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SGK1 is essential for meiotic resumption in mammalian oocytes

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Abstract

In mammalian females, oocytes are stored in the ovary and meiosis is arrested at the diplotene stage of prophase I. When females reach puberty oocytes are selectively recruited in cycles to grow, overcome the meiotic arrest, complete the first meiotic division and become mature (ready for fertilization). At a molecular level, the master regulator of prophase I arrest and meiotic resumption is the maturation-promoting factor (MPF) complex, formed by the active form of cyclin dependent kinase 1 (CDK1) and Cyclin B1. However, we still do not have complete information regarding the factors implicated in MPF activation.

In this study we document that out of three mammalian serum-glucocorticoid kinase proteins (SGK1, SGK2, SGK3), mouse oocytes express only SGK1 with a phosphorylated (active) form dominantly localized in the nucleoplasm. Further, suppression of SGK1 activity in oocytes results in decreased CDK1 activation via the phosphatase cell division cycle 25B (CDC25B), consequently delaying or inhibiting nuclear envelope breakdown. Expression of exogenous constitutively active CDK1 can rescue the phenotype induced by SGK1 inhibition. These findings bring new insights into the molecular pathways acting upstream of MPF and a better understanding of meiotic resumption control by presenting a new key player SGK1 in mammalian oocytes.

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Ethics statement
All animal work was conducted according to Act No 246/1992 for the protection of animals against cruelty; from 25.09.2014 no. CZ02389, issued by the Ministry of Agriculture.

CRediT authorship contribution statement
EDL and AS designed the experiments. EDL, AS and MK drafted and revised the manuscript. EDL was involved in all experiments and performed most of them. RY and DA performed oocyte microinjections. RY, MD, TM and MP prepared samples and polysomal fractions. ZJ sequences and analysed polysome bound RNA. AS and MK supervised the study.

Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2022.151210.

Keywords

Oocyte; MPF; CDK1; SGK1; Meiosis; Nuclear envelope breakdown

1. Introduction

In women, oocyte quality is an essential factor for a successful fertilization, pregnancy and embryo development. Consequently, poor oocyte quality is one of the most common hindrances to natural and assisted reproduction (Homer, 2020; Keefe et al., 2015; Krisher, 2004). Unlike somatic cells, oocytes undergo a meiotic cell division instead of mitosis. Therefore, in order to tackle poor oocyte quality, a better understanding of the mechanisms orchestrating the oocyte meiotic divisions is needed. In mammals, oocyte formation and entry into meiosis occur during the early stages of development, meaning that mammalian females are born with a determined pool of oocytes in their ovaries. Interestingly, the reserve of oocytes in the ovaries are arrested at the diplotene stage of prophase of the first meiotic cell division (prophase I) (van den Hurk and Zhao, 2005). At this stage, also referred to as the germinal vesicle (GV) stage, the chromatin is still not fully condensed and the nuclear envelope is intact and visible. This arrest continues until the female reaches puberty. From that point onwards, oocytes are selected in cycles to develop further and ovulate, resuming their meiotic cell divisions and becoming able to be fertilized (Edson et al., 2009).

The maturation-promoting factor (MPF) complex is the master regulator of this release from the prophase I arrest and subsequent meiotic resumption. It is a heterodimer composed of Cyclin Dependent Kinase 1 (CDK1) and Cyclin B1 (Gautier et al., 1990; Sharma et al., 2018; Pan and Li, 2019). Up to date several other proteins have been identified as regulators of MPF activity during meiosis, mainly related to the inhibitory phosphosites of CDK1 (Thr14 and Tyr15) and the amount of Cyclin B1 in the cell. In order to activate MPF, Cyclin B1 levels increase during M phase and the above-mentioned residues must be dephosphorylated. The prophase I arrest is maintained by protein kinase A (PKA), which activates the WEE1 kinase (which phosphorylates Thr14 and Tyr15) and inactivates CDC25 (responsible for dephosphorylating these inhibitory sites). At the time of ovulation, a drop in cGMP levels allows PDE3A to reduce cAMP in the oocyte. With low cAMP, PKA becomes inactive which ultimately results in the activation of MPF (Tripathi et al., 2010). Active MPF triggers meiotic resumption and the release of oocytes from the prophase I block characterized by nuclear envelope breakdown (NEBD), chromosome condensation and the subsequent first meiotic division (MI) (Norris et al., 2009; Sharma et al., 2018).

The Phosphoinositide 3-kinase/ Protein Kinase B (PI3K/AKT) pathway is also involved in meiotic resumption. In mammals, PI3K/AKT has been reported to be involved in Cyclin B1 expression and CDK1 activation (Roberts et al., 2002). Specifically, when AKT activity is suppressed in mouse oocytes, their meiotic resumption potential is significantly diminished (Kalous et al., 2006). Interestingly, in starfish oocytes Hiraoka et al. (2016a) observed that the PI3K/AKT pathway alone may not be enough to activate CDK1 and therefore other pathways should be involved. Later, the same group discovered that serum-glucocorticoid-regulated kinase (SGK) was indispensable for CDC25 phosphorylation and Myt1 inactivation (Hiraoka et al., 2019).

Until now, studies on SGK function in oocytes have been performed only on starfish (Hiraoka et al., 2019; Hosoda et al., 2019) leaving the role of SGKs in mammalian oocytes largely unknown. As SGK proteins are evolutionary conserved in mammals it is highly possible that they also have functional roles in higher animal species. In this study using the mouse model, we have shown for the first time the role of SGKs for the resumption of meiosis in mammalian oocytes.

Our results show that only SGK1 isoform is expressed in fully grown oocytes. Moreover, we demonstrate that SGK1 inhibition delays NEBD via negative influence of CDK1 activation. Our findings strengthen the hypothesis that SGK (SGK1 in mammals) is essential for MPF activation and oocyte meiotic resumption.

