Revisiting the multisite phosphorylation that produces the M-phase supershift of key mitotic regulators

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Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 2022-04-06 |
| Editorial Decision     | 2022-05-05 |
| Revision Received      | 2022-07-11 |
| Accepted               | 2022-08-08 |

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E22-04-0118
TITLE: Revisiting the multisite phosphorylation that produces the M-phase supershift of key mitotic regulators

Dear Dr. Kuang:

Your work has now been seen by two experts in the field. As you will see from their reports, both reviewers find the work intriguing, as they appreciate the significance of the findings in terms of cell cycle regulation, but both reviewers also raise a number of significant concerns. Reviewer 1 lays out a couple of specific experiments that will help alleviate the concerns, and I agree with this reviewer that these are reasonable control experiments to include. Reviewer 2 shares some of the same concerns as Reviewer 1 but has a much more dramatic suggestion that the only sure way to alleviate the concern is to identify the unknown kinase. While that would be ideal, that is a major undertaking that in my opinion is beyond the scope of the paper. I would ask that in your revision, you do your best to address the major concerns of reviewer 2 about the artificial system with phosphatase inhibitors.

In your revision, please include a point by point discussion of how the reviewer concerns were addressed.

Sincerely,

Claire Walczak
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Kuang,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
Reviewer #1 (Remarks to the Author):

see attached.

Reviewer #2 (Remarks to the Author):

This paper provides insights into a technical problem in the field of mitotic protein phosphorylation: several proteins phosphorylated during mitosis display an unusually large mobility shift on SDS-PAGE. This shift is known to be due to hyperphosphorylation, and well-known mitotic kinases, particularly Cdk1, have been implicated. The key result in the current paper is that the hyperphosphorylation is not entirely due to known mitotic kinases but might depend on other factors, which remain unidentified. These results raise the possibility that our understanding of the key regulators of mitosis is incomplete. In essence, however, the paper documents a negative result, which is difficult to prove and might leave some readers unsatisfied.

The central approach in the paper is to analyze hyperphosphorylation of several well-studied mitotic proteins in oocyte extracts (called MEE) containing the PP2A inhibitor okadaic acid (OA) plus ATP-g-S, which generates thiophosphorylated proteins that are resistant to phosphatases. The paper includes a deep and comprehensive analysis of the phosphorylation sites that trigger the gel mobility ‘supershift’ in these extracts. The key phosphorylated regions of multiple mitotic proteins are identified (Fig 2) and shown to be regions of predicted disorder (Fig 3). Phosphorylation site analysis by mass spectrometry then reveals that these regions are densely packed with highly phosphorylated serines, threonines, and even some tyrosines (Figs 4, 5). These dense patterns of phosphorylation were also seen in previous phosphosite studies of human proteins (Fig 6). Figure 7 contains clear evidence that the large mobility shift in one substrate, Cdc25C, depends on the additive effects of gradual changes in mobility with individual sites. Remarkably, gradual addition of phosphomimetic mutations results in a gradual retardation of gel mobility.

Figures 8-10 address the identity of the kinases responsible for the supershift. In Figure 8, the major mitotic kinases are shown to have little impact on the mobility shift of multiple proteins, suggesting that they are not sufficient for the shift. Similarly, immunodepletion of these kinases has little impact on the MEE-induced shift, also arguing that these kinases are not required.

Based on my reading of the Methods, the recombinant kinases used in Figure 8 are produced by translation in vitro, and not purified before use. Therefore they will contain phosphatases from the transcription/translation extract, which will reverse their effects. What is the effect of adding OA and ATP-g-S to the recombinant kinases before these reactions?

A key concern is that most of the evidence in this paper depends on studies of phosphorylation in ‘MEE’ extracts containing phosphatase inhibitor. Presumably, in untreated extracts PP2A reverses the inevitable background phosphorylation from the many kinases in the concentrated extract, ensuring that extensive phosphorylation occurs only when mitotic kinase activities rise in mitosis. In an MEE extract or oocyte where dephosphorylation is prevented, there is a concern that this background phosphorylation will accumulate, even when these mitotic kinases are mostly inhibited by chemicals or immunodepletion.

