guan K-L 2004 Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz–Jeghers syndrome. *Genes and Development* **18** 1533–1538. (doi:10.1101/gad.1199104)

Davies SP, Reddy H, Caivano M & Cohen P 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **351** 95–105. (doi:10.1042/0264-6021:3510095)

Djouder N, Tuerk RD, Suter M, Salvioni P, Thali RF, Scholz R, Vaahtomeri K, Auchli Y, Rechtsteiner H, Brunisholz RA *et al.* 2010 PKA phosphorylates and inactivates AMPKα to promote efficient lipolysis. *EMBO Journal* **29** 469–481. (doi:10.1038/emboj.2009.339)

Dokladda K, Green KA, Pan DA & Hardie DG 2005 PD98059 and U0126 activate AMP-activated protein kinase by increasing cellular AMP:ATP ratio and not via inhibition of the MAP kinase pathway. *FEBS Letters* **579** 236–240. (doi:10.1016/j.febslet.2004.11.084)

Downs SM, Hudson ER & Hardie DG 2002 A potential role for AMP-activated protein kinase in meiotic induction in mouse oocytes. *Developmental Biology* **245** 200–212. (doi:10.1006/dbio.2002.0613)

Downs SM, Mosey JL & Klinger J 2009 Fatty acid oxidation and meiotic resumption in mouse oocytes. *Molecular Reproduction and Development* **76** 844–853. (doi:10.1002/mrd.21047)

Dupont J, Chabrolle C, Rame C, Tosca L & Coyral-Castel S 2008 Role of peroxisome proliferator-activated receptors, adenosine monophosphate-activated kinase, and adiponectin in the ovary. *PPAR Research* **2008** 176275. (doi:10.1155/2008/176275)

Fogarty S & Hardie DG 2009 C-terminal phosphorylation of LKB1 is not required for regulation of AMP-activated protein kinase, BRSK1, BRSK2, or cell cycle arrest. *Journal of Biological Chemistry* **284** 77–84. (doi:10.1074/jbc.M806152200)

Font de Mora J, Uren A, Heidara M & Santos E 1997 Biological activity of $p27^{Kip1}$ and its amino- and carboxy-terminal domains in G2/M transition in *Xenopus* oocytes. *Oncogene* **15** 2541–2551. (doi:10.1038/sj.onc.1201420)

Funahashi K, Cao X, Yamauchi M, Kozaki Y, Ishiguro N & Kambe F 2009 Prostaglandin E_2 negatively regulates AMP-activated protein kinase via protein kinase A signaling pathway. *Prostaglandins & Other Lipid Mediators* **88** 31–35. (doi:10.1016/j.prostaglandins.2008.09.002)

Hardie DG 2007 AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature Reviews. Molecular Cell Biology* **8** 774–785. (doi:10.1038/nrm2249)

Higaki Y, Hirshman MR, Fujii N & Goodyear LJ 2001 Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle. *Diabetes* **50** 241–247. (doi:10.2337/diabetes.50.2.241)

Holz MK & Belnis J 2005 Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. *Journal of Biological Chemistry* **280** 26089–26093. (doi:10.1074/jbc.M504045200)

Horman S, Vertommen D, Heath R, Neumann D, Mouton V, Woods A, Schlattner U, Wallimann T, Carling D, Hue L *et al.* 2006 Insulin antagonizes ischemia-induced Thr^{172} phosphorylation of AMP-activated protein kinase α-subunits in heart via hierarchical phosphorylation of $Ser^{485/491}$. *Journal of Biological Chemistry* **281** 5335–5340. (doi:10.1074/jbc.M506850200)

Hurley RL, Barre LK, Wood SD, Anderson KA, Kemp BE, Means AR & Witters LA 2006 Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. *Journal of Biological Chemistry* **281** 36662–36672. (doi:10.1074/jbc.M606676200)

Inoki K, Zhu T & Guan K-L 2003 TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115** 577–590. (doi:10.1016/S0092-8674(03)00929-2)

Kayampilly PP & Menon KMJ 2009 Follicle-stimulating hormone inhibits adenosine 5′-monosphosphate-activated protein kinase activation and promotes cell proliferation of primary granulosa cells in culture through an Akt-dependent pathway. *Endocrinology* **150** 929–935. (doi:10.1210/en.2008-1032)