2. Results

2.1. Of the SGK genes only SGK1 is expressed in the mouse oocyte and its inhibition hinders nuclear envelope breakdown

In mammals, there are three genes coding for different SGK proteins: SGK1, SGK2 and SGK3. Although all proteins have a very similar structure, they differ in specific regions and their expression are dynamic throughout various tissues (Bruhn et al., 2010; Kobayashi et al., 1999; Lang and Cohen, 2001). To determine which SGKs are expressed in the mammalian oocyte, we first performed RT-PCR to verify the presence or absence of their respective mRNAs. The results showed that mRNAs coding for all SGKs are present in the mouse kidney and brain while ovaries and oocytes contain only *Sgk1* and *Sgk3* (Fig. S1A, B). Furthermore, we performed Western Blot (WB) to detect SGK protein expression in mouse oocytes. As expected, all SGK proteins were expressed in the mouse kidney while ovaries expressed SGK1 and SGK3. Interestingly, despite the presence of both *Sgk1* and *Sgk3* mRNAs in oocytes, only the SGK1 protein was expressed at similar levels throughout all oocyte maturation stages (Fig. 1A). Furthermore, polysomal datasets showed that *Sgk1* mRNA has the highest translation in the GV oocyte (Fig. S1C) while mRNA coding for SGK3 is absent. However, SGK3 translation was significantly increased in the 2 cell embryo (Fig. S1C).

The detection of SGK1 indicated its potential role in the oocyte. To unveil this role, we treated GV oocytes with a specific SGK1 inhibitor (GSK-650394, Merck, Darmstadt, Germany; Sherk et al., 2008) which restricts SGK1 activity (and SGK2 with less affinity) and has been already used in several fields of research (Berdel et al., 2014; Bomberger et al., 2014; Xiao et al., 2019). We applied concentrations of 0.01 mM and 0.03 mM of SKG1 inhibitor based on previously published results to keep cells viable for 48 h (Alamares-Sapuay et al., 2013). Initially, we validated the effect of the SGK1 inhibitor on oocytes by checking the phosphorylation status of the known SGK1 substrate NDRG1 (Thr346) (Murray et al., 2004). The results confirmed that the inhibitor treatment suppressed SGK1 activity, as phosphorylation of NDRG was significantly reduced (Figure S2). When SGK1 was inhibited, 88% of oocytes treated with 0.01 mM concentration underwent nuclear envelope breakdown (NEBD), which was similar as the control group, however, when treated with the 0.03 mM concentration, only 26% of oocytes underwent NEBD (Fig. 1B). Nonetheless, although most of the oocytes from the 0.01 mM group underwent NEBD, there was a significant delay compared to the control oocytes (211 ± 98 min and 67 ± 15 min, respectively) (Fig. 1C). To analyse the reversibility of the SGK1 inhibitor, oocytes were cultured in the presence of the inhibitor (0.01 mM) for one hour and then released. Those oocytes were able to undergo NEBD in 97 ± 14 min, that is, 30 min later than the control.

Moreover, we noticed a significant delay in polar body extrusion (PBE) when SGK1 was inhibited (Fig. 1D). To determine if this effect is due to the reported NEBD delay itself or whether SGK1 inhibition affects further meiotic stages, we introduced the SGK1 inhibitor at different time points during meiosis. The results show that SGK1 inhibition has a delaying effect on PB extrusion when oocytes were treated with the inhibitor up to four hours after IBMX removal (Fig. 1D). However, the timing of PBE was not affected when SGK1 was inhibited later (Fig. 1D).

In conclusion, our results show that only one member of the SGKs family (SGK1) is expressed in mouse oocytes and also suggest a role in the regulation of NEBD and PBE up to the first 4 h after meiotic resumption.

2.2. The active form of SGK1 is concentrated in the oocyte nucleus and its expression decreases along the first meiotic division

SGK1 becomes active when phosphorylated at Thr256 (Kobayashi and Cohen, 1999; Chen et al., 2009). To better understand the role of SGK1 in the mammalian oocyte we further focused on the localization of its active form by immunocytochemistry (ICC) at different meiotic stages. We found that SGK1 (Thr256) is dominantly localized in the nucleus of the GV oocyte and at the subsequently newly forming spindle (Fig. 2A and B). Similarly, the highest SGK1 phosphorylation levels were detected in the GV oocyte with a continuous significant decrease during meiotic progression to minimum in the MII stage (Fig. 2A and B). These results are in accordance with the previous live cell experiments, which show that SGK1 inhibition has its strongest effect on meiotic GV-NEBD transition (Fig. 1C and D).

2.3. Inhibition of SGK1 impairs CDK1 activation through CDC25B (Cell Division Cycle 25B) phosphatase in the oocyte prior to NEBD

The delay of NEBD caused by SGK1 inhibition pointed towards a possible effect of SGK1 on the master regulator of meiosis, CDK1. To test this hypothesis, we performed WB experiments to detect the inactive form of CDK1 (Tyr15) in oocytes in absence (control, DMSO 0.02%) or presence of SGK1 inhibitor (0.01 mM) at different time points after an IBMX wash (0, 30 and 60 min) (Fig. 3A and B). It is well known that the phosphorylation of CDK1 at Tyr15 must be removed to activate the kinase in order to resume meiosis (Coleman and Dunphy, 1994; Schmidt et al., 2017). The GV arrested oocyte group (0 min) was incubated for one hour in the presence of IBMX and treated with SGK1 inhibitor (or DMSO). Both groups showed maximal levels of CDK1 (Tyr15) as expected without any major differences. However, after 30 min post IBMX wash, inactive CDK1 levels (phosphorylated on Tyr 15) significantly decreased in non-treated oocytes while oocytes in

SGK1 inhibitor continued to show high levels. After one hour, the differences were even more pronounced between the two groups, as non-treated oocytes were already at the NEBD stage and treated oocytes were still at the GV stage (Fig. 3A and B).

As SGK1 is a protein kinase and CDK1 activation occurs through dephosphorylation (of Thr14 and Tyr15), we hypothesised that it must act through other proteins. Based on the literatures (Cazales et al., 2005; Pirino et al., 2009; Hiraoka et al., 2016b) and our *in silico* prediction interaction (Supplementary Table 1) the phosphatase CDC25B (which is known to dephosphorylate CDK1 on Tyr 15) proved to be a potential candidate as an SGK1 substrate. Therefore, we conducted a similar WB analysis to detect the activation of CDC25B phosphatase. The obtained results were in positive correlation with the previously detected activity of CDK1 (Fig. 3A and B). Arrested GV oocytes (0 min) showed no difference between the control and SGK1 inhibition with regards to the level of total CDC25B nor to its phosphorylation state (represented by two shifted bands) (Fig. 3C and D). However, when the oocytes were released from the IBMX block, differences became apparent. After 30 min, the lower band of control oocytes was fainter in comparison with SGK1 inhibited oocytes, indicating the activation of CDC25B. This shift was even more profound at 45 min after meiotic resumption, when a new higher band (representing the hyperphosphorylated CDC25B) appeared in control oocytes, while the lowest (hypophosphorylated) band disappeared. On the other hand, the oocytes cultured in the presence of SGK1 inhibitor still showed the presence of the lower hypophosphorylated band without any apparent hyperphosphorylated band visible (Fig. 3C and D).