This point is illustrated by the experiments in which the authors analyze protein phosphorylation in the physiological context of progesterone-stimulated oocyte maturation (Fig. 1D-F, Fig 10). The supershift is less dramatic in progesterone treatment than it is in the MEE treatment (Fig 1E-F), and the progesterone-dependent shift is prevented by mutation of Cdk1 sites (Fig 1F) or inhibition of Cdk1 and MEK (Fig 10A). Thus, under physiological conditions, these kinases are responsible. My interpretation of the MEE experiments is that OA stabilizes the low level of background phosphorylation that is normally turned over by phosphatases; even a small amount of Cdk1 and MEK activity might be sufficient in this case to promote significant phosphorylation. And the cell contains numerous other protein kinases that modify these proteins at a low rate, which could lead to high levels of phosphorylation when dephosphorylation is blocked.

Nevertheless, the authors might be correct in their proposal that other kinases or factors are required for mitotic entry. The only effective way to address this possibility is to identify those other factors and demonstrate their importance. Until then, there will continue to be doubts about results based on studies with phosphatase inhibitors.
POINT-BY-POINT RESPONSE TO REVIEWERS’ COMMENTS

Reviewer #1 (Remarks to the Author):

In this paper, Kuang and colleagues examine the multisite phosphorylation and electrophoretic mobility shifts of five key mitotic phosphoproteins. For the most part, the authors use what they call MEE—an M-phase Xenopus egg extract supplemented with ATP-gamma-S and 1 μM okadaic acid—to catalyze the phosphorylations. They find that MEE can cause substantial mobility shifts on five Myc-tagged recombinant substrate proteins (Cdc25C, Gwl, Myt1, Wee1, and APC3), and on Myc-tagged regulatory regions of these proteins. The shifts are similar to those seen in M-phase lysates, and the authors map the phosphorylation sites. Similar studies have been done by several other groups, but arguably the present work is the best, by far, on these issues. The authors have been able to obtain more complete coverage than anyone else has, and by examining more than one of these proteins they make a more convincing case for drawing general conclusions than previous studies examining one or another of the substrates have. Many but not all of the phosphorylations appear to contribute to the mobility shift. The results from this part of the paper (Figs 1-7) probably won’t surprise anyone—the phosphorylations happen almost exclusively in the regions of the proteins predicted to be intrinsically disordered, they happen both at SP/TP sites and at other sites, and there are many sites in each of the proteins—but the data of high quality will be valuable to the field.

The more surprising results are presented in Figs 8-10. First, the authors show that a mixture of four active, recombinant mitotic kinases—CycB1/Cdk1/p9, Plx1, MAPK, and Rsk2—is substantially less able to generate an electrophoretic band shift in Cdc25C 9-375, Myt1 406-548, Wee1A 1-199, Gwl 179-745, and APC3 147-457 than MEE is, even in the presence of the phosphatase inhibitor microcystin. My first thought was that maybe the authors had not managed to produce as much active kinase in the in vitro transcription/translation system as is present in MEE, and this remains a concern for me, but they do show that their recombinant Cyc B1/Cdk1/p9 is similar in activity toward histone H1 as MEE; that the recombinant Plx1, MAPK, and Rsk2 proteins are similar in concentration to that seen in MEE; and that their Plx1, MAPK, and Rsk2 proteins can phosphorylate Myc-Cdc25C pretty well. Whether or not they are just as active as their MEE counterparts is another question, but it is still surprising that they were not more effective at phosphorylating the five substrates (Fig S8). Something seems to be missing from the recombinant proteins, and it could be an overlooked mitotic kinase.

Furthermore, they show that immunodepletion of any of the four mitotic kinases from MEE has no discernible effect on the phosphorylation of these five substrates (Fig 8D). I suppose it is possible that the immunodepletion is not as complete it appears to be from the blots at the top of Fig 8D, or that at earlier time points a decrease in substrate phosphorylation would be more apparent. But perhaps I am being overly skeptical because of a reluctance to abandon the idea that these four kinases, plus Aurora A and B, really are the main M phase kinases in frog eggs.

