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Phosphocysteine in the PRL-CNNM pathway mediates magnesium homeostasis

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Author's response to original referees' comments – EMBO Journal

Thank you and the reviewers for their reviews of our manuscript. We are pleased to submit a revised manuscript that incorporates many of the reviewers' suggestions. Specifically, we have added new experiments to show that mutations of the catalytic and adjoining cysteine residues in PRL2 do not prevent binding of the CNNM3 CBS domain and that mutation of the PRL3 catalytic cysteine does prevent phosphorylation of PRL3 expressed in mammalian cell culture. We have also added statistic analysis of the magnesium efflux assays and an extended view movie showing the overlay of the PRL1 trimeric structure and the PRL2•CNNM3 CBS complex. We trust with these improvements that the manuscript is suitable for publication in the EMBO Journal. A detailed list of our responses to the reviewer concerns and suggestions follows.

Referee #1:

The manuscript describes the detection of the phospho-cysteine intermediate of PRLs and suggests that the modification that is usually known as intermediate step in the dephosphorylation mechanism of PTPs could in case of the PRLs function as a negative regulation of the interaction between CNNMs and PRLs. Furthermore, the manuscript contains the crystal structure of PRL-2 with CNNM3, which is to my knowledge the first crystal structure of PRL-2.

The idea of the slow second step of the dephosphorylation reaction being a regulatory mechanism for PRLs is very interesting. PRLs have an alanine instead of the commonly conserved serine in the p-loop, which is responsible for hydrolysis of the phosphocysteine intermediate. Its absence is responsible for a slower hydrolysis, as shown previously in several publications. In agreement with this, the respective A111S mutant (for PRL-3) has been shown to enhance hydrolysis against unnatural substrates. The main novelties of the work are the crystal structure of the complex and the proposed regulatory role of the phospho-cysteine.

Crystal structure: While it is interesting, it does not really add much knowledge to the binding parameters on the CNNM3 side. It confirms the mutational analysis done previously, and gives some insights into PRL-2 and its interaction with the CNNM protein. However, what is striking is that it does not explain why the C101S mutation or the C46A mutation in PRL-2 leads to loss of binding to the CNNM, at least not in the currently presented form. In figure 4c the two residues are not involved in the interaction.

Response: We are surprised by the reviewer's comment since we feel that the crystal structure is highly informative and a significant contribution to the literature. Its value is several-fold. First, the structure is the first of a complex of a PRL phosphatase and reveals that the CNNM acts as a substrate mimic by inserting an aspartic acid residue into the PRL2 catalytic site. Secondly, the structure leads to the hypothesis that the long-lived phospho-cysteine intermediate regulates binding between the two proteins. Not only does the structure explain the CNNM mutagenesis, it suggests new experiments, which are further developed in the paper.

We thank the reviewer for the suggestion. We have examined the effects of two mutations in the PRL2 catalytic site on CNNM binding and present the data as a new Figure 2e. In agreement with the crystal structure, pull-down assays with the CNNM3 GST-CBS domain do not show a strong effect of mutating the catalytic cysteine (C101) or the adjacent cysteine (C49) to alanine. Both mutant proteins were efficiently pulled down by the CBS domain. At present, it is not clear why our results differ from those reported by other groups. These could be due to the different context (full-length protein versus CBS-pair domain), the presence of factors in the mammalian cell extracts, or the presence of protein modifications of the full-length protines (e.g. PRL prenylation).

It is intriguing that both disulfide formation and the mutations that prevent disulfide formation prevent binding to CNNM, and I would have hoped to get insight about this from the crystal structure. If the disulfide formation would be modeled on this structure based on the PRL-1 crystal structure, that could explain why the disulfide formation inhibits binding. The authors only mention these points in the discussion with a very general blurry explanation: "the structure shows CNNM is stringently optimized for recognizing the active phosphatase. Structural changes due to disulfide bond formation, replacement of sulfur by oxygen, or most dramatically cysteine phosphorylation oppose binding." With the exception of the phospho-cysteine intermediate (which they call cysteine phosphorylation), the authors do not show that.

Response: As the reviewer points out, it is intriguing that disulfide formation decreases the affinity of CBS binding (Figure 2f) although it is important to note that the effect is not as dramatic as the block by phosphorylation. Our best explanation is that PRL oxidation leads to a conformational change within the binding site. For the phosphatase PTP1B, conformation-sensing antibodies were generated that sense the oxidized form of the phosphatase and even inhibit its activity (Haque et al, Cell. 2011). Disulfide bond formation in PRL2 likely causes a similar conformational change.

Quite the opposite, as in the PRL-1 structure that they use for comparison the C104S mutation is present (Figure 4d: typo, it says PRL3 there), and the structural change is minimal as evident from the comparison to the PRL-2 structure (4c). As I do not have access to the crystal structure, it is hard to judge this, but it is curious that the authors neglected this point in their analysis of the structure while making it a strong point later on in the discussion.

Response: There is indeed a great deal of interest in oxidation of PTPs; however, we see a larger effect from cysteine phosphorylation. As the focus of the current manuscript is phosphocysteine and the analysis of cysteine oxidation is problematic due to the multiple different oxidation states and

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their instability, we have modified the discussion of mutations of the catalytic cysteine and disulfide bond formation. We thank the reviewer for pointing out the typographical error in Figure 4d.

Phospho-cysteine as regulatory mechanism: Starting with the reduction of the burstkinetics in figure 1f and then following with the data in figure 2 and S2&3, this is very interesting and convincing that the authors can detect the phospho-cysteine intermediate. The enhancement of the signal with OMFP is a useful method to support the data. There is no doubt that the phospho-cysteine is formed, as also shown previously for PTP1B. They show that the presence of OMFP blocks the interaction with CNNM in vitro, and removal of the phosphate from the cysteine over time leads to regain of the binding. In the cellular data in Figure 5, unfortunately the authors do not repeat many of the controls that they had done in vitro, such as MS analysis and identification of the phospho-Cyscontaining peptide was not done at all (as for example done in Sun et al, PNAS 2012). No mutants were used in cells, like the C104/1S mutant as control for Cys phosphorylation.

Response: We have added a new figure panel that shows the analysis of the C104S and C49S mutants of PRL1 in cultured cells. The new Figure 5b shows that the catalytic cysteine (C104) is required for phosphorylation while the adjacent cysteine (C49) is not.

This has to be done as otherwise the endogenous phosphorylation can be on any other residue of the protein (PRL-3 was shown to be phosphorylated by Src for example), which was not at all considered here. Enforcing the phospho-Cys by OMFP treatment and then removing the signal through hydrolysis over time is not a proof that this happens endogenously.

Response: The sensitivity of the cysteine phosphorylation to boiling distinguishes it from other types of phosphorylation and excludes Src or other protein kinases as the origin of the PRL phosphorylation observed in cells. Taken together with the stimulation of the modification is induced by OMFP, which is not a kinase inducer, the evidence of phosphocysteine in mammalian cells is very strong. The modification leads to a shift of PRL proteins on phos-tag gels but not standard SDS-PAGE. The modification is chemically labile and reversed by boiling in SDS loading buffer. The modification requires the phosphatase catalytic cysteine.

The data on PRL-2 in figure 5a looks rather week and I assume that this is why PRL-1 was used in the following. There is, however, no in vitro data on PRL-1. The antibody used in 5c is not specific for PRL-1 or -2, so it is unclear if PRL-2 behaves the same in cells.

In general, the authors do not consider that differences may exist in the PRLs. Although most of the work is performed with PRL-2, some experiments include a different PRL. However, the authors generally refer in the text to PRLs in most of the cases. This can lead the reader to the interpretation that there is in general a redundancy of the physiological function of the three PRLs, but this is a question that is not yet clarified and it is under discussion in the field. Thus, for a better understanding of the manuscript and for accuracy, in every case the specific PRL used on every particular experiment should be named. For example, in figure 5d, they do not mention which PRL was tested, neither in the figure nor text nor captions.

Response: We apologize for the oversight. Figure 5d (now 5e) has been modified to indicate the antibody detects all three PRL species. While differences undoubtedly do exist between the different PRL isoforms, the proteins are more similar than they are different. Previous studies have shown that all three PRL phosphatases interact with CNNM4 and that PRL1/2 interact with CNNM3. The proteins are highly similar with ~85% sequence identity.

In experiments with endogenous proteins, it is difficult to distinguish between the different isoforms due to cross-reactivity between antibodies. Despite this ambiguity, the data showing phosphorylation of PRLs in cells are robust and highly reproducible. We obtained identical results in two different laboratories with different antibodies and different cell lines. Experiment 5c (now 5d) was performed in Montreal and used the anti-PRL2 antibody from Millipore, which also detects PRL1. Experiment 5d (now 5e) was performed in Osaka and used an in-house antibody that detects all three PRL isoforms. In all the experiments, we observed high levels (~50%) of cysteine phosphorylation under normal culture conditions and a decrease in phosphorylation upon magnesium deprivation.

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The major concern however is that the physiological relevance of this potential regulatory mechanism is missing. Figure 5d intends to show the physiological relevance, giving the impression that the phospho-cysteine intermediate formation is regulated by Mg2+ presence. However, as reported previously, depletion of Mg induces PRL-2 expression, as seen here. This means that constantly new protein is produced. How can the authors exclude that these new PRL proteins just not have found the substrate to form the phospho-cysteine intermediate yet? Or, that depletion of Mg leads to higher PRL activity removing the phosphate through some allosteric or oligomerization mechanism (PRL-1 and -3 were reported to oligomerize)? A functional experiment is missing. For example, the A108S / A111S mutant could be used and compared to PRL-2 / -1 WT in overexpression for Mg contents in the cell. The mutant should remove the phosphate quickly, and should therefore show a stronger interaction with CNNM leading to higher Mg in the cells or less Mg efflux. This mutant should be tested for its binding capacity with CNNM first, because the C101S mutant cannot be used, as it does not bind to CNNM.

