Cdk1 phosphorylation of fission yeast paxillin inhibits its cytokinetic ring localization

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(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 #E20-12-0807
TITLE: Cdk1 phosphorylation of fission yeast paxillin inhibits its cytokinetic ring localization

Dear Kathy,

Your paper has now been reviewed by two experts in the field. You will see that they both find your study interesting and point out the solid biochemistry, but also mention some difficulties in understanding the role of Pxl1 (de-)phosphorylation in the cell. They make suggestions for relatively simple experiments to consolidate this understanding.

I will be very happy to consider a revised manuscript that addresses the reviewers' comments. I don't think point 3 of reviewer 2 needs to be fully addressed experimentally.

Best wishes,

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gould,

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.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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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):

This paper investigates phosphorylation of the cytokinetic ring protein Pxl1 (paxillin) in fission yeast. Building on earlier papers, the authors show that CDK directly phosphorylates Pxl1. They next show that dephosphorylation of Pxl1 at the end of the cell cycle is mediated by the phosphatases Clp1 and PP1, each of which partially contribute to this dephosphorylation. The authors generate 9A (non-phosphorylatable) and 9D (phosphomimetic) versions of Pxl1 to study the impact of phosphorylation in vivo. The 9A mutant causes precocious Pxl1 recruitment to the cytokinetic ring along with changes in the timing of cytokinesis, while the 9D was largely similar to wild type. Finally, the authors use in vitro binding assays to show that phosphorylation of Pxl1 regulates its interaction with Cdc15, a known binding partner for Pxl1 at the cytokinetic ring.

Overall, the paper provides new information regarding phospho-regulation of a conserved protein that associates with the cytoskeleton. A strength is the identification of CDK and Clp1/PP1 as the kinase and phosphatases that regulate Pxl1 phosphorylation in cells. All of these players are conserved and well-studied in other organisms, so identification of this regulatory mechanism is likely to draw broad interest. However, the physiological role of the mechanism remains unclear. The cellular assays largely rely on the 9A and 9D mutants, and unfortunately the 9A mutant dramatically increases Pxl1 protein levels in cells for unknown reasons. Higher protein concentration likely drives Pxl1 biochemical interactions beyond typical levels, which could lead to secondary effects such as precocious recruitment to the cytokinetic ring. The 9D mutant is also difficult to interpret and the authors "suspect...pxl1(9D) is not a true phosphomimetic." Therefore, these mutations appear to have effects beyond phospho-regulation, and these caveats prevent a clear interpretation of how phospho-regulation by CDK/Clp1/PP1 might regulate Pxl1 function and its interaction with Cdc15 in cells. It should be noted that the authors provide a balanced discussion of these strengths and weaknesses in the manuscript.

Specific Comments:

1. Due to difficulties interpreting the 9A and 9D mutants, the authors should monitor changes in Pxl1-mNG in dis2Δ and clp1Δ mutants (and the double mutant, if viable) by microscopy. Similarly, they could use CDK mutants to test changes in the timing of Pxl1 recruitment to the ring. Perhaps they could address the role of Pxl1 phosphorylation in cells using such mutants, although it would be difficult to know if Pxl1 defects in these mutants are due to direct effects from Clp1, PP1, and CDK.

2. What happens to Pxl1 dephosphorylation in a dis2Δ clp1Δ double mutant? The authors' results predict that Pxl1 dephosphorylation might be completely lost in this double mutant.

3. PPase result in Figure 2D (right panel) is not very clear. It looks like the mutants might shift after phosphatase treatment? As presented, this result was inconclusive to me.

4. In reading the methods section, I became confused regarding the 3' UTR of pxl1 mutant alleles. The authors describe that their integration DNA contained "the desired pxl1 allele, a kanR cassette, and 500 bp of pxl1 3' noncoding region." This wording suggests that the 3' UTR has been separated from the coding region by insertion of kanR, which might alter stability of the transcript. Can the authors clarify the position of kanR in this scheme, and whether the pxl1 3' UTR remains adjacent to the coding sequence? I doubt this comment explains expression differences in 9A versus other pxl1 constructs because they all use the same scheme, but it is worth clarifying.