Kimura N, Tokunaga C, Dala S, Richardson C, Yoshin K-I, Hara K, Kemp BE, Witters LA, Mimura O & Yonezawa K 2003 A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes to Cells* **9** 65–79. (doi:10.1046/j.1365-2443.2003.00615.x)

Kishimoto T 2003 Cell cycle control during meiotic maturation. *Current Opinion in Cell Biology* **15** 654–663. (doi:10.1016/j.ceb.2003.10.010)

Kraiss LW, Weyrich AS, Alto NM, Dixon DA, Ennis TM, Modur V, McIntyre TM, Prescott SM & Zimmerman GA 2000 Fluid flow activates a regulator of translation, p70/p85 S6 kinase, in human endothelial cells. *American Journal of Physiology. Heart and Circulatory Physiology* **278** H1537–H1544.

LaRosa C & Downs SM 2005 MEK inhibitors block AICAR-induced maturation in mouse oocytes by a MAPK-independent mechanism. *Molecular Reproduction and Development* **70** 235–245. (doi:10.1002/mrd.20200)

LaRosa C & Downs SM 2006 Stress stimulates AMP-activated protein kinase and meiotic resumption in mouse oocytes. *Biology of Reproduction* **74** 585–592. (doi:10.1095/biolreprod.105.046524)

LaRosa C & Downs SM 2007 Meiotic induction by heat stress in mouse oocytes: involvement of AMP-activated protein kinase and MAPK family members. *Biology of Reproduction* **76** 476–486. (doi:10.1095/biolreprod.106.057422)

Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R, Bier E *et al.* 2010 Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* **327** 1223–1228. (doi:10.1126/science.1182228)

Liangpunsakul S, Wou S-E, Zeng Y, Ross RA, Jayaram HN & Crabb DW 2008 Effect of ethanol on hydrogen peroxide-induced AMPK phosphorylation. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **295** G1173–G1181. (doi:10.1152/ajpgi.90349.2008)

Mayes MA, Laforest MF, Guillemette C, Gilchrist RB & Richard FJ 2007 Adenosine 5′-monophosphate kinase-activated protein kinase (PRKA) activators delay meiotic resumption in porcine oocytes. *Biology of Reproduction* **76** 589–597. (doi:10.1095/biolreprod.106.057828)

Mehlmann LM 2005 Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction* **130** 791–799. (doi:10.1530/rep.1.00793)

Motoshima H, Goldstein BJ, Igata M & Araki E 2006 AMPK and cell proliferation – AMPK as a therapeutic target for atherosclerosis and cancer. *Journal of Physiology* **564** 63–71. (doi:10.1113/jphysiol.2006.108324)

Nakayama K, Nagahama H, Minamishima YA, Miyake S, Ishida N, Hatakeyama S, Kitagawa M, Iemura S-I, Natsume T & Nakayama KI 2004 Skp2-mediated degradation of p27 regulates prgression into mitosis. *Developmental Cell* **6** 661–672. (doi:10.1016/S1534-5807(04)00131-5)

Pellettieri J & Seydoux G 2002 Anterior–posterior polarity in *C. elegans* and *Drosophila* – PARallels and differences. *Science* **298** 1946–1950. (doi:10.1126/science.1072162)

Petritisch C, Wosholski R, Edlmann HML, Parker PJ & Ballou LM 1995 Selective inhibition of p70 S6 kinase activation by phosphatidylinositol 3-kinase inhibitors. *European Journal of Biochemistry* **230** 431–438. (doi:10.1111/j.1432-1033.1995.0431h.x)

Ratchford AM, Chang AS, Chi MM, Sheridan R & Moley KH 2007 Maternal diabetes adversely affects AMP-activated protein kinase activity and cellular metabolism in murine oocytes. *American Journal of Physiology. Endocrinology and Metabolism* **293** E1198–E1206. (doi:10.1152/ajpendo.00097.2007)

Richard FJ 2007 Regulation of meoitic maturation. *Journal of Animal Science* **85** E4–E6. (doi:10.2527/jas.2006-475)

Richardson MC, Ingamells S, Simonis CD, Cameron IT, Sreekumar R, Vijendren A, Sellahewa L, Coakley S & Byrne CD 2009 Stimulation of lactate production in human granulosa cells by metformin and potential involvement of adenosine 5′ monophosphate activated protein kinase. *Journal of Clinical Endocrinology and Metabolism* **94** 670–677. (doi:10.1210/jc.2008-2025)