Response: We respectfully disagree with the reviewer. Two published papers show the proteins directly interact and that PRL-CNNM interaction is relevant in cancer (Funato et al, J Clin Inv, 2014; Hardy et al, Oncogene, 2015). Here, we show that disrupting the interaction by mutating the aspartic acid residue in CNNM4 blocks inhibition of Mg2+ efflux by PRL3 (Figure 3b). Further, we show that PRL2 cysteine phosphorylation prevents the association with the CBS-pair domain of CNNM3 in vitro and PRL1 phosphorylation prevents CNNM4 binding in cell extracts. We further show that PRL phosphorylation levels change in cells in response to Mg2+ deprivation. Together the data are highly evocative of a physiological feedback pathway.

We agree that further studies are merited. The experiments suggested by the reviewer are important for future studies. The origin of the phosphate needs to be determined, the role of PRL oligomerization should be investigated, additional mutants tested, and the mechanism of inhibition of Mg2+ efflux determined. However, these experiments are out of the scope of the current paper. The revised manuscript already has 30 figure panels and presents a complete story spanning enzymatic assays, a crystal structure, cell assays of Mg2+ efflux, evidence of cysteine phosphorylation in cells and changes in response to environmental conditions.

We have added a paragraph and expanded view movie that describe how PRL trimerization is compatible with our PRL•CNNM complex.

The data shown in Figure 1 (with the exception of Figure 1f) adds useful biophysical constants to previously described facts (for example that aspartic acid 426 is essential for binding CNNM, that has been shown in mouse experiments before, so the ITC does not identify this fact as the authors claim). The experiments in the chapter "PRL-CNNM interaction regulates magnesium efflux in cells" related to Figure 3 confirm that PRL-3 interacts with CNNM4 as described before. In addition, they newly show through testing different PRL3 mutants in the Mg efflux assay that the interaction involves the same amino acid residues as previously described for PRL-2.

A valid question is where the phosphate group of the intermediate is coming from. However, in the PRL field it is accepted that this question has not been conclusively answered yet, and that it is very hard to find any substrate for PRLs, and few substrates have been suggested. While I would not expect the authors to answer that question conclusively due to the challenges, I completely disagree with their explanation of where the substrate could come from. They state: "We observed that recombinant PRL2 purified from E. coli is partially phosphorylated in the absence of added substrate (Fig. 2d, PRL2 input lane). This suggests that PRL2 has broad substrate specificity. Using detection of phosphorylated PRL as an activity assay, we observed phosphatase activity with a variety of low molecular weight compounds that are unlikely to be physiological substrates (data not shown)." 1) If it were true that PRL2 has broad substrate specificity like other phosphatases, there would be many more proposed substrates, but there are indeed very few. Also, E. coli could contain specific substrates of PRL-2, so the cysteine of PRL2 could be in the phospho-cysteine intermediate state without adding external substrate. 2) Many phosphatases accept many small molecule substrates, so the author's not shown observation does not explain anything. The authors also state later on that PRLs behave as pseudophosphatases. This statement needs to be clearly rephrased to be meant for the interaction with CNNMs only, otherwise the authors would need to disprove all previous publications on PRL substrates.

Response: We have revised the text to remove the confusing statements about PRL substrates. The reviewer is correct that many small molecules can act as substrates of phosphatases. The sentence referring to PRLs as pseudophosphatase as been rephrased to specify that it referred to the interaction with CNNMs and not imply that PRLs do not have catalytic activity as phosphatases.

The term phosphorylation in this manuscript should be explained earlier in the text (for example in the summary), and I am not even sure if this is the correct term. It should not be used in the title. The detected cysteine phosphorylation on the PRLs is a phosphocysteine intermediate from a phosphatase reaction rather than a kinase-mediated phosphorylation, as described for the bacterial phospho-cysteine (Sun et al. PNAS 2012). So PRL2 is not being phosphorylated on cysteine like the bacterial protein, and that needs to be clarified.

Response: We have reworded the title to use phospho-cysteine rather than phosphorylation. The abstract has been modified to clarify that the phosphorylation of cysteine arises from the phosphatase catalytic cycle. The discussion includes the statement that phosphorylation of PRLs is not the direct result of kinase activity.

The authors jump between showing the interaction in general and the phospho-cysteine intermediate regulation, and that makes the manuscript a bit difficult to read. Taken together, the authors confirm data published previously by other groups and add more information to it, providing the PRL-2/CNNM3 crystal structure, which unfortunately does not yet explain fully the abovementioned aspects. The authors provide the very intriguing idea that the phospho-cysteine intermediate in PRLs could have an inhibitory function. The physiological relevance of this is unclear, and it is not convincingly shown that this is not only part of the dephosphorylation reaction or really a negative regulation in cells. Other phosphatases like DUSP19 (mouse LDP2) have the same alanine mutation: could they also be regulated by a longer lasting intermediate? If so, would that be a more general mechanism? Or, is this rather a mutation that could specify substrate specificity or be involved in structural integrity (as previously suggested for PRL3 and LDP2)? These possibilities are not mentioned in the discussion. For all these reasons the data is, in my opinion, over-interpreted at this point and preliminary, and the authors wrote a selective discussion for their purposes. I therefore cannot recommend considering this work for publication in EMBO J. It could be reconsidered if the authors thoroughly and convincingly address the concerns detailed above, but at the moment it should not be published.

Response: We are pleased that the reviewer found the work "very interesting", "interesting", "intriguing", and "very interesting and convincing". We agree that the manuscript raises many important questions and hope the paper will stimulate other groups to extend our studies. However, the mechanism of dephosphorylation and the question whether other phosphatases such as DUSP19 also form a long lasting intermediates are outside of the scope of the current study. As requested, we have added a sentence to the Discussion raising the possibility that other phosphatases may show a similar phenomenon.

Referee #2:

The manuscript by Gehring and colleagues reports an interesting study relating the state of phosphorylation of the catalytic site of PRL phosphatases with regulation of CNNM Mg2+ transporters and the level of intracellular Mg2+ concentration. The authors show that a high proportion of PRLs are phosphorylated on catalytic site in vivo. PRLs interact with CNNM via an extended loop within the CBS domain of the transporter. Mutation of a critical Asp residue in this loop virtually abolishes PRL-CNNM interactions, and the capacity of PRL to inhibit CNNM Mg2+ transport activity. PRL-CNNM interactions are blocked by the phosphocysteine that is an intermediate in PRL substrate dephosphorylation reaction. Oxidation of the PRL catalytic Cys also blocks PRL-CNNM interactions.

The crystal structure of PRL in complex with the CBS domain peptide is convincing, showing the critical Asp426 residue of the CNNM loop interacts with the Arg107 of the catalytic site loop (Cx5R motif). This interaction would be blocked by a cysteinyl phosphate group and by oxidation of the catalytic Cys.

Finally the authors show that a reduction in Mg2+ levels decreases PRL phosphorylation.

Overall this is a very interesting and provocative manuscript with the highly novel finding that cysteine phosphorylation is a regulatory mechanism for controlling proteinprotein interactions. The mechanism of Cys phosphorylation is also novel in that it involves the catalytic intermediate of a Cys-dependent protein phosphatase. This could potentially be a more general mechanism than commonly known. The manuscript would be suitable for publication in the EMBO Journal subject to revision.

1. How does reduced magnesium decreased levels of PRL phosphorylation? Does it suppress PRL phosphatase activity, or stimulate dephosphorylation of phospho-PRL? These alternatives could easily be tested using their in vitro assays.

Response: The dephosphorylation in cells could be due to intrinsic hydrolysis, the synthesis of new unphosphorylated protein, or a combination of both. We have not observed conditions that markedly affect the rate of intrinsic hydrolysis although this is an area for continued investigation.

2. What is the substrate that is responsible for PRL phosphorylation in vivo? The authors speculate that phosphatidylinositol phosphates might be substrates. Has this been tested?

Response: The physiological substrates of PRL proteins remain unclear. We have tested the activity of PRL2 against phosphoinositide phosphates in vitro but the results were inconclusive.

3. The authors find that an enlarged loop within the CBS domain binds PRL. Is this loop sufficient for binding?

Response: Yes, we observed weak affinity for two peptides. Based on the sequence of the CBS loop, we tried three peptides cyclized by cysteine disulfides: VCNEGEGDPCY, CVNNEGEGDPFYC, and CVNNEGEG(pS)PFYC (with phosphoserine). By NMR spectroscopy, we were able to detect binding of the longer peptides to PRL2 but the estimated Kd's were above 2 mM.

4. The crystal structure of PRL in complex with the CBS-domain peptide is convincing. Did the authors test this interaction by mutation of Arg 107 and or Leu 105?

Response: Not yet. We have focused our studies on the role of phosphorylation. Additional studies are planned to quantify the role of other residues in the PRL-CNNM interface.

5. The statement on line 322 isn't clear. Why should the C49S mutation of PRL2 lose oncogenic potential? Does it fail to bind CNNM? And if so what is the mechanism?