These results indicate that SGK1 plays a regulatory role in CDK1 activation and meiotic resumption upstream of CDC25B, and that CDC25B may in fact be its direct substrate.

2.4. The phenotype resulting from SGK1 inhibition can be reversed by activation of CDK1

Based on the above presented data which show that activation of CDK1 by SGK1 inhibition is negatively influenced (Fig. 3A and B) and that CDK1 activation is a key event for the timing and promoting of NEBD (Koncicka et al., 2018), we sought to confirm that the SGK1 effect in oocytes is upstream of CDK1 activation. To that end, we microinjected oocytes with mRNA coding for CDK1-AF, a constitutively active CDK1 which cannot be phosphorylated on Tyr15 or Thr14 and therefore, allows oocytes to overcome meiotic arrest even in the presence of IBMX (Fig. 4A) (Adhikari et al., 2016; Akaike and Chibazakura, 2020; Hagting et al., 1998). WB of injected oocytes confirmed that the CDK1 protein was overexpressed compared to non-injected controls (Figure S3). First we expressed CDK1-AF in oocytes by microinjecting RNA coding for CDK1-AF + H2B-GFP in the presence of a higher concentration of SGK1 inhibitor (0.03 mM) as seen in Fig. 1B. The meiotically arrested phenotype caused by SGK1 inhibition was successfully overcome after CDK1-AF overexpression as 83% (± 0.35) of these oocytes went through NEBD compared to only 27% (± 0.75) of control oocytes (microinjected with RNA coding for H2B-GFP) (Fig. 4A).

Next, we performed experiments with a smaller concentration of SGK1 inhibitor (0.01 mM), which caused oocyte meiotic resumption delay (Fig. 1C). For this experiment, one group of oocytes was microinjected with *H2b-gfp* RNA as a negative control and was cultivated in the presence of SGK1 inhibitor. The other two groups were microinjected with *Cdk1-AF* +

H2b-gfp RNA; one group was cultivated in the presence of a solvent vehicle (0.02% DMSO) and another group in the presence of SGK1 inhibitor. All oocyte groups were arrested at the NEBD stage for 4 h in the presence of IBMX after microinjection. After IBMX release, oocytes without expression of the constitutively active form of CDK1 showed a significant NEBD delay (214 ± 15 min) similarly as seen in Fig. 1C. On the other hand, oocytes expressing CDK1-AF underwent NEBD significantly faster (127 ± 8 min) even in the presence of SGK1 inhibition (Fig.4B). Altogether, these results suggest a role of SGK1 in the regulation of NEBD in mammalian oocytes by influencing the regulatory pathway involved in CDK1 activation.

3. Discussion

Oocyte meiotic arrest and timely resumption are fundamental steps in mammalian meiosis. After much research, MPF has been accepted as a master regulator of such events. However, so far only a few key elements have been identified and described in detail as being involved in the MPF pathway (Edson et al., 2009, 2019). Data of the present study suggest SGK1 as a new player in mammalian oocyte meiotic resumption which is of great importance for the better understanding of the regulation of meiosis.

Up to now, no SGK protein has ever been linked to the process of meiosis (Bruhn et al., 2010; Lien et al., 2017; Di Cristofano, 2017). Only the recent studies by Hiraoka et al. (2019) have demonstrated that SGK protein was needed to overcome prophase I arrest at the GV stage in starfish oocytes (*Asterina pectinifera*). According to their work, SGK phosphorylates and activates CDC25, which in turn leads to the activation of MPF (cyclinB-CDK1) so the oocyte can proceed through the G2/M phase and continue meiosis. However, there are no reports on the matter outside of the starfish and, despite its advantages to study early reproduction, it is evolutionary far from vertebrates including humans. Therefore, our study provides much-needed information by focusing on the SGK role in mammals using the mouse model (*Mus musculus*).

Compared to the starfish whose genome codes for a single SGK protein, the mouse genome contains three different genes coding for three known SGK isoforms (SGK1, SGK2 and SGK3). These proteins share a sequence identity of 80% in their catalytic domain but only SGK3 contains an N-terminal phosphoinositide-binding Phox homology (PX) domain (Bruhn et al., 2010; Kobayashi et al., 1999; Lang and Cohen, 2001). Interestingly, despite their high similarity, SGKs have different tissue expression: SGK1 and SGK3 seem to be found in all tissues but tightly regulated, whereas SGK2 expression is dominant in the liver, pancreas, brain and kidney (Kobayashi et al., 1999). Accordingly, our results document the presence of *Sgk1* and *Sgk3* mRNAs in both ovaries and oocytes. However, despite both SGK1 and SGK3 proteins being expressed in mouse ovaries, we detected only the SGK1 in mouse oocytes. These results correlate with previously published oocyte translatome data (del Llano et al., 2020; Masek et al., 2020). Importantly, *Sgk3* mRNA is absent from oocyte polysomes but it starts to have a stronger polysomal presence after fertilization (Masek et al., 2020; Potireddy et al., 2006) and Figure S1C concomitantly with its transcription (Zeng et al., 2004). This suggests SGK1 as the sole isoform present in mouse oocytes and functioning in meiosis regulation while SGK3 is become translated after fertilization.

