JCB

PPM1F controls integrin activity via a conserved phospho-switch

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Re: JCB manuscript #202001057

Dr. Christof R Hauck
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Dear Dr. Hauck,

Thank you for submitting your manuscript entitled "The Phosphatase PPM1F controls integrin activity via a conserved phospho-switch in the integrin β subunit". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

All of the reviewers commented favourably on the potential of your studies to advance the field but, as you will see, they each raised a number of concerns and they requested further experimentation. After careful consideration, I have decided that the points raised by the reviewers currently preclude publication. However, in view of the potential importance of your work, I would like to give you the chance to respond to the comments, and I would therefore be willing to re-review a revised manuscript. I consider all of the points raised by reviewers to be within the scope of your manuscript and therefore any resubmission should address the comments in full. Since a significant number of concerns have been expressed, it is likely that extensive additional experimentation will be required.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Martin Humphries, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This manuscript describes PPM1F as a phosphatase that controls phosphorylation of a critical TT-motif in the beta1-integrin cytoplasmic domain. In vitro, phosphorylation of this motif inhibits filaminA association with the integrin, thereby allowing talin binding. Deletion of the phosphatase increased phosphorylation of the beta1-integrin TT motif in cells, increased talin association and increased cell adhesion. Whilst the regulation of filaminA binding to integrin beta-cytoplasmic domain by phosphorylation, and talin/filamin competition is not new, the description of PPM1F as an integrin phosphatase is an interesting and novel finding. Also the increased talin association with integrins and increased adhesion in PPM1F KO cells is potentially interesting. However, there are some major issues to be addressed/clarified.

1. FilaminA regulation by phosphorylation of the integrin beta-chain is not a new finding (Takala et al, Blood, 2008). Also talin binding to the integrin beta-chain is already known not to be affected by integrin phosphorylation status (Takala et al, 2008), but can be outcompeted by filamin (Klema et al 2006) and also by 14-3-3 proteins (Takala et al, 2008). Please modify the text in the results and discussion sections to reflect this and refer to relevant publications. In addition, although the role of talin in integrin activation and cell adhesion is clear, the role of filamin in integrin regulation in vivo is more conflicting. Please show adhesion assay and beta1-integrin activation assays in FlnA knock-

down cells to show whether filamin plays a role in beta1-integrin regulation in cells (Fig S2).

2. Critically, the TT-site in the beta-integrins is also the kindlin and 14-3-3 protein binding site (and also binds several other proteins). Especially kindlin has been shown to be critical for regulating integrin-mediated cell adhesion. How is kindlin binding to the beta1-integrin affected by the AA and DD-mutation and by direct phosphorylation? Does kindlin and talin compete or cooperate for binding and how is this regulated by phosphorylation? What about 14-3-3 proteins? Please carefully examine these issues experimentally in vitro and in cells to clarify these issues.

3. Are TT/DD-beta1-integrin expressing cells more adherent than WT integrin expressing cells? Is the beta1-integrin more active? Please clarify.

4. How does direct phosphorylation of the beta1-integrin tail impact filamin, filamin, kindlin and 14-3-3 binding (eg rather than charge-mimicking DD mutations)? This can be studied with phosphorylated peptides. Please confirm the data (filamin/talin binding) with phosphorylated and nonphosphorylated beta1-integrin peptides.

5. Why is there no filamin recruitment to the wt integrin construct in cells (Fig 2)? The integrin is certainly not phosphorylated to 100% stoichiometry in cells. Is filamin binding to integrin beta-tails an in vitro artefact?

6. Phosphatases such as PP2A has been previously implicated in dephosphorylation of the threonines of the beta1-integrin (Kim et al, JBC 2004). Please include as a control here.

7. What is the general effect of PPM1F deletion on Ser/Thr phosphorylation in cells/of proteins in focal adhesions? Is the increased threonine phosphorylation of the beta1-integrin unique? Please examine experimentally.