Response: We have now tested the effects of mutating the catalytic cysteine (C101) and adjacent cysteine (C46) in PRL2. Although in vivo studies with full-length proteins expressed in cultured cells had shown that both residues were essential, neither mutation affected binding CBS binding in pulldown assays with purified proteins (new Figure 2E). At present, it is not clear if the differences are specific to the particular the PRL isoforms studied, the protein context (full-length or CBS-pair domain), or other experimental differences. Experimentally, the C49S mutation of PRL3 leads to loss of oncogenicity (Funato et al, J. Clin. Inv., 2014). This could be due to the sensitivity of C49S PRL3 to inactivation by over-oxidation. The text in the discussion has been reworded to clarify our current understanding.

Minor points:
1. Explain CNNM and CBS in the Abstract.
2. One line 77, 'the interaction between PRLs and CNNM is regulated...' would be clearer as 'the interaction between PRLs and CNNM is negatively regulated...'
3. Line 90, affinity is 25 nM should be stated as a Kd of 25 nM. A similar correction is required in Fig. 1c.
4. Line 163. Refer to Fig. 2c.
5. Line 228. Label binds in Fig. 5a.

Response: We thank the reviewer for the corrections. The suggested changes have been made.

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Referee #3:

Gulerez et al. further explore the mechanism of interaction between members of the CNMM3 family of Mg transporters, and the PRL sub-family of PTPs. Using a combination of in vitro binding, mutagenesis, and ultimately, structural studies, they identify a loop within the CBS domains of the former that mediates interaction with latter by means of a pseudo-substrate interaction. The confirm previous studies with other members of the PRL and CNMM family by showing that CNMM4 promotes Mg efflux, PRL3 blocks this effect of CNNM4, and PRL3/CNNM4 interaction is required for efflux inhibition. Moreover, they confirm that PRL2 shows "burst kinetics," in which rapid hydrolysis and release of a small molecule substrate is followed by extremely slow hydrolysis of the cysteine-phosphate intermediate. They then cleverly explore the potential physiological significance of this fairly stable intermediate. Consistent with the above results, they show that PRL cysteine phosphorylation (mediated by hydrolysis of a small molecule substrate) blocks interaction with CNMM CBS domains in vitro, and provide evidence that PRL 1 and 2 occur in cysteine-phosphorylated and dephosphorylated forms in vivo. Furthermore, the ratio of PRL cysteine-phosphorylated and de-phosphorylated forms deceases following Mg depletion whereas the total amount of PRL increases.

This is a very interesting and generally well-performed paper that is certainly worthy of consideration by Embo J. The finding that PRLs might be endogenously cysteinephosphorylated, and that this state might have regulatory implications, is of wide potential interest. Nevertheless, there are some technical and conceptual issues that should be addressed before publication.

Major Points:

1) The authors should be careful in the text to point out when they are monitoring interactions between the CBS domain of CNNMs (most experiments) and the whole protein (only the in vivo experiments). They are a bit lax in their descriptions of these experiments.

Response: We have modified the text as requested. The purpose was not to mislead but simply to shorten the text and avoid unnecessary repetition.

2) It is a bit disconcerting that the authors switch back and forth between different PRLs and CNNMs. Although I imagine that they did these for convenience/available constructs, I think it would be important to demonstrate the key findings with the same pair.

Response: We agree that it could be confusing but we were limited in our choices by technical aspects. We generated the same set of three mutations in the CBS loop of CNNM3 and CNNM4 so that the results between the proteins would be directly comparable. The crystal structure was obtained with PRL2 and CNNM3 so it made sense to carry out the in vitro studies with those two proteins. Binding studies (both ours and published studies) show there is nothing unusual about the association of PRL2 and CNNM3. We show that all three PRL isoforms bind to CNNM4 and the group of Michel Tremblay has shown that PRL2 and PRL3 bind to CNNM3. We previously showed that PRL3 has burst kinetics due to the long-lived phosphocysteine intermediate (Kozlov et al, JBC, 2004) and we show here that all three PRLs are partially cysteine phosphorylated when expressed in mammalian cells (Figure 5a). PRL2 and PRL3 isoforms are 78% identical overall and are almost 100% identical in the sequences of their active sites.

For functional assays, we chose to use PRL3 and CNNM4 because of the previous published work from our (Miki) laboratory that showed PRL3 inhibition of CNNM4 Mg^{2+} efflux (Funato et al, J. Clin Invest, 2014). CNNM3 is inactive in the cell Mg^{2+} efflux assay (Hirata, JBC, 2014). The CBS domains of CNNM3 and CNNM4 show similar overall identity and again are 100% identical in the regions that interact with PRLs (Figure 1C).

3) I think that Figure 1b adds little to the manuscript, whereas Supp Figure 1 is quite important. If they want to keep the former, I would move that to the supplement and Supp Fig 1 to the text. Likewise, Supp Figure 5 belongs in the main text in my opinion.

Response: We thank the reviewer for the suggestions. Figure 1b has been removed and part of the supplemental figure 1 incorporated to the main figure. Similarly, Supplemental Figure 5 has been moved to Figure 4.

4) Referring to Fig. 1F, the authors state that the CNMM3 CBS interaction with PRL "abolishes the initial burst and decreases the steady state rate by 50%." Although this statement accurately describes the appearance of the graph, I think it misleads the reader in terms of the mechanism. Presumably, in a mixture of CNMM3 and PRL, the vast majority of PRL is in a complex. According to the authors' model, when bound to CNMM3, PRL is completely inactive. Upon dissociation, free PRL3 presumably exhibits the same initial burst; it's just that the amount of free PRL3 is small at any given time. Indeed, if I understand their model, what is being measured by the slope of the PRL hydrolysis reaction in the presence of CNNM3 is the sum of the off rate for thePRL/CNNM complex plus k4 in the scheme in Fig. 1d.

Response: We agree and have modified the text. The "steady-state" rate observed in the presence of the CNNM CBS domain is unrelated to the rate of hydrolysis of the phosphocysteine intermediate. We have added a reference in the introduction to the article (Zhang & VanEtten, JBC, 1991) that describes protein phosphatase burst kinetics in terms of chemical rate constants.

5) The authors describe (lines 158 ff) experiments with diamide and state that formation of the intramolecular cysteine could be detected on non-reducing gels. I don't see their data anywhere in the manuscript. Did they mean to state data not shown?

Response: The sentence has been removed to avoid the reference to unpublished results.

6) According to their analysis of the CNMM/PRL structure, phosphorylation of PRL C101 would be expected to inhibit CNMM interaction because of repulsion between D426 and the charged phosphate. They should also point out why the C101S mutant is expected to disrupt the interaction (presumably because of loss of the D426 hydrogen bond network).

Response: As discussed above, we now show that the PRL2 C101A mutant binds the CNNM3 CBS-pair domain as well as the wild-type PRL2 protein in a pulldown assay with purified proteins.

7) The authors' show that CNNM3 inhibits PRL3, and PRL3 inhibits CNMM3. Abrogation of mutual inhibition is known to confer switch-like behavior to biological processes. Do the authors have any evidence for this type of regulation? In this regard, change in phospho-/dephospho ratio in response to Mg depletion is quite slow; I would have thought one would want quicker regulation to prevent critical energy depletion. Is the regulation by oxidation faster? And if so, can oxidants get in to the complex (again, any regulation would appear to depend on the off-rate for the PRL/CNMM complex, which seems like a relatively inefficient means of regulation. These issues merit discussion.

Response: The idea that the PRL-CNNM interaction acts like a switch is intriguing but, at present, we do not have any evidence beyond their reciprocal inhibition. The nature of the substrate, the mechanism of dephosphorylation (passive or active), and the role of oxidation are all important questions for future investigation. A number of groups have looked for physiological substrates but at present there is no consensus (reviewed in Rios et al, FEBS J, 2013). Studies of oxidation of protein phosphatases is a large field with hundreds of publications. In vitro, PRLs can only be phosphorylated when fully reduced. In cells, PRLs are expected to be partially oxidized but this has not been carefully measured. Although cysteine disulfide formation reduces the affinity of PRLs for CNNM proteins, the modification also protects the protein from phosphorylation and over-oxidation. So paradoxically, disulfide bond formation might promote inhibition in cells.

We do not know how PRLs are dephosphorylated in cells although the rate we observed is similar to the spontaneous rate observed in vitro. The discussion has been revised to mention this.

8) The data on Mg efflux should be quantified and subjected to error analysis.

Response: Figure 3C has been revised to show error bars and the statistical significance.

9) Related to point 5, the authors' data on in vivo PRL cysteine-phosphorylation could be stronger. At the least, they should show that the Cys 101 mutant does not get phosphorylated (as monitored by phos-tag shift). Also, the claim that there is a change in the ratio of phospho- and de-phospho-PRL forms, as well as that there is an increase in total PRL levels requires quantification and a loading control (for the latter).

Response: We have added a figure panel (Fig. 5B) to show that the C101 mutant of PRL1 is not phosphorylated in cells. The cell extracts in Fig. 5E were normalized by total protein loaded.

10) PRLs have been reported to form trimers. Is trimerization incompatible with CNNM association? What about phosphorylation?

Response: PRL trimerization does appear to be compatible with binding the CNNM CBS domain if one allows a small conformational shift in the angle of PRL-binding loop. We have added a paragraph (starting at line 214) to describe this and a movie as an expanded view that shows an overlay of our structure with the PRL1 timer structure. Cysteine phosphorylation in the catalytic site is not expected to affect PRL trimerization.

11) In their Discussion, the authors state that PRLs are not regulated by exogenous phosphorylation (of their active site cysteines) but rather by substrate hydrolysis. How can they be sure? Also, given the previous data on phospholipids as potential PRL substrates, the mutual antagonism of PRLs and CNMMs suggested by their model, and the role of CNMMs in Mg efflux, are there any data on particular membrane microenvironments being particularly favorable for Mg transporter function?