Hiraoka et al. (2019) speculated that SGK3 could be involved in mammalian oocyte meiosis based on the fact that it is the isoform most related to starfish SGK as both contain the N-terminal PX domain. In their experiments SGK was knocked-down from starfish oocytes causing a perpetually arrested GV phenotype which was later successfully reversed by exogenously expressing human SGK3. However, our findings suggest that SGK1 and not SGK3 is present in mouse oocytes. This seeming contradiction might be explained by the fact that only the catalytic domain may have a role in oocyte meiosis from both starfish and mammals, whereas the N-terminal PX domain would be irrelevant. This indicates that both SGK1 and SGK3 with 80% similarity of the catalytic domain can phosphorylate similar targets (Kobayashi et al., 1999; Bruhn et al., 2010). Therefore, it would be interesting to repeat Hiraoka et al. (2019) rescue experiments expressing human SGK1 or SGK2 instead of SGK3 and analyse the effect on oocyte meiosis. This could prove the conclusion that the N-terminal PX domain is not necessary for oocyte meiotic resumption.

To investigate the potential role of SGK1 in the fully grown mammalian GV oocyte, we decided to perform several experiments using a selective SGK1 inhibitor (GSK-650394). The inhibitor concentrations of 0.01 mM and 0.03 mM were selected according to a previously published study reporting them as being able to keep cells viable for 48 h (Alamares-Sapuay et al., 2013). Surprisingly, our results after SGK1 inhibition at 0.03 mM were similar to those obtained by Hiraoka et al. (2019): meiotic resumption (G2/M transition) was suppressed and most oocytes did not continue through NEBD. In other words, selective SGK1 inhibition in mammalian oocytes had a similar effect as inhibition of SGK in starfish oocytes. Our results reinforce the essential role of SGK in meiotic resumption in both starfish and mouse oocytes. Moreover, smaller amounts of SGK1 inhibitor (0.01 mM) allowed oocytes to go through NEBD but at a much slower pace, pointing out that even small amounts of SGK1 can phosphorylate the necessary levels of G2/M transition key players if given enough time. The inhibitory effect was fully reversible for both concentrations as removing the inhibitor from the media allowed the oocytes to reach the MII stage. It is also noteworthy to mention that in our previous research we showed that these oocytes which underwent the first meiotic division in the presence of the inhibitor at 0.01 mM suffered from significantly abnormal cytokinesis (del Llano et al., 2020). Whether these abnormalities are the result of SGK1 acting on the oocyte spindle itself or the result of a delayed meiotic resumption is not clear and needs further investigation, however, the new results presented here point towards the latter possibility.

Furthermore, we were able to uncover the time window of action of SGK1 in meiotic resumption thanks to the slower meiotic division caused by SGK1 inhibitor (0.01 mM). By adding inhibitor at different time points and following the timing of PB extrusion we concluded that SGK1 activity is necessary up to 4 h after meiotic resumption. Nonetheless, its role is most relevant at the beginning of the resumption of meiosis.

At the molecular level, we found that the cause of meiotic arrest (or delay) in GV oocytes treated with SGK1 inhibitor was caused by a failure in MPF activation, more specifically by impeding the removal of the inhibitory Tyr15 phosphorylation of CDK1. However, as a protein kinase, SGK1 cannot act directly to dephosphorylate CDK1. To that end, we further investigated and proved that SGK1 inhibition also had an effect on CDC25B activation, the upstream phosphatase of CDK1 at Tyr15 (Cazales et al., 2005; Pirino et al., 2009; Hiraoka et al., 2016b). This also positively correlated with the data on SGK in starfish oocytes, where it was proven that SGK inhibition blocked meiotic resumption by preventing the activation of CDC25 and therefore MPF remained inactive (Hiraoka et al., 2019). Surprisingly, we observed that fully-grown GV oocytes already displayed high levels of active SGK1 (phosphorylated at Thr256). At this stage, the activator phosphosites of CDC25B are not yet phosphorylated and it is not until oocytes are released from a high cAMP environment that they are “allowed” to be phosphorylated (Coleman and Dunphy, 1994; Norris et al., 2009). The fact that SGK1 is active already in the GV oocyte might seem contradictory at first glance as it could be able to keep CDC25B phosphorylated and active the whole time. However, we also noticed that at that stage SGK1 (Thr256) is strongly localized in the nucleus, while CDC25B is known to remain in the cytoplasm before meiotic resumption and it is not until PKA is inhibited (by low cAMP levels) that CDC25B is quickly translocated to the oocyte nucleus right before NEBD (Lincoln et al., 2002; Solc et al., 2008; Ferencova et al.,). Therefore, we hypothesize that SGK1 (Thr256) is active but restricted to the nucleus, which keeps it physically apart from CDC25B, which would further activate it. When cAMP levels decline CDC25B translocates to the nucleus, where SGK1 (Thr256) could phosphorylate and activate it, allowing the further dephosphorylation of CDK1 inhibitory sites. This makes MPF active and capable to induce meiosis resumption (Fig. 5). Our hypothesis can be further strengthened by the fact that SGK1 and CDC25B display a high degree of interaction potential according to the online software PSOPIA. However, it must be taken into account that the evidence presented here together with the published data on starfish oocytes, are indirect and need to be addressed more specifically to be fully proven. Otherwise, despite the clear relation between SGK1 and CDC25B in oocytes, it is not possible to exclude the possibility that they do not interact directly but that there is a longer pathway, which connects them both through other proteins.

Furthermore, it is important to note that research groups studying SGK1 in kidneys reported that mouse homozygous knockouts for SGK1 are subfertile (Fejes-Tóth et al., 2008; Faresse et al., 2012). On one hand, this highlights the potential importance of this protein in female reproductive cells, adding support to our data. On the other hand, however, it is not possible to exclude that the effect on litter size was due to SGK1 absence affecting other reproductive tissues (testes, ovary, uterus, etc.) as the mice were full KO.

In summary, we present evidence that SGK1 has an important and previously unknown role in mammalian meiosis, specifically for the process of meiotic resumption. We suggest SGK1 acts through the phosphorylation of CDC25B, which ultimately leads to MPF activation. This role might be extrapolated to other species as it seems to be evolutionary conserved between the mouse and starfish. This research contributes to further understanding of the pathways controlling MPF, the master regulator of oocyte meiotic resumption.