Reviewer #2 (Comments to the Authors (Required)):

This study addresses and very interesting and largely overlooked aspect about integrin biology, the role of integrin cytoplasmic tail phosphorylation in regulating their function. Through a small shRNA screen of the described adhesome PPASEs the authors find that PPM1F silencing strongly augments adhesion of HEK293 cells. They go on to show that PPM1F impacts on cell spreading in a phosphatase activity dependent manner. They also demonstrate that PPM1F directly regulates b1-tail TT site phosphorylation. The authors suggest that PPM1F, through regulation of these sites, regulates the ability of filamin to bind to the b1-tail and the recruitment of talin to integrins. This is an interesting and predominantly carefully conducted study. The authors should, however, consider addressing the following issues.

1) Figure 1B. The authors may want to consider validation their "pseudophosphorylation mutant integrin tail data" (Figure 1B,C) with biotinylated recombinant peptides that are phosphorylated at these sites. These are commercially available (and have been used by the authors in figure 6c) and would directly show that role of phosphorylation (rather than negative charge) in regulating the binding.

2) (Figure 3 and 4) The authors should consider strengthening their data on integrin activation upon PPM1F silencing/knock-out by employing additional b1-integrin activation epitope antibodies (such as 12G10) and using golden-standard integrin activation assay where binding of labelled FN

fragments to integrins are investigated with flow cytometry. The extent of activation should be compared to the maximum activation achieved with Mn2+. Possible off-target effects need to be controlled for by using at least a second independent shRNA or with rescue experiments.

3) Figure 5. The data with increased integrin phosphorylation upon PPM1F are rather convincing. However, it seems that the TTpp positive band corresponds to the lower MW migration beta1 band. This is considered in the field to correspond to the immature/ER resident form of beta1. Is PPM1F regulating phosphorylation of the immature b1-integrin ? How would this be linked to integrin activity on the cell surface/in adhesion regulation?

4) Why is the phenotype of the shPPM1F cells (Fig G) and the ppm1f-/- cells (Increased active b1 positive FAs) and the phenotype of PPM1F KO A172 cells (poor spreading and integrin/talin ring) so different if the mechanism is the same? Is the cell spreading phenotype of A172 PPM1FKO cells sensitive to the ECM ligand density such that on very low ECM ligand density these cells would spread better than the ctrl cells? Can the phenotype be reverted by titrating in low amounts of b1-integrin antagonists?

5) "Enhanced integrin activity in the absence of PPM1F correlated with impaired spreading (Fig. 9F)" The enhanced integrin activity in the mouse -/- cells needs to be demonstrated directly, not just through increased cell adhesion.

6) Is the integrin b1TTpp signal increased in the ppm1f-/- cells?

Minor points:
The last paragraph of the introduction seems to be repeating the abstract and could be shortened/modified
The authors should mention in the introduction that the role of the double TT motif in integrin activity regulation and filamin binding is not entirely new and has been demonstrated for b2-integrin earlier.
Filamin binding to β2 integrins is also inhibited by 14-3-3 proteins that bind to β2 integrin tails phosphorylated on Thr758 ((Takala, H. et al. β2 integrin phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. Blood 112, 1853-1862 (2008).)

Page 10, typo "In contrast to
wildtpe integrin β1,"

Reviewer #3 (Comments to the Authors (Required)):

This manuscript puts forward the idea that integrin phosphorylation at a TT motif displaces filamin binding hence favoring talin binding, integrin activation and adhesion. They identify a phosphatase that may be responsible for dephosphorylating the TT motif and argue that this phosphatase regulates adhesion. Overall, the manuscript is clearly written and logically presented and the relatively straightforward message is appealing. There are however some concerns about over interpretation of the results.

While the in vitro biochemistry showing phospho-switching of talin and filamin binding is generally convincing little evidence was presented with the integrin mutants to support the idea that this switching alters adhesion or recruitment at focal adhesions. The effect of removing filamin (or mutating it to prevent integrin binding) is not explored in functional assays.

I appreciate that the manuscript already includes a large body of data but it is very surprising that kindlin binding is not considered here. Modifications of the threonine residues are likely to impact kindlin binding and hence integrin function and cell adhesion. The exclusive focus on talin and filamin seems questionable.

The screening assay which led to identification of PPM1F is also poorly controlled. Subsequent validation of the hit proves it to be interesting, so at one level deficiencies in the screen may not be important, but the choice of 293T cells without any validation that the phospho-mimicking/blocking mutations examined in Fig 1 and 2 have any impact on adhesion is highly questionable. Is there any evidence that the increased adhesion seen in the screen is linked to alterations in integrin phosphorylation in 293T cells? Or that loss of filamin can enhance 293T cell adhesion?