Fig 9 argues against the possibility that more p9 might be responsible for the good phosphoshifts seen in extracts, and Fig 10 shows that small molecule inhibitors of Cdk1/2 and MEK do not affect okadaic acid-induced oocyte maturation or protein phosphorylation (although they do affect progesterone-induced maturation).

I suspect that despite all of the various controls, the interpretation of Figs 8-10 will still be met with a good deal of skepticism by the field. Nevertheless, provided the experiments I suggest as my two major points give the hoped-for results, I would support publication of this provocative work. If the authors’ interpretation is right, it will be an important advance, and even if it is not right, there is enough evidence to support it that should be considered carefully.

We appreciate the thorough, fair, constructive and open-minded review by this reviewer. While this reviewer appreciated the solid and good quality parts of our work and recognized their contributions to the field, the weak aspects of our study were also critically pointed out with specific suggestions on how to amend the problem. Following these helpful suggestions, we have both added kinase inhibition experiments to complement the kinase depletion experiments and done detailed time course examinations in both approaches (see new Figs. 10&11 and new Figs. S11&S12). Largely consistent
with each other, results from kinase inhibition and depletion experiments indicate that none of the four major mitotic kinases examined is required for MEE-induced supershift of the five supershift fragments.

Although we could not think of a good experiment that directly addresses the concern of this reviewer that TNT-produced kinases may not be as active as their MEE counterparts, we have done a new experiment to demonstrate that activation of all four of the major mitotic kinases in diluted immature oocyte extracts by both OA and activated Cdc25C still failed to induce the M-phase supershift of endogenous Cdc25C, whereas 1:16- and 1:32-diluted MEE still quantitatively and partially induced the M-phase supershift of recombinant Cdc25C, respectively, under the same buffer condition. Using the two homologous cell free systems made it easier to demonstrate that the four major mitotic kinases were activated to similar levels. The results strengthen conclusion that phosphorylation with the four major mitotic kinases is insufficient to produce the M-phase supershift.

We thank this review for the time and effort in doing this thorough review. We hope that the revised version of the paper has successfully addressed the main concerns of this reviewer and that, by doing so, we significantly increase the quality of this paper.

Major points:

1. Please complement the Immunodepletion experiments with an additional experiment using pharmacological inhibitors to see if the various kinases are required for the MEE-induced phosphoshifts. For Cdk1/2 inhibitors, you could use a range of inhibitor concentrations and monitor the effect on histone H1 phosphorylation vs. TNT-produced substrate phosphorylation. For MEK inhibitors you could look at MAPK phosphorylation and Rsk2 phosphorylation vs. substrate phosphorylation. For Pkl1 inhibitors, the substrate used in Fig S8D would be a good control. Note that because you are adding the inhibitors to essentially undiluted cytoplasm, you will need to use higher concentrations than are needed in, say, immune complex kinase assays or tissue culture cells overlaid with a surfact of inhibitor-containing medium. That’s why running the gold-standard substrates in parallel under the same incubation conditions is important. This would be a good way of further testing, and hopefully corroborating, the surprising immunodepletion results. I know the authors already have data from oocytes treated with inhibitors, but it takes a long time for inhibitors to completely equilibrate throughout the oocyte. I think the inhibitor + extract experiment is cleaner and essential.

