Journal of Cell Science

Cofilin regulates actin network homeostasis and microvilli length in mouse oocytes

Anne Bourdais, Benoit Dehapiot and Guillaume Halet
DOI: 10.1242/jcs.259237

Editor: Michael Way

Review timeline
Original submission: 9 August 2021
Editorial decision: 13 September 2021
First revision received: 10 November 2021
Accepted: 18 November 2021

Original submission

First decision letter

MS ID#: JOCES/2021/259237

MS TITLE: Cofilin regulates actin network homeostasis and microvilli length in mouse oocytes

AUTHORS: Anne Bourdais, Benoit Dehapiot, and Guillaume Halet
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, reviewer 1 only has some minor points. In contrast, while reviewer 2 is positive they do raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns with addition experiments and controls. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Review of Bourdais et al., JOCES-2021-259237 Cofilin regulates actin network homeostasis and microvilli length in mouse oocytes

In this paper, the authors show the impact of cofilin-mediated actin dynamics during maturation of mouse oocytes. They inhibited the cofilin activity by expressing LIMK1 because of little effect of RNAi to deplete cofilin and found that the cofilin activity is dispensable for actin dynamics in ProI-arrested GV oocytes but becomes necessary for cytoplasmic and cortical actin filament dynamics during meiotic resumption.

The most prominent effect of LIMK1 expression in MI oocytes is the overgrowth of microvilli without recovery of the cytoplasmic actin network. This effect was abolished by co-expression of XAC(A3), indicating that microvilli overgrowth is dependent on depletion of the cofilin activity. The microvilli overgrowth was also exacerbated by the treatment with CK-666, an inhibitor of Arp2/3 complex, that disassembles the cortical actin network. In addition, the authors clearly showed that the microvilli overgrowth requires actin monomer supply, formin family proteins and CDK1-activating machinery.

In MII oocytes, LIMK1-injection induced a dramatic overgrowth of the actin cap rather than microvilli.

Kymographic and STICS analyses clearly demonstrated that cofilin inhibition freezes turnover of global actin filament networks. Furthermore, the authors carefully examined the relation between Arp2/3-dependent cortical actin network and formin-dependent microvilli overgrowth in MII oocytes and conclude that actin cap disassembly promotes microvilli elongation.

Thus, the current study will be of great interest to cell biologists, as it convincingly demonstrates a role for cofilin in mouse oocyte meiotic resumption. The authors argue, intriguingly, that microvilli functions as a sink for actin monomers in a balance between cytoplasmic and cortical actin network turnover. Experimental analyses and methods are well suitable for the journal. In summary, this study breaks important new ground in intracellular actin dynamics by showing the transition of regulatory mechanisms on actin network turnover during meiotic resumption in mouse oocytes. Therefore, I recommend the manuscript for publication.

I have minor concerns:
(1) From page 8, last paragraph to Page 9 The authors examined effects of CK-666 in LIMK1-injected MII oocytes and describe that the absence of microvilli elongation cannot be attributed to monomer sequestration into the oversized actin cap (Fig. 5E).
However, they again examined the effect of actin cap disassembly on microvilli elongation in the last section (Actin cap disassembly promotes microvilli elongation, Fig. 6). At first glance, it may look like the former statement negated the relationship between the actin cap and microvilli. This may appear contradictory to Fig. 6 and some readers could be misinterpreted. It will be helpful to the readers that the authors apparently describe the differences between Fig.5E and Fig. 6.

(2) Page 11, Line 11: Nishimura et al., 2021).

(3) Fig.2A, middle panel:
It will be helpful to the reader that intranuclear bulky F-actin structures are represented by an arrow.

Comments for the author

I have minor concerns:
(1) From page 8, last paragraph to Page 9 The authors examined effects of CK-666 in LIMK1-injected MII oocytes and describe that the absence of microvilli elongation cannot be attributed to monomer sequestration into the oversized actin cap (Fig. 5E).
However, they again examined the effect of actin cap disassembly on microvilli elongation in the last section (Actin cap disassembly promotes microvilli elongation, Fig. 6). At first glance, it may look like the former statement negated the relationship between the actin cap and microvilli. This

may appear contradictory to Fig. 6 and some readers could be misinterpreted. It will be helpful to the readers that the authors apparently describe the differences between Fig.5E and Fig. 6.

(2) Page 11, Line 11: Nishimura et al., 2021).

(3) Fig.2A, middle panel:
It will be helpful to the reader that intranuclear bulky F-actin structures are represented by an arrow.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Bourdais et al have studied the requirement of Cofilin for integrity of the cytoplasmic actin network and cortical actin in mouse oocytes.
These actin structures are essential for proper meiotic maturation of oocytes including spindle migration and chromosome segregation. Understanding the mechanisms that drive dynamics and organisation of such specialised actin networks is an active area of research within and beyond the oocyte biology field. The authors find that gradual activation of Cofilin is required for accurate meiosis and by overexpression of Cofilin inactivating kinases perform a range of experiments to address its function in oocyte meiosis. The work is overall interesting to the oocyte field.

Comments for the author

I firstly wish to disclose that I reviewed this manuscript for another journal.
While this revised version is significantly improved, I find that some outstanding major issues need to be addressed before publication.

Major points
- It is mentioned that reduction in phospho-cofilin was not due to downregulation of total cofilin, which is shown in figure 1A – since there is no loading control used in this blot, it is impossible to make this conclusion and the authors will need to provide a better controlled experiment here. I would also suggest using alpha-tubulin as a loading control instead of GAPDH, as this is more reliable for various reasons to do with oocyte biology. I would also like to see this loading control as a re-probe of a cofilin blot with an alpha-tubulin antibody, which should not be a problem since there is enough separation in molecular weight between the two proteins.
- Is Cofilin the only target of LIMK1 and LIMK2 (I don't imagine so)? If not, how are the authors able to rule out that all the observed meiotic defects are non-specific effects of blocking these kinases? Does overexpression of Cofilin mutants produce similar defects? Are other targets of LIMK1/2 unaffected?
- I am not convinced by the use of water microinjection as a control in the LIMK1/2 mRNA overexpression experiments. Oocytes microinjected with mRNA have their translation machinery fully engaged and this will likely change the transcriptome profile. This does not necessarily cause problems, but the correct control is at least microinjection of EGFP mRNAs – their data that microinjection of LIMK1/2 mutant encoding mRNA already reduces polar body extrusion efficiency relative to water microinjection is a clear indication of this.
- Chromosome spreads in figure 1E – please quantify these spreads and provide example images of what is a separated versus non-separated homolog. This is not clear at all from the figure.
- It is mentioned that LIMK overexpressing oocytes were largely blocked in anaphase configuration. However, the provided example in figure 4B actually shows an oocyte near completion of anaphase. Furthermore, the control egg has a metaphase II spindle that is perpendicularly positioned to the imaging place and it is not possible to demonstrate this anaphase configuration to readers who are not experts in imaging oocyte meiosis.
- Although it is clear that the majority of LIMK oocytes fail to undergo cytokinesis, the focus of some experiments on anaphase and CDK1 does raise question about whether the timing of anaphase onset is affected in these oocytes.
- '…accumulated at the distal tips of elongated microvilli (Figure S4A)' – I strongly recommend showing these images in color vision-deficient friendly colors.