Loss of PPMF1 does appear to increase cell adhesion and integrin activation (assessed by 9EG7 binding) and the similarity between shRNA and KO results is encouraging but extensive rescue experiments are not well documented. This is a significant weakness.

Even if rescue phenotypes are established it is difficult to determine that the effect is directly due to integrin phosphorylation as opposed to via indirect effects (presumably PPMF1 has other substrates). And if it is due to a loss of integrin phosphorylation is filamin part of the story?

Loss of PPM1F clearly impairs cell spreading, conceivably due to "intensified integrin-matrix interaction" but the causal link is not definitively established here so care is needed in the discussion of these results.

Specific points requiring clarification/correction:

In Fig 2 use of the OPTIC assay supports the talin-integrin binding data but the results with filamin-integrin are much weaker. Only the phospho-blocking mutant integrins showed filamin recruitment but this was barely above the 2.0 threshold, despite apparently occurring in most cells. The authors should comment on the very weak filamin recruitment in this assay.

In discussing the results of Fig 1 and 2 on page 6 the authors conclude that "These results demonstrated that the phosphorylation status of the integrin β1 T788/T789 motif dictates the association with integrin activity regulators in intact cells" - this conclusion seems premature as at this stage they have only assessed T/A and T/D mutants, not phosphorylation. Phospho-mimicking mutations are useful, but imperfect, models so maybe a more conservative interpretation is needed here.

In discussing the results from the initial shRNA screen the authors state that "Compared to control cells, depletion of the protein tyrosine phosphatases PTP-1B and PTP-PEST as well as depletion of the serine/threonine phosphatase PPM1F (...) resulted in enhanced cell adhesion to collagen and fibronectin, but not poly- L-lysine (Fig. 3A and B)." However, while it appears that PPM1F and PTP-PEST enhance binding to both collagen and fibronectin (and probably also to poly-L-lysine) PTP-1B seemed to only impact fibronectin binding. RPTP1 also seemed to increase binding to collagen and fibronectin but was not mentioned. This needs to be explained. Better statistical analysis of the results here might help - the legend seems to indicate that the results shown are for a single experiment with averaging over 3 well, are the screening results reproducible and are changes statistically significant?

The rescue experiments in Fig 5E are very important but the quantitation shown seems to be of a single blot. The experiment should be repeated several times and results of replicates plotted to provide an idea of the variability in the results.

The experiments in Fig 7 designed to show that PPM1F activity controls integrin tail interaction with talin and filamin A raise a series of questions. What is the basal phosphorylation levels of the chimeric integrin constructs in 293T cells? The large effects observed would seem to require a relatively high stoichiometry of phosphorylation - is there any evidence for this? It is also somewhat unclear why the over-expressed GFP-talin is displaced by PPM1F as talin apparently binds to both the phosphorylated and unphosphorylated tails - presumably the authors invoke competition with the endogenous filamin but are endogenous filamin levels sufficient to compete the over-expressed tagged talin? If filamin is knocked down/out does this prevent the PPM1F effect?

The embryonic lethal phenotype of PPM1F knockout mice confirms it importance but it remains to be determined whether this lethality is related to alterations in integrin activity. This should be made clear in the manuscript.

More information on PPM1F, its domain architecture and subcellular localization would help in this manuscript. Does it localize in adhesions in the cells used here?

The manuscript by Wennerberg (PMID: 9512507) seems relevant to this manuscript.

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20.7.2020

Re-Submission of JCB manuscript #202001057 for publication in *The Journal of Cell Biology*

Dear Dr. Humphries and Dr. O'Donnell,

Thank you very much for your kind letter and the opportunity to re-submit a revised version of our manuscript #202001057 entitled "*The Phosphatase PPM1F controls integrin activity via a conserved phospho-switch in the integrin β subunit*" to **Journal of Cell Biology**. We are also thankful for the extra time granted for preparing a revised manuscript. Similar to many other researchers, we were affected by lockdown measures and interrupted supply chains (e.g. the delivery of phospho-peptides from China was significantly delayed) and we did not have access to the laboratory in regular terms for over three months (March-June).