We appreciate these fair and constructive suggestions. Since kinase inhibition and kinase depletion approaches have different pros and cons, we agree with this reviewer that additional experiments with kinase inhibitors will strengthen our conclusion that the four major mitotic kinases are not required for the M-phase supershift. Following these suggestions, we have added two sets of experiments to determine the effect of four specific inhibitors on the M-phase supershift in the revised manuscript. In one set of experiments, GST-Cdc25C or myc-Myt1 was phosphorylated with TNT-produced kinases in the presence of different concentrations of the corresponding kinase inhibitor, i.e., 2 or 10 µM roscovitine for Cdk1/2, 0.02 or 0.1 µM VX-11e for MAPK, 10, 50 and 250 µM SL0101 for RSK1/2, and 0.4 and 2 µM BI2536 for Pkl1. While phosphorylation of GST-Cdc25C by Cdk1, MAPK and RSK2 was monitored by immunoblotting with phosphospecific antibodies for identified phosphorylation sites, phosphorylation of myc-Myt1 by Pkl1 was analyzed by gel mobility shift assays. Results from these experiments showed clearly that 2 µM roscovitine, 0.02 µM VX-11e, 10 µM SL0101 and 0.4 µM BI2536 were sufficient to potently inhibit the activity of TNT-produced Cdk1, MAPK, RSK2 and Pkl1, respectively (see new Figs. S11A-S11D). Based on these results, we felt that 5-fold higher of these inhibitor concentrations should be sufficient to potently inhibit their target kinase activities in MEE even if TNT-produced kinases were actually not as active as their counterparts in MEE, as was concerned by this reviewer. Consistent with this prediction, 10 µM roscovitine potently inhibited histone H1 kinase activity in MEE (new Fig. S11E). In another set of experiments, 5-fold higher of these concentrations of the specific kinase inhibitors, i.e., 10 µM roscovitine, 0.4 µM VX-11e, 50 µM SL0101 and 2.0 µM BI2536, were added to MEE along with 25 µM of the pan kinase inhibitor staurosporine and their individual and collective effects on MEE-induced supershifts of myc-tagged five supershift fragments were determined by time course examinations (Figs. 10B-10D). Clearly, none of the specific kinase inhibitors used
individually affected the magnitude or kinetics of the MEE-induced supershifts of the five supershift fragments, whereas the pan kinase inhibitor dramatically inhibited the supershifts (new Fig. 10B). While a combinational use of the four specific kinase inhibitors still did not affect MEE-induced supershifts of Cdc25C\textsuperscript{9-375}, Myt1\textsuperscript{406-546} or Wee1A\textsuperscript{1-199} (new Fig. 10C), it moderately retarded MEE-induced supershifts of APC3\textsuperscript{147-457} and Gwl\textsuperscript{179-745}, which shifted more slowly than the other three proteins (new Figs. 10D&10E).

At this stage, we do not know whether these moderate inhibitory effects were due to off target effects of the high concentrations of the kinase inhibitors or non-essential involvement of these kinases in the process. Nonetheless, these results are sufficient to indicate that the four major mitotic kinases are not required for MEE-induced supershifts.

2. From the data in Fig 8D, it looks like the phosphorylation of the 5 substrate proteins is not affected at all by depletion of any of the 4 kinases. How long was the incubation? Might it be possible that under these conditions--very very low phosphatase activity--if you wait long enough, you will obtain complete phosphorylation even from a low amount of kinase activity? If you look at earlier time points too, you should be able to say something like “depletion of Cdk1 by at least 90% caused no more than a 20% slowing of Cdc25C phosphorylation as judged by the phosphoprotein” (or whatever).

We appreciate this fair and constructive comment. To answer the question on incubation time, the data in Fig 8D were side-by-side immunoblotting of samples from the first and last timepoint (120 min) of 5 pairs of time course examinations (15, 30, 60, 90, 120). Regrettably, we did not present the whole set of data from time course examinations simply because the results were largely negative and omitting them saved a lot of space. However, this reviewer is right that since immunodepletion is hardly 100%, very very low phosphatase activity may allow very low kinase activity to generate complete phosphorylation given enough time. To eliminate this possibility, time course examination is actually needed to demonstrate that the phosphorylation of the 5 supershift fragments is not affected by depletion of any of the 4 kinases. Thus, in the revised manuscript, we have presented the whole set of the data from time course examinations in the new Fig. 11 and moved the data in the original Fig. 8D&8E to new Fig. S12.