It will be extremely difficult to judge this claim in a red and green image. Some quantification of this accumulation such as linescans of multiple microvilli would strengthen the data.
- Can the authors validate some of the key CDK1 results using the more widely-used CDK1 inhibitor Roscovitine? Alternatively, can they provide evidence that CDK1 is indeed inhibited in oocytes treated with Flavopiridol?
- '…cofilin was suggested to regulate cytoplasmic F-actin dynamics in mouse oocytes (Jang et al., 2014; Montaville et al., 2014).' – I find citation of Montaville et al here inaccurate as this paper does not examine the role of Cofilin in oocytes!
- 'Remarkably, LIMK1-injected GV oocytes exhibited a dense cytoplasmic actin network indistinguishable from controls, and showing similarly fast dynamics (Movie 1)' - This statement is highly unsupported by this movie. It is clear that over the 27 min duration of the movie that both the control and LIMK1 oocyte undergo nuclear envelope disassembly (thus this are no longer GV oocytes) and that the that the cytoplasm increasingly becomes static in LIMK1 oocytes (note some of the filaments becoming less mobile over time and the chromosomes are less mobile after GVBD in comparison to control oocytes).
- The authors should provide uncropped blots for all western blot data.

Minor points
- I would advise the authors to cite seminal literature and not just reviews in their introduction and discussion sections – e.g. During meiotic maturation mouse oocytes experience a profound remodelling of their actin cytoskeleton allowing symmetry breaking and polar body formation (Uraji et al., 2018; Duan and Sun, 2019) – here there is some ground breaking work that has been done by labs in the field and I find a blanket citation of reviews is insufficient. In addition without mentioning specifics, it seems to me there is a preference throughout the manuscript to cite work from certain labs and not others and the authors should do their best to avoid this.
- Western blots presented throughout the manuscript are sound and convey a clear message. However, since these are not truly quantitative blots as fluorescence blots, I would suggest moving the quantification data to supplementary section.

First revision

Author response to reviewers' comments

Response to Reviewers

We wish to thank the Reviewers for their constructive comments and suggestions, that allowed us to strengthen our conclusions, and improve the manuscript. We provide below a point-by-point response to all comments that have been raised. On some occasions, we refer to the literature, and the relevant references are listed at the end of each section.

Reviewer 1

In this paper, the authors show the impact of cofilin-mediated actin dynamics during maturation of mouse oocytes. They inhibited the cofilin activity by expressing LIMK1 because of little effect of RNAi to deplete cofilin and found that the cofilin activity is dispensable for actin dynamics in ProI-arrested GV oocytes but becomes necessary for cytoplasmic and cortical actin filament dynamics during meiotic resumption.

The most prominent effect of LIMK1 expression in MI oocytes is the overgrowth of microvilli without recovery of the cytoplasmic actin network. This effect was abolished by co-expression of XAC(A3), indicating that microvilli overgrowth is dependent on depletion of the cofilin activity. The microvilli overgrowth was also exacerbated by the treatment with CK-666, an inhibitor of Arp2/3 complex, that disassembles the cortical actin network. In addition, the authors clearly showed that the microvilli overgrowth requires actin monomer supply, formin family proteins and CDK1-activating machinery.

In MII oocytes, LIMK1-injection induced a dramatic overgrowth of the actin cap rather than microvilli. Kymographic and STICS analyses clearly demonstrated that cofilin inhibition freezes turnover of global actin filament networks. Furthermore, the authors carefully examined the

relation between Arp2/3-dependent cortical actin network and formin-dependent microvilli overgrowth in MII oocytes and conclude that actin cap disassembly promotes microvilli elongation.

Thus, the current study will be of great interest to cell biologists, as it convincingly demonstrates a role for cofilin in mouse oocyte meiotic resumption. The authors argue, intriguingly, that microvilli functions as a sink for actin monomers in a balance between cytoplasmic and cortical actin network turnover. Experimental analyses and methods are well suitable for the journal. In summary, this study breaks important new ground in intracellular actin dynamics by showing the transition of regulatory mechanisms on actin network turnover during meiotic resumption in mouse oocytes. Therefore, I recommend the manuscript for publication.

➢ We are grateful to Reviewer 1 for their kind encouragements and suggestions for improvement.

Reviewer 1 Comments for the Author...
I have minor concerns:

(1) From page 8, last paragraph to Page 9
The authors examined effects of CK-666 in LIMK1-injected MII oocytes and describe that the absence of microvilli elongation cannot be attributed to monomer sequestration into the oversized actin cap (Fig. 5E). However, they again examined the effect of actin cap disassembly on microvilli elongation in the last section (Actin cap disassembly promotes microvilli elongation, Fig. 6). At first glance, it may look like the former statement negated the relationship between the actin cap and microvilli. This may appear contradictory to Fig. 6 and some readers could be misinterpreted. It will be helpful to the readers that the authors apparently describe the differences between Fig.5E and Fig. 6.

➢ This is a fair point as these two sets of experiments may appear contradictory. These distinct results are explained by the fact that the effects of CK-666 are time-dependent, and ectopic microvilli are a transient phenomenon. In the first 2-3 hours after CK-666 addition, the actin cap will slowly disassemble, and the MII spindle will progressively detach from the cortex. At this stage, the MII spindle can be found in a tilted, or perpendicular orientation, but still in the vicinity of the cortex (as seen in Fig. 6B and S5C). In these oocytes (3 hours treatment), the actin cap has not yet fully disassemble, and ectopic microvilli are generated, fueled by the monomers released from the disassembling cap. However, if the oocytes are maintained in culture for longer (4 hours or more), the actin cap has now fully disappeared, and the MII spindle has continued to detach, eventually reaching the center of the oocyte (see Figs. 5E and 6H). These oocytes don't show ectopic microvilli any more, suggesting the latter are transient structures. Therefore, in our experiments, we have used two distinct time points after CK-666 addition :

1) in Fig. 6, we fixed the oocytes after only 3 hours of treatment with CK-666. In doing so, we managed to catch the actin cap while it was disassembling, and this is how we discovered the ectopic microvilli outgrowth. Accordingly, these oocytes still exhibit a distinct polarized F-actin accumulation (phalloidin staining), reflecting the actin cap not yet fully disassembled (e.g. Fig. 6B);

2) in Fig. 5E in contrast, our aim was instead to fully disassemble the actin cap, to ensure it would not compete with microvilli for monomers. Therefore, we incubated oocytes with CK-666 for 4 hours. Accordingly, these oocytes have completely disassembled their actin cap, and the MII spindle has moved deeper into the inner cytoplasm. These oocytes did not show any microvillar elongation. We could therefore conclude that the lack of microvilli elongation in these oocytes was not due to actin monomers being diverted to the actin cap. However, it is expected that these oocytes must have experienced ectopic microvilli outgrowth when the cap started to disassemble, but the phenomenon was terminated by the time of the fixation (4 h).

   To highlight the fact that ectopic microvilli are transient and eventually disappear as the actin cap vanishes, we examined oocytes fixed after 5 h treatment with CK-666, and we show that these oocytes don't show ectopic microvilli any more (see Fig. 6H,I).