4. Material and methods

4.1. Oocyte collection and culture

ICR mice (bred in-house) were injected 46 h prior to oocyte collection to be primed with 5 IU pregnant mare serum gonadothropin (PMSG HOR 272, ProSpec, Rehovot, Israel). All oocytes were collected at the GV stage from the mice ovaries in the presence of transfer media supplemented with 100 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, Darmstadt, Germany) to block meiotic resumption (as described in Tetkova and Hancova, 2016). From the GV collected oocytes, only the fully grown were selected, denuded by pipetting and transferred to M16 media (Sigma-Aldrich, Darmstadt, Germany) with IBMX at 37 °C, 5% CO2. For oocyte samples at further advanced meiotic stages than GV, the oocytes were placed in M16 media (Sigma-Aldrich, Darmstadt, Germany) at 37 °C, 5% CO2 without IBMX. For SGK1 inhibitor treatments, the oocytes were transferred in M16 media (without IBMX) supplemented with 0.02% or 0.06% Dimethyl Sulphoxide (DMSO) for solvent vehicle control or 0.01 mM or 0.03 mM GSK-650394 (Merck, Darmstadt, Germany) inhibitor.

All animal work was conducted according to Act No 246/1992 for the protection of animals against cruelty; from 25.09.2014 no. CZ02389, issued by Ministry of Agriculture.

4.2. Live cell imaging

Oocytes were transferred from M16 media to a 4-well culture chamber (Sarstedt, Prague, Czech Republic) in 15 μl of M16 covered with mineral oil (M8410; Sigma-Aldrich) so they could be cultivated further under an inverted microscope Leica DMI 6000B (Leica Microsystems, Wetzlar, Germany) under the same culture conditions (Tempcontroller 2000–2 Pecon, and a CO2 controller, Pecon, Erbach, Germany) and monitored live. The live cell time lapse images were taken using LAS X software (Leica microsystems, Wetzlar, Germany) every 5 and 15 min.

4.3. RNA isolation and RT-PCR

RNA was extracted from oocytes using RNeasy Plus Micro kit (74034, Qiagen, Hilden, Germany) which includes a step for genomic DNA depletion using gDNA Eliminator columns. Afterwards, RT-PCR was performed using a qPCRBIO cDNA synthesis kit (PCR BIOSYSTEMS, London, UK). For regular PCR the PPP Mastermix kit (Top-Bio, Vestec, Czech Republic) was used. Primer sequences are listed in Table S2A.

4.4. Immunoblotting

Oocyte samples were lysed using 10 ul 1x Reducing SDS Loading Buffer (lithium dodecyl sulphate sample buffer NP 0007 and reduction buffer NP 0004 [Thermo Fisher Scientific, Waltham, MA, USA]) and heated at 100 °C for 5 min. Separation of proteins was carried out in gradient precast 4–12% SDS–PAGE gels (NP 0323, Thermo Fisher Scientific) and transferred onto an Immobilon P membrane (IPVD 00010, Millipore, Merck group, Darmstadt, Germany) using a semidry blotting system (Biometra GmbH, Analytik Jena, Jena, Germany) for 25 min at 5 mA per cm^{-2}. Blocking was done using 5% skimmed milk dissolved in 0.05% Tween-Tris buffer saline (TTBS) with pH 7.4 for 1 h. The membranes were then briefly washed with TTBS and incubated with 1% milk/TTBS diluted primary antibodies (see Table S2B) at 4 °C O/N. Secondary antibodies, Peroxidase Anti-Rabbit Donkey and Peroxidase Anti-Mouse Donkey (711–035–152 and 715–035–151, Jackson ImmunoResearch, West Grove, PA, USA) were diluted 1:7500 in 1% milk/TTBS. Membranes were incubated with secondary antibodies for 1 h at room temperature. Proteins were visualised by chemiluminescence using ECL (Amersham) and imaged on Azure 600 Imager (Azure Biosystems) and acquired signals were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

4.5. Immunocytochemistry

Oocytes were fixed in 4% paraformaldehyde (PFA, Alfa Aesar, Thermo Fisher Scientific, Waltham, MA, USA) in PBS/PVA and left for 15 min followed by permeabilization in 0.1% Triton (X-100, Sigma-Aldrich) PBS/PVA for 10 min. The oocytes were then washed in PBS/PVA and incubated with primary antibodies (see Table S2B) at 4 °C O/N. The next day, two washes in PBS/PVA were applied followed by incubation with the corresponding secondary antibody and conjugation with Alexa Fluor 488 or 647 (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature protected from light. Next, the oocytes were washed in PBS/PVA twice and mounted on glass slides using ProLong™ Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). Images of samples were taken with a Leica SP5 inverted confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were assembled in software LAS X (Leica Microsystems) and signal intensity from the spindle area was quantified with ImageJ.

4.6. RNA synthesis and microinjection

Cdk1-AF and *H2b:gfp* RNAs were in vitro transcribed by using the correspondent plasmid templates (Cdk1-AF: pcDNA3-CDC2-AF (718) was a gift from Jonathon Pines (Addgene plasmid # 39872; http://n2t.net/addgene:39872; RRID: Addgene 39872); H2B-GFP: provided by Dr Martin Anger, Laboratory of Cell Division Control, IAPG CAS) and mMESSAGE mMACHINE™ Transcription Kit (Invitrogen, Carlsbad, CA, USA). In vitro transcribed RNA was then injected into GV oocytes at a final concentration of 50 ng/μl in the presence of transfer media and IBMX. Microinjection of GV oocytes was performed using FemtoJet (Eppendorf) and TransferMan NK2 (Eppendorf, Hamburg, Germany) using an inverted microscope Leica DMI 6000B (Leica Microsystems, Wetzlar, Germany). Afterwards, injected oocytes in IBMX were incubated at 37 °C, 5% CO2 for 6 h to give them enough time to translate the injected RNAs.

4.6.1. Polysome fractionation and RNA sequencing—Polysome fractionation followed by RNA isolation was carried out according to the Scarce Sample Polysome profiling (SSP-profiling) method from Masek et al. (2020). Then, polysomal fractions (P; fractions 6–10) were pooled and subjected to qRT-PCR (QuantStudio 3 cycler, Applied Biosystems). Sequencing libraries were prepared using SMART-seq v4 ultra low input RNA kit (Takara Bio). Sequencing was performed with HiSeq 2500 (Illumina) as 150-bp paired-ends. Reads were trimmed using Trim Galore v0.4.1 and mapped onto the mouse GRCm38 genome assembly using Hisat2 v2.0.5. Gene expression was quantified as fragments per kilobase per million (FPKM) values in Seqmonk v1.40.0.