Minor points:

p 6 line 5. I think this is cyclin B1. That should be specified since cyclin B2 does undergo an M-phase phosphoshift (see Kobayashi et al. CSHSQB 1991). Also there are two different cyclin B1 genes in Xenopus and both are expressed in eggs, so it would be best to say which B1 is being examined here.

We appreciate this critique and apologize for not spelling out the specific form of cyclin B. In the revised manuscript, we clearly state that while Plx1, MEK1 and MAPK were all from Xenopus, cyclin B was from sea urchin (cyclin B \textsuperscript{A90}) and Pin 1 was from human. The difference was simply due to the availability of expression constructs for myc-tagged proteins in our reagent stock. Nonetheless, sea urchin cyclin B \textsuperscript{A90} behaves similarly as Xenopus cyclin B1 in having no M-phase shift in Xenopus systems (PMID: 25669118). Human Pin1, which has great sequence similarity with Xenopus Pin1, undergoes no M-phase shift in mitotic cell cycles (PMID: 9499405). We thus felt that they were legitimate representatives of non-supershift proteins for this part of the study.

p 8 bottom and Fig S2. Actually cyclin B1 has an enrichment of S/TP residues and a small enrichment of S’s and T’s in its regulatory region.

We appreciate this comment, which made us realize that the original description did not emphasize enough that the supershift domain has substantial enrichment of BOTH S/T residues and S/TP motifs. In fact, although none of the five non-supershift proteins examined contains a long intrinsically disordered region that is enriched in BOTH S/T residues and S/TP motifs, both Plx1 and Pin1 contains a long intrinsically disordered region that has significant enrichment of S/T residues, whereas both cyclin B1 (long form) and MEK1 contain a long intrinsically disordered region that has significant enrichment of S/TP motifs. These descriptions are included in the revised manuscript to avoid potential confusions.
p 10 line 4: Replace “inevitable” with “inevitably”.

We appreciate this catch, and a correction has been made in the revised manuscript.

p 11, Fig 5: Please specify the duration of the incubation. Was this phosphorylation measured after 6 h of incubation, as in Fig S4? If so you may want to explain that you are using such a long incubation because you are trying to phosphorylate higher-than-physiological concentrations of the substrate proteins (if that is in fact the case).

We thank this reviewer for this comment, which pressed me to sort out exactly how long the incubation with MEE was for mass spec work in this particular study and why. As described below, we have done more mass spectrometry work than is presented in this paper, and some of the information was actually for mass spectrometry work not presented in this paper. We apologize for these mismatching mistakes and have provided specific information on the phosphorylation time in the revised manuscript.

(i) Although not described in this paper, we initially used GST-tagged near full-length Cdc25C (GST-Cdc25C) as a model molecule to characterize the supershift-producing phosphorylation by mass spectrometry. To ensure that the phosphorylation of GST-Cdc25C with MEE was both robust and not an overkill, the contributing author for this part of work (Dr. Wu) phosphorylated GST-Cdc25C with MEE for 3, 6 and 15 in our pilot studies and found that the shift was still increasing from 3 to 15 h (Fig. 1A). To determine whether the 3-h phosphorylation was acceptable, she then phosphorylated GST-Cdc25C and myc-Cdc25C in parallel with MEE for 3 h and made side-by-side comparison of their gel mobility shifts. On the same blot, immobilized GST-Cdc25C shifted about 10-kDa less than soluble myc-Cdc25C (Fig. 1B), presumably due to much higher concentration and much less mobility of GST-Cdc25C than myc-Cdc25C. Thus, the 3-h phosphorylation time seemed to be too short for GST-Cdc25C. To partially compensate for this deficiency, Dr. Wu decided to phosphorylate GST-Cdc25C with MEE for 6 h for mass spectrometry, even though the time was still not long enough for GST-Cdc25C to reach the same amount of shift as myc-Cdc25C. Regrettably, although the 6-h phosphorylation time was only for GST-Cdc25C, I mistakenly assumed that it was for GST-tagged five supershift fragments as well. As a result, I provided incorrect information both in the text and figure legends for Fig. S4. I apologize for these mistakes due to my insufficient care in details and have corrected them in the revised manuscript.