To clarify this discrepancy, we have amended the text describing the use of CK-666 in Figure 6, as follow :

"To test this prediction, we treated MII oocytes with CK-666 (100 µM) in order to promote spontaneous actin cap disassembly and monomer release. Oocytes were fixed after 3 h of CK-666 treatment, at which time the actin cap had not yet fully disassembled. Remarkably, …" Results, page 9

(2) Page 11, Line 11: ; Nishimura et al., 2021).
➢ This typo is now corrected Discussion, page 10

(3) Fig.2A, middle panel: It will be helpful to the reader that intranuclear bulky F-actin structures are represented by an arrow.
➢ This is a good point – we have added an arrow, and refer to it in the Legend :
"Note the thick F-actin bundles in the nucleoplasm of the LIMK1-expressing GV oocyte (arrow)." Figure 2, legend, page 26

Reviewer 2

In this manuscript, Bourdais et al have studied the requirement of Cofilin for integrity of the cytoplasmic actin network and cortical actin in mouse oocytes. These actin structures are essential for proper meiotic maturation of oocytes including spindle migration and chromosome segregation. Understanding the mechanisms that drive dynamics and organisation of such specialised actin networks is an active area of research within and beyond the oocyte biology field. The authors find that gradual activation of Cofilin is required for accurate meiosis and by overexpression of Cofilin inactivating kinases perform a range of experiments to address its function in oocyte meiosis. The work is overall interesting to the oocyte field.

➢ We are grateful to Reviewer 2 for their helpful suggestions for improving the quality of the manuscript.

Reviewer 2 Comments for the Author...
I firstly wish to disclose that I reviewed this manuscript for another journal. While this revised version is significantly improved, I find that some outstanding major issues need to be addressed before publication.

Major points

- It is mentioned that reduction in phospho-cofilin was not due to downregulation of total cofilin, which is shown in figure 1A – since there is no loading control used in this blot, it is impossible to make this conclusion and the authors will need to provide a better controlled experiment here.

➢ In fact, there is a loading control.
All our WB experiments were realized with the monitoring of GAPDH levels in all samples, as a loading control. In Figure 1A, both phospho-cofilin and total cofilin are displayed against a GAPDH loading control. This is also clearly referred to in the legend for Figure 1A : "GAPDH was used as a loading control". In the corresponding bar graph, band intensities were normalized against the GAPDH level in the sample, as stated in the legend.

I would also suggest using alpha-tubulin as a loading control instead of GAPDH, as this is more reliable for various reasons to do with oocyte biology. I would also like to see this loading control as a re-probe of a cofilin blot with an alpha-tubulin antibody, which should not be a problem since there is enough separation in molecular weight between the two proteins.

➢ As stated above, our experiments all already include a loading control. We do not believe there is any ground to state that GAPDH is unreliable. GAPDH is a classic WB loading control, and it has already been used by others in mouse oocyte WB experiments across maturation

stages (e.g. [1-3]). These published studies, and our own data, show that GAPDH protein levels do not vary significantly between the four landmark stages examined (i.e. GV, NEBD, MI and MII) [1-3]. We therefore consider that GAPDH is a reliable loading control for WB experiments in mouse oocytes. While GAPDH is mostly known for its "house-keeping" role in the glycolytic pathway, non-metabolic functions have also been described. Expression of GAPDH in oocytes (which have little glycolysis activity) may reflect these non-metabolic functions.

[1] Homer et al. (2005). Genes Dev. 19 : 202-207.
[2] Gómez-Fernández et al. (2012). Mol. Hum. Reprod. 18 : 194-203.
[3] Zhou et al. (2020). Sci. Adv. 6 : eaax3969.

- Is Cofilin the only target of LIMK1 and LIMK2 (I don't imagine so)? If not, how are the authors able to rule out that all the observed meiotic defects are non-specific effects of blocking these kinases? Does overexpression of Cofilin mutants produce similar defects? Are other targets of LIMK1/2 unaffected?

➢ There seems to be a misunderstanding : we do not block LIMK kinases in this study. We aimed to block Cofilin, and therefore performed overexpression of LIMK1, in order to increase Cofilin phosphorylation (= inactivation). The observed meiotic defects are consecutive to overexpression of LIMK1. We did not perform overexpression of LIMK2.

➢ LIM kinases are recognised for their exquisite specificity for Ser3 of ADF/Cofilin [4]. The molecular basis for this highly selective kinase-substrate interaction was recently investigated in two insightful structural studies of LIMK1-Cofilin complexes. Intriguingly, this unique specificity appears to result from an atypical LIMK-cofilin interface, that does not follow the canonical linear motif recognition paradigm [5]. Instead, Cofilin interacts with LIMK1 in such a way that the phosphoacceptor site Ser3 is precisely oriented toward the catalytic center in close vicinity to the ATP γ phosphate, for phosphotransfer to occur. A second structural study from another laboratory came to the same conclusions [6]. This unique mode of kinase-substrate interaction, whereby phosphorylation occurs at the tip of the polypeptide chain (here, Ser3), defines LIMK1-Cofilin as a distinct class of kinase-substrate recognition [5,6].

We realize these are significant findings for the reader to capture the exquisite specificity of LIMK1-Cofilin interaction. Therefore, we have added a sentence in the Introduction, with reference to the two above cited structural studies :

"Intriguingly, structural studies revealed a noncanonical kinase-substrate interaction mode, which may account for the exquisite selectivity of LIMK toward cofilin (Hamill et al., 2016; Salah et al., 2019)." **Introduction, page 3**

- Hamill S, Lou HJ, Turk BE and Boggon TJ (2016). Structural basis for noncanonical substrate recognition of Cofilin/ADF proteins by LIM kinases. Mol. Cell 62: 397-408. **References, page 20**
-
- Salah E, Chatterjee D, Beltrami A, Tumber A, Preuss F, Canning P, Chaikuad A, Knaus P, Knapp S, Bullock AN and Mathea S (2019). Lessons from LIMK1 enzymology and their impact on inhibitor design. Biochem. J. 476: 3197-3209. **References, page 24**

[4] Prunier et al. (2017). Oncotarget 8 : 41749-41763
[5] Hamill et al. (2016). Mol. Cell 62 : 397-408.
[6] Salah et al. (2019). Biochem. J. 476 : 3197-3209.

Nonetheless, it seems reasonable to speculate that LIM kinases may have additional substrates. Over the last decade, a few proteins have been suggested to be novel LIMK substrates. However these studies were mostly single observations, only supported by indirect evidence such as co-immunoprecipitation or in vitro kinase assays. For instance, it was suggested that TPPP1/p25alpha, a regulator of microtubule polymerization, was a novel LIMK substrate, but this claim was later

disproved [7]. We have analysed the literature exhaustively, and we list below the novel substrates for which direct evidence was provided, i.e. identification of the targeted residues. Two proteins are candidate LIMK1 substrates, with however little relevance to our study. Beside, another two proteins have recently been identified as bona fide LIMK2 substrates, but there is no evidence that these are also LIMK1 substrates :

- MT1-MMP (Mmp14) : a matrix-degrading protease, involved in metastasis. This is an essentially extracellular protein, anchored to the plasma membrane via a C-terminal hydrophobic stretch. Evidence suggests MT1- MMP is phosphorylated by LIMK1, presumably at Tyr573, promoting matrix degradation [8]. Note however that RNAseq data indicate that mouse oocytes do not express MT1-MMP [9,10]. For these reasons, we find it unlikely that the effects of LIMK1 overexpression in our study could be mediated by MT1-MMP.