Response: While in theory the catalytic cysteine might be phosphorylated by a protein kinase, this seems unlikely. The cysteine is ensconced in the catalytic site and not accessible to kinases. Instead, we suggested that access to phosphatidylinositol phosphates or other substrates might regulate the phosphorylation. We do not know of any evidence for CNNM proteins in membrane microenvironments although that is an intriguing idea.

12) Also in the Discussion, the authors note previous studies showing that the C49S mutation in PRL3 retains catalytic potential but loses CNMM binding ability: does their crystal structure provide an explanation for the latter?

Response: The C49S mutation does retain catalytic activity although in cells it may be more sensitive to inactivation by hyperoxidation to sulfinyl or sulfonyl forms. The C104- C49 disulfide is thought to be protective since it reversible. As described above, we still see binding of the CNNM3 CBS domain to PRL2 with a C46A mutation. The text has been updated to clarify our findings.

Minor Points:

1) Re: the summary, all cancers are, by definition, malignant. Either remove "malignant" or change to malignant tumors. Also, although I agree that little is known about the PRLs, it is not at all clear that this is because of their low activity.

Response: The redundant word has been removed. The sentence in the abstract has been modified and now indicates that their low activity is in part responsible.

2) Figure 1e is described/discussed before Figure 1d. Also, why did the authors show napthyl phosphate as the substrate in Figure 1d, when they used DiFMUP and OMFP in the text?

Response: The figure has been changed to show a phosphotyrosine substrate.

3) Referring Fig. 5b, the authors state that the experiments were performed using 293 and HeLa cells, but the figure says 293 and COS7 cells. Please clarify/correct.
Response: We apologize for the error. The experiments were performed using 293 and COS7 cells as indicated in the figure. The text has been corrected.

Editorial Decision – *EMBO Journal*

Thank you for submitting your revised manuscript for our consideration. Please excuse the delay in getting back to you with a decision, but unfortunately the re-evaluation by the referees was not straightforward and thus prompted additional consultations from our side, as I will try to explain in the following.

All three of the original reviewers have once more looked at your study. As you will see from their comments below, only referee 3 has been fully satisfied by your revisions and now strongly supports publication. However, I am afraid that neither of the other two referees felt that there have been sufficient efforts to decisively address their original concerns. In particular, referee 1 feels that some of their original comments have been misinterpreted or taken out of context, and therefore - while appreciating certain improvements such as the confirmation of phosphocysteine on PRLs in cells - still cannot support publication. Moreover, referee 1 already initially referred to prior mutagenesis data on PRL-2/CNNM3 interaction and its physiological relevance, and notes that the relevant work published by the Tremblay lab earlier this year is still neither referenced nor discussed. Given this, and various other remaining concerns, I am afraid we had -after several discussions within our team- to conclude that the study is still not acceptable at this stage, and that we will thus ultimately not be able to publish it.

Given the only limited improvements achieved during this comparably short revision, it is apparent that satisfactorily addressing persisting experimental concerns would now still require another major round of revision. However, in my first decision letter, I had clearly emphasized our single-major-revision-round policy and the resulting importance of adequately addressing referee points during its course, offering both an extended revision deadline as well as pre-discussion of revision plans. Moreover, I assume based on your rapid resubmission as well as on your additional correspondence that you would at this stage prefer to publish this work rapidly and without further involved revision experiments.

I have therefore taken the liberty to discuss your manuscript and its review history with my colleagues at our sister journal, EMBO reports, to explore the possibility of publishing your manuscript in more or less its current version in their pages. Following these discussions, my colleague Dr. Martina Rembold (cc'd in this email) agreed in principle to publishing this work without further rounds of review, however pending further careful rewriting to address both referee 1 and 3's remaining points, with particular attention to presenting precedent findings as well as the current results and conclusions in the most appropriate and circumspect manner. Should you be interested in this option, please simply utilize the hyperlink at the end of this email to directly channel the manuscript to EMBO reports, or get in touch with Dr. Rembold via email.

I am sorry that the outcome of the re-review did not allow me to be more positive regarding publication in The EMBO Journal, but very much hope that you will consider the possibility of publishing this work in EMBO reports.

REFEREE REPORTS

Referee #1:

------The authors have addressed some of my concerns, but they have not addressed my major concern about showing that the presence of the phospho-cysteine intermediate actually leads to an effect in cells that depends on the interaction with CNNMs. I address their comments below and mark mine with "------". I still cannot recommend this work for publication in EMBO J.------

(from 1st review:) The manuscript describes the detection of the phospho-cysteine intermediate of PRLs and suggests that the modification that is usually known as intermediate step in the dephosphorylation mechanism of PTPs could in case of the PRLs function as a negative regulation of the interaction between CNNMs and PRLs. Furthermore, the manuscript contains the crystal structure of PRL-2 with CNNM3, which is to my knowledge the first crystal structure of PRL-2. The idea of the slow second step of the dephosphorylation reaction being a regulatory mechanism for PRLs is very interesting. PRLs have an alanine instead of the commonly conserved serine in the p-loop, which is responsible for hydrolysis of the phosphocysteine intermediate. Its absence is

responsible for a slower hydrolysis, as shown previously in several publications. In agreement with this, the respective A111S mutant (for PRL-3) has been shown to enhance hydrolysis against unnatural substrates. The main novelties of the work are the crystal structure of the complex and the proposed regulatory role of the phospho-cysteine.

(from 1st review:) Crystal structure: While it is interesting, it does not really add much knowledge to the binding parameters on the CNNM3 side. It confirms the mutational analysis done previously, and gives some insights into PRL-2 and its interaction with the CNNM protein. However, what is striking is that it does not explain why the C101S mutation or the C46A mutation in PRL-2 leads to loss of binding to the CNNM, at least not in the currently presented form. In figure 4c the two residues are not involved in the interaction.

Authors: We are surprised by the reviewer's comment since we feel that the crystal structure is highly informative and a significant contribution to the literature. Its value is several-fold. First, the structure is the first of a complex of a PRL phosphatase and reveals that the CNNM acts as a substrate mimic by inserting an aspartic acid residue into the PRL2 catalytic site. Secondly, the structure leads to the hypothesis that the long-lived phospho-cysteine intermediate regulates binding between the two proteins. Not only does the structure explain the CNNM mutagenesis, it suggests new experiments, which are further developed in the paper.

----- I think the authors' surprise stems from the fact that they overlooked this publication, at least it is not cited nor discussed: “Inhibition of PRL-2·CNNM3 Protein Complex Formation Decreases Breast Cancer Proliferation and Tumor Growth.” Kostantin E, Hardy S, Valinsky WC, Kompatscher A, de Baaij JH, Zolotarov Y, Landry M, Uetani N, Martínez-Cruz LA, Hoenderop JG, Shrier A, Tremblay ML. *J Biol Chem.* 2016 May 13;291(20):10716-25. doi: 10.1074/jbc.M115.705863. Epub 2016 Mar 11.

This publication contains a detailed mutational analysis of the CNNM3-PRL-2 interaction, including a structural model that predicts that CNNM3 binds PRL-3 in a pseudosubstrate fashion. D426 is recognized as binding in the active site, and the D426A variant is studied in mice. Thus, while this is a new structure, the first of PRL-2 as I mentioned before, and the first one of this complex (PRL-1 was crystallized in complex with a peptide), it confirms the major finding of D426 binding in the active site as a pseudosubstrate but does not really reveal it. Indeed, a thorough discussion on how the crystal structure compares to the model would be interesting to have. Kostatin et al. also show that the interaction is disrupted by an inhibitor (similar to what the authors attempt to show here for the phospho-cysteine) and that the biological consequence is a reduction of cell proliferation. While this is an indirect read-out as opposed to for example measuring Mg^{2+} efflux, at least they do show a biological consequence, which is completely missing in the current manuscript.-----

Authors: We thank the reviewer for the suggestion. We have examined the effects of two mutations in the PRL2 catalytic site on CNNM binding and present the data as a new Figure 2e. In agreement with the crystal structure, pull-down assays with the CNNM3 GST-CBS domain do not show a strong effect of mutating the catalytic cysteine (C101) or the adjacent cysteine (C49) to alanine. Both mutant proteins were efficiently pulled down by the CBS domain. At present, it is not clear why our results differ from those reported by other groups. These could be due to the different context (full-length protein versus CBS-pair domain), the presence of factors in the mammalian cell extracts, or the presence of protein modifications of the full-length protines (e.g. PRL prenylation).

----I appreciate that the authors looked into this further, as it clarifies that the structure does not explain the finding that in cells the C101 and C49 mutations lead to loss of binding.-----

(from 1st review:) It is intriguing that both disulfide formation and the mutations that prevent disulfide formation prevent binding to CNNM, and I would have hoped to get insight about this from the crystal structure. If the disulfide formation would be modeled on this structure based on the PRL-1 crystal structure, that could explain why the disulfide formation inhibits binding. The authors only mention these points in the discussion with a very general blurry explanation: "the structure shows CNNM is stringently optimized for recognizing the active phosphatase. Structural changes due to disulfide bond formation, replacement of sulfur by oxygen, or most dramatically cysteine phosphorylation oppose binding." With the exception of the phospho-cysteine intermediate (which they call cysteine phosphorylation), the authors do not show that.

