SGK phosphorylates Cdc25 and Myt1 to trigger cyclin B-Cdk1 activation at the meiotic G2/M transition

Daisaku Hiraoka[1*], Enako Hosoda[2], Kazuyoshi Chiba[2], and Takeo Kishimoto[1*]

[1]Science & Education Center, Ochanomizu University, Tokyo 112-8610, Japan.

[2]Department of Biological Sciences, Ochanomizu University, Tokyo 112-8610, Japan.

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*Correspondence to:

Daisaku Hiraoka: E-mail: hiraoka.daisaku@ocha.ac.jp, Tel: +81-3-5978-5370

Takeo Kishimoto: E-mail: kishimoto.takeo@ocha.ac.jp, Tel: +81-3-5978-5370

Abstract

The kinase cyclin B-Cdk1 complex is a master regulator of M-phase in both mitosis and meiosis. At the G2/M transition, cyclin B-Cdk1 activation is initiated by a trigger that reverses the balance of activities between Cdc25 and Wee1/Myt1, and is further accelerated by autoregulatory loops. In somatic cell mitosis, this trigger was recently proposed to be the cyclin A-Cdk1/Plk1 axis. However, in the oocyte meiotic G2/M transition, in which hormonal stimuli induce cyclin B-Cdk1 activation, cyclin A-Cdk1 is non-essential and hence the trigger remains elusive. Here, we show that SGK directly phosphorylates Cdc25 and Myt1 to trigger cyclin B-Cdk1 activation in starfish oocytes. After hormonal stimulation of the meiotic G2/M transition, SGK is activated by cooperation between the Gβγ-PI3K pathway and an unidentified pathway downstream of Gβγ, called the atypical Gβγ pathway. These findings identify the trigger in oocyte meiosis and provide insights into the role and activation of SGK.

Introduction

Activation of cyclin-dependent kinase 1 complexed with cyclin B (cyclin B-Cdk1) induces entry into M-phase during somatic cell mitosis and germ cell meiosis[1-3]. Cyclin B-Cdk1 is regulated by synthesis and degradation of cyclin B, and by inhibitory phosphorylation of Cdk1 at Thr14 and Tyr15. These residues are phosphorylated by kinases belonging to the Wee1/membrane-associated tyrosine/threonine 1 (Myt1) family, but dephosphorylated by cell division cycle 25 (Cdc25)[4]. Cyclin B accumulates before and/or during M-phase entry. However, Wee1/Myt1 activity is dominant over Cdc25 activity before M-phase; therefore, cyclin B-Cdk1 remains inactive due to inhibitory phosphorylation. At M-phase entry, a small population of cyclin B-Cdk1 is first activated by a trigger that reverses the balance between Cdc25 and Wee1/Myt1 activities, thereby making Cdc25 activity predominant. Thereafter, cyclin B-Cdk1 itself further accelerates dephosphorylation of Cdk1 via feedback loops, leading to maximal activation[4-8]. Although the molecular identity of the trigger of cyclin B-Cdk1 activation has received great attention, it remains elusive.

In mitotic M-phase entry (G2/M transition), the trigger may be affected by various factors, such as checkpoints[5,9,10], and may involve redundant or stochastic processes[5,9]. At least in normal mitotic cell cycles, polo-like kinase 1 (Plk1) is activated by Aurora A in a cyclin A-Cdk1-dependent manner, and in turn phosphorylates Cdc25 to trigger activation of cyclin B-Cdk1[11-13]. Consistently, the cyclin A-Cdk1/Plk1 axis functions as the trigger for cyclin B-Cdk1 activation in the first embryonic cell division cycle[14].

In contrast with mitotic cell cycles in proliferating somatic cells, meiotic cell cycles in animal oocytes mostly arrest at prophase of meiosis I (prophase-I), which corresponds to late mitotic G2 phase[15]. Release from this arrest is also induced by cyclin

58 B-Cdk1, which is activated downstream of extracellular hormonal stimuli[16]; hence this
59 is known as the meiotic G2/M transition. However, this transition has not been reported
60 to require cyclin A[16-21]. Furthermore, Plk1[14,22,23] and Aurora[24-26] are non-essential in
61 some oocytes. Thus, mechanisms other than the cyclin A-Cdk1/Plk1 axis likely trigger
62 cyclin B-Cdk1 activation at the meiotic G2/M transition in oocytes.

63 In vertebrate oocytes, meiotic G2 arrest requires cAMP-dependent protein kinase
64 A (PKA), and downregulation of this kinase leads to meiotic G2/M transition[16,20,27,28].
65 In mice, PKA appears to directly upregulate Wee1B and downregulate Cdc25; however,
66 it remains unclear how downregulation of PKA reverses the balance between Wee1B
67 and Cdc25 activities to trigger cyclin B-Cdk1 activation[29]. In *Xenopus*, the hormone
68 progesterone stimulates protein synthesis of Moloney sarcoma oncogene (Mos) and
69 cyclin B, which redundantly trigger cyclin B-Cdk1 activation[30]. This redundant process
70 is possibly mediated in two manners: activating phosphorylation of Cdc25 by
71 Mos/mitogen activated protein kinase (MAPK) cascade, and inhibitory phosphorylation
72 of Myt1 by newly synthesized cyclin B-associated Cdk1[23]. However, the molecular link
73 between downregulation of PKA and *de novo* protein synthesis of Mos and cyclin B
74 remains elusive[31,32].

75 In contrast with vertebrate oocytes, the mechanisms by which activation of cyclin
76 B-Cdk1 is triggered at the meiotic G2/M transition have been well studied in
77 invertebrate starfish oocytes since the existence of a trigger kinase was first
78 reported[16,21,33]. In starfish, cAMP and PKA are likely not involved in meiotic G2
79 arrest[34]. The physiological maturation-inducing hormone 1-methyladenine (1-MeAde)
80 induces the meiotic G2/M transition[35] without a requirement for new protein synthesis[36].
81 Cyclin A, Wee1, and Mos are not present, and Aurora and Plk are not required for

82 cyclin B-Cdk1 activation, in the meiotic G2/M transition[14,19,25,37]. In unstimulated
83 oocytes, cyclin B is already accumulated[38], but Myt1 inactivates cyclin B-Cdk1[39].
84 Stimulation by 1-MeAde is mediated by an unidentified cell surface G-protein-coupled
85 receptor (GPCR), which induces dissociation of Gβγ from Gαi on the plasma
86 membrane[40-43]. Gβγ binds to and activates phosphoinositide-3-kinase (PI3K), resulting
87 in production of phosphatidylinositol 3,4,5-triphosphate (PI-345P_3)[44,45]. Dependent on
88 PI-345P_3, phosphoinositide-dependent kinase 1 (PDK1) and target of rapamycin
89 complex 2 (TORC2) phosphorylate Akt in its activation loop (A-loop) and hydrophobic
90 motif (HM), respectively, leading to its activation[46,47]. Thereafter, Akt inactivates Myt1
91 by directly phosphorylating Ser75 (ref. 39) and activates Cdc25 by directly
92 phosphorylating multiple residues including Ser188 (ref. 48), resulting in reversal of the
93 balance of Cdc25 and Myt1 activities, and consequently initial activation of cyclin
94 B-Cdk1 (refs 33,39). These observations suggest that Akt likely represents the trigger
95 kinase at the meiotic G2/M transition in starfish oocytes[39].
96 Nevertheless, our recent observations suggested that the PI3K-Akt pathway is
97 insufficient[48]. Expression of a constitutively active form of Akt (CA-Akt) induces the
98 meiotic G2/M transition, but the required expression level of CA-Akt is 40-fold higher
99 than that of endogenous Akt. Furthermore, expression of a constitutively active form of
100 PI3K (CA-PI3K) induces Akt activation, but results in low levels of Cdc25 and Myt1
101 phosphorylation and therefore fails to induce the meiotic G2/M transition. We reported
102 that another pathway, called "the atypical pathway", which is activated by Gβγ in
103 parallel with PI3K activation, compensates for this insufficiency[48]. Although the
104 molecular details of this pathway remain unclear, we proposed that it cooperates with
105 the PI3K-Akt pathway to trigger cyclin B-Cdk1 activation[48]. One possibility is that

106 another kinase, which can redundantly phosphorylate Akt substrates, functions in these
107 pathways to trigger cyclin B-Cdk1 activation.

108 One such candidate kinase is serum- and glucocorticoid-regulated kinase
109 (SGK)[49,50]. SGK is a member of the AGC kinase family, which includes Akt[51], and has
110 the same substrate consensus motif (RXRXXS/T, where X represents any amino acid)
111 as Akt[52]. In mammalian cells, similar to Akt, SGK family members are activated in a
112 PI3K-dependent manner, dependent on phosphorylation of the A-loop and HM by
113 PDK1 and TORC2, respectively[52,53]. However, the contribution of SGK to the meiotic
114 G2/M transition has not been investigated.

115 Here, we carefully evaluated whether Akt and SGK play redundant roles in the
116 meiotic G2/M transition in starfish oocytes. We showed that SGK is necessary and
117 sufficient for the initial phosphorylation of Cdc25 and Myt1 to trigger cyclin B-Cdk1
118 activation upon 1-MeAde stimulation. By contrast, although contradictory to our
119 previous report[39], inhibition of Akt did not affect the meiotic G2/M transition. Several
120 experiments supported the importance of SGK, rather than of Akt. This discrepancy is
121 discussed. Furthermore, as upstream pathways, we found that PI3K and the atypical
122 Gβγ pathway cooperatively activate SGK. All these observations clearly explain the
123 insufficiency of the PI3K-Akt pathway. Thus, our findings indicate that SGK is the
124 trigger kinase at the meiotic G2/M transition in starfish oocytes, and provide new
125 insights into SGK activation.

126 **Results**

127 **A newly generated phospho-specific antibody detects A-loop phosphorylation of**
128 **endogenous starfish Akt (sfAkt)**

129 In previous studies[47,48], we monitored phosphorylation of the HM by immunoblotting as
130 a marker of starfish Akt (sfAkt) activity because attempts to generate an antibody that
131 detects the phosphorylated A-loop were unsuccessful. Nevertheless, it is important to
132 analyze A-loop phosphorylation to precisely evaluate Akt activation. Thus, we again
133 attempted to generate a phospho-specific antibody against a 17-amino acid
134 phospho-peptide derived from the A-loop of sfAkt that includes phospho-Thr at the site
135 of phosphorylation by PDK1 (Fig. 1a). In immunoblot analysis of whole-oocyte
136 samples using this antibody, phosphorylation of sfAkt was detectable following
137 1-MeAde stimulation only when Akt was overexpressed (Fig. 1b, closed arrowhead).
138 When endogenous Akt was concentrated by immunoprecipitation using an antibody
139 raised against a C-terminal fragment of sfAkt (anti-sfAkt-C antibody), the
140 anti-phospho-A-loop antibody detected a band at the same size as Akt after 1-MeAde
141 stimulation (Fig. 1c for input and flow-through, d for precipitate, closed arrowhead).
142 The band disappeared upon treatment with the PDK1 inhibitor BX795 (Fig. 1d, closed
143 arrowhead), indicating that the anti-phospho-A-loop antibody successfully detected
144 A-loop phosphorylation of endogenous Akt by PDK1.

145

146 **SGK is activated after 1-MeAde stimulation in a PDK1- and TORC2-dependent**
147 **manner**

148 The phospho-A-loop antibody detected another band that migrated slightly slower (~60
149 kDa) than Akt (Fig. 1b, c, open arrowhead). This band was still observed in

150 flow-through samples in which Akt had been removed by immunoprecipitation (Fig. 1c,
151 open arrowhead) and disappeared in the presence of BX795 (Fig. 1c, open arrowhead).
152 Thus, we speculated that the band corresponds to PDK1-dependent A-loop
153 phosphorylation of a kinase other than Akt.

154 In parallel with our research, Hosoda et al. found that starfish SGK (sfSGK) is
155 activated after 1-MeAde stimulation in starfish oocytes in a study of intracellular pH
156 regulation[54]. They isolated a cDNA encoding sfSGK and collaborated with us to
157 generate an anti-sfSGK neutralizing antibody raised against a C-terminal peptide of
158 sfSGK including the HM (anti-sfSGK-C antibody). A predicted molecular weight of
159 sfSGK is 56 kDa, and eleven of the seventeen residues in the antigen peptide used to
160 raise the anti-phospho-A-loop antibody against sfAkt are conserved in the A-loop of
161 SGK (Fig. 1a). Thus, the other band detected by the anti-phospho-A-loop antibody may
162 correspond to A-loop phosphorylation of SGK. The mobility of this band was the same
163 as that of the top band of SGK detected by the anti-sfSGK-C antibody in
164 1-MeAde-treated oocytes (Fig. 1e, open arrowhead). In addition, the band disappeared
165 after depletion of SGK from oocyte extracts (Fig. 1f), whereas it was detected in a
166 sample of immunoprecipitated SGK (Fig. 1f), indicating that the anti-phospho-A-loop
167 antibody also detects A-loop phosphorylation of sfSGK. Taken together, these results
168 confirmed that SGK is activated after 1-MeAde stimulation.