- CREB : a transcription factor that regulates cAMP-responsive genes. Evidence suggests CREB is phosphorylated by LIMK1, presumably at Ser133, in differentiating neuronal progenitors, with effect to promote gene transcription [11]. While active CREB (phospho-Ser133) is readily detected in surrounding granulosa cells, there is no evidence for CREB activation in mouse oocytes [12]. Moreover, oocytes undergoing meiotic maturation are transcriptionally inactive. For these reasons, we find it unlikely that the effects of LIMK1 overexpression in our study could be mediated by CREB.

- TWIST1 : a transcription factor that regulates gastrulation and mesoderm specification; also a potent oncogene involved in metastasis. TWIST1 was recently shown to be phosphorylated by LIMK2 on four identified Ser residues, thereby preventing TWIST1 degradation [13]. However, this was not shown for LIMK1. Note that RNAseq data indicate that mouse oocytes do not express TWIST1 [9,10]. For these reasons, we find it unlikely that the effects of LIMK1 overexpression in our study could be mediated by TWIST1.

- PTEN : a lipid phosphatase that oppose PI3K/Akt signalling; also a tumour suppressor. PTEN was recently shown to be phosphorylated by LIMK2 on five identified Ser residues, thereby promoting PTEN ubiquitylation and degradation [14]. However, this was not shown for LIMK1. Moreover, fully grown oocytes lacking PTEN do not show noticeable defects, and achieve maturation normally, leading to unaltered fertility [15]. For these reasons, we find it unlikely that the effects of LIMK1 overexpression in our study could be mediated by PTEN loss.

None of these reported LIMK substrates appear relevant to oocyte maturation, actin filament turnover, microvilli dynamics or cytokinesis. Therefore we stand by the view that the meiotic defects reported in our study are consecutive to cofilin inhibition by LIMK1.

[7] Schofield et al. (2012). J. Biol. Chem. 287 : 43620-43629
[8] Lagoutte et al. (2016). Sci. Rep. 6 : 24925
[9] Tang et al. (2009). Nat. Methods 6 : 377-382.
[10] Pfender et al. (2015). Nature 524 : 239-242.
[11] Yang et al. (2004). J. Biol. Chem. 279 : 8903-8910.
[12] Li et al. (2020). Histochem. Cell Biol. 154 : 287-299.
[13] Nikhil et al. (2019). Cancer Lett. 448 : 182-196
[14] Nikhil et al. (2021). Cancer Lett. 498 : 1-18
[15] Jagarlamudi et al. (2009). PLoS One 4 : e6186.

➢ As suggested, we investigated the possibility to observe similar defects using mutant Cofilin overexpression. To obtain a similar effect as LIMK1 overexpression (which leads to Cofilin hyperphosphorylation), the only experiment that appeared relevant to us was to overexpress a phosphomimetic mutant Cofilin, which is considered inactive because it cannot bind actin. We obtained Cofilin-S3D (phosphomimetic) from Prof. Iryna Ethell (Univ. California Riverside) and overexpressed it in oocytes as we did for LIMK1. Perhaps not surprisingly, we could not reproduce the effects seen with LIMK1 (PB1 failure, microvilli elongation, actin cap

overgrowth). This could be explained by the fact that endogenous Cofilin is still present. In fact, we show that endogenous Cofilin is a rather abundant and stable protein, resistant to siRNA knockdown (Figure S1). Therefore, it is likely that endogenous Cofilin functions normally (= binds to and severs actin) even if Cofilin-S3D is expressed. The lack of a "dominant-negative" effect of cofilin-S3D, and the fact that such mutant does not interfere with endogenous cofilin, have been described by others [16-18]. In addition, it was shown that in vitro, phosphomimetic Cofilin still binds weakly to actin filaments, and exhibits residual severing activity that can be further enhanced if filaments are also decorated with Tropomyosin-3 [19]. Therefore, the phosphomimetic Cofilin mutant does not appear as a reliable tool to mimic the inactivation of endogenous Cofilin in cells. For these reasons, we did not mention our experiements with cofilin-S3D in the manuscript. In our view, the overexpression of the upstream kinase LIMK1 remains a valuable strategy to achieve acute inactivation of endogenous cofilin, as was shown previously in other cells.

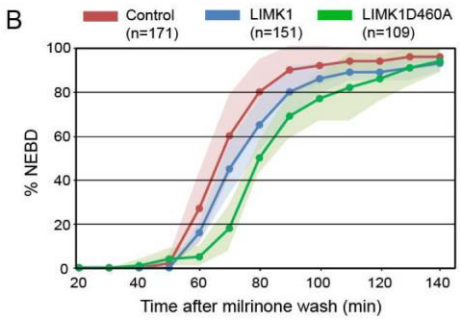
[16] Blangy et al. (2012). PloS One 7 : e45909.
[17] Peverelli et al. (2017). Cancer Lett. 406 : 54-63.
[18] Havekes et al. (2016). Elife 5: e13424.
[19] [19] Elam et al. (2017). J. Biol. Chem. 292 : 19565-19579.

- I am not convinced by the use of water microinjection as a control in the LIMK1/2 mRNA overexpression experiments. Oocytes microinjected with mRNA have their translation machinery fully engaged and this will likely change the transcriptome profile. This does not necessarily cause problems, but the correct control is at least microinjection of EGFP mRNAs – their data that microinjection of LIMK1/2 mutant encoding mRNA already reduces polar body extrusion efficiency relative to water microinjection is a clear indication of this.

We agree that injection of an mRNA is a better control than water injection when describing the meiotic defects consecutive to LIMK1 overexpression. Hence, we have used the mRNA encoding kinase-dead LIMK1-D460A as a dual purpose control : 1) as a control for mRNA injection, and 2) to demonstrate the requirement for LIMK1 kinase activity in the observed phenotype, where relevant. However, as noticed by this Reviewer, water-injected control oocytes are shown on two occasions. We have now corrected this, using LIMK1-D460A mRNA injection, as follow :

- In Figure 1B, the kinetics of NEBD in the control population was initially obtained using water-injected oocytes. To provide a better control, and to match the data shown in Figure 1C, we have now performed a new set of experiments where we monitored the rate of NEBD in oocytes injected with LIMK1-D460A mRNA, against controls (water). These new data points have been added on Figure 1B, labeled as "LIMK1D460A". Note that for clarity, we have displayed the error (S.D.) as shaded areas instead of error bars. We also corrected a typo error in the x axis (the first time point illustrated is t=20min, not t=0). The figure legend and main text have been modified accordingly, as follow :

New Figure 1B :

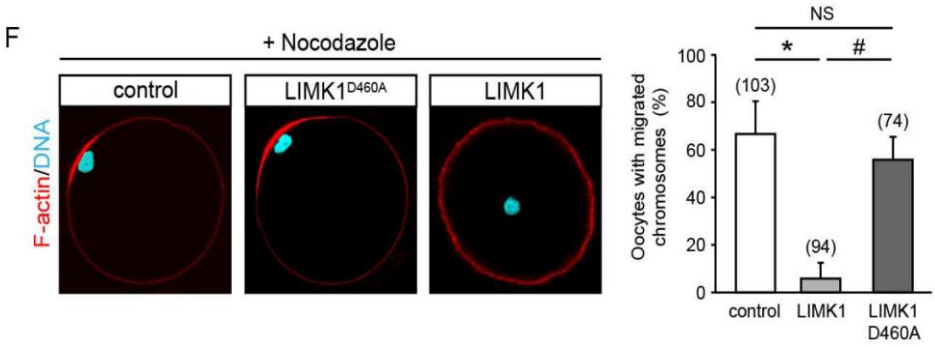


"Spontaneous meiosis resumption was not overtly affected by LIMK1, or LIMK1^{D460A} overexpression (Fig. 1B)" **Results, page 4**