Authors: As the reviewer points out, it is intriguing that disulfide formation decreases the affinity of CBS binding (Figure 2f) although it is important to note that the effect is not as dramatic as the block by phosphorylation. Our best explanation is that PRL oxidation leads to a conformational change within the binding site. For the phosphatase PTP1B, conformation-sensing antibodies were generated that sense the oxidized form of the phosphatase and even inhibit its activity (Haque et al, Cell. 2011). Disulfide bond formation in PRL2 likely causes a similar conformational change.

------I agree that the disulfide formation could lead to a local conformational change that could interfere with the CNNM binding. I would however not compare this to the oxidation of the catalytic cysteine of PTP1B, as the mechanism of oxidation and the chemical groups that result from the oxidation (disulfide bond vs. cyclic sulfenyl-amide) and the residues involved in this (C101 and C46 in PRL-2 and C215 and the backbone of S216 in PTP1B) are completely different. Formation of this sulfenyl-amide intermediate causes profound conformational changes in the active site (Haque et al 2011) whereas for PRL-1 such large differences were not observed (Rios et al, 2013; Jeong et al 2005). ------

(from 1st review:) Quite the opposite, as in the PRL-1 structure that they use for comparison the C104S mutation is present (Figure 4d: typo, it says PRL3 there), and the structural change is minimal as evident from the comparison to the PRL-2 structure (4c). As I do not have access to the crystal structure, it is hard to judge this, but it is curious that the authors neglected this point in their analysis of the structure while making it a strong point later on in the discussion.

Authors: There is indeed a great deal of interest in oxidation of PTPs; however, we see a larger effect from cysteine phosphorylation. As the focus of the current manuscript is phosphocysteine and the analysis of cysteine oxidation is problematic due to the multiple different oxidation states and their instability, we have modified the discussion of mutations of the catalytic cysteine and disulfide bond formation. We thank the reviewer for pointing out the typographical error in Figure 4d.

------I appreciate that the authors have modified the discussion and removed this speculation. ------

(from 1st review:) Phospho-cysteine as regulatory mechanism: Starting with the reduction of the burstkinetics in figure 1f and then following with the data in figure 2 and S2&3, this is very interesting and convincing that the authors can detect the phospho-cysteine intermediate. The enhancement of the signal with OMFP is a useful method to support the data. There is no doubt that the phospho-cysteine is formed, as also shown previously for PTP1B. They show that the presence of OMFP blocks the interaction with CNNM in vitro, and removal of the phosphate from the cysteine over time leads to regain of the binding. In the cellular data in Figure 5, unfortunately the authors do not repeat many of the controls that they had done in vitro, such as MS analysis and identification of the phospho-Cys containing peptide was not done at all (as for example done in Sun et al, PNAS 2012). No mutants were used in cells, like the C104/1S mutant as control for Cys phosphorylation.

Authors: We have added a new figure panel that shows the analysis of the C104S and C49S mutants of PRL1 in cultured cells. The new Figure 5b shows that the catalytic cysteine (C104) is required for phosphorylation while the adjacent cysteine (C49) is not.

------I appreciate that the authors addressed this issue and I am now convinced that they detect the phospho-cysteine intermediate also in cells. This shows that the PRLs are active enzymes dephosphorylating substrates in cells, and that the second step of the dephosphorylation reaction is slow as previously shown in vitro, but it does not show anything else.------

(from 1st review:) This has to be done as otherwise the endogenous phosphorylation can be on any other residue of the protein (PRL-3 was shown to be phosphorylated by Src for example), which was not at all considered here. Enforcing the phospho-Cys by OMFP treatment and then removing the signal through hydrolysis over time is not a proof that this happens endogenously.

Authors: The sensitivity of the cysteine phosphorylation to boiling distinguishes it from other types of phosphorylation and excludes Src or other protein kinases as the origin of the PRL phosphorylation observed in cells. Taken together with the stimulation of the modification is

induced by OMFP, which is not a kinase inducer, the evidence of phosphocysteine in mammalian cells is very strong. The modification leads to a shift of PRL proteins on phos-tag gels but not standard SDS-PAGE. The modification is chemically labile and reversed by boiling in SDS loading buffer. The modification requires the phosphatase catalytic cysteine.

------As stated above, I am now convinced that the authors detect the phospho-cysteine intermediate also in cells.------

(from 1st review:) The data on PRL-2 in figure 5a looks rather week and I assume that this is why PRL-1 was used in the following. There is, however, no in vitro data on PRL-1. The antibody used in 5c is not specific for PRL-1 or -2, so it is unclear if PRL-2 behaves the same in cells. In general, the authors do not consider that differences may exist in the PRLs. Although most of the work is performed with PRL-2, some experiments include a different PRL. However, the authors generally refer in the text to PRLs in most of the cases. This can lead the reader to the interpretation that there is in general a redundancy of the physiological function of the three PRLs, but this is a question that is not yet clarified and it is under discussion in the field. Thus, for a better understanding of the manuscript and for accuracy, in every case the specific PRL used on every particular experiment should be named. For example, in figure 5d, they do not mention which PRL was tested, neither in the figure nor text nor captions.

Authors: We apologize for the oversight. Figure 5d (now 5e) has been modified to indicate the antibody detects all three PRL species. While differences undoubtedly do exist between the different PRL isoforms, the proteins are more similar than they are different. Previous studies have shown that all three PRL phosphatases interact with CNNM4 and that PRL1/2 interact with CNNM3. The proteins are highly similar with ~85% sequence identity. In experiments with endogenous proteins, it is difficult to distinguish between the different isoforms due to cross-reactivity between antibodies. Despite this ambiguity, the data showing phosphorylation of PRLs in cells are robust and highly reproducible. We obtained identical results in two different laboratories with different antibodies and different cell lines. Experiment 5c (now 5d) was performed in Montreal and used the anti-PRL2 antibody from Millipore, which also detects PRL1. Experiment 5d (now 5e) was performed in Osaka and used an in-house antibody that detects all three PRL isoforms. In all the experiments, we observed high levels (~50%) of cysteine phosphorylation under normal culture conditions and a decrease in phosphorylation upon magnesium deprivation.

------I do not doubt that all PRLs interact with CNNMs. However, the authors are still generalizing too much.

1) It was never shown that PRL-3 expression is induced by Mg2+ depletion, that was only shown for PRL-1 and -2 (Hardy et al 2015). Since gene expression depends on more factors than sequence similarity, the authors cannot include PRL-3 in their conclusion that PRLs are induced by Mg2+ depletion when using a general anti-PRL antibody. Why do the authors not use a PRL-1/-2 antibody for this crucial experiment? This would be much cleaner. Alternatively, they could knock down PRL-1 and -2 and use the antibody that recognizes all PRLs, and they would obtain the answer if PRL-3 is also induced upon Mg2+ depletion.

2) In Figure 5A it is clearly visible, but not mentioned by the authors, that PRL-3 is much less found in the phospho-cysteine intermediate state than PRL-1 and -2. Thus, in spite of the high sequence similarity there is a difference visible between the phosphatases even in their own data. -------

(from 1st review:) The major concern however is that the physiological relevance of this potential regulatory mechanism is missing. Figure 5d intends to show the physiological relevance, giving the impression that the phospho-cysteine intermediate formation is regulated by Mg2+ presence. However, as reported previously, depletion of Mg induces PRL-2 expression, as seen here. This means that constantly new protein is produced. How can the authors exclude that these new PRL proteins just not have found the substrate to form the phospho-cysteine intermediate yet? Or, that depletion of Mg leads to higher PRL activity removing the phosphate through some allosteric or oligomerization mechanism (PRL-1 and -3 were reported to oligomerize)? A functional experiment is missing. For example, the A108S / A111S mutant could be used and compared to PRL-2 / -1 WT in overexpression for Mg contents in the cell. The mutant should remove the phosphate quickly, and should therefore show a stronger interaction with CNNM leading to higher Mg in the cells or less

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Mg efflux. This mutant should be tested for its binding capacity with CNNM first, because the C101S mutant cannot be used, as it does not bind to CNNM.

Authors: We respectfully disagree with the reviewer. Two published papers show the proteins directly interact and that PRL-CNNM interaction is relevant in cancer (Funato et al, J Clin Inv, 2014; Hardy et al, Oncogene, 2015). Here, we show that disrupting the interaction by mutating the aspartic acid residue in CNNM4 blocks inhibition of Mg2+ efflux by PRL3 (Figure 3b). Further, we show that PRL2 cysteine phosphorylation prevents the association with the CBS-pair domain of CNNM3 in vitro and PRL1 phosphorylation prevents CNNM4 binding in cell extracts. We further show that PRL phosphorylation levels change in cells in response to Mg2+ deprivation. Together the data are highly evocative of a physiological feedback pathway.