169 SGK and Akt phosphorylate a Ser or Thr residue in the same consensus motif[52]
170 and may therefore play a redundant role in 1-MeAde signaling. We next analyzed the
171 dynamics of SGK activation in starfish oocytes in detail. As previously reported[48],
172 phosphorylation of Akt in the HM and phosphorylation of Cdc25 at Ser188 were
173 detected within 2 min after 1-MeAde addition (Fig. 1g). SGK was activated at a similar

174 time (Fig. 1g, pA-loop). Thereafter, Tyr15 of Cdk1 was dephosphorylated, which is
175 indicative of cyclin B-Cdk1 activation (Fig. 1g, 12 min and later). In mammalian cells,
176 SGK is activated by phosphorylation of its A-loop and HM, which is catalyzed by
177 PDK1 and mTORC2, respectively[52,53]. In addition, A-loop phosphorylation depends on
178 HM phosphorylation[52]. In starfish oocytes, a mobility shift of SGK was still observed
179 even when A-loop phosphorylation was abolished by BX795 (Fig. 1c). Using chemical
180 inhibitors, Hosoda et al. suggested that this mobility shift represents TORC2-dependent
181 phosphorylation[54]. Consistently, inhibition of TOR by microinjection of an anti-TOR
182 neutralizing antibody[47] completely abolished the mobility shift of SGK after 1-MeAde
183 stimulation (Fig. 1h). In these oocytes, A-loop phosphorylation of SGK was also
184 abolished (Fig. 1h). Therefore, A-loop phosphorylation of SGK likely depends on
185 TORC2-dependent HM phosphorylation in starfish oocytes, as reported in mammalian
186 cells.
187
188 **SGK, but not Akt, is required for the meiotic G2/M transition**
189 Next, we examined the involvement of Akt and SGK in the meiotic G2/M transition. To
190 this end, we specifically inhibited each kinase using antibodies. Non-specific bands
191 were detected by immunoblotting of starfish oocytes with the anti-sfAkt-C antibody
192 (Fig. 2a), but were not immunoprecipitated by this antibody (Fig. 2a). This indicates
193 that the anti-sfAkt-C antibody does not interact with these non-specific proteins in
194 non-denaturing conditions. In addition, although immunoprecipitates obtained using the
195 anti-sfAkt-C antibody contained a trace amount of SGK (Fig. 1d, SGK with long
196 exposure), this was not due to cross-reaction of SGK with this antibody because a
197 similar amount of SGK was detected in a control precipitation obtained using a control

198 IgG (Fig. 1d, SGK with long exposure). Furthermore, the amount of SGK was not
199 reduced in extracts after immunoprecipitation of Akt (Fig. 1c). These observations
200 confirm the specificity of the anti-sfAkt-C antibody. The anti-sfSGK-C antibody
201 specifically detected SGK by immunoblotting (Fig. 1e). In addition, the protein level of
202 Akt remained constant before and after depletion of SGK using this antibody (Fig. 1f),
203 confirming the specificity of the anti-sfSGK-C antibody.

204 The antigens of these antibodies include the phosphorylation site in the HM of
205 each protein. Thus, the antibodies were expected to bind to the HM and block its
206 phosphorylation. As expected, microinjection of the anti-sfAkt-C antibody abolished
207 HM phosphorylation of Akt after 1-MeAde-stimulation (Fig. 2b). We previously
208 suggested that A-loop phosphorylation of Akt depends on its HM phosphorylation in
209 starfish oocytes based on analysis using exogenously expressed human Akt[47].
210 Consistently, A-loop phosphorylation was also disrupted by injection of the
211 anti-sfAkt-C antibody (Fig. 2c, closed arrowhead). SGK was activated as normal in
212 these oocytes (Fig. 2b, open arrowhead). Thus, the anti-sfAkt-C antibody specifically
213 inhibits Akt. On the other hand, microinjection of the anti-sfSGK-C antibody disrupted
214 both A-loop phosphorylation and the mobility shift of SGK after 1-MeAde stimulation
215 (Fig. 2d) without affecting phosphorylation of Akt (Fig. 2d for HM, e for A-loop, closed
216 arrowhead), indicating that this antibody specifically inhibits SGK.

217 We next examined the effect of microinjection of these antibodies on the meiotic
218 G2/M transition. Oocytes were injected with the anti-sfAkt-C antibody and then treated
219 with 1-MeAde. As a marker of the meiotic G2/M transition, we monitored germinal
220 vesicle breakdown (GVBD), which is equivalent to nuclear envelope breakdown in
221 somatic cells. Surprisingly, GVBD occurred as normal in these Akt-inhibited oocytes

222 (Fig. 2f). By contrast, microinjection of the anti-sfSGK-C antibody suppressed GVBD
223 in a dose-dependent manner (Fig. 2g), suggesting that SGK, but not Akt, is required for
224 the meiotic G2/M transition.

225 We previously reported that microinjection of an anti-sfAkt antibody inhibits the
226 meiotic G2/M transition[39]. The antibody used in that previous study was raised against
227 an 88-amino acid C-terminal fragment of sfAkt. In the present study, there were no
228 stocks of the antiserum left and therefore we used an antibody purified from an
229 antiserum raised against the same fragment of sfAkt in another rabbit. Thus, the
230 specificity of the previously used antibody may differ from that of the presently used
231 antibody. In addition, 33 of the 88 residues in the antigen peptide are conserved in
232 sfSGK and therefore the previously used antibody may have cross-reacted with sfSGK
233 and consequently inhibited SGK as well as Akt. Check of the specificity of this
234 antibody was limited during our previous study because we lacked an anti-sfSGK
235 antibody and phospho-specific antibodies against both sfAkt and sfSGK at that time.
236

237 **SGK directly phosphorylates Cdc25 and Myt1 to trigger cyclin B-Cdk1 activation**

238 To trigger activation of cyclin B-Cdk1, we previously demonstrated that Cdc25 and
239 Myt1 are activated and inactivated, respectively, by phosphorylation of Akt/SGK
240 consensus motifs, which correspond to multiple sites including Ser188 in Cdc25 (ref.
241 48), and Ser75 in Myt1 (ref. 39). Consistent with these previous findings and with the
242 requirement of SGK for the meiotic G2/M transition, phosphorylation of Cdc25 at
243 Ser188 and Myt1 at Ser75 was inhibited by injection of the anti-sfSGK-C antibody (Fig.
244 2d), but not by injection of the anti-sfAkt-C antibody (Fig. 2b). Furthermore, in
245 Phos-tag SDS-polyacrylamide gel electrophoresis (PAGE) analysis, which emphasizes

246 the mobility shifts of phosphorylated proteins, the upward shifts of Cdc25 and Myt1
247 after 1-MeAde stimulation were completely inhibited by the anti-sfSGK-C antibody,
248 but not by the anti-sfAkt-C antibody (Fig. 3a). This suggests that the regulatory
249 phosphorylation of Cdc25 and Myt1 to trigger activation of cyclin B-Cdk1 depends on
250 SGK, but not on Akt.

251 To investigate whether SGK directly phosphorylates Cdc25 and Myt1, *in vitro*
252 phosphorylation experiments were performed using recombinant proteins. Attempts to
253 prepare active recombinant sfSGK were unsuccessful; therefore, we used commercially
254 available recombinant human SGK3 (hSGK3), which is the best related to sfSGK
255 among the three isoforms of human SGK. Ser188 of starfish Cdc25 (sfCdc25) was
256 phosphorylated upon incubation with hSGK3 (Fig. 3b). In addition, the mobility shift
257 was still observed when Ser188 of sfCdc25 was substituted with Ala (Fig. 3b),
258 suggesting that hSGK3 directly phosphorylates sfCdc25 at multiple residues including
259 Ser188. This is consistent with the fact that sfCdc25 has five Akt/SGK consensus motifs.
260 hSGK3 also phosphorylated Ser75 of starfish Myt1 (sfMyt1), leading to a slight
261 mobility shift (Fig. 3c). Consistent with the fact that sfMyt1 has only one Akt/SGK
262 consensus motif, the mobility shift of sfMyt1 was not observed when Ser75 was
263 substituted with Ala (Fig. 3c), suggesting that Ser75 is the only site phosphorylated by
264 hSGK3 in sfMyt1. These results suggest that Cdc25 and Myt1 are direct substrates for
265 SGK in starfish oocytes.

266 The importance of SGK in 1-MeAde signaling was further supported by
267 experiments using oocyte extracts. A GST-conjugated Akt/SGK substrate peptide
268 (GST-AS peptide), which contained an Akt/SGK consensus motif, was incubated in
269 oocyte extracts. Thereafter, phosphorylation of the peptide was evaluated by

270 immunoblotting with an anti-pan-phospho-Akt/SGK substrate antibody. The GST-AS
271 peptide was not phosphorylated in the extract of unstimulated oocytes, but was
272 phosphorylated in the extract of 1-MeAde-treated oocytes (Fig. 4a, b). This
273 phosphorylation of the GST-AS peptide was not affected by immunodepletion of Akt
274 (Fig. 4c for confirmation of Akt depletion and d, e for activity), but was abolished upon
275 depletion of SGK (Fig. 4f for confirmation of SGK depletion and g, h for activity).
276 These results suggest that SGK is a major kinase activated after 1-MeAde stimulation
277 and phosphorylates the Akt/SGK consensus motif in starfish oocytes.
278 Taken together, these observations support the conclusion that SGK, but not Akt,
279 is required for the regulatory phosphorylation that makes Cdc25 activity dominant over
280 Myt1 activity to trigger cyclin B-Cdk1 activation.
281
282 **SGK is sufficient for the regulatory phosphorylation of Cdc25 and Myt1 to trigger**
283 **cyclin B-Cdk1 activation at the meiotic G2/M transition**
284 Next, we investigated whether sfSGK is sufficient to trigger cyclin B-Cdk1 activation.
285 Mammalian SGK3 and Akt have a regulatory domain N-terminal to their catalytic
286 domain, which is named the PX domain and PH domain, respectively[51]. Binding of
287 these domains to phosphoinositides on the cellular membrane contributes to activation
288 of these kinases[55,56]. Membrane targeting of Akt via substitution of the PH domain with
289 a myristoylation sequence results in its activation without extracellular stimulation[39,47,57].
290 To generate constitutively active sfSGK (CA-SGK), we substituted the PX domain of
291 sfSGK with a myristoylation sequence. Furthermore, Thr479, which is the conserved
292 phosphorylation site by TORC2, was substituted with Glu to mimic phosphorylation of
293 the HM. Injection of mRNA encoding CA-SGK into unstimulated oocytes induced

294 GVBD (Fig. 5a). In these oocytes, the level of A-loop phosphorylation of CA-SGK at
295 GVBD was comparable to that of endogenous SGK after 1-MeAde stimulation (Fig. 5b,
296 c), suggesting that CA-SGK is activated in the absence of 1-MeAde stimulation and that
297 activation of SGK is sufficient to induce the meiotic G2/M transition.
298 We then examined whether CA-SGK expression induces the regulatory
299 phosphorylation of Cdc25 and Myt1 to trigger cyclin B-Cdk1 activation. To analyze
300 CA-SGK-induced signaling without any effect of Cdk-dependent feedback pathways,
301 CA-SGK was expressed in the presence of the Cdk inhibitor roscovitine. These oocytes
302 were collected at the time point at which 50% of CA-SGK-expressing oocytes exhibited
303 GVBD in the absence of roscovitine (5 h and 45 min in Fig. 5a). Although expression
304 and A-loop phosphorylation levels of CA-SGK were lower than those of endogenous
305 SGK (Fig. 5d, pA-loop), phosphorylation of Cdc25 at Ser188 and of Myt1 at Ser75
306 were detectable in these oocytes (Fig. 5d). Taken together, these findings suggest that
307 activation of SGK is sufficient for the regulatory phosphorylation of Cdc25 and Myt1 to
308 trigger cyclin B-Cdk1 activation.
309
310 **SGK is activated by cooperation of the Gβγ-PI3K and atypical Gβγ pathways in**
311 **starfish oocytes**
312 In a previous report[48], we proposed the existence of an atypical Gβγ pathway that is
313 activated by Gβγ and cooperates with the Gβγ-PI3K pathway to induce the meiotic
314 G2/M transition. Therefore, we examined whether SGK is activated by these pathways.
315 First, we investigated whether SGK is activated by signaling pathways
316 downstream of Gβγ. Expression of exogenous Gβγ induced the meiotic G2/M transition
317 as previously reported[48], whereas Gβγ-induced GVBD was inhibited by microinjection