4.7. Statistical Analysis

Data are mean ± standard error of mean (SEM) of (n) replicates. All percentage data are first subjected to arcsine squire-root transformation and then subjected to statistical analysis. Data were analyzed either by Student’s t- test or One-way ANOVA using GraphPad Prism

Software (San Diego, California, USA) with post-hoc analyses with a 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

No data was used for the research described in the article.

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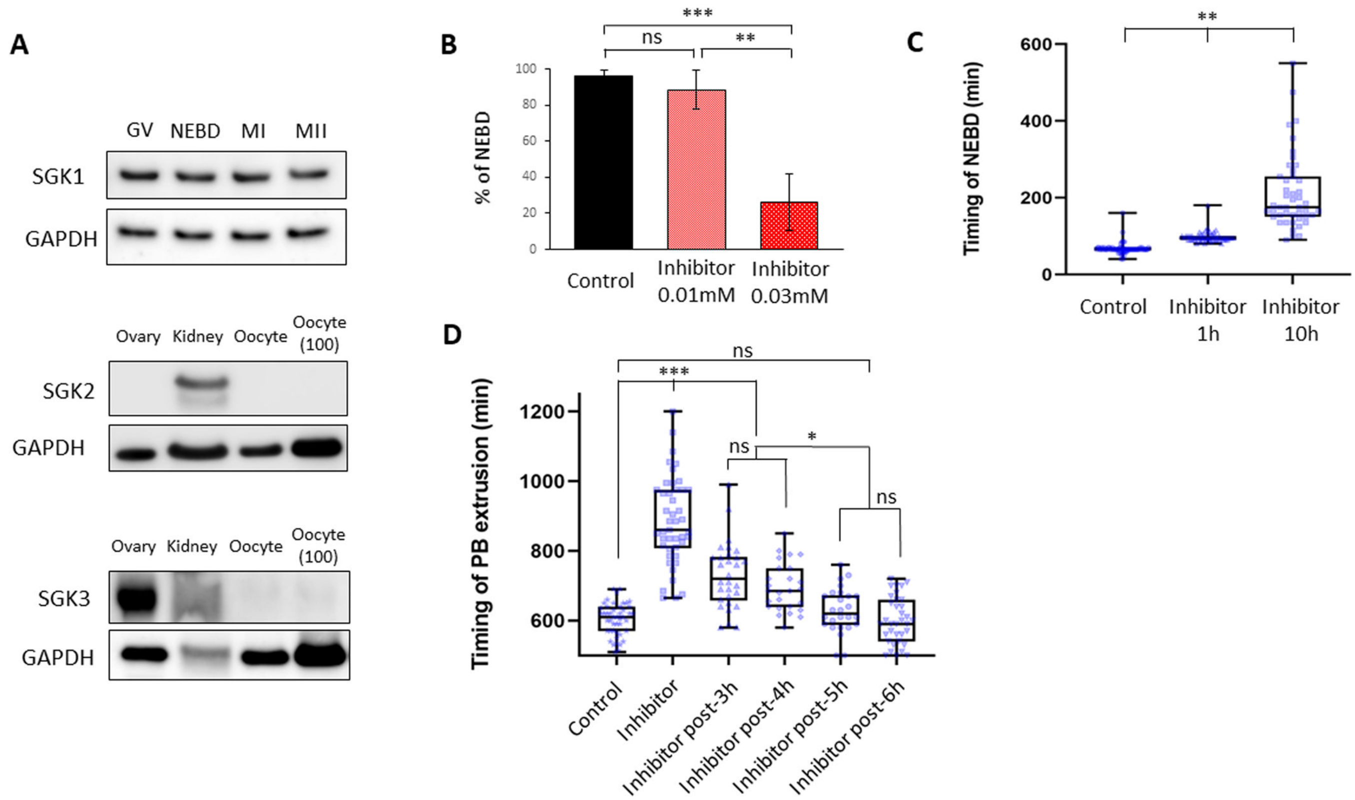

Fig. 1.

Of the SGK genes only SGK1 is expressed in the mouse oocyte and its inhibition hinders nuclear envelope breakdown. A) WB analysis of the expression of the three SGK proteins in oocytes (30 or 100 per sample) and control tissue; GAPDH was used as loading control. The images are representative from at least three biological replicates. For mRNA expression see Fig. S1. B) Quantification of oocytes undergoing nuclear envelope breakdown (NEBD) in the control (0.06% vehicle, DMSO) and presence of SGK1 inhibitor (GSK-650394). Data are represented as the mean ± SEM of at least three independent experiments; $n \geq 44$ oocytes; ns, not significant; *** $p < 0.001$; ** $p < 0.01$ according to One-way ANOVA after arcsine transformation. For inhibitor validation see Fig. S2. C) Timing of oocyte NEBD after IBMX wash in absence (Control, 0.06% vehicle DMSO) or presence of SGK1 inhibitor (0.01 mM) for 10 h and 1 h. Box plot displays mean, 25th and 75th percentile and ± SD of at least three independent experiments; $n \geq 45$ oocytes; ns, not significant; ** $p < 0.01$ according One-way ANOVA. D) Timing of oocyte cytokinesis (polar body extrusion) in absence (control, 0.06% vehicle DMSO) or presence of SGK1 inhibitor (0.01 mM) added at different time points post-IBMX wash. Box plot displays mean, 25th and 75th percentile and ± SD of at least three independent experiments; $n \geq 26$ oocytes; ns, not significant; * $p < 0.05$; *** $p < 0.001$; according One-way ANOVA.