-------I respectfully disagree with the authors. Yes, it was previously shown that the proteins interact and that this is important in cancer. But this does not show that the disruption by formation of the phospho-cysteine intermediate has an effect on Mg2+ efflux or concentration in the cell. This is speculation based on correlating in vitro data to an in-cell setting. By showing that the C101A mutant still binds to CNNM(CBS) in vitro but not in cells, they actually show that it is not always feasible to correlate an in vitro finding with an in-cell effect (that may or may not have biological relevance). They even point out themselves that "the difference between our in vitro assay and results in cultured cells is indicative additional complexity in the PRL•CNNM interaction". Furthermore, it is unclear why the authors mutate in all experiments in figure 3 only the CNNM protein when they want to show that a change in the PRL protein leads to disruption of the interaction. Why not applying a mutant like the one I suggested and include it into the cell-based Mg2+ efflux assays? Another interesting mutant could be PRL-2 R107A as the other reviewer suggests, as this is also according to the crystal structure involved in the binding, but this was not tested through mutagenesis. Towards showing that the disruption of the interaction is also happening in cells, they carry out a pull-down experiment (Figure 5c). I think it would be crucial to show that they detect the phospho-cysteine containing PRL-1 in the supernatant of the pull-down, as it might get lost during the pull-down procedure since the phosphate gets hydrolyzed over time. Finally, the experiment that supposedly shows that "PRL" phosphorylation levels change is not convincing. My previous comments already shed doubt on the clear interpretation of the experiment by the authors, and this was not addressed. In addition to my comment about the induction of PRL-3 expression above, contrary to Fig. 5a in fig. 5c all PRLs are found at the same size. How can the authors be sure that what they see is the phospho-cysteine form of PRLs? How can they be sure that Mg2+ deprivation does not lead to PRL substrate down-regulation or that not enough substrate is there to get dephosphorylated by them, as their levels are higher? Based on this, how do they know that this is an intended negative feedback loop and not just an artifact, without knowing if it has a consequence on the function of the CNNM-PRL interaction? I do not think that this is highly evocative of a physiological feedback at this point, but speculation. As this is the major point and novelty of the manuscript (also according to the authors), for publication in EMBO J this has to be addressed.------

Authors: We agree that further studies are merited. The experiments suggested by the reviewer are important for future studies. The origin of the phosphate needs to be determined, the role of PRL oligomerization should be investigated, additional mutants tested, and the mechanism of inhibition of Mg2+ efflux determined. However, these experiments are out of the scope of the current paper. The revised manuscript already has 30 figure panels and presents a complete story spanning enzymatic assays, a crystal structure, cell assays of Mg2+ efflux, evidence of cysteine phosphorylation in cells and changes in response to environmental conditions. We have added a paragraph and expanded view movie that describe how PRL trimerization is compatible with our PRL•CNNM complex.

--------I did not say that there were not many experiments done, and I did not ask to determine where the substrate is coming from (see below). But as I discussed above, the story is not complete, and the interpretation is still not warranted for the major story that brings the novelty: the role of the phospho-cysteine intermediate. It is unclear why the authors focused in figure 3 on the CNNM mutations, that were published (which is not mentioned here) for PRL-2 and CNNM3 before, and why they did not focus on the story about the phospho-cysteine intermediate. My points below from the 1st revision already point out that parts of what the authors have done here was published similarly before, but the authors did not discuss the paper of Kostantin et al., JBC 2016.-------

(from 1st review:) The data shown in Figure 1 (with the exception of Figure 1f) adds useful biophysical constants to previously described facts (for example that aspartic acid 426 is essential for binding CNNM, that has been shown in mouse experiments before, so the ITC does not identify this fact as the authors claim). The experiments in the chapter "PRL-CNNM interaction regulates magnesium efflux in cells" related to Figure 3 confirm that PRL-3 interacts with CNNM4 as described before. In addition, they newly show through testing different PRL3 mutants in the Mg efflux assay that the interaction involves the same amino acid residues as previously described for PRL-2.

A valid question is where the phosphate group of the intermediate is coming from. However, in the PRL field it is accepted that this question has not been conclusively answered yet, and that it is very hard to find any substrate for PRLs, and few substrates have been suggested. While I would not expect the authors to answer that question conclusively due to the challenges, I completely disagree with their explanation of where the substrate could come from. They state: "We observed that recombinant PRL2 purified from E. coli is partially phosphorylated in the absence of added substrate (Fig. 2d, PRL2 input lane). This suggests that PRL2 has broad substrate specificity. Using detection of phosphorylated PRL as an activity assay, we observed phosphatase activity with a variety of low molecular weight compounds that are unlikely to be physiological substrates (data not shown)." 1) If it were true that PRL2 has broad substrate specificity like other phosphatases, there would be many more proposed substrates, but there are indeed very few. Also, E. coli could contain specific substrates of PRL-2, so the cysteine of PRL2 could be in the phospho-cysteine intermediate state without adding external substrate. 2) Many phosphatases accept many small molecule substrates, so the author's not shown observation does not explain anything. The authors also state later on that PRLs behave as pseudophosphatases. This statement needs to be clearly rephrased to be meant for the interaction with CNNMs only, otherwise the authors would need to disprove all previous publications on PRL substrates.

Authors: We have revised the text to remove the confusing statements about PRL substrates. The reviewer is correct that many small molecules can act as substrates of phosphatases. The sentence referring to PRLs as pseudophosphatase as been rephrased to specify that it referred to the interaction with CNNMs and not imply that PRLs do not have catalytic activity as phosphatases.

-----I appreciate that the authors addressed this comment.-----

(from 1st review:) The term phosphorylation in this manuscript should be explained earlier in the text (for example in the summary), and I am not even sure if this is the correct term. It should not be used in the title. The detected cysteine phosphorylation on the PRLs is a phosphocysteine intermediate from a phosphatase reaction rather than a kinase-mediated phosphorylation, as described for the bacterial phospho-cysteine (Sun et al. PNAS 2012). So PRL2 is not being phosphorylated on cysteine like the bacterial protein, and that needs to be clarified.

Authors: We have reworded the title to use phospho-cysteine rather than phosphorylation. The abstract has been modified to clarify that the phosphorylation of cysteine arises from the phosphatase catalytic cycle. The discussion includes the statement that phosphorylation of PRLs is not the direct result of kinase activity.

-------The authors still mix the two terms, but it is a bit clearer now. -------

(from 1st review:) The authors jump between showing the interaction in general and the phospho-cysteine intermediate regulation, and that makes the manuscript a bit difficult to read. Taken together, the authors confirm data published previously by other groups and add more information to it, providing the PRL-2/CNNM3 crystal structure, which unfortunately does not yet explain fully the above mentioned aspects. The authors provide the very intriguing idea that the phospho-cysteine intermediate in PRLs could have an inhibitory function. The physiological relevance of this is unclear, and it is not convincingly shown that this is not only part of the dephosphorylation reaction or really a negative regulation in cells. Other phosphatases like DUSP19 (mouse LDP2) have the same alanine mutation: could they also be regulated by a longer lasting intermediate? If so, would that be a more general mechanism? Or, is this rather a mutation that could specify substrate specificity or be involved in structural integrity (as previously suggested for PRL3 and LDP2)?

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These possibilities are not mentioned in the discussion. For all these reasons the data is, in my opinion, over-interpreted at this point and preliminary, and the authors wrote a selective discussion for their purposes. I therefore cannot recommend considering this work for publication in EMBO J. It could be reconsidered if the authors thoroughly and convincingly address the concerns detailed above, but at the moment it should not be published.

Authors: We are pleased that the reviewer found the work "very interesting", "interesting", "intriguing", and "very interesting and convincing". We agree that the manuscript raises many important questions and hope the paper will stimulate other groups to extend our studies. However, the mechanism of dephosphorylation and the question whether other phosphatases such as DUSP19 also form a long lasting intermediates are outside of the scope of the current study. As requested, we have added a sentence to the Discussion raising the possibility that other phosphatases may show a similar phenomenon.

-------The authors took my statements of what I found very interesting etc. out of context and misinterpreted them in their favour. I clearly did not use these terms for the full work described in this paper. It is all written above, I will not repeat this here.

Minor points:
- Line 160: 2F should be 2E
- Line 164: 2F should be 2E
- Line 169: 2E should be 2F
- Line 290: Fig. 2D & E should be 2D & F
- Abstract: line 29: this sentence is misleading, it should be rephrased to show that they did not test PRL mutations in the Mg^{2+} efflux assay. The whole abstract is again too general w.r.t. CNNMs and PRLs.
- Line 374: "We observed that the recombinant PRL2 purified from e.coli is partially phosphorylated in the absence of added substrate (...), which rules out the action of a specific kinase". The figure shows that recombinant PRL-2 purified from bacteria is partially phosphorylated in the absence of OMFP. This phosphorylation could come from different sources, including a kinase. This sentence is confusing.

A comment on the answers to reviewers 2 and 3: I do not think that the authors have addressed many of the major concerns adequately, as some of them would require further experimentation.

Referee #2:

I was positive about the original manuscript, but am a bit disappointed by the lack of effort to address the questions in my review, specifically 1,2,4.

Still I think the manuscript is worthy of publication.

Referee #3:

This is a revised version of a manuscript that I reviewed earlier and thought was quite interesting and potentially appropriate for publication in Embo J. In this revised version, the authors have addressed most of my concerns and those of the other reviewers. I do have three very minor comments that can be easily addressed without need for additional review:

1) The authors state, line 117, "...PRL2 shows burst kinetics the rapid turnover of on molecule of substrate...." As written, this sentence is an apparent run-on. There should be a comma after "kinetics."

2) I think that their explanation of the effect of CNNMs on PRL kinetics still is not clear to the general reader. On lines 122-3, they state that "...the initial burst of activity was completely inhibited and the steady-state rate was decreased by ~50%." This statement is accurate, but as the authors noted in response to my initial comments, "The "steady-state rate" in the presence of the CNMN

CBS domain is unrelated to the rate of hydrolysis of the phosphocysteine intermediate. I think that they way that they revised the text still does not make the mechanism clear enough for the general reader (in fact, I am pretty sure that given the low level of knowledge of most PIs these days about basic enzymology, even some specialists might be confused). It is, of course, up to the authors, but I suggest they state much more explicitly what is going on: that the rate of hydrolysis of the p-Cys intermediate is not affected by the CBS domain, but instead, the decrease in rate reflects the amount of free PRL.