318 of the anti-sfSGK-C antibody, but not by injection of the anti-sfAkt-C antibody (Fig.
319 6a). These findings indicate that signaling from exogenous Gβγ induces the meiotic
320 G2/M transition in a SGK-dependent manner, similar to 1-MeAde stimulation.
321 Consistently, immunoblot analysis showed that SGK was activated upon expression of
322 Gβγ (Fig. 6b). Furthermore, when Gβγ was expressed in the presence of the Cdk
323 inhibitor roscovitine, thereby avoiding any effect of cell cycle progression on signal
324 transduction, the level of A-loop phosphorylation of sfSGK was comparable to that
325 induced by 1-MeAde (Fig. 6c), suggesting that sfSGK is activated downstream of Gβγ
326 independently of Cdk activity. However, 1-MeAde- and Gβγ-induced SGK activation
327 was abolished upon inhibition of PI3K by wortmannin (Fig. 6d), suggesting that
328 activation of sfSGK by Gβγ requires PI3K.
329 Next, to determine whether PI3K activation solely induces SGK activation,
330 CA-PI3K was expressed in unstimulated oocytes. As previously reported[48], CA-PI3K
331 induced HM phosphorylation of Akt (Fig. 6e), but not the meiotic G2/M transition. In
332 these oocytes, A-loop phosphorylation of SGK was only detectable by immunoblotting
333 with a long time exposure for acquisition of the chemiluminescent image (Fig. 6e,
334 pA-loop with long exposure, open arrowhead), indicating that activation of PI3K barely
335 induces activation of SGK.
336 Taken together, Gβγ activates SGK in a PI3K-dependent manner, but activation of
337 PI3K alone is insufficient for SGK activation. These observations suggest that Gβγ
338 activates not only PI3K but also another pathway(s), which we call the atypical Gβγ
339 pathway, and that cooperation of the Gβγ-PI3K and atypical Gβγ pathways leads to
340 activation of SGK.

341 As to activation of Akt, CA-PI3K induced HM phosphorylation of Akt (Fig. 6e),
342 as previously reported[48]. In these oocytes, the anti-phospho-A-loop antibody detected a
343 weak band at the same mobility as Akt by immunoblotting after a long exposure (Fig.
344 6e, pA-loop with long exposure, closed arrowhead) even in whole-oocyte samples. This
345 band disappeared after immunodepletion of Akt from the oocyte extract (Fig. 6e),
346 showing that it corresponds to A-loop phosphorylation of Akt. Given that the level of
347 1-MeAde-induced A-loop phosphorylation of Akt was below the threshold of detection
348 in whole-oocyte samples (Fig. 1b–d), Akt is activated more strongly by CA-PI3K than
349 by 1-MeAde. This further indicates that activation of endogenous Akt is insufficient for
350 the meiotic G2/M transition. By contrast, in Gβγ-expressing oocytes, A-loop
351 phosphorylation of Akt was undetectable even in immunoprecipitated Akt samples (Fig.
352 6f, closed arrowhead), although HM phosphorylation was comparable to that in
353 1-MeAde-treated oocytes (Fig. 6c, Akt-pHM in Input). This suggests that Gβγ signaling
354 is insufficient for A-loop phosphorylation of Akt. This finding will be discussed later.

355 **Discussion**

356 This study showed that SGK is necessary and sufficient for the regulatory
357 phosphorylation of Cdc25 and Myt1 to trigger activation of cyclin B-Cdk1 in the
358 meiotic G2/M transition. Furthermore, we revealed that SGK is activated by
359 cooperation of the Gβγ-PI3K and atypical Gβγ pathways downstream of 1-MeAde
360 stimulation (Fig. 7). These findings clarify the molecular identity of the trigger kinase,
361 and offer important clues to elucidate the 1-MeAde signaling pathway. Moreover, they
362 provide new insights into the role and regulation of SGK.

363 In *Xenopus* oocytes, multiple pathways are proposed to reverse the balance
364 between the activities of Cdc25 and Myt1 (refs 23,30). Therefore, it is difficult to
365 determine the relative contribution of each pathway. In this context, SGK is the only
366 trigger kinase in starfish oocytes because it is necessary and sufficient for the regulatory
367 phosphorylation of Cdc25 and Myt1. Particularly, the phosphorylation of Cdc25 and
368 Myt1 was undetectable in SGK-inhibited oocytes (Fig. 2). In mouse oocytes, Cyclin
369 B-Cdk1 activation might be triggered by a phosphatase antagonistic to PKA, rather than
370 by kinases, and it is unclear whether the activity of this phosphatase is regulated at the
371 meiotic G2/M transition[29]. Thus, our present study is the first time that the trigger
372 kinase in oocyte meiosis has been clearly identified. To the best of our knowledge, this
373 is the first report that SGK is involved in M-phase entry.

374 In parallel with the present study, Hosoda et al. showed that sfSGK is required for
375 the increase in intracellular pH after 1-MeAde stimulation in starfish oocytes[54]. They
376 suggested that the increase in intracellular pH is not involved in cyclin B-Cdk1
377 activation, but is required for progression of GVBD after full activation of cyclin
378 B-Cdk1 in ovarian oocytes. The functions of SGK have been mainly studied in

379 mammalian cells, which express three SGK isoforms: SGK1, SGK2, and SGK3[51].
380 Although mammalian SGKs are reportedly involved in various processes, such as cell
381 survival, stress responses, and tumorigenesis, in somatic cells[58,59], their functions in
382 germ cells remain unclear. To the best of our knowledge, studies presented here and in
383 the paper by Hosoda et al.[54] are the first indication of the functions of SGK in germ
384 cells.

385 Among the three mammalian SGK isoforms, only SGK3 contains a regulatory
386 domain, called the PX domain, N-terminal to the kinase domain[51,60]. sfSGK also has a
387 PX domain and is therefore likely an ortholog of mammalian SGK3 (ref. 54). The PX
388 domain of mammalian SGK3 binds to phosphatidylinositol 3-phosphate (PI-3P), which
389 is a phosphorylated derivative of phosphatidylinositol (PI) at the 3-position of the
390 inositol ring[56]. This interaction is essential for the localization and activation of SGK3
391 (refs 50,56). PI-3P is generated by dephosphorylation of PI-345P_3 at the 4- and
392 5-positions of the inositol ring[50]. PI3K, which produces PI-345P_3 from PI-45P_2, is
393 activated in signaling pathways downstream of receptor tyrosine kinases and GPCRs[45].
394 Thus, production of PI-3P from PI-345P_3 may occur downstream of these receptors.
395 Indeed, a recent report suggested that this mechanism is implicated in activation of
396 SGK3 after stimulation by IGF-1, which is an agonist for a receptor tyrosine kinase[61]. In
397 this context, SHIP2 and INPP4A/B were proposed to be phosphatases for PI-3P
398 production, although the regulatory mechanisms remain elusive[61]. Furthermore, it is
399 unclear whether PI-345P_3 is a source of PI-3P for SGK activation in GPCR-dependent
400 signaling. We previously reported that PI-345P_3 is generated on the plasma membrane
401 after 1-MeAde stimulation in starfish oocytes[48]. This PI-345P_3 may be metabolized to
402 PI-3P for SGK activation. Our present results suggest that the atypical Gβγ pathway

403 cooperates with PI3K to activate sfSGK (Fig. 6). Thus, we propose that the atypical
404 Gβγ pathway activates phosphatases that produce PI-3P from PI-345P_3 downstream of
405 GPCR-mediated signaling.

406 Another mechanism for PI-3P production is phosphorylation of PI at the
407 3-position, which occurs on early endosomes during intracellular vesicle trafficking and
408 endocytosis[45,50]. GPCRs are frequently endocytosed after agonist stimulation[62]. Thus,
409 1-MeAde may induce endocytosis, which increases the level of early endosomes. If this
410 is the case, the endocytic pathway may also contribute to activation of SGK in starfish
411 oocytes. Many aspects of these scenarios, such as the dynamics of PI-3P, the
412 localization of SGK, and the molecular identity of the atypical Gβγ pathway, remain
413 unclear. Nonetheless, these working models could be tested in future studies and may
414 help to elucidate the regulatory mechanisms of SGK3 activation in mammalian somatic
415 cells.

416 SGKs and Akt reportedly play redundant roles in some contexts[59]. In starfish
417 oocytes, Akt has been reported to have a potential to trigger cyclin B-Cdk1 activation[39].
418 Indeed, an excess amount of CA-Akt expression can induce the meiotic G2/M
419 transition[39,48]. Furthermore, regulatory effects of phosphorylation of the Akt/SGK
420 consensus motifs on Myt1 and Cdc25 activities were originally showed using Akt as a
421 kinase in *in vitro* experiments[39,48]. Nonetheless, our present results showed that SGK is
422 predominantly responsible for these phosphorylation *in ovo*. Contribution of Akt was, if
423 any, undetectable (Fig. 2) possibly due to its weak activation, as supported by low
424 levels of A-loop phosphorylation (Figs. 1) and by the kinase assay using oocyte extracts
425 (Fig. 5). The role of Akt may be other than the triggering of cyclin B-Cdk1 activation.
426 Even so, we cannot exclude the possibility that Akt makes a significant contribution to

427 the triggering as a redundant kinase with SGK in a limited number of unknown
428 circumstances in which Akt is more abundant than SGK in oocytes.
429 Contrary to our present conclusion, we previously reported that Akt is necessary
430 for the meiotic G2/M transition[39]. In our previous report, in addition to the anti-sfAkt
431 antibody, we also used competitive peptides derived from the A-loop or HM of Akt to
432 inhibit Akt activation. Akt and SGK have common upstream kinases; therefore, peptide
433 competition likely inhibited activation of both Akt and SGK. In the present study,
434 successful generation of anti-phospho-A-loop and anti-sfSGK-C antibodies enabled us
435 to evaluate the specificities of the neutralizing antibodies more precisely. In addition,
436 Akt was dispensable at least in all cases tested in the current study, which used oocytes
437 isolated from six starfish collected at three different areas in Japan. Thus, our current
438 conclusions are more convincing than our previous conclusions.
439 We made some interesting observations regarding activation of Akt. Distinct from
440 SGK, activation of Akt depends on binding of PI-345P_3 to its PH domain[59]. Expression
441 of CA-PI3K, but not of Gβγ, induced A-loop phosphorylation of Akt (Fig. 6e, f). We
442 previously reported that a comparable level of PI-345P_3 is produced upon 1-MeAde
443 stimulation and Gβγ expression, while CA-PI3K induces a higher level of PI-345P_3 (ref.
444 48). Thus, it is possible that a high level of PI-345P_3 alone can induce Akt activation,
445 whereas a low level of PI-345P_3 requires an additional mechanism to enhance A-loop
446 phosphorylation. In addition, it should be noted that the levels of HM phosphorylation
447 induced by CA-PI3K and exogenous Gβγ were comparable to that induced by 1-MeAde
448 stimulation (Fig. 6c, e). Thus, the threshold level of PI-345P_3 required for HM
449 phosphorylation may be lower than that required for A-loop phosphorylation.
450 In summary, we identified SGK as the trigger kinase for activation of cyclin

451 B-Cdk1 at M-phase entry and as the target of the collaborative actions of PI3K and the
452 atypical Gβγ pathway. These findings not only demonstrate the novel role of SGK in
453 the triggering of M-phase, but also provide insights into the regulatory mechanisms of
454 SGK activation, particularly with regard to PI-3P production.

455 **Methods**

456 **Chemicals.** Roscovitine (Calbiochem), wortmannin (LC Laboratories), and BX795
457 (Enzo Life Sciences) were dissolved in DMSO at concentrations of 50, 20, and 6 mM,
458 respectively, as stock solutions and used at final concentrations of 45, 40, and 6 μM,
459 respectively.

460

461 **DNA constructs.** For CA-SGK, an N-terminal fragment of sfSGK containing the PX
462 domain (Met1–Asp132) was substituted with a myristoylation sequence
463 (MGSSKSKPKDPSQR) by PCR. Thereafter, the insert was cloned into a modified
464 pSP64-S vector, in which the SP6 promoter for *in vitro* transcription had been
465 substituted by a T7 promoter sequence[48,63], using an In-Fusion kit (Takara Bio). Finally,
466 Thr479 in the HM was substituted with Glu by PCR, and the point mutation was
467 verified by sequencing. Constructs encoding untagged sfAkt, CA-PI3K in which Flag
468 tag and plasma membrane-targeting sequence including a CAAX motif fused to the N-
469 and C-terminus of the p110 catalytic subunit of sfPI3K, respectively, untagged starfish
470 Gβ (sfGβ), and untagged starfish Gγ (sfGγ) for mRNA preparation were prepared as
471 described previously[48].