Rosner M, Freilinger A & Hengstchlaeger M 2006 The tuberous sclerosis genes and regulation of the cyclin-dependent kinase inhibitor p27. *Mutation Research* **613** 10–16. (doi:10.1016/j.mrrev.2006.03.001)

Rosner M, Siegel N, Valli A, Fuchs C & Hengstschlaeger M 2010 mTOR phosphorylated at S2448 binds to raptor and rictor. *Amino Acids* **38** 223–228. (doi:10.1007/s00726-008-0230-7)

Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F & Ido Y 2010 AMPK and SIRT1: a long-standing partnership? *American Journal of Physiology. Endocrinology and Metabolism* **298** E751–E760. (doi:10.1152/ajpendo.00745.2009)

Sakamoto K, Goransson O, Hardie DG & Alessi DR 2004 Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *American Journal of Physiology. Endocrinology and Metabolism* **287** E310–E317. (doi:10.1152/ajpendo.00074.2004)

Salt I, Cellar JW, Hawley SA, Prescott A, Woods A & Carling D 1998 AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the α2 isoform. *Biochemical Journal* **334** 177–187.

Sanders MJ, Grondin PO, Hegarty BD, Snowden MA & Carling D 2007 Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochemical Journal* **403** 139–148. (doi:10.1042/BJ20061520)

Shackelford DB & Shaw RJ 2009 The LKB1–AMPK pathway: metabolism and growth control in tumour suppression. *Nature Reviews. Cancer* **9** 563–575. (doi:10.1038/nrc2676)

Sherr CJ & Roberts JM 1999 CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes and Development* **13** 1501–1512. (doi:10.1101/gad.13.12.1501)

Smythe TL & Stricker SA 2005 Germinal vesicle breakdown is not fully dependent on MAPK activation in maturing oocytes of marine nemertean worms. *Molecular Reproduction and Development* **70** 91–102. (doi:10.1002/mrd.20188)

Stricker SA 1987 Phylum Nemertea. In *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast,* pp 129–137. Ed. M Strathmann. Seattle: University of Washington Press.

Stricker SA 2009*a* Roles of protein kinase C isotypes during seawater- versus cAMP-induced oocyte maturation in a marine worm. *Molecular Reproduction and Development* **76** 693–707. (doi:10.1002/mrd.20993)

Stricker SA 2009*b* Interactions between mitogen-activated protein kinase and protein kinase C signaling during oocyte maturation and fertilization in a marine worm. *Molecular Reproduction and Development* **76** 708–721. (doi:10.1002/mrd.21032)

Stricker SA & Schatten G 1989 Nuclear disassembly and nuclear lamina depolymerization during germinal vesicle breakdown in starfish. *Developmental Biology* **135** 87–98. (doi:10.1016/0012-1606(89)90160-7)

Stricker SA & Smythe TL 2000 Multiple triggers of oocyte maturation in nemertean worms: the roles of calcium and serotonin. *Journal of Experimental Zoology* **287** 243–261. (doi:10.1002/1097-010X(20000801)287:3<243::AID-JEZ6>3.0.CO;2-B)

Stricker SA & Smythe TL 2001 5-HT causes an increase in cAMP that stimulates, rather than inhibits, oocyte maturation in marine nemertean worms. *Development* **128** 1415–1427.

Stricker SA & Smythe TL 2003 Endoplasmic reticulum reorganizations and Ca^{2+} signaling in maturing and fertilized oocytes of marine protostome worms: the roles of MAPKs and MPF. *Development* **130** 2867–2879. (doi:10.1242/dev.00508)

Stricker SA & Smythe TL 2006*a* Differing mechanisms of cAMP- versus seawater-induced oocyte maturation in marine nemertean worms I. The roles of serine/threonine kinases and phosphatases. *Molecular Reproduction and Development* **73** 1578–1590. (doi:10.1002/mrd.20597)

Stricker SA & Smythe TL 2006*b* Differing mechanisms of cAMP- versus seawater-induced oocyte maturation in marine nemertean worms II. The roles of tyrosine kinases and phosphatases. *Molecular Reproduction and Development* **73** 1564–1577. (doi:10.1002/mrd.20596)

Stricker SA, Smythe TL, Miller L & Norenburg JL 2001 Comparative biology of oogenesis in nemertean worms. *Acta Zoologica* **82** 213–230. (doi:10.1046/j.1463-6395.2001.00080.x)