With regard to our initial submission, we would like to thank the reviewers for their very constructive and insightful criticism, which we appreciate and value. Prompted by their expert advice, we have i) expanded the scope of our investigation and conducted additional experiments with Kindlin-2; ii) conducted integrin β1 phospho-peptide pulldown assays with all investigated proteins; iii) performed additional rescue experiments for A172 cells and mouse embryonic fibroblasts; iv) investigated phenotypic effects of filaminA silenced A172 cells; v) added suggested citations, which were omitted beforehand due to space limitations; and vi) re-phrased several paragraphs according to the suggestions of the reviewers.

You will see, that we produced a substantial amount of novel data, which in total comprise more than three completely novel multi-panel figures. As already our initial submission was at the limit of the allowed space for main figures and supplementary figures of a regular article, we were not able to combine all data into a single article. Accordingly, we have splitted our results now into two separate, but of course strongly interconnected manuscripts:

ChemicalBiology
University of Konstanz

Manuscript A) a regular article comprising 10 Figures and 5 Suppl. Figures detailing the main biochemical findings of the role of PPM1F as the cellular phosphatase directed towards the conserved threonine motif in the integrin β subunit. A major aspect are the consequences of PPM1F action/integrin T788/T789 phosphorylation for talin, kindlin2, and filamin association and for integrin activity and cell adhesion/cell spreading/cell migration.

Manuscript B) a short report comprising 3 Figures and 2 Suppl. Figures detailing the consequences of the PPM1F knock-out in mice and the phenotype of the derived knock-out and reconstituted primary fibroblasts.

Though each study stands on its own, they are strongly complementary and together combine the data presented in our initial submission plus all the additional material requested by the reviewers. Therefore, we strongly believe that these two studies should be published side-by-side and we hope for your and the reviewers support in this regard.

You will find our detailed point-by-point response (marked in blue) to the questions of the reviewers (marked in red) from the next page on. Overall, we feel that we have fully addressed the issues raised in the initial review and that the additional experiments, data, and explanations not only substantially improve our manuscripts, but also further corroborate our original hypothesis. Also thanks to the reviewers insight and their request for experiments addressing kindlin in this context, we believe that our data now also provide a fascinating novel framework, which integrates integrin β1 phosphorylation to explain the cooperative action of talin and kindlin during integrin inside-out signaling. Therefore, we hope that our revised manuscripts might now be suitable for publication in **Journal of Cell Biology**.

Thank you again for all your efforts.

With best regards from Konstanz

Prof. Dr. Christof R. Hauck

Reviewer Comments:

Reviewer #1 (Required):

References cited in our response to Reviewer 1 can be found at the end of this section (page 13)

1. FilaminA regulation by phosphorylation of the integrin beta-chain is not a new finding (Takala et al, Blood, 2008). Also talin binding to the integrin beta-chain is already known not to be affected by integrin phosphorylation status (Takala et al, 2008), but can be outcompeted by filamin (KIema et al 2006) and also by 14-3-3 proteins (Takala et al, 2008). Please modify the text in the results and discussion sections to reflect this and refer to relevant publications.

The reviewer is completely right. It was shown before that filaminA is not able to interact with integrin β2 phospho-T758 peptides in biochemical assays, while talin binding was not affected by integrin phosphorylation (Takala et al., 2008). Takala et al. also supported their conclusion by solving the crystal structure of the IgFLNa21/ β2 complex (Takala et al., 2008). Additionally, Kiema et al. showed for integrin β7 (T783-T785) that filaminA could outcompete talin at the wildtype integrin tail, but that filaminA was unable to bind pseudo-phosphorylated integrin (Kiema et al., 2006). By deduction, Kiema et al. concluded that filamin would not compete with talin in the case of a phosphorylated integrin β subunit, but that was not assessed experimentally. Therefore, we now directly tested this idea by competition experiments with filaminA and talin using both unphosphorylated as well as phosphorylated integrin β1, thereby not only providing the experimental evidence, but also extending these findings from the hematopoietic integrin β subunits β2 and β7 to the ubiquitously expressed β1 subunit.
Importantly, we cite these prior publications directly in the introduction section and re-phrased the paragraph on page 4, line 79ff. of **Manuscript A** to read:

"Besides talin and kindlin as positive regulators of integrin function, several negative regulators of integrin activity such as filaminA, Dok1, Sharpin, or ICAP-1 have been described (Bouvard et al., 2003; Kiema et al., 2006; Liu et al., 2015; Oxley et al., 2008; Rantala et al., 2011). These non-enzymatic proteins are thought to act by competitive binding to the integrin β subunit, where they displace positive regulators of integrin activity. For example, filaminA and talin have overlapping binding sites in the leukocyte-specific integrin subunits β2 and β7, which they occupy in a mutually exclusive manner (Kiema et al., 2006; Takala et al., 2008)...[…] These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown."

We also modified several passages in the results and discussion parts accordingly to now read on page 6, line 117ff. of **Manuscript A**:

"*Previous studies using the leukocyte-specific integrins β2 and β7 already suggested that these threonine residues could operate as a phospho-switch to control binding of talin versus filaminA (Kiema et al., 2006, Takala et al., 2008).*"

Page 7, line 148: "*These structural models are in line with data reported previously for β2 and β7 (Kiema et al., 2006, Takala et al., 2008) and strongly support our conclusion that phosphorylation of the integrin β1 T788/T789 motif disrupts filaminA binding, but does not impact talin association.*"

Page 17, line 487: "*The proposed phospho-switch mechanism in the integrin β1 subunit and the role of filaminA in this context is in line with previous reports on the integrin β7 and integrin β2 subunits (Kiema et al., 2006; Takala et al., 2008).*"

We also want to stress that the studies mentioned by the reviewer do not contain experiments in intact cells. Therefore, the relevance of these biochemical observations had not been tested in a cellular context. By identifying the phosphatase responsible for the dephosphorylation of the conserved threonine motif in integrin β1, we have now been able to manipulate the integrin T788/T789 phosphorylation levels in intact cells, which allows us to report the recruitment of talin and filamin to the integrin β1 tail in the cellular environment. Therefore, we feel that these data are not mere repetitions, but rather add important novel insight.

2. In addition, although the role of talin in integrin activation and cell adhesion is clear, the role of filamin in integrin regulation in vivo is more conflicting. Please show adhesion assay and beta1-integrin activation assays in FlnA knock-down cells to show whether filamin plays a role in beta1-integrin regulation in cells (Fig S2).

The reviewer is touching on the particular role of filamin in integrin activity regulation, as filaminA is not only able to associate with integrins, but has various interaction partners inside mammalian cells. Due to these multiple interactions with membrane proteins and cytoskeletal components, filaminA could influence complex cellular behavious such as spreading and migration in multiple ways (Zhou et al., 2010; Kim et al., 2011; Razinia et al., 2012). Nevertheless, the role of filaminA for regulating integrin activity is well documented by a number of prior studies, which demonstrate that filaminA

stabilizes the low affinity state of the heterodimeric integrin receptor by tightly binding to different integrin β cytoplasmic tails (β3, β2, β7) (Kiema et al., 2006; Chatterjee et al., 2018; Takala et al., 2008). FilaminA appears to be particularly suited to keep integrin heterodimers in a closed conformation as it contacts both the β and the α subunits (Liu et al., 2015).
The integrin β subunits share a conserved filaminA binding site, which in integrin β1 centers around the T788/T789 motif. In cells, filaminA knock-down results in stronger integrin activation and enhanced cell adhesion in Jurkat T cells, NIH3T3, and 293T cells and leads to reduced cell spreading and a reduced number of cell extensions in a β2-, β3- and in a β1-dependent manner (Kim et al., 2010; Kim et al., 2008; Kim et al., 2010; Takala et al., 2008; Kiema et al.,2006; Baldassarre et al., 2009; Hu et al., 2017). Accordingly, integrin non-binding versions of filaminA were reported to promote β1, β2 or β3 integrin activation and cell adhesion in different cell types, while overexpression of filaminA has been shown to impair integrin activity and cell migration (Ithychanda, 2009; Das et al., 2011; Waldt et al., 2018; Liu et al., 2015; Calderwood et al., 2001). Finally, also talin-enrichment at active integrins was reported in filaminA knock-down cells, which is totally in line with our data for PPM1F depleted cells (Kumar et al., 2019).