472

473 **Preparation of recombinant proteins.** Recombinant sfMyt1 (N229A for the
474 kinase-dead mutant or S75A mutant) cloned into the pGEX-4T-1 vector was expressed
475 in *Escherichia coli* (*E. coli*) strain BL21 (DE3) (Invitrogen) as an N-terminal
476 GST-tagged protein, purified using glutathione-Sepharose 4B beads (GE Healthcare),
477 and dialyzed against storage buffer (20 mM PIPES pH 6.8, 200 mM sucrose, and 1 mM
478 DTT). For His_6-Cdc25 (wild-type or S188A mutant), *E. coli* strain BL21 (DE3) was

479 transformed with the pET21a construct. Expressed His_6-Cdc25 were purified from
480 inclusion bodies under denaturing conditions (6 M urea) using Probond-Resin
481 (Invitrogen) and then refolded via stepwise reduction of the urea concentration by
482 dialysis using EasySep (TOMY) for 2 h against Tris-buffered saline (TBS; 50 mM Tris
483 and 150 mM NaCl, pH 7.5) containing 4 M urea, for 2 h against TBS containing 2 M
484 urea, and for 2 h against TBS lacking urea. To prepare the recombinant GST-fused AS
485 peptide (AGRPRAATFIESG), *E. coli* strain BL21-CodonPlus-RIL (Agilent
486 Technologies) was transformed with the pGEX-4T-1 construct. The expressed GST-AS
487 peptide was purified using glutathione-Sepharose 4B and dialyzed against
488 phosphate-buffered saline (137 mM NaCl, 2.68 KCl, 10 mM Na_2HPO_4, and 1.76 mM
489 KH_2PO_4, pH 7.4). A recombinant C-terminal 88-amino acid fragment of sfAkt was
490 prepared as previously described[39].
491
492 **Antibody generation.** Antibodies were generated by immunizing rabbits (Biologica)
493 and then purified using antigens. Antigens used for immunization and antibody
494 purification were a phospho-peptide of the A-loop of sfAkt (Thr304–Pro320,
495 phosphorylated at Thr315) for the anti-phospho-A-loop antibody, a C-terminal peptide
496 of sfSGK (Ser473–Asp489) for the anti-sfSGK-C antibody[54], and a C-terminal
497 88-amino acid fragment of sfAkt for the anti-sfAkt-C antibody. Control IgG for
498 microinjection or immunoprecipitation was purified from rabbit pre-immune serum
499 using protein A-Sepharose 4B (Sigma).
500
501 **Oocyte preparation.** Starfish *Asterina pectinifera* (renamed *Patiria pectinifera* in the
502 2007 National Center for Biotechnology Information Taxonomy Browser) was collected

503 during the breeding season and kept in laboratory aquaria supplied with circulating
504 seawater at 14°C. Fully grown unstimulated oocytes with follicle cells were manually
505 isolated from ovaries using forceps. Thereafter, the follicle cells were removed by
506 washing oocytes with calcium-free artificial seawater (476 mM NaCl, 10 mM KCl, 36
507 mM $MgCl_2$, 18 mM $MgSO_4$, and 20 mM H_3BO_3, pH 8.2). All treatments with 1-MeAde
508 or other chemicals, as well as microinjections, were performed in artificial seawater
509 (ASW; 462 mM NaCl, 9 mM $CaCl_2$, 10 mM KCl, 36 mM $MgCl_2$, 18 mM $MgSO_4$, and
510 20 mM H_3BO_3, pH 8.2) at 23°C. Oocytes were stimulated with 0.5 μM 1-MeAde.
511
512 **Immunoblotting.** Oocytes were lysed by vortexing in Laemmli sample buffer (LSB)
513 and then heated at 95°C for 5 min. Proteins were separated by SDS-PAGE[64] on an 8%
514 or 8.5% gel (separating gel: 8% or 8.5% acrylamide, 0.22% or 0.23%
515 N,N'-methylenebisacrylamide, 375 mM Tris, 0.1% SDS, 0.1% APS, and 0.1% TEMED,
516 pH 8.8; stacking gel: 3.6% acrylamide, 0.096% N,N'-methylenebisacrylamide, 120 mM
517 Tris, 0.1% SDS, 0.2% APS, and 0.1% TEMED, pH 6.8; and electrophoresis buffer: 25
518 mM Tris, 192 mM glycine, and 0.1% SDS) and transferred to a PVDF membrane
519 (Millipore)[65] using transfer buffer (100 mM Tris, 192 mM glycine, 0.1% SDS, and 20%
520 methanol). Phos-tag SDS-PAGE was performed in accordance with the manufacturer's
521 protocol using an 8% polyacrylamide gel containing 4 μM Phos-tag (FUJIFILM) and 8
522 μM $MnCl_2$. A modified 15% gel and buffer were used to detect Gγ (separating gel: 15%
523 acrylamide, 0.41% N,N'-methylenebisacrylamide, 750 mM Tris, 0.1% SDS, 0.1% APS,
524 and 0.1% TEMED, pH 8.8; stacking gel: 3.6% acrylamide, 0.096%
525 N,N'-methylenebisacrylamide, 240 mM Tris, 0.1% SDS, 0.2% APS, and 0.1% TEMED,
526 pH 6.8; and electrophoresis buffer: 50 mM Tris, 384 mM glycine, and 0.1% SDS). The

527 membranes were then blocked by incubation in blocking buffer (5% skimmed milk
528 prepared in TBS containing 0.1% Tween-20 (TBS-T)) for 1 h for the
529 anti-phospho-sfMyt1-Ser75, anti-sfMyt1, and anti-GST antibodies, but not for the other
530 antibodies. The primary antibodies were anti-phospho-A-loop (purified, 1:50 in TBS-T),
531 anti-sfAkt-C (purified, 1:1,000 in Can Get Signal Solution 1),
532 anti-phospho-sfAkt-Ser477 (ref. 47, purified, 1:1,000 in Can Get Signal Solution 1),
533 anti-sfSGK-C (purified, 1:2,000 in Can Get Signal Solution 1),
534 anti-phospho-sfCdc25-Ser188 (ref. 48, serum, 1:1,000 in Can Get Signal Solution 1),
535 anti-sfCdc25 (ref. 33, serum, 1:2,000 in Can Get Signal Solution 1),
536 anti-phospho-Cdk1-Tyr15 (Cell Signaling Technology, 1:1,000 in Can Get Signal
537 Solution 1), anti-PSTAIR[47] to detect Cdk1 (1:50,000 in Can Get Signal Solution 1),
538 anti-phospho-sfMyt1-Ser75 (ref. 48, purified, 1:100 in blocking buffer), anti-sfMyt1[39]
539 (purified, 1:200 in TBS-T), anti-GST (GE Healthcare, 1:500 in blocking buffer),
540 anti-pan-phospho-Akt/SGK substrate (Cell Signaling Technology, 1:1,000 in Can Get
541 Signal Solution 1), anti-sfp110β to detect sfPI3K[48] (purified, 1:1,000 in Can Get Signal
542 Solution 1), anti-sfGβ[48] (serum, 1:1,000 in Can Get Signal Solution 1), and anti-sfGγ[48]
543 (purified, 1:100 in Can Get Signal Solution 1). Horseradish peroxidase-conjugated
544 secondary antibodies were anti-goat IgG (Sigma, 1:500 in blocking buffer for anti-GST
545 antibody), anti-mouse IgG (Dako, 1:2,000 in Can Get Signal Solution 2 for
546 anti-PSTAIR antibody), and anti-rabbit IgG (GE Healthcare; 1:2,000 in TBS-T for
547 anti-phospho-A-loop, anti-sfSGK-C, anti-phospho-sfMyt1-Ser75, and
548 anti-pan-phospho-Akt/SGK substrate antibodies; in blocking buffer for the anti-sfMyt1
549 antibody; and in Can Get Signal Solution 2 for the other antibodies). When the signal
550 derived from IgG heavy chains hampered detection, TrueBlot (ROCKLAND, 1:2,000 in

551 Can Get Signal Solution 2) was used instead of the secondary antibody. Signals were
552 visualized with ECL Prime (GE Healthcare), and digital images were acquired on a
553 LAS4000 mini imager (Fujifilm). Quantitation was performed using ImageJ (National
554 Institutes of Health, USA). Graphs were generated using Microsoft Excel. Brightness
555 and contrast were adjusted using ImageJ.
556
557 **Microinjection.** Microinjection was performed as described previously[66]. mRNAs for
558 exogenous protein expression in starfish oocytes were transcribed from pSP64-S
559 constructs using a mMESSAGE mMACHINE kit (Ambion), dissolved in water, and
560 injected into unstimulated oocytes (20 pg for sfAkt, 20 pg for CA-SGK, and 40 pg for
561 CA-PI3K). To express starfish Gβγ, 50 pg of an equimolar mixture of mRNAs encoding
562 sfGβ and sfGγ was injected. The incubation time for protein expression was determined
563 based on the amount of time taken to induce GVBD or to induce HM phosphorylation
564 of Akt to a comparable extent as that induced by 1-MeAde. For microinjection of
565 antibodies, concentration of antibodies and buffer exchange with phosphate-buffered
566 saline were performed using a 50 k cutoff Amicon Ultra filter (Millipore). Nonidet P-40
567 (NP-40) was added at a final concentration of 0.05%. Unstimulated oocytes were
568 injected with 230 pg of the anti-TOR antibody, 85 pg of the anti-sfAkt-C antibody, or
569 200 pg of the anti-sfSGK-C antibody. 1-MeAde was added after incubation for 1 h.
570
571 **Immunoprecipitation and immunodepletion.** To prepare antibody-bound beads,
572 antibodies (anti-sfAkt antibody, 2.5 μg/μl beads, 0.83 μg for 10 oocytes; and
573 anti-sfSGK antibody, 2 μg/μl beads, 2.7 μg for 10 oocytes) were incubated with Protein
574 A-Sepharose (Sigma) for more than 2 h on ice. Antibody-bound beads were washed

575 with TBS and lysis buffer (80 mM β-glycerophosphate, 20 mM EGTA, 10 mM MOPS
576 pH 7.0, 100 mM sucrose, 100 mM KCl, 1 mM DTT, 1× cOmplete EDTA-free (Merck),
577 0.5 mM sodium orthovanadate, 1 μM okadaic acid (Calbiochem), and 0.5% NP-40) and
578 used for immunoprecipitation. For cross-linking, antibody-bound beads were washed
579 with TBS and borate buffer (0.2 M NaCl and 0.2 M boric acid pH 9.0) and then
580 incubated with 3.75 mM disuccinimidyl suberate prepared in borate buffer at 24°C for
581 30 min. Thereafter, the supernatant was discarded and the beads were further incubated
582 in 1 M ethanolamine (pH 8.0) at 24°C for 15 min and finally washed with TBS and lysis
583 buffer. For immunoprecipitation, freeze-thawed oocytes in ASW (10 oocytes/μl of
584 ASW for immunoprecipitation of Akt and SGK) were lysed by adding 6 volumes of
585 lysis buffer and incubating samples on ice for 30 min, followed by gentle vortexing.
586 Lysates were centrifuged at 20 k × g at 4°C for 15 min. The supernatant represented the
587 oocyte extract. Antibody-bound beads were incubated with the oocyte extract (60
588 oocytes for immunoprecipitation of Akt, 30 oocytes for depletion of Akt, and 15
589 oocytes for immunoprecipitation and depletion of SGK) for 90 min on ice, separated
590 from flow-through extracts, and washed with lysis buffer. For immunoprecipitation of
591 Akt from oocytes that had been injected with the anti-sfSGK antibody, extracts were
592 pre-incubated with protein A-Sepharose 4B to remove injected IgG. LSB was added to
593 the input extracts, flow-through extracts, and beads for immunoblot analysis. All
594 samples were then heated at 95°C for 5 min and subjected to SDS-PAGE. To prepare
595 mock-, Akt-, and SGK-depleted extracts for the GST-AS peptide kinase assay,
596 freeze-thawed oocytes in ASW (5 oocytes/μl of ASW) were lysed by adding 6.5
597 volumes of lysis buffer. The extract was then prepared as described above and mixed
598 with beads bound to control IgG, the anti-sfAkt-C antibody, or the anti-sfSGK-C