Stricker SA, Swiderek L & Nguyen T 2010*a* Stimulators of AMP-activated kinase inhibit seawater- but not cAMP-induced oocyte maturation in a marine worm: implications for interactions between cAMP and AMPK signaling. *Molecular Reproduction and Development* **77** 497–510. (doi:10.1002/mrd.21177)

Stricker SA, Escalona JR, Abernathy S & Marquardt A 2010*b* Pharmacological analyses of protein kinases regulating egg maturation in marine nemertean worms: a review and comparison with mammalian eggs. *Marine Drugs* **8** 2417–2434. (doi:10.3390/md8082417)

Stricker SA, Carroll DJ & Tsui WL 2010*c* Roles of Src family kinase signaling during fertilization and the first cell cycle in the marine protostome worm Cerebratulus. *International Journal of Developmental Biology* **54** 787–793. (doi:10.1387/ijdb.092918ss)

Tosca L, Froment P, Solnais P, Ferre P, Foufelle F & Dupont J 2005 Adenosine 5′-monophosphate-activated protein kinase regulates progesterone secretion in rat granulosa cells. *Endocrinology* **146** 4500–4513. (doi:10.1210/en.2005-0301)

Tosca L, Solnais P, Ferre P, Foufelle F & Dupont J 2006 Metformin-induced stimulation of adenosine 5′ monophosphate-activated protein kinase (PRKA) impairs progesterone secretion in rat granulosa cells. *Biology of Reproduction* **75** 342–351. (doi:10.1095/biolreprod.106.050831)

Tosca L, Uzbekova S, Chabrolle C & Dupont J 2007 Possible role of 5′ AMP-activated protein kinase in the metformin-mediated arrest of bovine oocytes at the germinal vesicle stage during *in vitro* maturation. *Biology of Reproduction* **77** 452–465. (doi:10.1095/biolreprod.107.060848)

Tosca L, Rame C, Chabrolle C, Tesseraud S & Dupont J 2010 Metformin decreases IGF1-induced cell proliferation and protein synthesis through AMP-activated protein kinase in cultured bovine granulosa cells. *Reproduction* **139** 409–418. (doi:10.1530/REP-09-0351)

Vlach J, Hennecke S & Amati B 1997 Phosphorylation-dependent degradation of the cyclin-dependent kinase p27. *EMBO Journal* **16** 5334–5344. (doi:10.1093/emboj/16.17.5334)

Voronina E & Wessel GM 2003 The regulation of oocyte maturation. *Current Topics in Developmental Biology* **58** 53–110.

Williams T & Brenman JE 2008 LKB1 and AMPK in cell polarity and division. *Trends in Cell Biology* **18** 193–198. (doi:10.1016/j.tcb.2008.01.008)

Woods A, Dickerson K, Heath R, Hong S-Y, Momcilovic M, Johnstone SR, Carlson M & Carling D 2005 Ca^{2+}/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metabolism* **2** 21–33. (doi:10.1016/j.cmet.2005.06.005)

Wullschleger S, Loewith R & Hall MN 2006 TOR signaling in growth and metabolism. *Cell* **124** 471–484. (doi:10.1016/j.cell.2006.01.016)

Xie M, Zhang D, Dyck JRB, Li Y, Zhang H, Morishima M, Mann DL, Taffet GE, Baldini A, Khoury DS *et al.* 2006 A pivotal role for endogenous TGF-β-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *PNAS* **103** 17378–17383. (doi:10.1073/pnas.0604708103)

Xie Z, Dong Y, Scholz R, Neumann D & Zou M-H 2008 Phosphorylation of LKB1 at Serine 428 by protein kinase C-ζ is required for metformin-enhanced activation of AMP-activated protein kinase in endothelial cells. *Circulation* **117** 952–962. (doi:10.1161/CIRCULATIONAHA.107.744490)

Zheng B, Jeong JH, Asara JM, Yuan Y-Y, Granter SR, Chin L & Cantley LC 2009 Oncogenic B-RAF negatively regulates the tumor suppressor LKB1 to promote melanoma cell proliferation. *Molecular Cell* **33** 237–247. (doi:10.1016/j.molcel.2008.12.026)

Zierath JR 2002 Exercise training-induced changes in insulin signaling in skeletal muscle. *Journal of Applied Physiology* **93** 773–781.

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