"(B) Time course of spontaneous meiosis resumption in oocytes injected with water (Control), LIMK1 cRNA or LIMK1^{D460A} cRNA, at the GV stage." **Figure 1, legend, page 26**

➢ In Figure 1F : the nocodazole experiments have been realised with both water- and LIMK1-D460A mRNA-injected oocytes as controls, as indicated in the bar graph. However, the illustration initially showed only a water-injected control. We have now added an image to show the staining pattern in a LIMK1-D460A-expressing oocyte as well, to provide a better control. The legend has been modified accordingly, as follow :

New Figure 1F :



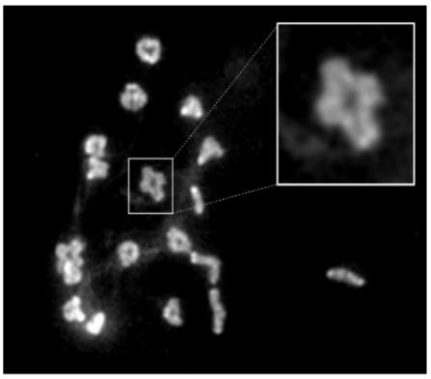
"Representative images of a control oocyte, and oocytes expressing LIMK1^{D460A} or LIMK1, are shown (left panel)." Figure 1, legend, page 26

- Chromosome spreads in figure 1E – please quantify these spreads and provide example images of what is a separated versus non-separated homolog. This is not clear at all from the figure.

➢ There may be a misunderstanding : the spread in Fig. 1E does not show a mixture of separated and non-separated homologs. In this spread, all 20 bivalents have segregated, resulting in a population of 40 monovalents (as verified by manual counting).

In contrast, an oocyte that would not have segregated its homologs would show a population of 20 bivalents – for illustration we show here a population of non-segregated bivalents, as can be seen in metaphase- I oocytes (the inset shows a magnification of a non-segregated bivalent, with the characteristic stretched configuration).

In our experiments, we aimed to find out whether oocytes that did not emit PB1 were simply blocked in metaphase-I (ie, 20 bivalents), or whether they achieved anaphase-I but failed to form a polar body. The fact that these oocytes contained 40 monovalents (Fig. 1E) is a demonstration that anaphase-I has occurred, indicating that LIMK1 overexpression did not interfere with the metaphase-anaphase transition. We cannot provide further quantification. However, we indicate the fraction of oocytes showing homolog segregation (ie 40 monovalents) in Fig. 1E.



Metaphase-I spread :
20 non-segregated bivalents

- It is mentioned that LIMK overexpressing oocytes were largely blocked in anaphase configuration. However, the provided example in figure 4B actually shows an oocyte near completion of anaphase. Furthermore, the control egg has a metaphase II spindle that is perpendicularly positioned to the imaging place and it is not possible to demonstrate this anaphase configuration to readers who are not experts in imaging oocyte meiosis.

➢ Thank you for spotting this misstatement. What we meant to say, indeed, is that oocytes were blocked after anaphase was completed with segregated homologs having reached the spindle poles. We did not see oocytes arrested with homologous half-way to the poles. The wording "blocked in anaphase" is thus misleading, and we have therefore rephrased to "late anaphase" in the main text :

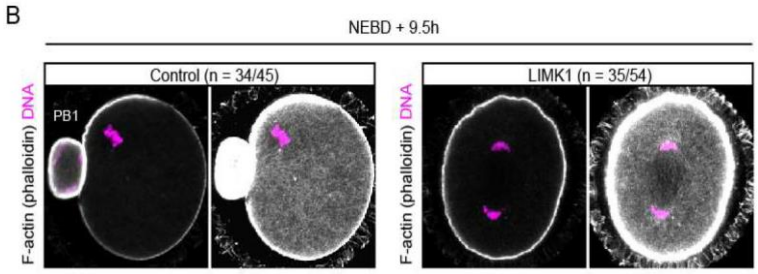
"a large fraction (35/54 oocytes; 65%) of these oocytes were blocked in a late anaphase-I configuration, while the majority of time-matched control…" **Results, page 6**

This configuration is sometimes referred to as "telophase-I" by others. However we do not agree to this denomination, as telophase is classically regarded as the cell cycle phase when chromosomes decondense and the nuclear envelope reforms [19], which is not the case here.

[19] Pines and Rieder (2001). Nat. Cell Biol. 3 : E3-E6.

➢ To facilitate the comparison between the two configurations (metaphase-II vs late anaphase-I), we have changed the image of the control egg for another one where the MII spindle is parallel to the image plane, and the metaphase alignment of chromosomes is more obvious :

New Figure 4B :



- Although it is clear that the majority of LIMK oocytes fail to undergo cytokinesis, the focus of some experiments on anaphase and CDK1 does raise question about whether the timing of anaphase onset is affected in these oocytes.

➢ Thank you for the suggestion. We have now investigated the kinetics of anaphase onset in control uninjected oocytes, vs. oocytes overexpressing LIMK1 or LIMK1-D460A. Oocytes were cultured in vitro in M16 medium and anaphase-I was assessed by imaging chromosome configuration with Hoechst-33342 staining.

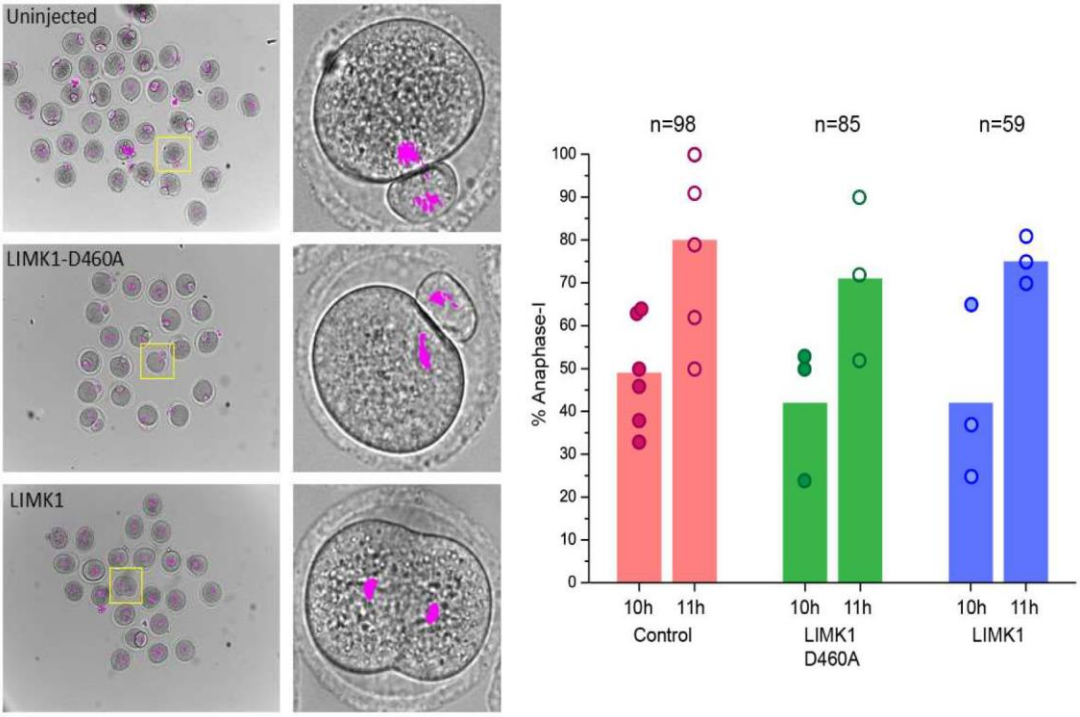
In mouse oocytes cultured in vitro, anaphase-I typically occurs ~10 h after release from meiotic arrest [20]. Thus, in order to evaluate the onset and progression into anaphase-I, we scored oocytes at two successive time points : 10 h and 11 h after release from meiotic arrest (milrinone wash).