However, despite the ample experimental evidence for a role of filaminA in suppressing integrin activity, we have followed the suggestion of the reviewer. Thus, we have conducted cell adhesion and integrin β1 activity experiments with A172 cells treated with shRNA directed against human filaminA or a control shRNA to evaluate the consequences of filaminA depletion in this cell type. Furthermore, we have taken advantage of this newly made shRNA-encoding lentivirus to also knock-down filaminA expression in PPM1F-deficient A172 cells. The idea behind this epistasis experiment was to see, if indeed PPM1F and filaminA work together in the same regulatory pathway to control integrin activity.
Importantly, we could confirm a negative regulatory role of filaminA for β1-integrin in A172 cells. In particular, filaminA knock-down strongly resembled the PPM1F knock-out phenotype in terms of reduced cell spreading, elevated integrin-based cell adhesion and increased integrin activity (novel Figure 4 H-J and Figure S4 A-I). Furthermore, the epistasis experiment showed that filaminA knock-down did not further enhance the adhesion and integrin-activation phenotype of PPM1F KO cells, substantiating our hypothesis that PPM1F works via filaminA to regulate integrin β1 activity. Also these results are now contained in novel Figure 4 H-J and Figure S4 A-I of **Manuscript A**.

These novel data are now described on page 10, line 248ff of **Manuscript A**:

"*To further confirm that the increased cell adhesion of PPM1F-deficient cells is connected to filamin-dependent activity regulation of integrins we performed epistasis experiments. Therefore, A172 control cells and PPM1F KO cells received either a control shRNA or shRNA targeting human filaminA (Fig. 4H). Similar to the knock-out of PPM1F and consistent with the known inhibitory role of filaminA (Liu et al., 2015; Takala et al., 2008; Waldt et al., 2018), shRNA-mediated knock-down of filaminA in A172 control cells increased cell adhesion, reduced cell spreading, and elevated integrin activity (Fig. 4, I and J and Fig. S4, A-D). However, depletion of filaminA in PPM1F KO cells did not further elevate the increased integrin-dependent adhesion or the enhanced integrin activity in these cells, nor did it further reduce cell spreading (Fig. 4, I and J and Fig. S4, A-D). The results of these epistasis experiments highlight the strong similarities in the phenotype of PPM1F KO cells and filaminA knock down cells and suggest that PPM1F and filaminA work together in the same pathway controlling integrin activity (Fig. S4 E).*"

3. Critically, the TT-site in the beta-integrins is also the kindlin and 14-3-3 protein binding site (and also binds several other proteins). Especially kindlin has been shown to be critical for regulating integrin-mediated cell adhesion. How is kindlin binding to the beta1-integrin affected by the AA and DD-mutation and by direct phosphorylation? Does kindlin and talin compete or cooperate for binding and how is this regulated by phosphorylation? What about 14-3-3 proteins? Please carefully examine these issues experimentally in vitro and in cells to clarify these issues.

We were well aware of the potential role of TT-phosphorylation for regulating access of kindlin to the integrin β1 tail and of the high interest of the integrin field in this particular question. Therefore, we are thankful for the reviewer to bring this point up, even though these interconnected questions demanded a large amount of additional experimentation. We have concentrated on kindlin2 and investigated its binding behaviour *in vitro* to pseudophosphorylated as well as directly phosphorylated integrin β1 tails and also analysed the competition between filaminA and kindlin. Finally, we also investigated the binding behaviour, when the four critical components, talin, kindlin, filamin and the phosphorylated or unphosphorylated integrin β1 tail, are brought together. We believe that our unexpected results are highly interesting and might help to resolve some of the open questions with regard to the talin-kindlin cooperation in integrin inside-out signaling.
These results are now presented in novel Figures 7 and 8 of **Manuscript A**, and summarized in the novel scheme in Fig. 10. They are described in the results section starting from page 12, line 332, to page 14 of **Manuscript A**.