Reviewer #2 (Remarks to the Author):

The authors of this work find that Cdk1 phosphorylates Pxl1 during mitosis exit. Phosphorylation occurs on nine residues at Pxl1 N-terminus and affects its role in cytokinesis. Additionally, the authors show that Pxl1 can be dephosphorylated by Clp1 and Dis2 (PP1), and also demonstrate that preventing Pxl1 phosphorylation results in increased Pxl1 levels, precocious recruitment to the division site, and increase in the duration of CR constriction. In vitro, Cdk1-mediated phosphorylation of Pxl1 inhibits its interaction with the F-BAR domain of the cytokinetic scaffold Cdc15.
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Comments:

1. In vitro, Cdk1-mediated phosphorylation of Pxl1 inhibits its interaction with the F-BAR domain of the cytokinetic scaffold Cdc15, thereby disrupting a major mechanism of Pxl1 recruitment.

Is there less GFP-Pxl1 in the CR of clp1▯ or dis2▯▯or in the double mutant? or is GFP-Pxl1 recruited later in these mutants? These experiments are easy to perform and might add important information.

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Minor points:

- Pg4. Iqg1/Rng2 is confusing, change to: Iqg1 and Rng2 respectively
...and S. cerevisiae C2 domain protein Inn1/Fic1 (Palani et al., 2012; Kuilman et al., 2015). Please, change to: Inn1, the ortholog of S. pombe Fic1.
- Pg 4 and 5. "pxl1Δ cells have severe cytokinesis defects, displaying CR sliding and splitting during anaphase (Ge and Balasubramanian, 2008; Pinar et al., 2008)". The CR sliding phenotype was described in Cortés et al. PLOS Genetics 2015, not in the mentioned articles. Please, include the reference.
- Figure 2E. It would be better if the blot for Pxl1-91A were changed for other blot with a protein load similar to those of Pxl1 and Pxl1-9D blots.
- Figure 3A. Why was the treatment with the CN inhibitor FK506, tested if it prevented Pxl1 dephosphorylation, done 30 min after release of G2 arrest and not at the time of release (time 0)?
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We thank Reviewer #1 for his/her thoughtful summary and assessment of our manuscript and for their experimental suggestions. Both *clp1*Δ and *dis2*Δ have altered cytokinetic timings (Trautmann, S. et al. 2001; Buttrick, G.J. et al 2011, Tang, N.H. and Toda, T. 2015, and our preliminary experiments), which limit interpretation of Pxl1 temporal dynamics compared to wild-type. We did quantify Pxl1 abundance in interphase and M phase cells in *clp1*Δ and *dis2*Δ (as we did for the phosphomutants), which showed an increase in mNG-Pxl1 cytokinetic ring (CR) intensity in both phosphatase deletions compared to wild-type, as well as a statistically significant increase in whole cell (WC) intensity in *dis2*Δ and trend to increase in WC intensity in *clp1*Δ, leading to comparable CR:WC ratios across all strains (Figure S3, D-F), similar to what was seen in *pxl1-9A* mutant (Figure S3, A-C).

Buttrick, G.J., Meadows, J.C., Lancaster, T.C., Vanoosthuyse, V., Shepperd, L.A., Hoe, K.-L., Kim, D.-U., Park, H.-O., Hardwick, K.G., and Millar, J.B.A. (2011). Nsk1 ensures accurate chromosome segregation by promoting association of kinetochores to spindle poles during anaphase B. Mol Biol Cell 22, 4486-4502.
Tang, N.H., and Toda, T. (2015). Alp7/TACC recruits kinesin-8-PP1 to the Ndc80 kinetochore protein for timely mitotic progression and chromosome movement. J Cell Sci 128, 354-363.

Trautmann, S., Wolfe, B.A., Jorgensen, P., Tyers, M., Gould, K.L., and McCollum, D. (2001). Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. Curr Biol 11, 931-940.

2. What happens to Pxl1 dephosphorylation in a dis2Δ clp1Δ double mutant? The authors' results predict that Pxl1 dephosphorylation might be completely lost in this double mutant.

We examined Pxl1 phosphorylation in *clp1Δdis2Δ* double mutant (Figure S2C), which showed that the phosphorylation of Pxl1 in the double mutant is similar to both single mutants. This suggests that Dis2 and Clp1 likely dephosphorylate overlapping sites on Pxl1, which is consistent with both participating in reversal of CDK phosphorylation either directly (Clp1) or indirectly (Dis2).