[20] Holt and Jones (2009). Mol. Hum. Reprod. 15: 139-147.

The results of these experiments are shown below. All three populations of oocytes had initiated anaphase-I at t=10h, with, on average, around 40% of injected oocytes showing anaphase-I configurations (vs. 50% of uninjected controls). Likewise, all three populations of oocytes showed further progression into anaphase-I at t=11h, with over 70 % of injected oocytes having achieved anaphase-I (vs. 80% of uninjected controls). Representative images of oocytes stained with Hoechst-33342 (magenta) are shown.

From these experiments, we note that, while the overall rate of anaphase-I was slightly decreased in injected oocytes, presumably reflecting the microinjection procedure and/or the

expression of an exogenous protein, the overexpression of LIMK1 or LIMK1-D460A did not significantly alter the onset and progression into anaphase-I. Interestingly, these experiments confirmed our initial observation that LIMK1-expressing oocytes failed to achieve cytokinesis.



We realize it seems like a natural question to ask about the timing of anaphase onset, and we feel these results should therefore be included in the manuscript, to complement the data on NEBD and maturation rates. We have therefore added a new supplementary figure (New Figure S3) containing this new set of data, and we refer to this figure in the main text, Figure legends, and Materials and Methods, as follow :

"Likewise, the onset, and progression into anaphase-I were little affected by exogenous LIMK1 or LIMK1^{D460A} (Fig. S3). We noticed however that LIMK1-expressing oocytes undergoing anaphase-I failed to form a polar body, arguing for a delay or inhibition of cytokinesis (Fig. S3). Accordingly,…" **Results, page 4**

Figure S3. LIMK1 overexpression does not affect the timing of anaphase-I.
Oocytes arrested at the GV stage with milrinone were injected with cRNA encoding LIMK1 or LIMK1^{D460A}, or were left uninjected (Control). After milrinone washout, oocytes were cultured in M16 medium until anaphase-I. Oocytes were scored for anaphase-I at 10 h (t=10h) and 11 h (t=11h) post-milrinone washout, using Hoechst-33342 staining to visualize chromosome configuration. The left panel shows representative images of all 3 populations of oocytes, taken at t=11h. Chromosomes are labeled with Hoechst 33342 (magenta). Magnified images of individual oocytes (as indicated by the yellow square) are shown. Note the absence of polar body protrusion in LIMK1-expressing oocytes. The right panel shows the rate of anaphase-I as observed at t=10h and t=11h, expressed as percentages of the total number of oocytes scored in each experiment. Data points represent individual experiments, and the bars represent the corresponding mean value. The total number of oocytes scored is indicated above each data sets. **Supplementary information, Figure S3 legend**

Chromosome staining for monitoring anaphase-I
To evaluate the kinetics of anaphase-I, GV-stage oocytes (uninjected, or expressing LIMK1 or LIMK1^{D460A}) were washed from milrinone and allowed to resume meiosis and mature in vitro in a small drop of M16 medium layered with mineral oil (Sigma M8410). Ten hours after

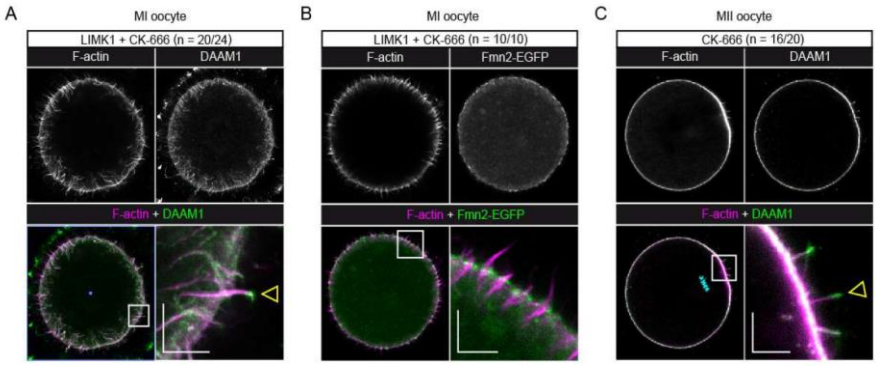
milrinone washout, oocytes were stained with Hoechst-33342 (5 µM; Invitrogen H3570) for 10 min in M2 medium, followed by wash, and transferred to glass-bottom dishes (MatTek, Ashland, MA). Oocytes were examined for chromosome configuration using an inverted Leica DMI4000B microscope equipped with a 365nm LED module and a digital monochrome DFC345 FX camera (Leica). Images were aquired using the LAS AF 2.3.0 software (Leica). Oocytes were then quickly returned to a 37°C hot block and imaged again at 11 h post-milrinone washout. **Materials and Methods, page 14**

➢ Note that, as a consequence of this new Figure S3, the numbering of subsequent supplementary figures is shifted : old Figure S3 is now Figure S4, etc.

- '…accumulated at the distal tips of elongated microvilli (Figure S4A)' – I strongly recommend showing these images in color vision-deficient friendly colors. It will be extremely difficult to judge this claim in a red and green image. Some quantification of this accumulation such as linescans of multiple microvilli would strengthen the data.

➢ This is a good suggestion. We have followed the recommandations given on the imageJ website and replaced Red with Magenta (for F-actin staining) in Figure S5A-C (formerly Fig. S4). In Figure S5C, the chromosomes intially displayed in Magenta are now in Cyan. We hope this is now satisfactory for colorblind readers. The legend has been modified accordingly :

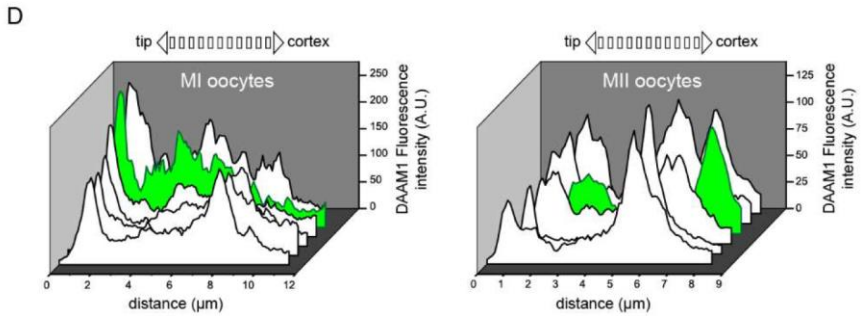
New Figure S5A-C :



"F-actin was stained with Alexa Fluor 568-phalloidin (magenta). Chromosomes in (C) are labeled with TO-PRO-3 (cyan)."
Supplementary information, Figure S5 legend