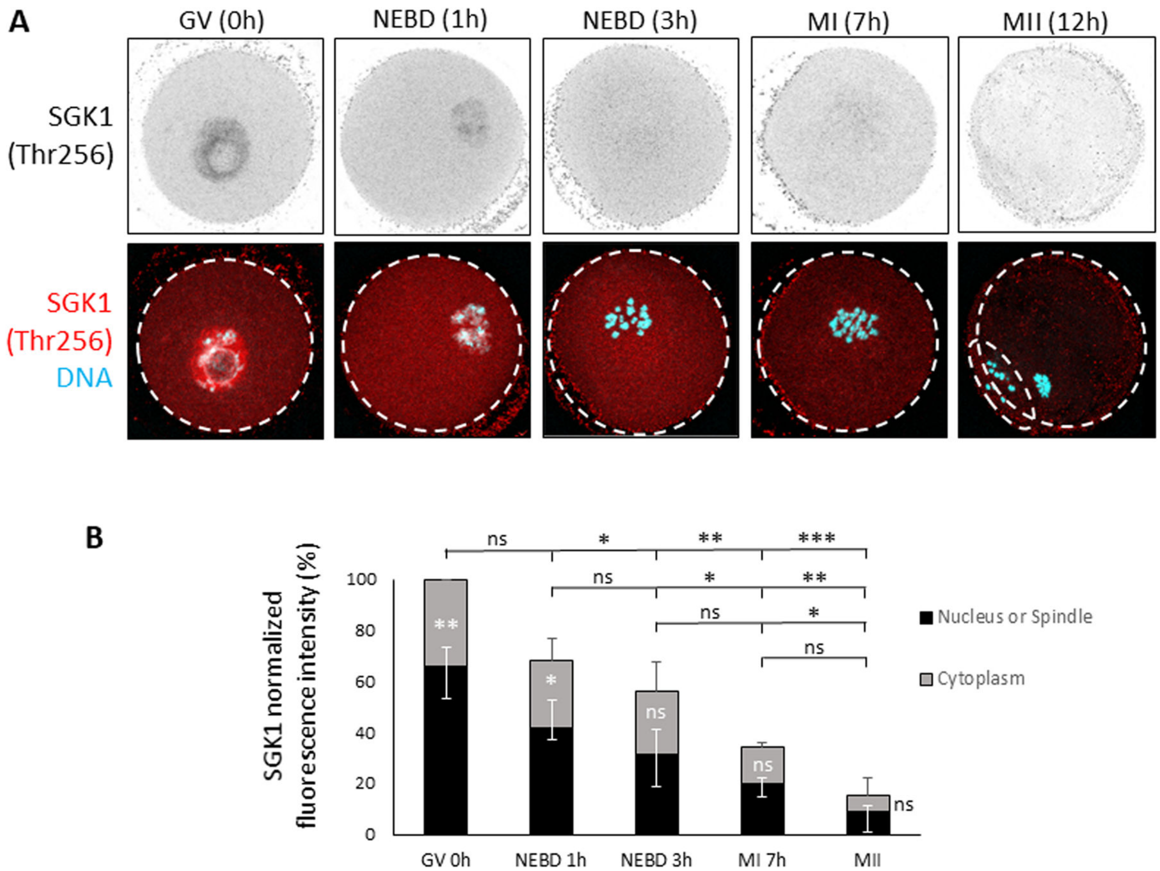

Fig. 2.

Active SGK1 is concentrated in the oocyte nucleus and its expression decreases along the first meiotic division. A) Immunocytochemistry shows dominant localization of SGK1 phosphorylated at Thr256 in the oocyte nucleus (grey and red). DAPI (blue), scale bar 15 µm. B) Quantification of SGK1 (Thr256) fluorescence at different oocyte areas and stages of meiosis. Data are represented as the mean ± SEM of at least three independent experiments normalized to the group with highest intensity (GV) as 100% fluorescence; n ≥ 40 oocytes; ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ according to One-way ANOVA for comparing oocyte stages and Student’s t test for comparing oocyte areas.

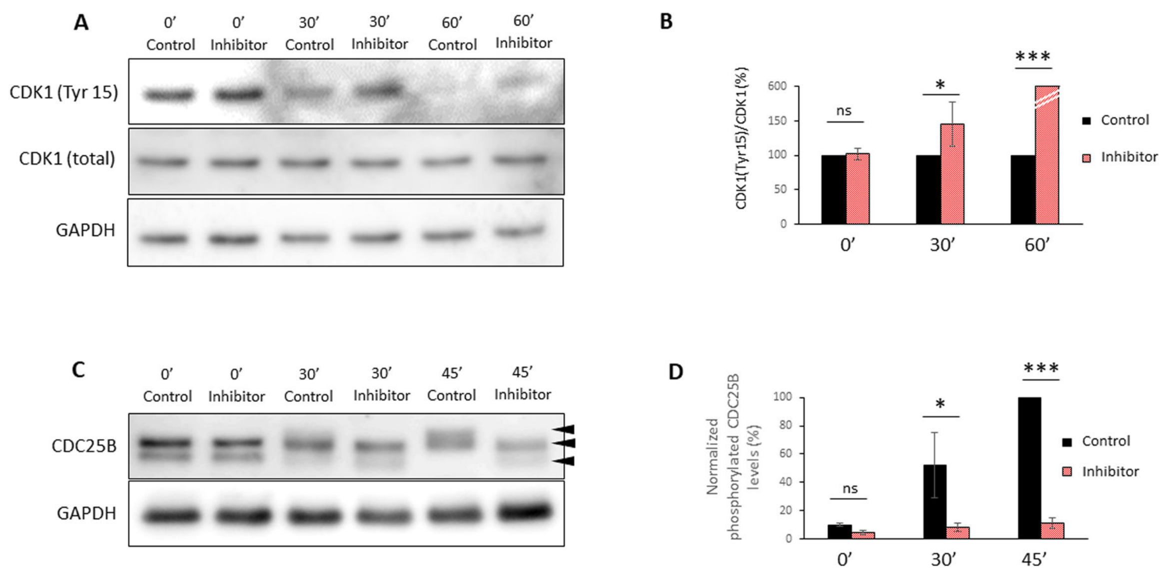

Fig. 3.