Heim, A., Konietzny, A., and Mayer, T.U. (2015). Protein phosphatase 1 is essential for Greatwall inactivation at mitotic exit. EMBO Rep 16, 1501-1510.
Martín, R., Stonyte, V., and Lopez-Aviles, S. (2020). Protein Phosphatases in G1 Regulation. International Journal of Molecular Sciences 21, 395.
Qian, J., Beullens, M., Huang, J., De Munter, S., Lesage, B., and Bollen, M. (2015). Cdk1 orders mitotic events through coordination of a chromosome-associated phosphatase switch. Nature communications 6, 10215.

3. PPase result in Figure 2D (right panel) is not very clear. It looks like the mutants might shift after phosphatase treatment? As presented, this result was inconclusive to me.

We agreed with the reviewer that this result was inconclusive, which is why we pursued analyzing the phosphorylation of Pxl1 phospho-mutants throughout the cell cycle as lysates from synchronized cells better highlights the dramatic loss of gel shift in both the 9A and 9D phosphomutants. We have moderated our language in the text to reflect this.

4. In reading the methods section, I became confused regarding the 3' UTR of pxl1 mutant alleles. The authors describe that their integration DNA contained "the desired pxl1 allele, a kanR cassette, and 500 bp of pxl1 3' noncoding region." This wording suggests that the 3' UTR has been separated from the coding region by insertion of kanR, which might alter stability of the transcript. Can the authors clarify the position of kanR in this scheme, and whether the pxl1 3' UTR remains adjacent to the coding sequence? I doubt this comment explains expression differences in 9A versus other pxl1 constructs because they all use the same scheme, but it is worth clarifying.

Yes, the coding region is separated from the 3' UTR by insertion of *kanR* cassette directly after the *pxl1* STOP codon, which is comparable to the strategy employed for C-terminal tagging with a fluorescent protein or affinity tag as published by Bahler et al., 1998.

Reviewer #2 (Remarks to the Author):

The authors of this work find that Cdk1 phosphorylates Pxl1 during mitosis exit. Phosphorylation occurs on nine residues at Pxl1 N-terminus and affects its role in cytokinesis. Additionally, the authors show that Pxl1 can be dephosphorylated by Clp1 and Dis2 (PP1), and also demonstrate that preventing Pxl1 phosphorylation results in increased Pxl1 levels, precocious recruitment to the division site, and increase in the duration of CR constriction. In vitro, Cdk1-mediated phosphorylation of Pxl1 inhibits its interaction with the F-BAR domain of the cytokinetic scaffold Cdc15.
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cytokinesis is very mild and the phospho-mimetic Pxl1-9D results in a functional protein similar to wild type. Perhaps the addition of some experiments would help us see in vivo the effects of Pxl1 phosphorylation.

Comments:

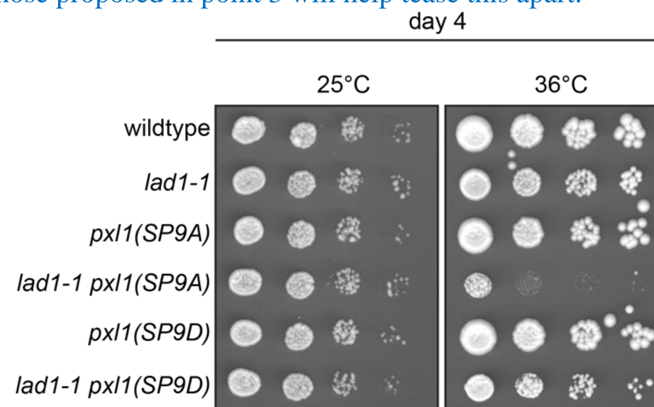
1. In vitro, Cdk1-mediated phosphorylation of Pxl1 inhibits its interaction with the F-BAR domain of the cytokinetic scaffold Cdc15, thereby disrupting a major mechanism of Pxl1 recruitment.
Is there less GFP-Pxl1 in the CR of clp1Δ or dis2Δ or in the double mutant? or is GFP-Pxl1 recruited later in these mutants? These experiments are easy to perform and might add important information.