➢ We have performed the linescan analysis as suggested. Figure S5 now includes two new panels (Figure S5D) showing typical DAAM1 fluorescence profiles across the length of individual microvilli, for both MI oocytes and MII oocytes. Six linescans obtained from six different oocytes are displayed in each panel. The Figure legend and Materials and methods section were modified accordingly :

New Figure S5D :



"(D) A selection of individual DAAM1 fluorescence profiles. The fluorescence intensity of DAAM1 staining (in arbitrary units) along individual microvilli was plotted as a function of the distance (µm). Six distinct profiles are shown, from six different MI oocytes (as in panel A) and six different MII oocytes (as in panel C). Fluorescence profiles highlighted in green refer to the elongated microvilli marked by an arrowhead in (A) and (C). Note the increased

intensity at microvillar tips, reflecting DAAM1 accumulation. **Supplementary information, Figure S5 legend**

"DAAM1 fluorescence profiles were generated using the segmented line tool and the Plot Profile function in FIJI"
Materials and Methods, page 16

➢ Regarding the particular shape of elongated microvilli, please note that we have added a reference to the work of Orly et al. (2014) in the Discussion, as their computational model fitted particularly well with our experimental observations:

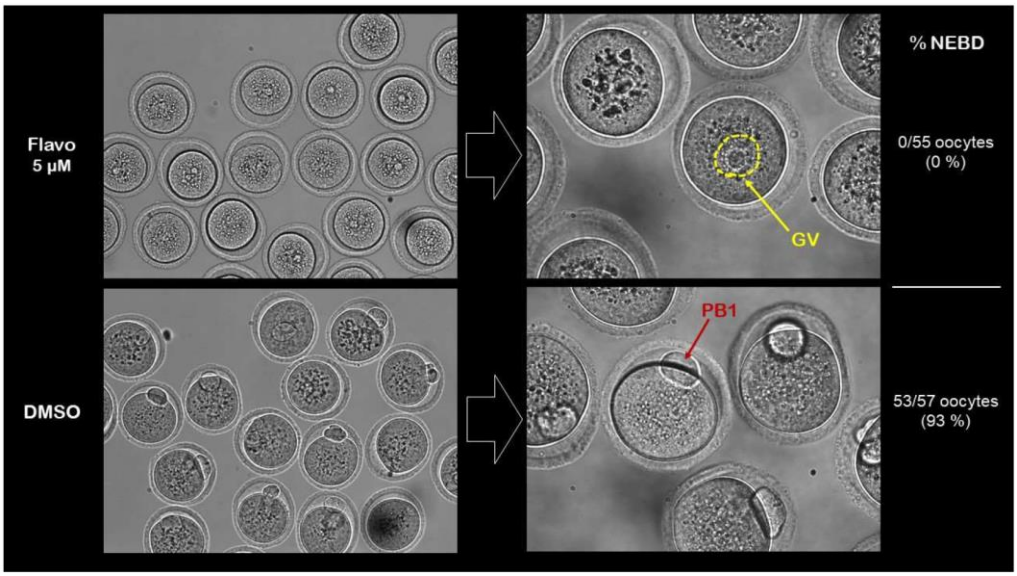
"Interestingly, computational modeling of actin-rich protrusion dynamics predicted that decreased severing at the base of microvilli would eventually increase the steady-state length of both the rootlet and the microvilli protrusion (Orly et al., 2014), which is corroborated by our experimental data." Discussion, page 10

- Can the authors validate some of the key CDK1 results using the more widely-used CDK1 inhibitor Roscovitine? Alternatively, can they provide evidence that CDK1 is indeed inhibited in oocytes treated with Flavopiridol?

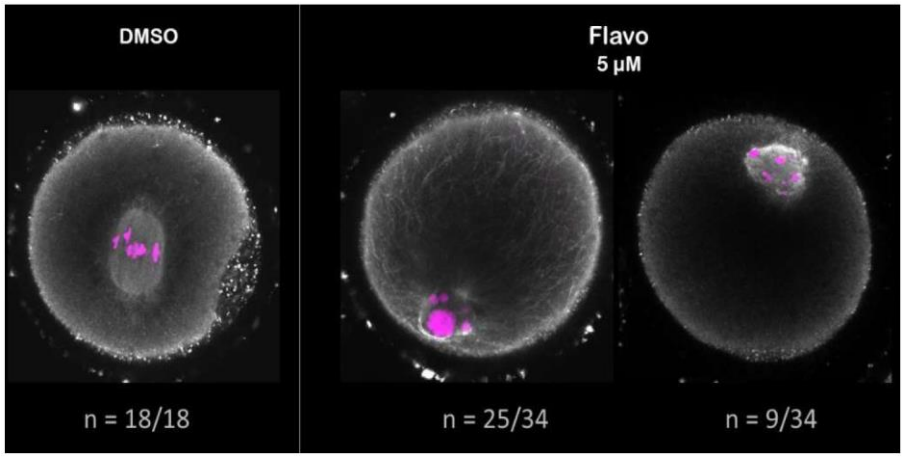
➢ Flavopiridol is a first generation pan-CDK inhibitor, like Roscovitine. It is currently being investigated in phase II clinical trials, for the treatment of various types of cancers. The use of Flavopiridol to inhibit CDK1 activity in mouse oocytes has been reported [21-24]. We decided to use Flavopiridol (5 µM) because it is described as "more potent" than Roscovitine in mouse oocytes [21], and because it was demonstrated to fully inhibit meiosis resumption/NEBD, indicating robust CDK1 inhibition [21,24].

In our study, Flavopiridol (5 µM) was used in a single set of experiments : to interfere with CDK1 activity during maturation, in order to examine whether microvilli elongation requires CDK1 activity. To make sure oocytes had resumed meiosis, Flavopiridol was added after NEBD was complete. In order to demonstrate the effectiveness of the inhibitor, we have performed two additional sets of experiments:

1) We cultured denuded GV-stage oocytes overnight in the presence of 5 µM Flavopiridol, or vehicule (DMSO). Culture was in M16 medium without milrinone, in order to promote spontaneous meiosis resumption. The results are depicted below. As expected, the great majority (93%) of control oocytes resumed meiosis and emitted the first polar body. In contrast, oocytes cultured in the presence of Flavopiridol all remained arrested at the GV stage, indicating a failure to resume meiosis. This result is identical to previous published work, and consistent with a robust inhibition of CDK1 [21,24].



2) As an additional demonstration of CDK1 inhibition, we have examined spindle formation. Oocytes were allowed to resume meiosis (NEBD) spontaneously, and were next split into two populations : Flavopiridol (5 µM) vs. DMSO (control). Oocytes were fixed at NEBD + 6 hours, and processed for tubulin immuno-labeling and chromosome staining with To-pro-3. The results are depicted below (gray : microtubules; magenta : chromatin).



As expected, vehicle-treated control oocytes exhibited a bipolar spindle with metaphase chromosomes arranged at the spindle equator (n = 18/18 oocytes). In contrast, oocytes treated with Flavopiridol failed to form a bipolar spindle. A minority of oocytes exhibited an apolar microtubule ball with scattered chromosomes (n = 9/34 oocytes), while the majority of oocytes (n = 25/34) were filled with an extended network of microtubules, resembling an interphase configuration, while DNA staining revealed a large mass of decondensed chromatin. These features are indicative of CDK1 inhibition, and are consistent with the previous observation that Flavopiridol induces an “interphase-like” state in mouse oocytes [24].