Inhibition of SGK1 impairs CDK1 activation through CDC25B in the oocyte prior to NEBD. A) WB analysis of CDK1 (Tyr 15) at different timing of oocyte meiotic resumption in absence (control, 0.02% vehicle DMSO) and presence of SGK1 inhibitor (GSK-650394; 0.01 mM). GAPDH and CDK1 (total) were used as a loading control. The images are representative from at least three biological replicates of 30 oocytes per sample. B) WB quantification of CDK1 (Tyr15) normalized to CDK1 (total). Data are represented as the mean ± SEM from at least three independent experiments; n = 30 oocytes per sample; ns, not significant, * $p < 0.05$, *** $p < 0.001$ according to Student's t test. C) WB analysis of CDC25B at different timing of oocyte meiotic resumption in absence (control, 0.02% vehicle DMSO) and presence of SGK1 inhibitor (0.01 mM). GAPDH was used as a loading control. The images are representative from at least three biological replicates of 30 oocytes per sample. The arrowheads depict phosphorylated variants of CDC25B. D) WB quantification of CDC25B protein normalized to GAPDH. Data are represented as the mean ± SEM from at least three independent experiments; 30 oocytes per sample; ns = not significant, * $p < 0.05$, *** $p < 0.001$ according to Student's t test.

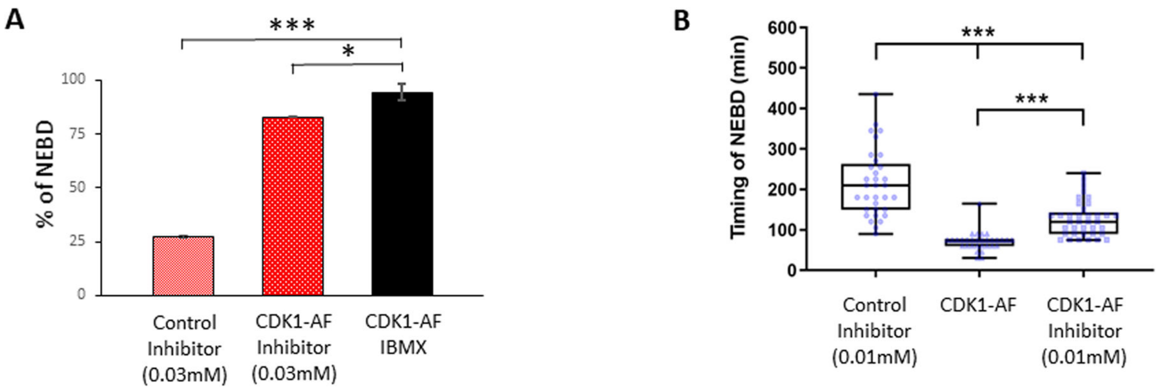

Fig. 4.

The phenotype resulting from SGK1 inhibition can be reversed by activation of CDK1.

A) Quantification of oocytes undergoing NEBD after microinjection with RNA coding for *H2b-gfp* RNA (control) in the presence of SGK1 inhibitor or microinjected with RNA coding for H2B-GFP + CDK1-AF RNA in the presence of SGK1 inhibitor or IBMX. Data are represented as mean ± SEM of at least three independent experiments; n = 39 oocytes per group; ns, not significant, * $p < 0.05$, *** $p < 0.001$ according to One-way ANOVA. B) Timing of NEBD after IBMX wash in oocytes microinjected with H2B-GFP RNA (control) in the presence of inhibitor or microinjected with H2B-GFP+CDK1-AF RNA in absence and presence of SGK1 inhibitor. Box plot displays mean, 25th and 75th percentile and ± SD of at least three independent experiments; n ≥ 30 oocytes per group; ns, not significant; *** $p < 0.001$ according to One-way ANOVA.

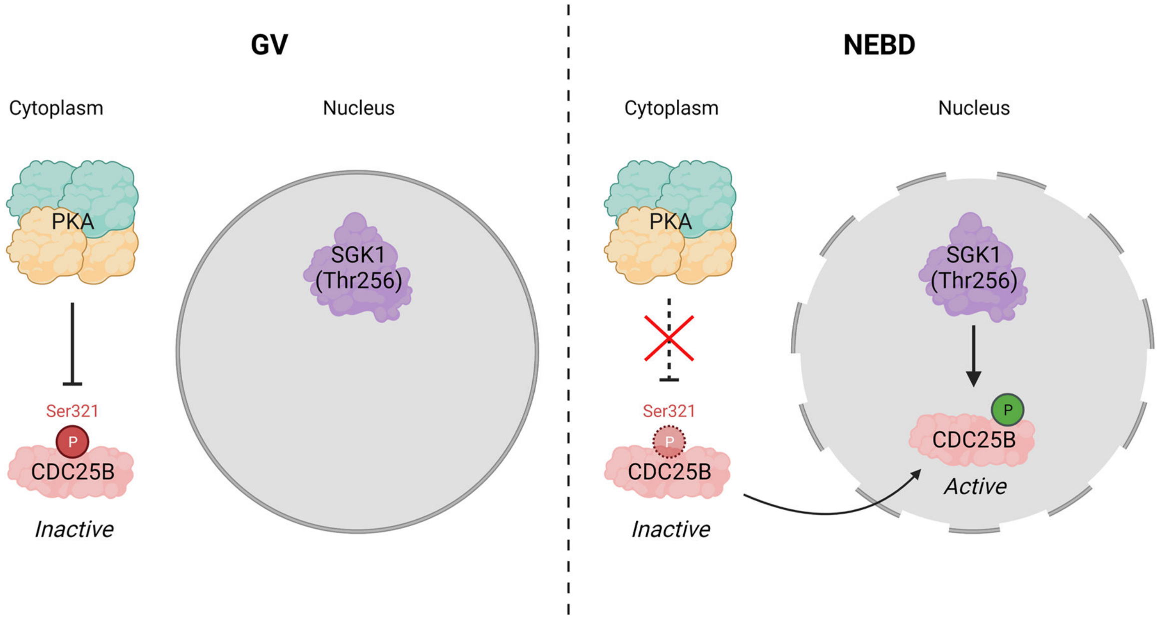

Fig. 5.

Hypothesis of role of the SGK1 in the resumption of meiosis. At the GV stage, SGK1 (Thr256) is active enclosed in the oocyte nucleus without effect on meiotic resumption. PKA is active when cAMP levels are high, phosphorylating CDC25B (Ser321), inhibiting this phosphatase and keeping it in the cytoplasm. Prior to nuclear envelope breakdown (NEBD), cAMP levels decline, PKA becomes inactive and CDC25B is dephosphorylated. Consequently, CDC25B localize to the nucleus where active SGK1 (Thr256) phosphorylate the activation sites of CDC25B, and thus promote the NEBD process.