(ii) After mass spec data from MEE-shifted GST-Cdc25C clearly indicated the robust region-specific but site nonspecific phosphorylations, we decided to systematically investigate the issue using five key mitotic regulators as model molecules. To define an optimal time for phosphorylation of GST-tagged five supershift fragments with MEE for mass spectrometry, the contributing author for this part of the work (also Dr. Wu) phosphorylated GST-Cdc25C9-374 and myc-Cdc25C9-374 in parallel with MEE for 3 h and made side-by-side comparison of their gel mobility shifts on the same blot. Although immobilized GST- Cdc25C9-374 still shifted less than soluble myc-Cdc25C9-374 (Fig. 1C), the difference was less...
dramatic than that for GST-Cdc25C, possibly due to absence of intramolecular interactions. Also further increasing the phosphorylation time from 3 to 4 to 6 h did not improve the magnitude of the phosphorylation (data not shown). Based on these results, Dr. Wu decided to phosphorylate all five of the GST-tagged supershift fragments with MEE for 3 h, rather than 6 h, for mass spec work. Again, I apologize for mismatched information in the original manuscript and have corrected these mistakes.

(iii) Since we have done more mass spectrometry work than is presented in this study, Dr. Wu wrote a general method to cover it all, that is “For mass spectrometry, GST-tagged proteins, which were affinity-absorbed onto glutathione sepharose, were phosphorylated with two volumes of MEE for 3-6 h”. Although it should be my responsibility to tailor the method to this particular paper, this general method was used directly in the original manuscript. This mistake has been corrected in the Method section of the revised manuscript.

(iv) Since 3 h is still a long time for phosphorylation, we have added in the revised manuscript that we were using such a long incubation time because GST-tagged substrate proteins were used at much higher concentrations than myc-tagged substrate proteins. Note that myc-tagged substrates were already of much higher concentrations than physiological concentrations.

Reviewer #2 (Remarks to the Author):

This paper provides insights into a technical problem in the field of mitotic protein phosphorylation: several proteins phosphorylated during mitosis display an unusually large mobility shift on SDS-PAGE. This shift is known to be due to hyperphosphorylation, and well-known mitotic kinases, particularly Cdk1, have been implicated. The key result in the current paper is that the hyperphosphorylation is not entirely due to known mitotic kinases but might depend on other factors, which remain unidentified. These results raise the possibility that our understanding of the key regulators of mitosis is incomplete. In essence, however, the paper documents a negative result, which is difficult to prove and might leave some readers unsatisfied.

We appreciate these comments and agree with this reviewer that negative results are more difficult to prove than positive results. However, since clearly showing that something is missing is a critical step in a multi-step process toward defining what is actually missing, we have added several new experiments in the revised manuscript to increase the strength of our fining that the well-known mitotic kinases are neither sufficient nor required for the M-phase supershift. We hope that adding these new experiments significantly increases the strength of our conclusion.

The central approach in the paper is to analyze hyperphosphorylation of several well-studied mitotic proteins in oocyte extracts (called MEE) containing the PP2A inhibitor okadaic acid (OA) plus ATP-γ-S, which generates thiophosphorylated proteins that are resistant to phosphatases. The paper includes a deep and comprehensive analysis of the phosphorylation sites that trigger the gel mobility ‘supershift’ in these extracts. The key phosphorylated regions of multiple mitotic proteins are identified (Fig 2) and shown to be regions of predicted disorder (Fig 3). Phosphorylation site analysis by mass spectrometry then reveals that these regions are densely packed with highly phosphorylated serines, threonines, and even some tyrosines (Figs 4, 5). These dense patterns of phosphorylation were also seen in previous phosphosite studies of human proteins (Fig 6). Figure 7 contains clear evidence that the large mobility shift in one substrate, Cdc25C, depends on the additive effects of gradual changes in mobility with individual sites. Remarkably, gradual addition of phosphomimetic mutations results in a gradual retardation of gel mobility.