599 antibody. After incubation at 4°C for 45 min, the supernatant was analyzed by
600 immunoblotting or used for the GST-AS peptide kinase assay.
601
602 ***In vitro* phosphorylation of sfCdc25 and sfMyt1.** Recombinant sfCdc25 (final
603 concentration of 5 ng/μl) or sfMyt1 (final concentration of 200 ng/μl) was incubated
604 with N-terminal GST-tagged human SGK3 (final concentration of 10 ng/μl,
605 SignalChem) in reaction buffer (80 mM β-glycerophosphate, 20 mM EGTA, 1 mM
606 MOPS pH 7.0, 1 mM DTT, 1 mM ATP, 5 mM $MgCl_2$, and 0.3% NP-40) at 30°C for 50
607 min. The reaction was stopped by adding LSB followed by heating at 95°C for 5 min.
608 Samples were analyzed by immunoblotting with phospho-specific antibodies.
609
610 **GST-AS peptide kinase assay.** Freeze-thawed oocytes in ASW (5 oocytes/μl of ASW)
611 were lysed by adding 6.5 volumes of lysis buffer and then incubating samples on ice for
612 30 min, followed by gentle vortexing. Lysates were centrifuged at 20 k × g at 4°C for
613 15 min. The supernatant was used for the kinase assay. Mock, Akt-, and SGK-depleted
614 extracts were prepared as described above. For the kinase assay, a reaction mixture
615 containing the GST-AS peptide as a substrate (80 mM β-glycerophosphate, 15 mM
616 $MgCl_2$, 20 mM EGTA, 10 mM MOPS, pH 7.0, 1 mM DTT, 1× cOmplete EDTA-free, 2
617 mM ATP, 0.5 mM sodium orthovanadate, 1 μM okadaic acid, 0.1 mg/ml GST-AS
618 peptide, and 0.3% NP-40) was added to an equal volume of the extract. After incubation
619 at 30°C, the reaction solution was mixed with LSB, heated at 95°C for 5 min, and then
620 analyzed by immunoblotting.
621

622 **Statistical analysis.** Statistical significance was calculated using the unpaired one-tailed
623 Student's t-test in Microsoft Excel with StatPlus (AnalystSoft). Statistical significance
624 was defined as $p < 0.05$.
625
626 **Accession numbers.** Accession numbers are as follows: LC430700 for sfSGK,
627 AB076395 for sfCdc25, AB060280 for sfMyt1, AB060291 for sfAkt, LC017891 for
628 sfGβ, LC017892 for sfGγ, and LC017893 for the sfPI3K catalytic subunit p110β.

Data availability

Data supporting the findings of this study are available within the article or upon reasonable requests to the corresponding authors.

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Author contributions

D.H. designed and performed all the experiments. E.H. and K.C. generated the anti-sfSGK-C antibody. All authors discussed the results. D.H. and T.K. wrote the manuscript. T.K. obtained funding and supervised the study.

Competing interests

The authors declare no competing interests.

Materials and correspondence

Correspondence and requests for materials should be addressed to D.H. and T.K.

649 **References**

650 1. Dunphy, W. G. & Newport, J. W. Unraveling of mitotic control mechanisms. *Cell*
651 **55,** 925-928 (1988).
652 2. Hunt, T. Maturation promoting factor, cyclin and the control of M-phase. *Curr.*
653 *Opin. Cell Biol.* **1,** 268-274 (1989).
654 3. Nurse, P. Universal control mechanism regulating onset of M-phase. *Nature* **344,**
655 503-508 (1990).
656 4. Lew, D.J. & Kornbluth, S. Regulatory roles of cyclin dependent kinase
657 phosphorylation in cell cycle control. *Curr. Opin. Cell Biol.* **8,**795-804 (1996).
658 5. Lindqvist, A., Rodríguez-Bravo, V. & Medema, R. H. The decision to enter
659 mitosis: feedback and redundancy in the mitotic entry network. *J. Cell Biol.* **185,**
660 193-202 (2009).
661 6. Qian, J., Winkler, C. & Bollen, M. 4D-networking by mitotic phosphatases. *Curr.*
662 *Opin. Cell Biol.* **25,** 697-703 (2013).
663 7. Kishimoto, T. Entry into mitosis: a solution to the decades-long enigma of MPF.
664 *Chromosoma* **124,** 417-428 (2015).
665 8. Hégarat, N., Rata, S. & Hochegger, H. Bistability of mitotic entry and exit switches
666 during open mitosis in mammalian cells. *Bioessays* 627-643 (2016).
667 9. O'Farrell, P. H. Triggering the all-or-nothing switch into mitosis. *Trends Cell*
668 *Biol.* **11,** 512-519 (2001).
669 10. Rieder, C. L. Mitosis in vertebrates: the G2/M and M/A transitions and their
670 associated checkpoints. *Chromosome Res.* **19,** 291-306 (2011).
671 11. Thomas, Y. et al. Cdk1 Phosphorylates SPAT-1/Bora to promote Plk1 activation
672 in *C. elegans* and human cells. *Cell Rep.* **15,** 510-518 (2016).

673 12. Gheghiani, L., Loew, D., Lombard, B., Mansfeld, J. & Gavet, O. PLK1 activation
674 in late G2 sets up commitment to mitosis. *Cell Rep.* **19,** 2060-2073 (2017).
675 13. Vigneron, S. et al. Cyclin A-cdk1-dependent phosphorylation of Bora is the
676 triggering factor promoting mitotic entry. *Dev Cell.* **45,** 637-650 (2018).
677 14. Okano-Uchida, T. et al. Distinct regulators for Plk1 activation in starfish meiotic
678 and early embryonic cycles. *EMBO J.* **22,** 5633-5642 (2003).
679 15. Masui, Y. Meiotic arrest in animal oocytes. *Biology of Fertilization* **1,** 189–219
680 (Academic Press, San Diego, 1985).
681 16. Kishimoto, T. MPF-based meiotic cell cycle control: Half a century of lessons
682 from starfish oocytes. *Proc. Jpn. Acad. Ser. B* **94,** 180-203 (2018).
683 17. Kobayashi, H. et al. On the synthesis and destruction of A- and B-type cyclins
684 during oogenesis and meiotic maturation in *Xenopus laevis*. *J. Cell Biol*. **114,** 755-765
685 (1991).
686 18. Minshull, J., Murray, A., Colman, A. & Hunt, T. *Xenopus* oocyte maturation does
687 not require new cyclin synthesis. *J. Cell Biol.* **114,** 767-772 (1991).
688 19. Okano-Uchida, T. et al. *In vivo* regulation of cyclin A/Cdc2 and cyclin B/Cdc2
689 through meiotic and early cleavage cycles in starfish. *Dev. Biol.* **197,** 39-53 (1998).
690 20. Nebreda, A. R. & Ferby, I. Regulation of the meiotic cell cycle in oocytes. *Curr.*
691 *Opin. Cell Biol.* **12,** 666-675 (2000).
692 21. Kishimoto, T. Cell-cycle control during meiotic maturation. *Curr. Opin. Cell Biol.*
693 2003 **15,** 654-663 (2003).
694 22. Pahlavan, G. et al. Characterization of polo-like kinase 1 during meiotic
695 maturation of the mouse oocyte. *Dev. Biol.* **220,** 392-400 (2000).
696 23. Gaffré, M. et al. A critical balance between Cyclin B synthesis and Myt1 activity

697 controls meiosis entry in *Xenopus* oocytes. *Development* **138,** 3735-3744 (2011).
698 24. Maton, G. et al. Cdc2-cyclin B triggers H3 kinase activation of Aurora-A in
699 *Xenopus* oocytes. *J. Biol. Chem.* **278,** 21439-21449 (2003).
700 25. Abe, Y., Okumura, E., Hosoya, T., Hirota, T. & Kishimoto, T. A single starfish
701 Aurora kinase performs the combined functions of Aurora-A and Aurora-B in human
702 cells. *J. Cell Sci.* **123,** 3978-3988 (2010).
703 26. Komrskova, P. et al. Aurora kinase A is not involved in CPEB1 phosphorylation
704 and cyclin B1 mRNA polyadenylation during meiotic maturation of porcine oocytes.
705 *PLoS One* **9,** e101222 (2014). doi: 10.1371/journal.pone.0101222.
706 27. Maller, J. L. & Krebs, E. G. Progesterone-stimulated meiotic cell division in
707 *Xenopus* oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of
708 adenosine 3':5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* **252,**
709 1712-1718 (1977).
710 28. Jaffe, L. A. & Egbert, J. R. Regulation of mammalian oocyte meiosis by
711 intercellular communication within the ovarian follicle. *Annu. Rev. Physiol.* **79,** 237-260
712 (2017).
713 29. Adhikari, D. & Liu, K. The regulation of maturation promoting factor during
714 prophase I arrest and meiotic entry in mammalian oocytes. *Mol. Cell. Endocrinol.* **382,**
715 480-487 (2014).
716 30. Haccard, O. & Jessus, C. Redundant pathways for Cdc2 activation in *Xenopus*
717 oocyte: either cyclin B or Mos synthesis. *EMBO Rep.* **7,** 321-325 (2006).
718 31. Haccard, O. & Jessus, C. Oocyte maturation, Mos and cyclins--a matter of
719 synthesis: two functionally redundant ways to induce meiotic maturation. *Cell Cycle* **5,**
720 1152-1159 (2006).

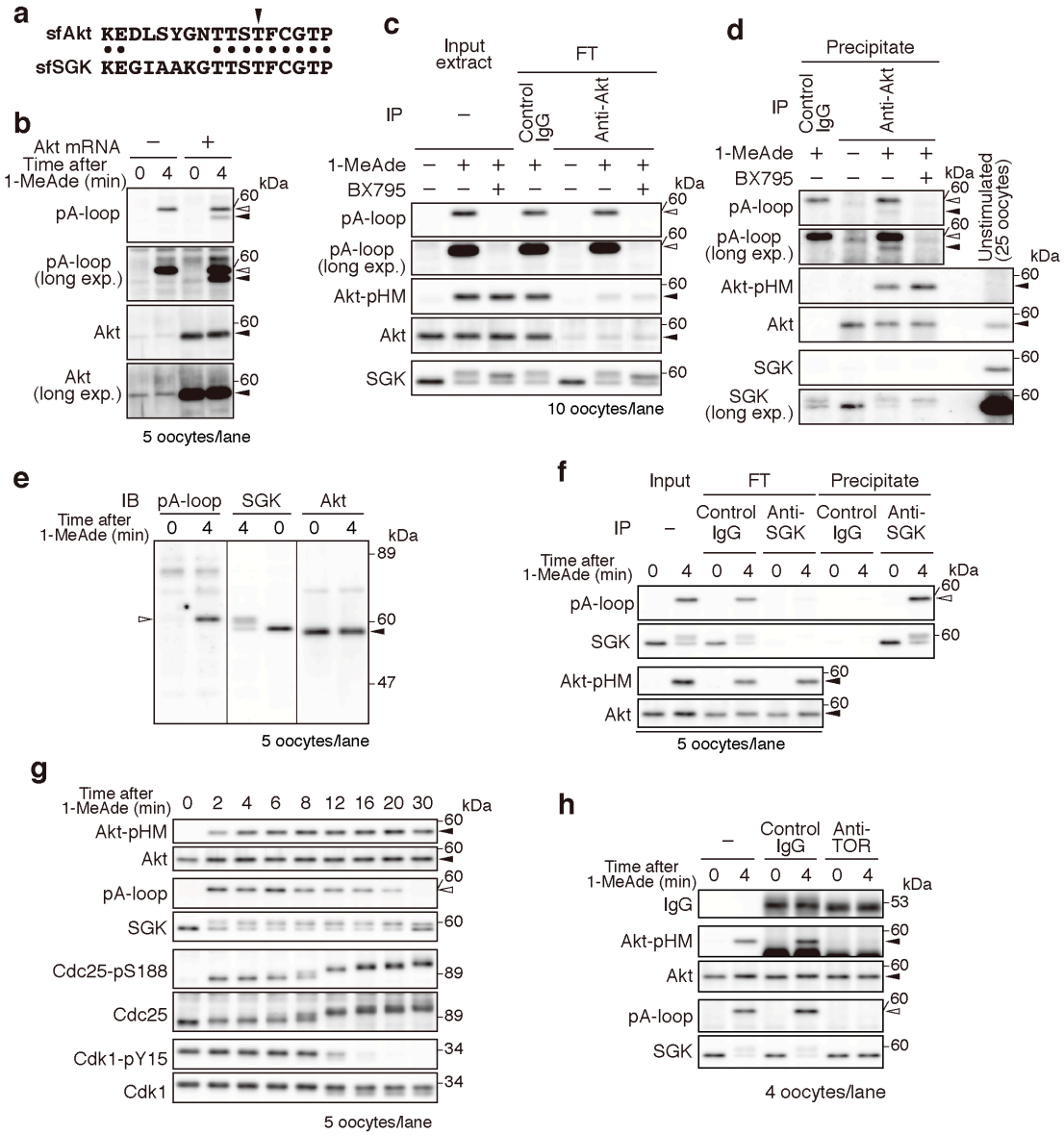
721 32. Dupré, A., Daldello, E. M., Nairn, A. C., Jessus, C. & Haccard, O.
722 Phosphorylation of ARPP19 by protein kinase A prevents meiosis resumption in
723 *Xenopus* oocytes. *Nat. Commun.* **5,** 3318 (2014). doi: 10.1038/ncomms4318.
724 33. Okumura, E., Sekiai, T., Hisanaga, S., Tachibana, K. & Kishimoto, T., Initial
725 triggering of M-phase in starfish oocytes: a possible novel component of
726 maturation-promoting factor besides cdc2 kinase. *J. Cell Biol.* **132,** 125-135 (1996).
727 34. Meijer, L. & Guerrier, P. Maturation and fertilization in starfish oocytes. *Int.*
728 *Rev. Cytol.* **86,** 129-196 (1984).
729 35. Kanatani, H., Shirai, H., Nakanishi, K. & Kurokawa, T. Isolation and
730 identification on meiosis inducing substance in starfish *Asterias amurensis*. *Nature*. **221,**
731 273-274 (1969).
732 36. Dorée, M. Protein synthesis is not involved in initiation or amplification of
733 the maturation-promoting factor (MPF) in starfish oocytes. *Exp. Cell Res.* **139,** 127-133
734 (1982).
735 37. Tachibana, K., Tanaka, D., Isobe, T., Kishimoto, T. c-Mos forces the mitotic cell
736 cycle to undergo meiosis II to produce haploid gametes. *Proc. Natl. Acad. Sci. USA* **97,**
737 14301-14306 (2000).
738 38. Ookata, K., Hisanaga, S., Okano, T., Tachibana, K. & Kishimoto, T. Relocation
739 and distinct subcellular localization of p34cdc2-cyclin B complex at meiosis reinitiation
740 in starfish oocytes. *EMBO J.* **11**, 1763–1772 (1992).
741 39. Okumura, E. et al. Akt inhibits Myt1 in the signalling pathway that leads to
742 meiotic G2/M-phase transition. *Nat. Cell Biol.* **4,** 111-116 (2002).
743 40. Kanatani, H. & Hiramoto, Y. Site of action of 1-methyladenine in inducing oocyte
744 maturation in starfish. *Exp. Cell Res.* **61,** 280-284 (1970).