These additional experiments confirm Flavopiridol (5 µM) as an effective inhibitor of CDK1 activity in mouse oocytes, producing the same defects as reported with Roscovitine [25,26]. Since CDK1 inhibition with Flavopiridol has already been described in mouse oocytes [21-24], we did not add these sets of experiments to the manuscript.

[21] Lane and Jones (2014). Nat. Commun 5 : 3444
[22] Wei et al. (2018). Nat. Commun. 9 : 4029

[23] Levasseur et al. (2019). Dev. Cell 48 : 672-684
[24] Mermillod et al. (2000). Mol. Reprod. Dev. 55 : 89-95
[25] Solc et al. (2015). PloS One 10 : e0116783
[26] Wang et al. (2004). Reproduction 128 : 493-502.

- '…cofilin was suggested to regulate cytoplasmic F-actin dynamics in mouse oocytes (Jang et al., 2014; Montaville et al., 2014).' – I find citation of Montaville et al here inaccurate as this paper does not examine the role of Cofilin in oocytes!

➢ It is true that the role of cofilin was not specifically investigated in this paper; the authors simply refered to ADF/cofilin as a likely regulator of cytoplasmic F-actin in their model (Fig. 9). We have removed this citation. **Results, page 5**

- Remarkably, LIMK1-injected GV oocytes exhibited a dense cytoplasmic actin network indistinguishable from controls, and showing similarly fast dynamics (Movie 1)' - This statement is highly unsupported by this movie. It is clear that over the 27 min duration of the movie that both the control and LIMK1 oocyte undergo nuclear envelope disassembly (thus this are no longer GV oocytes) and that the that the cytoplasm increasingly becomes static in LIMK1 oocytes (note some of the filaments becoming less mobile over time and the chromosomes are less mobile after GVBD in comparison to control oocytes).

➢ This was in fact intentional : we want the reader to see first-hand the striking difference in cytoplasmic F-actin density before and after NEBD. Hence, this movie conveys two informations : 1) cytoplasmic F-actin dynamics are similar when the oocytes are still at GV-stage (first half of the movie), and 2) both populations of oocytes experience meshwork collapse at NEBD (second half of the movie). However, we agree that this sentence is somewhat misleading as the actual quantification of F-actin density is addressed in Figure 2 and refers to fixed oocytes, and the extent of the drop in cytoplasmic F-actin appears stronger in LIMK1-expressing oocytes. To clarify our statements, we have rephrased this section, as well as the movie title, as follow :

> "Remarkably, GV oocytes overexpressing LIMK1 exhibited a dense cytoplasmic actin network indistinguishable from controls (Fig. 2A). In line with previous observations in metaphase oocytes (Azoury et al., 2008; Schuh and Ellenberg, 2008), cytoplasmic F-actin exhibited constant remodeling in a highly dynamic fashion, in both control and LIMK1-expressing oocytes, while at the GV stage (Movie 1). Moreover, and consistent with previous findings (Azoury et al., 2011), both control and LIMK1-expressing oocytes showed a significant drop in cytoplasmic F- actin density shortly before NEBD (Fig. 2B; Movie 1). These observations…" **Results, page 5**

> "Cofilin is dispensable for cytoplasmic F-actin dynamics in GV oocytes and during NEBD." **Supplementary information, Movie 1 legend**

➢ It is not entirely clear to us what this Reviewer means by "the cytoplasm increasingly becomes static […] filaments becoming less mobile…". We agree that in Movie 1 the two oocytes show some differences after NEBD, as the drop in cytoplasmic F-actin appears to be more severe in the LIMK1-expressing oocyte (while a baseline network seems to remain in control). This could reflect actin monomers being diverted to microvilli in cofilin-inhibited oocytes. However, after quantification in a population of oocytes, the difference was hardly significant (see Fig. 2B). In our view, the main information conveyed by these experiments is that cofilin-inhibited oocytes show similar features as control oocytes, i.e. they contain a highly dynamic F-actin network at the GV stage, and this network drops at NEBD.

- The authors should provide uncropped blots for all western blot data.

➢ Uncropped images of the Western blots are now provided in new Figure S6. Supplementary information, Figure S6

This is also mentioned in the Materials and Methods section :

"Uncropped images of Western blot data are provided in Fig. S6." Materials and Methods, page 14

Minor points

- I would advise the authors to cite seminal literature and not just reviews in their introduction and discussion sections –e.g. During meiotic maturation, mouse oocytes experience a profound remodelling of their actin cytoskeleton, allowing symmetry breaking and polar body formation (Uraji et al., 2018; Duan and Sun, 2019) – here there is some ground breaking work that has been done by labs in the field and I find a blanket citation of reviews is insufficient. In addition, without mentioning specifics, it seems to me there is a preference throughout the manuscript to cite work from certain labs and not others and the authors should do their best to avoid this.

➢ We feel it is exaggerated to state we cite just reviews.
Our manuscript contains 100 references, out of which 12 are reviews. Beside reviews, the Introduction also refers to 31 original research papers. Regarding the above mentioned review articles (Uraji et al., 2018; Duan and Sun, 2019), we cite them in an introductory sentence ("During meiotic maturation, mouse oocytes experience…") that is precisely followed by a detailed description of the key findings regarding the topic, with references to the relevant seminal literature. We went back to the seminal discoveries by Maro, Van Blerkom and Longo in 1985/1986.
Likewise, the Discussion refers to 43 original research articles. Therefore, we do not feel our manuscript makes a disproportionate use of review citations.

➢ It is quite bewildering to answer this reviewer's claim that we favor some labs in our reference list. Inevitably, we refer to previous seminal work in the mouse oocyte actin field, which originates from a relatively small number of labs worldwide. Therefore a number of citations come from a small cluster of labs. However, we truly feel that we have covered all the labs whose work is relevant to our study, and we refute strongly any deliberate attempt to favor some labs over others.

- Western blots presented throughout the manuscript are sound and convey a clear message. However, since these are not truly quantitative blots as fluorescence blots, I would suggest moving the quantification data to supplementary section.

➢ In our opinion, Western blot quantification data (i.e. semi quantitative) are easier to appreciate when displayed next to the gel images.

Second decision letter

MS ID#: JOCES/2021/259237

MS TITLE: Cofilin regulates actin network homeostasis and microvilli length in mouse oocytes

AUTHORS: Anne Bourdais, Benoit Dehapiot, and Guillaume Halet
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have accurately answered the comments and criticism of reviewers, in particular, the reviewer-2.

This second version of the manuscript is a great improvement. I recommend this manuscript in its current form for publication in JCS.

Comments for the author

The authors have accurately answered the comments and criticism of reviewers, in particular, the reviewer-2.
This second version of the manuscript is a great improvement. I recommend this manuscript in its current form for publication in JCS.

Reviewer 2

Advance summary and potential significance to field

Paper is of general interest to the oocyte field.

Comments for the author

The authors have adddressed most outstanding issues and the manuscript is now significantly improved and in my assessment is suitable for publication in JCS.
The authors have highlighted some misunderstanding in my previous comments. While I do respect that, with few exceptions these arose because of sentences that were open to misinterpretation. They have made significant improvements to such sentences throughout the manuscript which should make it more accessible to other readers.

I am not satisfied by the argument about representative citations or citation of reviews that are not comprehensive when refering to fundamental body of work.
Having 100 citations in the manuscript does not substitute for this. This was nonetheless a minor point and should not prevent publication of this work.