Figures 8-10 address the identity of the kinases responsible for the supershift. In Figure 8, the major mitotic kinases are shown to have little impact on the mobility shift of multiple proteins, suggesting that they are not sufficient for the shift. Similarly, immunodepletion of these kinases has little impact on the MEE-induced shift, also arguing that these kinases are not required.

Based on my reading of the Methods, the recombinant kinases used in Figure 8 are produced by translation in vitro, and not purified before use. Therefore they will contain phosphatases from the
transcription/translation extract, which will reverse their effects. What is the effect of adding OA and ATP-$\gamma$-S to the recombinant kinases before these reactions?

We appreciate this critique, which made us realize that some of descriptions in the method section was not sufficiently clear. To clarify this point, we routinely added okadaic acid or microcystin to all of TNT-produced kinases to eliminate this complication since we were also aware of the possible presence of opposing phosphatase activity in the TNT system. In the original manuscript, we did state inclusion of phosphatase inhibitors in TNT-produced kinases, although the information might be buried between lines based on this reviewer’s comment. To make this point clearer, we have revised the method section on this issue in the revised manuscript.

In term of ATP-$\gamma$-S, an established understanding from reading of the literature in the past is that that ATP-$\gamma$-S is about 10-20 fold less efficient in supporting phosphorylation than ATP. Consistent with this background information, ATP-$\gamma$-S was clearly less efficient than ATP in generating the M-phase supershift when we previously phosphorylated recombinant Cdc25C with MEE containing either ATP or ATP-$\gamma$-S in the presence of phosphatase inhibitors. This is why we did not add ATP-$\gamma$-S to either TNT-produced kinases or dilution buffer for MEE or p9-Cdk1. Of note, why ATP-$\gamma$-S stabilizes MPF activity is still an open problem.

A key concern is that most of the evidence in this paper depends on studies of phosphorylation in ‘MEE’ extracts containing phosphatase inhibitor. Presumably, in untreated extracts PP2A reverses the inevitable background phosphorylation from the many kinases in the concentrated extract, ensuring that extensive phosphorylation occurs only when mitotic kinase activities rise in mitosis. In an MEE extract or oocyte where dephosphorylation is prevented, there is a concern that this background phosphorylation will accumulate, even when these mitotic kinases are mostly inhibited by chemicals or immunodepletion. This point is illustrated by the experiments in which the authors analyze protein phosphorylation in the physiological context of progesterone-stimulated oocyte maturation (Fig. 1D-F, Fig 10). The supershift is less dramatic in progesterone treatment than it is in the MEE treatment (Fig 1E-F), and the progesterone-dependent shift is prevented by mutation of Cdk1 sites (Fig 1F) or inhibition of Cdk1 and MEK (Fig 10A). Thus, under physiological conditions, these kinases are responsible. My interpretation of the MEE experiments is that OA stabilizes the low level of background phosphorylation that is normally turned over by phosphatases; even a small amount of Cdk1 and MEK activity might be sufficient in this case to promote significant phosphorylation. And the cell contains numerous other protein kinases that modify these proteins at a low rate, which could lead to high levels of phosphorylation when dephosphorylation is blocked.

We understand this reviewer’s key concern that in the absence of opposing phosphatase activities, even a small amount of Cdk1 and other mitotic kinase activities might be sufficient to accomplish the supershift producing phosphorylation give enough time. To address this concern, we have fully activated all four of the major mitotic kinases in diluted immature oocyte extracts by both okadaic acid and activated Cdc25C (see new Fig. 9A) and then determined the effect on the M-phase supershift of endogenous Cdc25C by time course examination for up to 2.5 h (see new Fig. 9B). A premise for this approach was that even 1:16- and 1:32-diluted MEE still quantitatively and partially induced the supershift of myc-Cdc25C after 2-h phosphorylation reactions under the same buffer condition (Fig. S10C). Consistent with results obtained from phosphorylation of Cdc25C with multiple TNT-produced kinases (see Fig. 8B), robust activation of all four of the major mitotic kinases in the immature oocyte extracts did not induce the M-phase supershift of endogenous Cdc25C. Neither did it induce the burst of MPM-2 reactivity in numerous proteins, which is a reliable M-phase specific marker that tightly associates with the M-phase supershift of the five mitotic regulators. However, activation of the four major mitotic kinases did induce slight gel mobility shifts of endogenous Cdc25C. In our opinion, these results significantly strengthen the conclusion that the phosphorylation by the four major mitotic kinases is not sufficient to produce the M-phase supershift.