745 41. Chiba, K., Kontani, K., Tadenuma, H., Katada, T. & Hoshi, M. Induction of
746 starfish oocyte maturation by the βγ subunit of starfish G protein and possible existence
747 of the subsequent effector in cytoplasm. *Mol. Biol. Cell* **4,** 1027–1034 (1993).
748 42. Jaffe, L. A., Gallo, C. J., Lee, R. H., Ho, Y. K. & Jones, T. L. Oocyte maturation
749 in starfish is mediated by the βγ-subunit complex of a G-protein. *J. Cell Biol.* **121,**
750 775–783 (1993).
751 43. Shilling, F., Chiba, K., Hoshi, M., Kishimoto, T. & Jaffe, L. A. Pertussis toxin
752 inhibits 1-methyladenine-induced maturation in starfish oocytes. *Dev. Biol.* **133,**
753 605–608 (1989).
754 44. Sadler, K. C. & Ruderman, J. V. Components of the signaling pathway linking the
755 1-methyladenine receptor to MPF activation and maturation in starfish oocytes. *Dev.*
756 *Biol.* **197,** 25–38 (1998).
757 45. Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M. & Bilanges B. The
758 emerging mechanisms of isoform-specific PI3K signalling. *Nat. Rev. Mol. Cell Biol.* **11,**
759 329-341 (2010).
760 46. Hiraoka, D. et al. PDK1 is required for the hormonal signaling pathway leading to
761 meiotic resumption in starfish oocytes. *Dev. Biol.* **276,** 330-336 (2004).
762 47. Hiraoka, D., Okumura, E., Kishimoto, T. Turn motif phosphorylation negatively
763 regulates activation loop phosphorylation in Akt. *Oncogene* **30,** 4487-4497 (2011).
764 48. Hiraoka, D., Aono, R., Hanada, S., Okumura, E., & Kishimoto, T. Two new
765 competing pathways establish the threshold for cyclin-B-Cdk1 activation at the meiotic
766 G2/M transition. *J. Cell Sci.* **129,** 3153-3166 (2016).
767 49. Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C. & Firestone, G. L.
768 Characterization of sgk, a novel member of the serine/threonine protein kinase gene

769 family which is transcriptionally induced by glucocorticoids and serum. *Mol. Cell. Biol.*
770 **13,** 2031-2040 (1993).
771 50. Lien, E. C., Dibble, C. C. & Toker, A. PI3K signaling in cancer: beyond AKT.
772 *Curr. Opin. Cell Biol.* **45,** 62-71(2017).
773 51. Pearce, L. R., Komander, D. & Alessi, D. R. The nuts and bolts of AGC protein
774 kinases. *Nat. Rev. Mol. Cell Biol.* **11,** 9-22 (2010).
775 52. Kobayashi, T. & Cohen, P. Activation of serum- and glucocorticoid-regulated
776 protein kinase by agonists that activate phosphatidylinositide 3-kinase is
777 mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2.
778 *Biochem. J.* **339,** 319-328 (1999).
779 53. García-Martínez, J. M. & Alessi, D. R. mTOR complex 2 (mTORC2) controls
780 hydrophobic motif phosphorylation and activation of serum- and
781 glucocorticoid-induced protein kinase 1 (SGK1). *Biochem. J.* **416,** 375-385 (2008).
782 54. Hosoda, E. et al. SGK regulates pH increase and cyclin B-Cdk1 activation to
783 resume meiosis in starfish ovarian oocytes. Preprint at https://doi.org/10.1101/499699
784 (2018).
785 55. Andjelković, M. et al. Activation and phosphorylation of a pleckstrin homology
786 domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein
787 phosphatase inhibitors. *Proc. Natl. Acad. Sci. USA* **93,** 5699-5704 (1996).
788 56. Virbasius, J. V. et al. Activation of the Akt-related cytokine-independent survival
789 kinase requires interaction of its phox domain with endosomal phosphatidylinositol
790 3-phosphate. *Proc. Natl. Acad. Sci. USA* **98,** 12908-12913 (2001).
791 57. Kohn, A. D., Takeuchi, F. & Roth, R. A. Akt, a pleckstrin homology domain
792 containing kinase, is activated primarily by phosphorylation. *J. Biol. Chem.* **271,**

793 21920-21926 (1996).
794 58. Lang, F. et al. (Patho)physiological significance of the serum- and
795 glucocorticoid-inducible kinase isoforms. *Physiol. Rev.* **86,** 1151-1178 (2006).
796 59. Bruhn, M. A., Pearson, R. B., Hannan, R. D. & Sheppard, K. E. AKT-independent
797 PI3-K signaling in cancer - emerging role for SGK3. *Cancer Manag. Res.* **5,** 281-292
798 (2013).
799 60. Liu, D., Yang, X. & Songyang, Z. Identification of CISK, a new member of the
800 SGK kinase family that promotes IL-3-dependent survival. *Curr. Biol.* **10,** 1233-1236
801 (2000).
802 61. Malik, N. et al. Mechanism of activation of SGK3 by growth factors via the Class
803 1 and Class 3 PI3Ks. *Biochem. J.* **475,** 117-135 (2018).
804 62. Tsao, P. I. & von Zastrow, M. Diversity and specificity in the regulated endocytic
805 membrane trafficking of G-protein-coupled receptors. *Pharmacol. Ther.* **89,** 139-147
806 (2001).
807 63. Terasaki, M., Okumura, E., Hinkle, B. & Kishimoto, T. Localization and
808 dynamics of Cdc2-cyclin B during meiotic reinitiation in starfish oocytes. *Mol. Biol.*
809 *Cell.* **14,** 4685-4694 (2003).
810 64. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of
811 bacteriophage T4. *Nature* **227,** 680-685 (1970).
812 65. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from
813 polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc.*
814 *Natl. Acad. Sci. USA* **76,** 4350-4354 (1979).
815 66. Kishimoto, T. Microinjection and cytoplasmic transfer in starfish oocytes.
816 *Methods. Cell Biol.* **27,** 379–394 (1986).

817



818

819 **Fig. 1** SGK is activated after 1-MeAde stimulation in a PDK1- and TORC2-dependent
820 manner. **a** The region of sfAkt used to raise the anti-phospho-A-loop antibody (upper,
821 Lys304–Pro320 of sfAkt) is aligned with the corresponding region of sfSGK
822 (Lys301–Pro317). The arrowhead indicates the residue phosphorylated by PDK1
823 (Thr315 in sfAkt and Thr312 in sfSGK). Dots indicate identical residues. **b** A-loop
824 phosphorylation of endogenous sfAkt is undetectable level in whole oocyte extracts.
825 Uninjected oocytes and oocytes injected with mRNA encoding untagged sfAkt were
826 treated with 1-MeAde, and analyzed by immunoblotting with the anti-phospho-A-loop
827 (pA-loop) and anti-sfAkt-C (Akt) antibodies. Closed and open arrowheads indicate the
828 positions of Akt and A-loop-phosphorylated SGK (see **e** and **f**), respectively. **c, d**

829 A-loop phosphorylation of endogenous Akt is detectable in immunoprecipitates.
830 Oocytes were incubated with 1-MeAde in the presence or absence of a PDK1 inhibitor,
831 BX795, lysed, and subjected to immunoprecipitation with control IgG or the
832 anti-sfAkt-C antibody. Input and flow-through (FT) extracts (**c**), and precipitates (**d**)
833 were analyzed by immunoblotting (Akt-pHM represents phosphorylation of Ser477 in
834 the HM of Akt). A-loop phosphorylation of non-specifically included SGK was
835 detected in control and Akt immunoprecipitates (**d**, open arrowhead). **e** Unstimulated
836 and 1-MeAde-treated oocytes were subjected to immunoblotting with
837 anti-phospho-A-loop (left), anti-sfSGK-C (middle), and anti-sfAkt-C (right) antibodies.
838 **f** The anti-phospho-A-loop antibody against sfAkt cross-reacts with
839 A-loop-phosphorylated endogenous SGK. Unstimulated oocytes were treated with
840 1-MeAde. Immunoprecipitation was performed with control IgG or the anti-sfSGK-C
841 antibody. Input, flow-through, and precipitates were analyzed by immunoblotting. **g**
842 SGK is activated simultaneously with Akt activation. Oocytes were treated with
843 1-MeAde and collected at the indicated time points. Phosphorylation of Akt (Akt-pHM),
844 SGK (pA-loop), Cdc25 (pS188), and Cdk1 (pY15) were analyzed by immunoblotting.
845 GVBD occurred around 20 min. **h** SGK activation in starfish oocytes depends on TOR.
846 Unstimulated oocytes were injected with control IgG or the anti-TOR antibody, treated
847 with 1-MeAde, and analyzed by immunoblotting. The data are representative of two
848 independent experiments in **b**, **e**, and **f**; and three independent experiments in **c**, **d**, and **g**.
849 In all panels, closed and open arrowheads indicate the positions of Akt and
850 A-loop-phosphorylated sfSGK, respectively.