We also thank Reviewer #2 for taking time to review our paper and suggesting experiments to strengthen its in vivo significance. We did perform these experiments and refer you to our response to Reviewer #1, point 1.

2. The phospho-ablated mutation increases the amount of Pxl1 but it causes delay in cytokinesis. Is it possible that is the excess of Pxl1, not its lack of phosphorylation, that is affecting cytokinesis? Does overexpression of Pxl1 cause the same defects in cytokinesis?

Pxl1 overexpression has been shown to suppress the thermosensitivity (but not the morphologic defects) of *cdc42—1625* (Pinar et al., 2008). *pxl1*Δ rescues *ehs2*-1, which is a temperature-sensitive mutant of Rgf3 that activates Rho1 signaling during cytokinesis (Pinar et al., 2008). We had examined the genetic interactions of Pxl1-9A and Pxl1-9D with the *rgf3* temperature-sensitive mutant *lad1-1*, which lyses at 36°C. The *lad1-1 pxl1-9A* is synthetically sick, supporting that Pxl1-9A is a gain-of-function allele. *lad1-1 pxl1-9D* is also sicker than *lad1-1* single mutant, though marginally compared to *pxl1-9A*. This indicates that phosphorylated Pxl1 is not equivalent to loss-of-function of Pxl1.

We did not include this assay in the paper as it felt beyond the scope of our objective, which was to demonstrate a novel mechanism of phospho-regulation of Pxl1 and a novel substrate of CDK and propose a mechanism by which phosphorylation impacts Pxl1 molecularly. Our studies did not elaborate as to the specific cytokinetic function of Pxl1 that is impacted by phosphorylation, but we hope that further studies such as those proposed in point 3 will help tease this apart.



3. It would be informative to see how Pxl1-9A affects the binding and phenotypes of other Cdc15 partners like ppb1-GFP, fic1-GFP, cyk3-GFP... Is Pxl1-9A mutation synthetically lethal or sick with fic1Δ, ppb1Δ, cps1-191, or rga7Δ? It would be interesting to analyze the final steps of cytokinesis in these double mutants.

Given that Pxl1 is required for calcineurin localization and directly interacts with calcineurin, we tested if Pxl1-9A or Pxl1-9D alters abundance or timing of Cnb1, the calcineurin regulatory subunit. Timing of

Cnb1 arrival at the division site was not altered in 9A vs. 9D (data not shown). Given that mNG-Pxl1-9A is two-fold increased compared to wildtype, we hypothesized a two-fold increase in Cnb1-mNG at CR in *pxl1-9A* compared to wildtype. The WC intensity of Cnb1-mNG was reduced in Pxl1-9A and Pxl1-9D compared to wildtype, which increased the ratio of CR:WC intensity in both the Pxl1-9A and Pxl1-9D (Figure S3). However, these results suggest that Pxl1 localization alone is not sufficient for Cnb1 recruitment and there are other factors regulating their interaction.

Minor points:

- Pg4. Iqg1/Rng2 is confusing, change to: Iqg1 and Rng2 respectively
...and S. cerevisiae C2 domain protein Inn1/Fic1 (Palani et al., 2012; Kuilman et al., 2015). Please, change to: Inn1, the ortholog of S. pombe Fic1.

We have made the suggested changes – thank you!

- Pg 4 and 5. "pxl1Δ cells have severe cytokinesis defects, displaying CR sliding and splitting during anaphase (Ge and Balasubramanian, 2008; Pinar et al., 2008)". The CR sliding phenotype was described in Cortés et al. PLOS Genetics 2015, not in the mentioned articles. Please, include the reference.

We have added the reference.

- Figure 2E. It would be better if the blot for Pxl1-91A were changed for other blot with a protein load similar to those of Pxl1 and Pxl1-9D blots.

We have replaced the blot for Pxl1-9A so that the total protein load matches that of Pxl1 and Pxl1-9D.

- Figure 3A. Why was the treatment with the CN inhibitor FK506, tested if it prevented Pxl1 dephosphorylation, done 30 min after release of G2 arrest and not at the time of release (time 0)?