In addition to the above-described new results, we have also determined the effects of both kinase inhibition and kinase depletion on the kinetics of the MEE-induced supershifts of the five supershift
fragments by detained time course examination, as suggested by the other reviewer for a similar concern (see new Figs. 10&11 and new Figs. S11&S12). The results from these new experiments showed clearly that none of the single kinase inhibition or depletion significantly affected the kinetics or magnitude of the MEE-induced supershifts through the time course. Even when the four kinases were inhibited or depleted together, the M-phase induced supershift of the five supershift fragments were either nor affected at all or only mildly retarded, which could be due to nonspecific reasons. These results strongly argue against the possibility that the inability of the kinase inhibition/depletion to inhibit the M-phase supershift is due to amplified effects of residual levels of mitotic kinases due to the absence of opposing phosphatase activities. Together, we hope that these new experiments added to the revised manuscript are able to eliminate or significantly decrease the key concern of this reviewer.

Nevertheless, the authors might be correct in their proposal that other kinases or factors are required for mitotic entry. The only effective way to address this possibility is to identify those other factors and demonstrate their importance. Until then, there will continue to be doubts about results based on studies with phosphatase inhibitors.

We appreciate the open-minded attitude of this reviewer and agree that the most definitive way to demonstrate that other kinases or factors are required for mitotic entry is to identify those other factors and demonstrate their physiological importance. In our opinion, achieving this goal is inevitably a complex multi-step process that cannot be accomplished by one paper. On one hand, the supershift producing activity of MEE is always mysteriously lost during hardcore biochemical fractionation of MEE even in the presence of both ATP-γ-S and okadaic acid, although there is no difficulty to recover the activity of the four major mitotic kinases. Similar failures were also experienced by other labs, although these negative results were never published. The hint from these bewildering results is that we are dealing with a multifactorial problem that cannot be resolved by hardcore protein purifications or other traditional biochemical approaches. On the other hand, the M-phase supershift is a highly substrate specific phenomenon, which seems to be sensitive to substrate division (Fig. S3). These observations imply that the supershift domain contains cis-operating elements that enable the supershift producing phosphorylation. Although identification of the substrate determinants is beyond the scope of this paper, we have started the process and realized that the cis-operating determinant for the supershift producing phosphorylation is also multifactorial in location and function. Since different cis-operating elements in the supershift domain may bind different kinase and non-kinase factors to assemble a highly efficient molecular condensate that catalyzes the supershift producing phosphorylation, this may be why we and others could not purify the supershift producing activity. At this stage, identification and characterization of the cis-operating determinants in the supershift domain is our priority since we feel that it holds a key position in both establishing the framework for the M-phase supershift and generating an effective approach to identify the kinase and non-kinase factors that catalyze the M-phase supershift.
Dear Dr. Kuang:

Your manuscript has now been reviewed by the original two reviewers of your paper. Both reviewers found your revision addressed all of their major concerns, and I am happy to accept your paper for publication in Molecular Biology of the Cell.

Sincerely,
Claire Walczak
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Kuang:

Congratulations on the acceptance of your manuscript. A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Sincerely,
Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):
The authors have responded well to my review (and, in my opinion, to the other reviewer's review). I recommend publication.

Reviewer #2 (Remarks to the Author):
The authors have dedicated a lot of effort to performing new experiments that address most of my previous concerns. I continue
to be puzzled about the identity of the activity that is responsible for the supershift in the MEE. Nevertheless, the paper provides multiple lines of evidence to support their hypothesis about a separate kinase, and I believe that these results should be published to catalyze further study of this provocative idea.