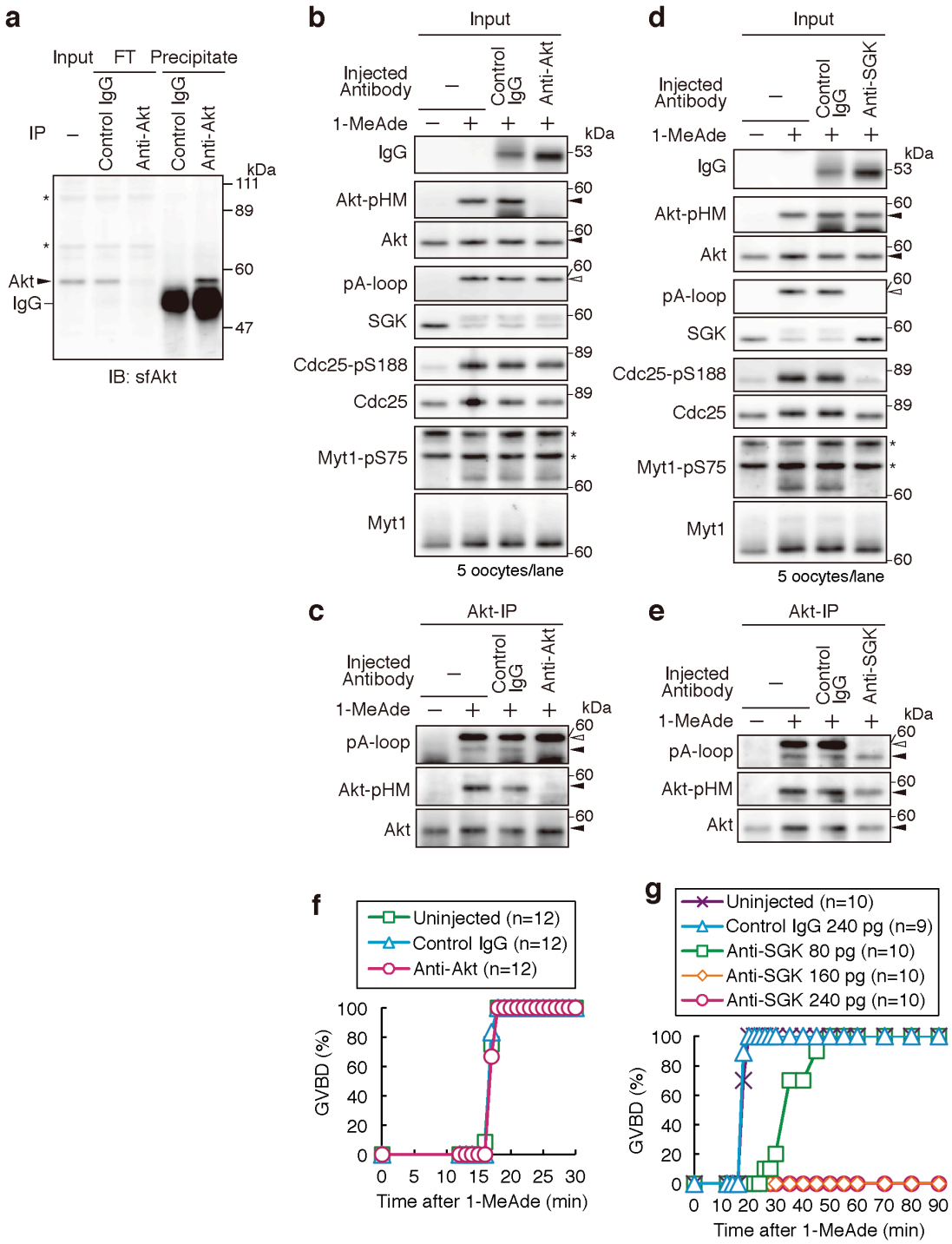


Fig. 2 SGK, but not Akt, is required for the meiotic G2/M transition. **a** Immunoprecipitation with control IgG or the anti-sfAkt-C antibody was performed using extracts prepared from unstimulated oocytes, followed by immunoblotting with the anti-sfAkt-C antibody. **b, c** The anti-sfAkt-C antibody inhibits activation of Akt. Unstimulated oocytes were injected with the anti-sfAkt-C antibody or control IgG and then treated with 1-MeAde for 4 min. Immunoprecipitation with the anti-sfAkt-C antibody was performed. Input extracts (**b**, Input) and precipitates (**c**, Akt-IP) were

859 analyzed by immunoblotting. **d, e** The anti-sfSGK-C antibody inhibits activation of
860 SGK. Unstimulated oocytes were injected with the anti-sfSGK-C antibody or control
861 IgG and then treated with 1-MeAde for 4 min. Extracts were prepared, and injected IgG
862 was removed by mixing samples with protein A-Sepharose beads. Thereafter, extracts
863 were subjected to immunoprecipitation with the anti-sfAkt-C antibody. Input extracts (**d**,
864 Input) and precipitates (**e**, Akt-IP) were analyzed by immunoblotting. **f** Akt is not
865 required for the meiotic G2/M transition. Unstimulated oocytes were injected with the
866 anti-sfAkt-C antibody or control IgG and then treated with 1-MeAde. GVBD was
867 monitored as a marker of the meiotic G2/M transition. The graph shows the percentage
868 of oocytes that had undergone GVBD by the indicated time points. "n" indicates the
869 number of oocytes observed. **g** SGK is required for the meiotic G2/M transition.
870 Unstimulated oocytes were injected with the indicated amount of the anti-sfSGK-C
871 antibody or control IgG and then treated with 1-MeAde. GVBD was monitored and
872 plotted as described in **f**. In all panels, open arrowheads indicate the position of
873 A-loop-phosphorylated SGK, closed arrowheads indicate the position of Akt, and
874 asterisks indicate non-specific bands. The data are representative of two independent
875 experiments in **a-c**, **f**, and **g**; and three independent experiments in **d** and **e**.

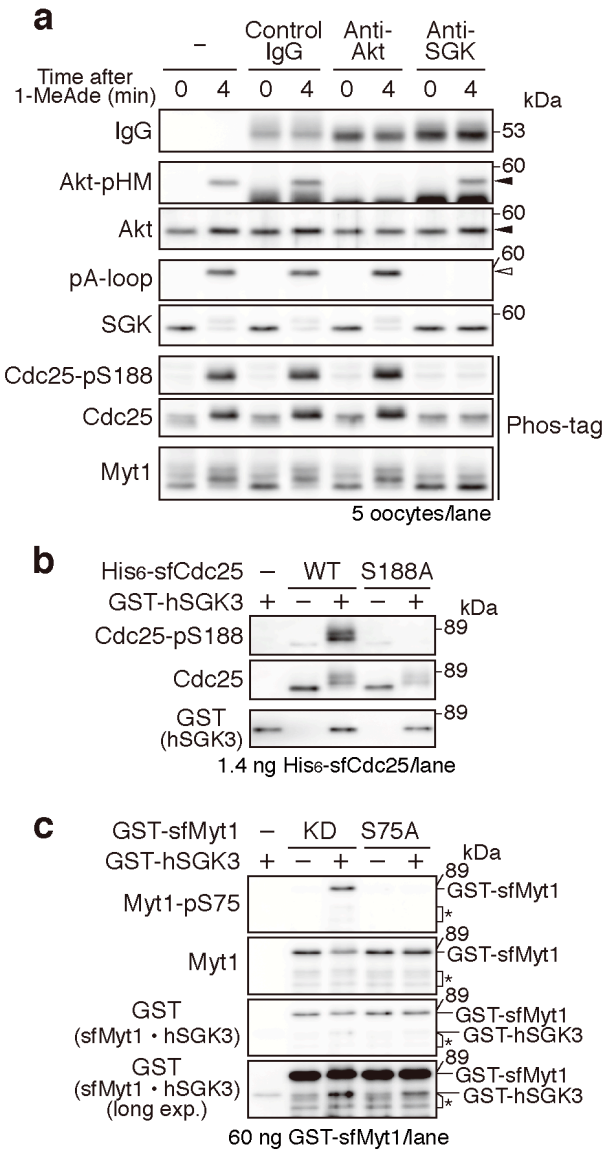


Fig. 3 SGK directly phosphorylates Cdc25 and Myt1. **a** SGK, but not Akt, is required for Cdk-independent phosphorylation of Cdc25 and Myt1 upon 1-MeAde stimulation. Unstimulated oocytes were injected with the indicated antibodies, treated with 1-MeAde in the presence of roscovitine, and then analyzed by immunoblotting using a normal or Phos-tag-containing SDS-PAGE gel. The open arrowhead indicates the position of A-loop-phosphorylated SGK, while the closed arrowheads indicate the position of Akt. **b** His-tagged recombinant wild-type (WT) sfCdc25 or the S188A mutant was incubated with or without GST-tagged active human SGK3 (hSGK3) in the presence of ATP and $MgCl_2$. Thereafter, immunoblotting was performed. **c** GST-tagged recombinant kinase-dead (KD) or the S75A mutant of sfMyt1 was phosphorylated by human SGK3 as described in **b**. Asterisks indicate minor fragments of recombinant Myt1. The data in all panels are representatives of each two independent experiments.

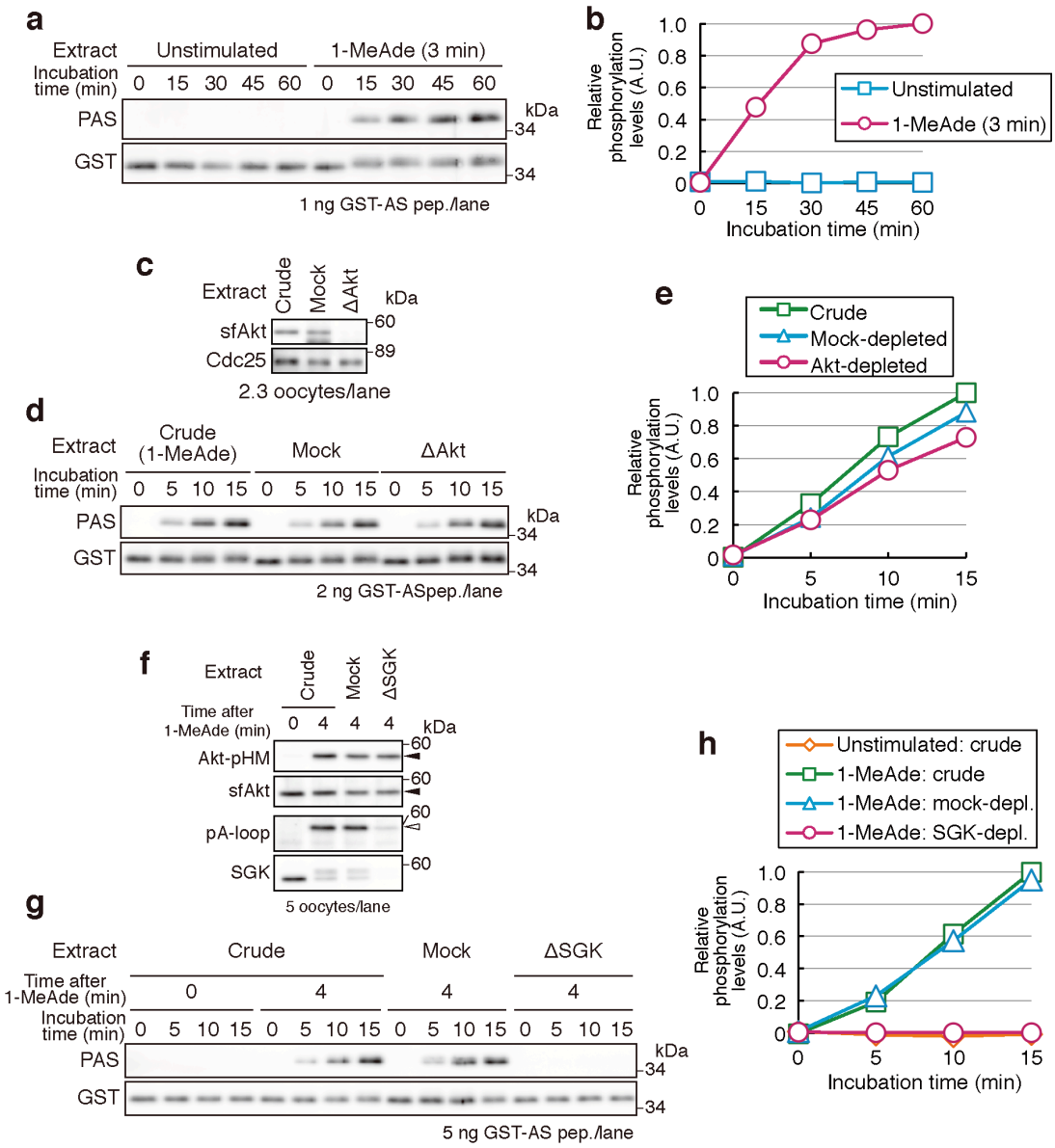


Fig. 4 SGK is a major kinase that phosphorylates the Akt/SGK consensus motif in starfish oocytes. **a, b** Kinase activity for the AS peptide is detectable in an extract of 1-MeAde-treated oocytes. Extracts were prepared from unstimulated or 1-MeAde-treated (3 min) oocytes and incubated with the GST-AS peptide for the indicated durations. Phosphorylation of the GST-AS peptide was detected by immunoblotting with an anti-pan-phospho-Akt/SGK substrate antibody (PAS), which recognizes phosphorylated Ser or Thr in the Akt/SGK consensus motif (**a**). Phosphorylation of the GST-AS peptide was quantitated from the immunoblots in **a** and normalized against the total amount of the GST-AS peptide. The graph shows the levels of phosphorylation relative to that at 60 min (**b**). **c–e** Akt is not responsible for the AS-peptide kinase activity in the extract. An extract was prepared from 1-MeAde-treated oocytes. Immunodepletion was performed with control IgG (mock) or

902 the anti-sfAkt-C antibody (ΔAkt). The crude and immunodepleted extracts were
903 analyzed by immunoblotting (**c**). The GST-AS peptide kinase assay was performed
904 using the extracts shown in **c** (**d**). Phosphorylation was quantitated as described in **a** and
905 **b**. The graph shows the levels of phosphorylation relative to that at 15 min in the crude
906 extract (**e**). **f–h** SGK is responsible for the AS-peptide kinase activity in the extract. An
907 extract was prepared from unstimulated or 1-MeAde-treated oocytes. Immunodepletion
908 was performed with the anti-sfSGK-C antibody (**f**), the GST-AS peptide kinase assay
909 was performed (**g**), and phosphorylation was quantitated (**h**) as described in **c**, **d**, and **e**.
910 The open arrowhead indicates the position of A-loop-phosphorylated SGK, while the
911 closed arrowheads indicate the position of Akt. The data in all panels are representatives
912 of each two independent experiments.