3) As I noted in my initial review, the authors shift back and forth between different CNMM/PRL combinations for different types of experiments. They provide a reasonable explanation for these choices in their rebuttal letter, but they do not discuss this issue at all in the text. Again, it's the authors' paper, and really up to them. But I am pretty sure that other readers are going to have the same questions about this that I did, and I suggest they provide a brief discussion (similar to that in their rebuttal letter) to explain why they made these choices.

Transfer to *EMBO Reports* 22 September 2016

Manuscript submitted via internal transfer system to *EMBO Reports.*

Editorial Decision 29 September 2016

Thank you for the submission of your research manuscript to EMBO reports. As my colleague Hartmut Vordermaier from our sister journal EMBO Journal has already outlined to you -given the potential interest of your findings and the support by two referees- we are interested in publishing your manuscript in its current form, given that the remaining referee requests are incorporated into the manuscript and the conclusions are presented and discussed in the most appropriate manner. Moreover, the work published by the Tremblay lab earlier this year has to be cited and discussed.

3rd Revision – authors' response to original comments 01 October 2016

Referee #1:

----- I think the authors' surprise stems from the fact that they overlooked this publication, at least it is not cited nor discussed: “Inhibition of PRL-2·CNNM3 Protein Complex Formation Decreases Breast Cancer Proliferation and Tumor Growth.” Kostantin E, Hardy S, Valinsky WC, Kompatscher A, de Baaij JH, Zolotarov Y, Landry M, Uetani N, Martínez-Cruz LA, Hoenderop JG, Shrier A, Tremblay ML. *J Biol Chem*. 2016 May13;291(20):10716-25. doi: 10.1074/jbc.M115.705863. Epub. 2016 Mar 11.

This publication contains a detailed mutational analysis of the CNNM3-PRL-2 interaction, including a structural model that predicts that CNNM3 binds PRL-3 in a pseudosubstrate fashion. D426 is recognized as binding in the active site, and the D426A variant is studied in mice. Thus, while this is a new structure, the first of PRL-2 as I mentioned before, and the first one of this complex (PRL-1 was crystallized in complex with a peptide), it confirms the major finding of D426 binding in the active site as a pseudosubstrate but does not really reveal it. Indeed, a thorough discussion on how the crystal structure compares to the model would be interesting to have. Kostatin et al. also show that the interaction is disrupted by an inhibitor (similar to what the authors attempt to show here for the phospho-cysteine) and that the biological consequence is a reduction of cell proliferation. While this is an indirect read-out as opposed to for example measuring Mg2+ efflux, at least they do show a biological consequence, which is completely missing in the current manuscript.-----

Response: We thank the reviewer for pointing out the paper by Kostantin et al. We have added a citation to that manuscript and to the 2015 MSc thesis that first reported the structure and the effects of D426A mutation. We note that we provided the preliminary atomic coordinates of a CNNM3 CBS • PRL2 complex to the Tremblay laboratory in 2012 and that the D426A mutation and structure were presented at the 2014 FASEB Phosphatase meeting. The molecular modeling by Kostantin is predictive and highly useful but it does not confer the same degree of certainty as the experimentally determined atomic model presented here. In particular, differences between the

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structures are most likely due to uncertainties in modeling rather than any biological effects. Irrespective of issues of priority, publication of the crystal structure of CNNM3 CBS • PRL2 complex is significant advance in our understanding of this novel pathway.

The paper by Kostantin does report important evidence of the biological consequences of inhibiting the CNNM/PRL interaction. They show that the disrupting the CNNM-PRL interaction has an effect on colony formation and that a phosphatase inhibitor decreases tumor growth in a xenograft mouse model. The concordance of the different approaches strengthens the conclusion that CNNM-PRL interaction is important physiologically.

------I agree that the disulfide formation could lead to a local conformational change that could interfere with the CNNM binding. I would however not compare this to the oxidation of the catalytic cysteine of PTP1B, as the mechanism of oxidation and the chemical groups that result from the oxidation (disulfide bond vs. cyclic sulfenyl-amide) and the residues involved in this (C101 and C46 in PRL-2 and C215 and the backbone of S216 in PTP1B) are completely different. Formation of this sulfenyl-amide intermediate causes profound conformational changes in the active site (Haque et al 2011) whereas for PRL-1 such large differences were not observed (Rios et al, 2013; Jeong et al 2005). ------

Response: We have removed the reference to the structural changes in PTP1B.

------I appreciate that the authors addressed this issue and I am now convinced that they detect the phospho-cysteine intermediate also in cells. This shows that the PRLs are active enzymes dephosphorylating substrates in cells, and that the second step of the dephosphorylation reaction is slow as previously shown in vitro, but it does not show anything else.------

Response: We show that there are two pools of PRL in cells. Unphosphorylated PRLs that are able to bind and inhibit CNNMs and phosphorylated PRLs that are not. We also show that the relative sizes of the pools change in response to changes in Mg++ availability.

------I do not doubt that all PRLs interact with CNNMs. However, the authors are still generalizing too much.

1) It was never shown that PRL-3 expression is induced by Mg2+ depletion, that was only shown for PRL-1 and -2 (Hardy et al 2015). Since gene expression depends on more factors than sequence similarity, the authors cannot include PRL-3 in their conclusion that PRLs are induced by Mg2+ depletion when using a general anti-PRL antibody. Why do the authors not use a PRL-1/-2 antibody for this crucial experiment? This would be much cleaner. Alternatively, they could knock down PRL-1 and -2 and use the antibody that recognizes all PRLs, and they would obtain the answer if PRL-3 is also induced upon Mg2+ depletion.

2) In Figure 5A it is clearly visible, but not mentioned by the authors, that PRL-3 is much less found in the phospho-cysteine intermediate state than PRL-1 and -2. Thus, in spite of the high sequence similarity there is a difference visible between
the phosphatases even in their own data. -------

Response: We agree that we cannot conclude isoform specific changes in expression. However even without this information the finding of overall changes in PRL phosphorylation are significant and evocative. Our findings enable future studies of the individual PRLs such as those suggested. We note that knockdown experiments aren't without their own caveats. Knocking-down PRL1 and 2 could change the expression or phosphorylation of PRL3. Unfortunately, there are no PRL3-specific antibodies available at present. With respect to Fig 5A, we do not see a large difference in levels of PRL3 phosphorylation in transfected cells. Perhaps the reviewer is referring to the difference in the OMFP control where PRL3 is incompletely phosphorylated. This could be due to the presence of oxidized PRL3 in the assay despite the presence of a reducing agent. As we reported in 2004, it is difficult to completely reduce PRL3.

-------I respectfully disagree with the authors. Yes, it was previously shown that the proteins interact and that this is important in cancer. But this does not show that the disruption by formation of the phospho-cysteine intermediate has an effect on Mg2+ efflux or concentration in the cell. This is

speculation based on correlating in vitro data to an in-cell setting. By showing that the C101A mutant still binds to CNNM(CBS) in vitro but not in cells, they actually show that it is not always feasible to correlate an in vitro finding with an in-cell effect (that may or may not have biological relevance). They even point out themselves that "the difference between our in vitro assay and results in cultured cells is indicative additional complexity in the PRL•CNNM interaction". Furthermore, it is unclear why the authors mutate in all experiments in figure 3 only the CNNM protein when they want to show that a change in the PRL protein leads to disruption of the interaction. Why not applying a mutant like the one I suggested and include it into the cell-based Mg2+ efflux assays? Another interesting mutant could be PRL-2 R107A as the other reviewer suggests, as this is also according to the crystal structure involved in the binding, but this was not tested through mutagenesis.

Response: The experiment suggested, mutating residue A108S (or R107A) to increase the hydrolysis of the phosphocysteine intermediate, is impractical for several reasons. Wild-type PRLs are roughly 65% unphosphorylated when transfected (Fig 5A) so that the maximum effect that could be observed for the mutants is only 1.5-fold (arising from a change of 65% to 100% unphosphorylated PRL.) Furthermore, while the A111S mutation does increase hydrolysis in vitro, it is not clear that the increase is sufficient to completely dephosphorylate all of the transfected protein in vivo. In this case, the difference in levels of unphosphorylated PRL3 between the wildtype and mutant protein would be smaller than 1.5-fold. Yet another issue is that it is not clear that the Mg++ efflux assay is in the linear regime (with respect to inhibition by PRL3) so that a 50% increase in the amount of unphosphorylated PRL3 might lead to a smaller change in inhibition. To measure this small change, the transfection experiments would have to be carefully controlled to ensure that the expression levels of the mutant and wild-type PRLs are precisely equal. Lastly, any differences observed would have to be controlled by in vitro studies to confirm that the mutations don't have a direct effect on the affinity of the PRL-CNNM interaction. A 50% change in affinity would completely confound the interpretation of the in vivo experiments.

Towards showing that the disruption of the interaction is also happening in cells, they carry out a pull-down experiment (Figure 5c). I think it would be crucial to show that they detect the phospho-cysteine containing PRL-1 in the supernatant of the pull-down, as it might get lost during the pull-down procedure since the phosphate gets hydrolyzed over time.

Response: We do show phospho-cysteine containing PRL1 is uniquely found in the supernatant of pull-downs (Fig 2D & F). The experiment in Fig 5c used cell lysates. While, in theory, it is possible that the immunoprecipitate contains an activity that dephosphorylates PRLs, this seems highly unlikely. The phosphorylated PRL1 is observed in the cell lysates and the anti-FLAG is a highly purified antibody and unlikely to affect the hydrolysis. The intrinsic rate of hydrolysis of PRLs (Fig EV1) is much slower than the incubation periods used for the immunoprecipitation.