We observed in our assays with wildtype Pxl1 (Figure 2E) that dephosphorylation occurs between 30 and 60 minutes after release from a G2 arrest. Therefore, to allow entry into mitosis and to minimize indirect effects of calcineurin inhibition, we chose to add FK506 at the 30-minute time point.

- The meaning of IDR, which is mentioned in the discussion section, is missing. Also LIM should appear in the abbreviations section.

We defined IDR and LIM domains, but we did not include in the abbreviations section since they only occur once each.

RE: Manuscript #E20-12-0807R
TITLE: "Cdk1 phosphorylation of fission yeast paxillin inhibits its cytokinetic ring localization"

Dear Kathy,

Thanks for submitting your revised manuscript. As you will see, the reviewers are satisfied with your revisions, except for one comment from reviewer 1.

I feel that comment is valid, as we have also observed in some cases significant reduction in protein levels when a 3' UTR was not present in the construct. My understanding is that you used the HA-Pxl1 from the Pinar et al 2008 study (which has 3' UTR and is expressed under endogenous promoter but at distinct genomic locus) in the first figure and then your new HA-Pxl1 at endogenous locus (but without 3' UTR) in the rest of the paper. I have the feeling, judging from relative levels of specific and non-specific anti-HA bands, that there are no major changes in protein levels between the two constructs, but perhaps you can directly compare Pxl1 levels between the two strains to reassure on this point. You may already have such a blot. For clarity, I would then simply ask that you state in the methods that the constructs lack a 3'UTR.

I should be able to make a rapid decision after this small modification.

Best wishes,
Sophie

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gould,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The authors have revised the text and added new data to address most of the reviewer concerns. The manuscript is improved based on these changes and will be a nice addition to the field. However, I remain uncomfortable about one aspect of the study that was not really addressed in the revision. If I understand correctly, all pxl1 constructs are expressed without a 3'UTR (i.e. terminator). In their rebuttal, the authors explain that the pxl1 coding sequence is followed directly by the kanR cassette. They state that this strategy is similar to the common pFA6a tag integration strategy published by Bahler et al 1998, but this isn't true. The pFA6a plasmids all contain the ADH1 terminator sequence following the tag, which means that a "tagged" gene is expressed with the ADH1 3'UTR to stabilize the transcript. It remains possible that the pxl1 constructs in this paper are impacted by the lack of any 3'UTR, which is predicted to change their translation and stability. This concern seems important because protein levels are altered in their various mutants e.g. 9E. This effect on protein levels could be impacted by the lack of the 3'UTR. It would be helpful for the authors to confirm that their pxl1 constructs lacking 3'UTR are expressed at a similar level to constructs containing a 3'UTR. For example, are there published versions of HA-pxl1 or mNG-pxl1 that contain a 3'UTR, so that they can compare their levels as a control experiment? I would also recommend that the authors discuss this caveat in their results. It remains possible that results in the paper are impacted by the lack of 3'UTR, which could cause confusion for the field when future studies examine the same proteins and pathways.

Reviewer #2 (Remarks to the Author):

The authors addressed sufficiently most of the specific comments and doubts from the previous revision of their manuscript. They also provided some additional data and improved the figures. Therefore, I believe the manuscript increased in quality and can be published in MBC.

Dear Sophie,

There seems to have been some confusion on the part of the reviewer and yourself about what strains were used in our manuscript. Our strain table lists all of the strains used in our paper, organized by figure, and referring to it you will notice that while the HA-Pxl1 strain from Dr. Perez's laboratory was used in Figure 1, it was not used elsewhere. Indeed, the HA-Pxl1 wildtype strain used throughout the remainder of our paper was constructed in the same way that the pxl1 phosphomutants were constructed so that it was the appropriate control for our experiments comparing the effects of mutating phosphosites.

There was also a confusion on our part. The kanR cassette used in constructing the wildtype and mutant constructs at the endogenous pxl1 locus was obtained from the pAF6 cassette and included the entirety of the cassette so it included the adh1 sequences.

We have clarified these two issues in our methods section. I hope this satisfies the concern.

Kathy

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Dear Kathy,

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell. I thank you for the clarifications and the text edit. This work makes a nice addition to our understanding of the regulation of cell division by the cell cycle machinery.

Best wishes,
Sophie

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gould:

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,

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