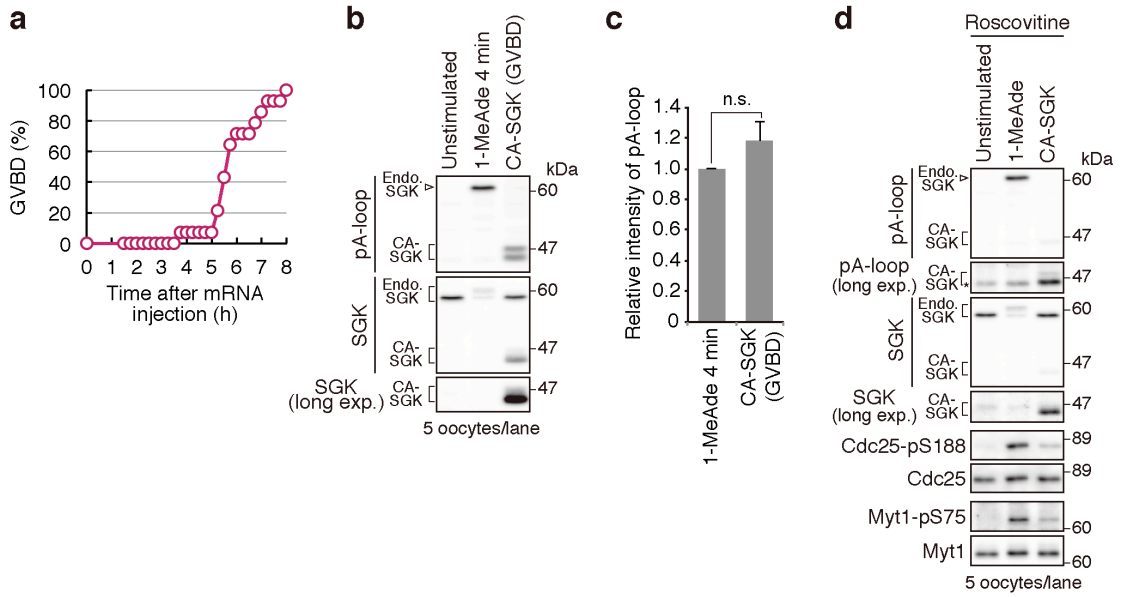


Fig. 5 SGK is sufficient for the meiotic G2/M transition. **a–c** CA-SGK expression induces the meiotic G2/M transition. Unstimulated oocytes were injected with mRNA encoding CA-SGK and incubated. GVBD in 14 oocytes was monitored every 15 min. The graph shows the percentage of oocytes that had undergone GVBD by the indicated time points (**a**). CA-SGK-expressing oocytes at GVBD, unstimulated oocytes, and 1-MeAde-treated oocytes were analyzed by immunoblotting (**b**). Phosphorylation levels of the A-loop of endogenous SGK in 1-MeAde-treated oocytes and CA-SGK at GVBD were quantitated from the immunoblots in **b**. The graph shows the relative phosphorylation levels (**c**). Data represent mean values ± standard deviation from three independent experiments. P = 0.06 (one-tailed t-test; not significant, n.s.). **d** CA-SGK expression induces regulatory phosphorylation of Cdc25 and Myt1 to trigger cyclin B-Cdk1 activation. Unstimulated oocytes were treated with 1-MeAde for 4 min in the presence of roscovitine. For expression of CA-SGK, unstimulated oocytes were injected with the mRNA, incubated with roscovitine, collected at the time point at which 50% of CA-SGK-expressing oocytes exhibited GVBD in the absence of roscovitine (5 h 45 min in **a**), and analyzed by immunoblotting. Note that, in the immunoblot of phospho-A-loop after a long exposure (pA-loop, long exp.), weak non-specific bands were detected at almost the same size as CA-SGK (asterisk). The data are representative of three independent experiments in **a**, **b**, and **c**; and two independent experiments in **d**.

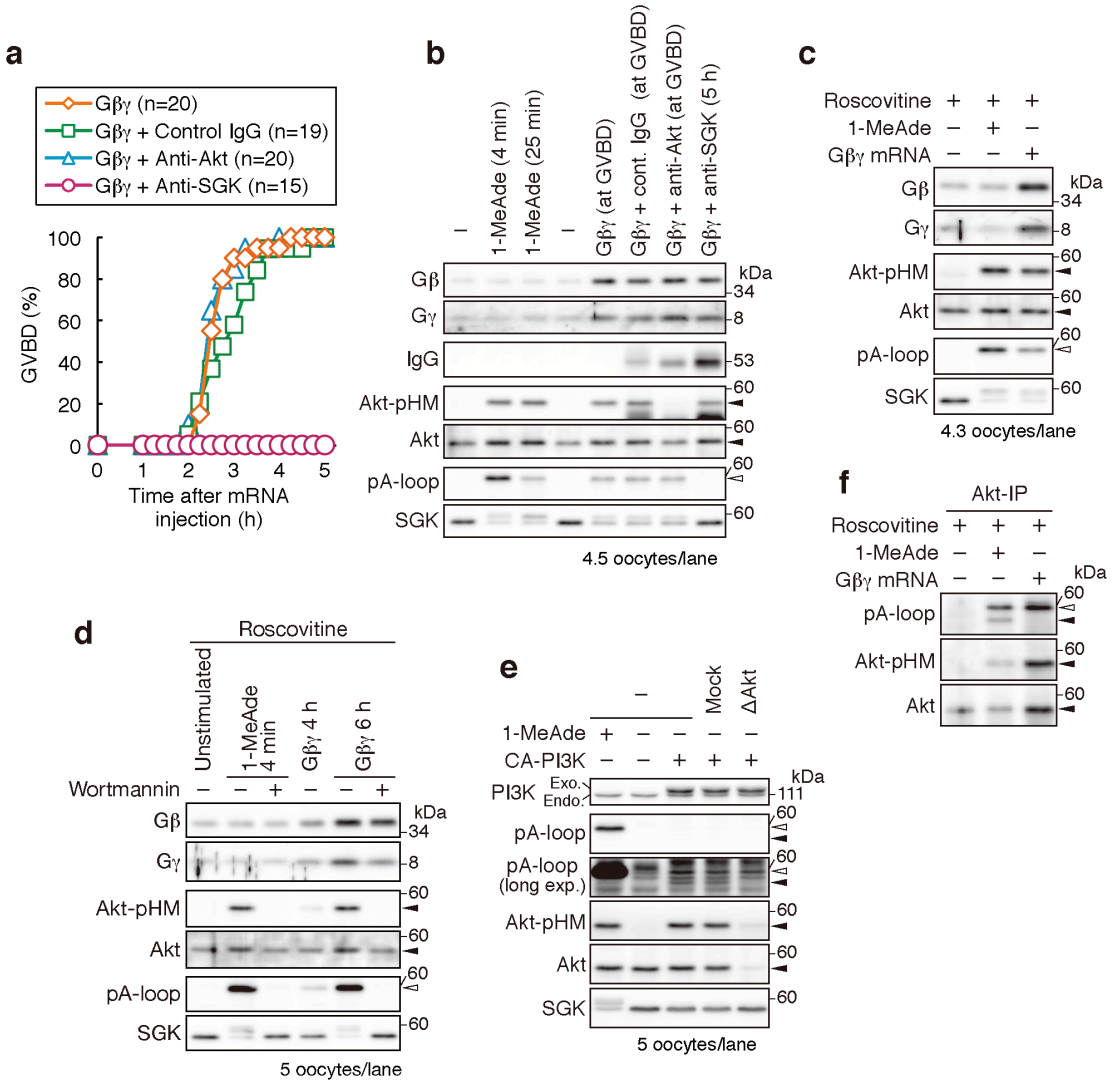


Fig. 6 SGK is activated by cooperation of the Gβγ-PI3K and atypical Gβγ pathways. **a, b** Exogenous expression of Gβγ induces the meiotic G2/M transition in a SGK-dependent manner. Unstimulated oocytes were injected with the indicated antibodies and subsequently injected with mRNA encoding Gβγ. Thereafter, GVBD was monitored (**a**). Oocytes were collected when GVBD occurred. Anti-sfSGK antibody-injected oocytes, which did not undergo GVBD, were collected at 5 h after mRNA injection. For 1-MeAde treatment, unstimulated oocytes were treated with 1-MeAde for 4 or 25 min (GVBD occurred at approximately 21 min) and then analyzed by immunoblotting (**b**). **c** Exogenous Gβγ expression induces SGK activation. Unstimulated oocytes were treated with 1-MeAde for 4 min in the presence of roscovitine or injected with mRNA encoding Gβγ and then incubated with roscovitine for 6 h. Thereafter, extracts were prepared from these oocytes and analyzed by immunoblotting. These extracts were also used for immunoprecipitation of Akt in **f**. **d** Gβγ-induced SGK activation requires PI3K. Unstimulated oocytes were treated with 1-MeAde or injected with mRNA encoding Gβγ, incubated in the presence of

949 roscovitine with or without wortmannin, and analyzed by immunoblotting. **e** PI3K is not
950 sufficient for activation of SGK. Unstimulated oocytes were treated with 1-MeAde for 4
951 min or injected with mRNA encoding CA-PI3K and incubated for 3 h. Extracts of
952 CA-PI3K-expressing oocytes were immunodepleted using control IgG (Mock) or the
953 anti-sfAkt-C antibody (ΔAkt). Thereafter, immunoblotting was performed. **f** Expression
954 of exogenous Gβγ fails to induce A-loop phosphorylation of Akt. Endogenous Akt was
955 immunoprecipitated from the extracts in **c** and analyzed by immunoblotting. In all
956 panels, open arrowheads indicate the position of A-loop-phosphorylated SGK, while
957 closed arrowheads indicate the position of Akt. The data are representative of two
958 independent experiments in **a**, **b**, **d**, and **e**; and three independent experiments in **c** and **f**.

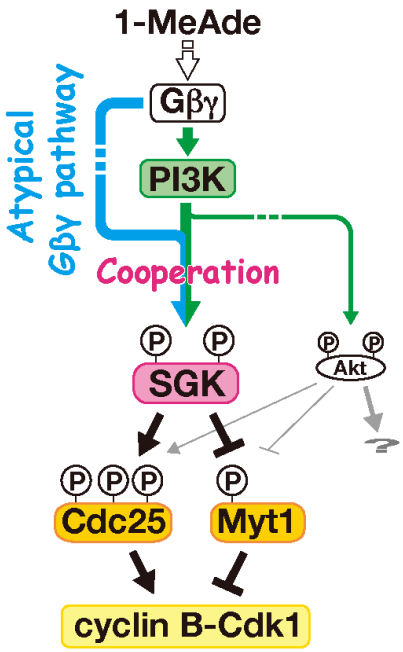


Fig. 7 SGK triggers cyclin B-Cdk1 activation in 1-MeAde signaling. Downstream of 1-MeAde stimulation, Gβγ activates the PI3K pathway (green arrow) and the atypical Gβγ pathway (cyan arrow). SGK is activated by cooperation of these pathways and subsequently activates and inactivates Cdc25 and Myt1, respectively, via direct phosphorylation. Consequently, reversal of the balance of Cdc25 and Myt1 activities triggers cyclin B-Cdk1 activation. Akt is weakly activated after 1-MeAde stimulation, and may phosphorylate Cdc25 and Myt1 at undetectable levels. However, Akt is dispensable for cyclin B-Cdk1 activation and may have any other function in oocytes.