Finally, the experiment that supposedly shows that "PRL" phosphorylation levels change is not convincing. My previous comments already shed doubt on the clear interpretation of the experiment by the authors, and this was not addressed. In addition to my comment about the induction of PRL-3 expression above, contrary to Fig. 5a in fig. 5c all PRLs are found at the same size. How can the authors be sure that what they see is the phospho-cysteine form of PRLs? How can they be sure that Mg2+ deprivation does not lead to PRL substrate downregulation or that not enough substrate is there to get dephosphorylated by them, as their levels are higher? Based on this, how do they know that this is an intended negative feedback loop and not just an artifact, without knowing if it has a consequence on the function of the CNNM-PRL interaction? I do not think that this is highly evocative of a physiological feedback at this point, but speculation. As this is the major point and novelty of the manuscript (also according to the authors), for publication in EMBO J this has to be addressed.------

Response: The points raised by the reviewer are valid and important for future investigation. It is indeed possible that changes in substrate levels or availability are responsible for the changes in PRL phosphorylation observed. Phosphorylation of PRLs could be a mechanism for temporal control or for inactivating PRLs that are not bound to CNNMs. As we show in Fig 1, binding of CNNM CBS domain to PRL2 inhibits its catalytic activity. This would also protect it from phosphoryation.

--------I did not say that there were not many experiments done, and I did not ask to determine where the substrate is coming from (see below). But as I discussed above, the story is not complete, and the interpretation is still not warranted for the major story that brings the novelty: the role of the phospho-cysteine intermediate. It is unclear why the authors focused in figure 3 on the CNNM mutations, that were published (which is not mentioned here) for PRL-2 and CNNM3 before, and why they did not focus on the story about the phosphocysteine intermediate. My points below from the 1st revision already point out that parts of what the authors have done here was published similarly before, but the authors did not discuss the paper of Kostantin et al., JBC 2016.-------

Response: We apologize for overlooking the citation of Kostantin et al., JBC 2016. As noted above, that paper provides important in vivo confirmation of the relevance of the PRL-CNNM interaction.

-------The authors took my statements of what I found very interesting etc. out of context and misinterpreted them in their favour. I clearly did not use these terms for the full work described in this paper. It is all written above, I will not repeat this here.

Minor points:
- Line 160: 2F should be 2E
- Line 164: 2F should be 2E
- Line 169: 2E should be 2F
- Line 290: Fig. 2D & E should be 2D & F
- Abstract: line 29: this sentence is misleading, it should be rephrased to show that they did not test PRL mutations in the Mg2+ efflux assay. The whole abstract is again too general w.r.t. CNNMs and PRLs.
- Line 374: "We observed that the recombinant PRL2 purified from e.coli is partially phosphorylated in the absence of added substrate (...), which rules out the action of a specific kinase". The figure shows that recombinant PRL-2 purified from bacteria is partially phosphorylated in the absence of OMFP. This phosphorylation could come from different sources, including a kinase. This sentence is confusing.

A comment on the answers to reviewers 2 and 3: I do not think that the authors have addressed many of the major concerns adequately, as some of them would require further experimentation.-----

Response: We thank the reviewer for the corrections and apologize if we misquoted him. We were merely pointing out that the reviewer stated several parts of the manuscript interesting. We have made the specific changes requested.

Referee #2:

I was positive about the original manuscript, but am a bit disappointed by the lack of effort to address the questions in my review, specifically 1,2,4. Still I think the manuscript is worthy of publication.

Response: We apologize for the apparent lack of effort. In fact, we had previously spent considerable time on two of the experiments suggested as explained below. As the results of those experiments were inconclusive, we did not include them in the manuscript. The questions are recopied below, along with more detailed responses.

<u>1. How does reduced magnesium decreased levels of PRL phosphorylation? Does it suppress PRL phosphatase activity, or stimulate dephosphorylation of phospho-PRL. These alternatives could easily be tested using their in vitro assays.</u>

We definitely agree that this is an interesting and relevant question. Prior to submission of the manuscript, we used in vitro experiments to screen for conditions (pH, magnesium, temperature) that affect the PRL dephosphorylation. While we did not identify any strong effects of buffer conditions, we did observe multiexponential decay. The dephosphorylation rate decreases with time. There are several possible explanations for this, such as heterogeneity in the protein preparation, which we hope to test once the current manuscript is accepted.

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<u>2. What is the substrate that is responsible for PRL phosphorylation in vivo? The authors speculate that phosphatidylinositol phosphates might be substrates. Has this been tested?</u>

Again, we agree that the experiments are important. We have carried out experiments to test various physiological, small molecular weight substrates. At present, the results are inconclusive and not suitable for inclusion in the manuscript. We were able to observe some activity with fluorescent phosphatidylinositol phosphates substrates as reported by McParland et al. in 2011; however, whether these are the physiological substrates of PRLs is unclear. Curiously, we observed significant (up to 50%) phosphorylation of PRL2 when purified from E. coli cells, which to my knowledge do not contain phosphatidylinositol phosphates. We are investigating the substrate specificity of PRLs further but are unable to do more than speculate at present.

<u>4. The crystal structure of PRL in complex with the CBS-domain peptide is convincing. Did the authors test this interaction by mutation of Arg 107 and or Leu 105?</u>

We have focused our PRL mutagenesis on the catalytic cysteine and the adjacent cysteine residue in order to assess the effect of redox signaling on the PRL-CNNM interaction. The Arg107 and Leu105 mutants are currently being tested. As reviewer #1 pointed out, the Arg107 could potentially be used to assess in vivo the role of cysteine phosphorylation.

Referee #3:

This is a revised version of a manuscript that I reviewed earlier and thought was quite interesting and potentially appropriate for publication in Embo J. In this revised version, the authors have addressed most of my concerns and those of the other reviewers. I do have three very minor comments that can be easily addressed without need for additional review:

1) The authors state, line 117, "...PRL2 shows burst kinetics the rapid turnover of on molecule of substrate...." As written, this sentence is an apparent run-on. There should be a comma after "kinetics."

Response: We thank the reviewer and have corrected the sentence.

2) I think that their explanation of the effect of CNNMs on PRL kinetics still is not clear to the general reader. On lines 122-3, they state that "...the initial burst of activity was completely inhibited and the steady-state rate was decreased by ~50%." This statement is accurate, but as the authors noted in response to my initial comments, "The "steady-state rate" in the presence of the CNMN CBS domain is unrelated to the rate of hydrolysis of the phosphocysteine intermediate. I think that they way that they revised the text still does not make the mechanism clear enough for the general reader (in fact, I am pretty sure that given the low level of knowledge of most PIs these days about basic enzymology, even some specialists might be confused). It is, of course, up to the authors, but I suggest they state much more explicitly what is going on: that the rate of hydrolysis of the p-Cys intermediate is not affected by the CBS domain, but instead, the decrease in rate reflects the amount of free PRL.

Response: We agree with the reviewer and have revised the paragraph as suggested.

3) As I noted in my initial review, the authors shift back and forth between different CNMM/PRL combinations for different types of experiments. They provide a reasonable explanation for these choices in their rebuttal letter, but they do not discuss this issue at all in the text. Again, it's the authors' paper, and really up to them. But I am pretty sure that other readers are going to have the same questions about this that I did, and I suggest they provide a brief discussion (similar to that in their rebuttal letter) to explain why they made these choices.

Response: We have expanded the explanation in lines 173-177.

2nd Editorial Decision 06 October 2016

Thank you for the submission of your revised manuscript to EMBO reports. I have gone through the manuscript and there are a few things from the editorial side that we need before we can proceed

with the official acceptance of your study.

- Please rename Movie 1 to Movie EV1 and provide a figure legend for the movie. Please upload a zip file that contains both, movie EV1 and the legend.
- Please rename the "Methods" section to "Materials and Methods".
- Please provide a "Conflict of interest" statement.

- We routinely perform a quality check of all submitted figure files and images. This scan indicated a sharp transition/non-continuous lanes in the Western blot in Figure 5A (rightmost column). While this is in principle ok, we do require that this is indicated with a line separator -in case the image was spliced. As EMBO press encourages the publication of source data, could you please provide the full image as source data for this figure?

I am looking forward to seeing a final version of your manuscript as soon as possible.

4th Revision – authors' response | 07 October 2016

The authors resubmitted the manuscript after making the requested changes.

3rd Editorial Decision | 13 October 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Kalle Gehring

Journal Submitted to: EMBO Reports
Manuscript Number: EMBO-2016-43393

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	A fixed number of cells were selected prior to the statistical analysis of the magnesium efflux assays in Figure 3B.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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For animal studies, include a statement about randomization even if no randomization was used.	N/A
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4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	The stasticial significance of the data in Figure 3C was assessed by one way ANOVA followed by Bonferroni's multiple comparison test.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-PRL monoclonal in Fig 5D was EMD Millipore 05-1583 | Anti-PRL-2 Antibody, clone 42. Anti-PRL polyclonal in Fig 5E was described by Yamazaki et al (2013) PLoS Genet 9: e1003983. Anti-FLAG for immunoblotting was F7425 (SIGM, Anti-FLAG for immunoprecipitation was 016-22784 (Wako). Anti-Myc was sc-789 (Santa Cruz).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell lines used in this study (COS7, HEK293, HeLa) are routinely used in our laboratories. Neither STR profiling nor mycoplasma test has been performed with these cell lines.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The structure factors and coordinates have been deposited to the Protein Data Bank under accession number 5K22.
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G- Dual use research of concern

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