In short, our data demonstrate that kindlin2 on its own does not bind to the integrin β1 tail, when the TT motiv is in the phosphorylated form, as integrin tails harbouring the phosphorylation mimicking TT/DD mutations as well as a phosphorylated synthetic integrin β1 tail peptide do not support kindlin2 binding (Fig. 7A-C). Only in the unphosphorylated form, the integrin β1 tail associates with kindlin2, but under these circumstances, kindlin2 is outcompeted by filaminA (Fig. 7D). However, if the TT motiv in the integrin β1 tail is phosphorylated, the presence of the talin head allows kindlin to associate with its binding site at the distal NPxY motif and in this constellation, filaminA can not displace kindlin from the integrin tail (Fig. 7F and G).

This binding behaviour of kindlin in the presence of talin is also seen in intact cells, where only the wildtype and the pseudophosphorylated integrin tails strongly recruit kindlin2 (Fig. 8A). Moreover, in PPM1F ko cells, where integrin β1 is constitutively phosphorylated at the TT motiv, kindlin shows strong peripheral accumulation together with active integrin, closely mimicking the distribution of talin in these cells (Fig. 8B). The functional cooperation between talin and kindlin does not depend on physical interaction between these two proteins, but talin binding to the membrane proximal NPxY motif seems to reorient the integrin β1 tail in a way, which allows access of kindlin to its membrane distal NPxY binding site despite phosphorylation of the TT motiv (Fig. 7F). Such a re-orientation of the integrin tail by the talinF3 domain, which allows kindlin2 to bind adjacent to talin without noticeably contacting the talinF3 domain itself is completely in line with the results of Bledzka et al (Bledzka et al., 2012). It is important to emphasize that the phosphorylation status of the TT motiv together with the binding behaviour of kindlin2 enforce co-operation between talin and kindlin and explain the different consequences of talin vs. kindlin overexpression on integrin activity (Montanez et al., 2008; Ye et al., 2013). Furthermore, phosphorylation of the TT motif in the integrin tail also can explain why mutations blocking talin binding inhibit both talin- and kindlin-driven integrin activation, while mutations that inhibit kindlin binding still permit talin-mediated activation, although they block the kindlin enhancement effect (Ye et al., 2010).

These data on kindlin-integrin association complement previous studies on talin/kindlin-mediated integrin activation (Montanez et al., 2008; Moser et al., 2009; Moser et al., 2008; Cluzel et al., 2005; He et al., 2011; Ye et al., 2013; Li et al., 2017) and help to explain how kindlin, which is clearly required for full integrin function (Theodosiou et al., 2016), fails to acitvate integrins in intact cells by itself.

The wealth of novel data (including the additional data on FilaminA knock-down; see point 2 above) were compiled into three addditional multi-panel figures and

this increase in data made it necessary to split the manuscript into two separate parts:
Manuscript A) an article focussing on the identification of PPM1F and on biochemical elucidation of interactions with the phosphorylated/unphosphorylated integrin β1 tail.
Manucript B) a report detailing the phenotype of PPM1F deletion in the mouse and the the integrin-dependent phenotype of the primary PPM1F-knock-out cells.
We hope that the reviewer agrees that both aspects, the investigation of the role of TT-phosphorylation for kindlin binding as well as the elucidation of the *in vivo* function of PPM1F in mice, are important aspects, which complement each other and which should be published side-by-side. Therefore, we hope you support our suggestion to split this huge amount of novel data into two separate manuscripts.

4. Are TT/DD-beta1-integrin expressing cells more adherent than WT integrin expressing cells? Is the beta1-integrin more active? Please clarify.

This important question has been addressed by different groups in several prior studies. Due to the limited space, we had not cited these prior studies adequately in our initial submission and we have to apologize for that omission. We have now included a complete paragraph in the introduction of **Manuscript A** starting on page 5, line 86:

"*Interestingly, an evolutionary conserved threonine motif within the context of the filaminA and talin core binding sites is located in the cytoplasmic tails of most integrin β subunits (T788/T789 in the human integrin β1, Fig. 1A, Fig. S1A) (Garcia-Alvarez et al., 2003; Gingras et al., 2009; Kiema et al., 2006; Liu et al., 2015; Wegener et al., 2007). Upon cell stimulation, these threonine residues are phosphorylated (Buyon et al., 1990; Chatila et al., 1989; Craig et al., 2009; Hibbs et al., 1991; Hilden et al., 2003) and mutations mimicking Ser/Thr phosphorylation lead to enhanced integrin activity and integrin-based cell adhesion in vitro (Craig et al., 2009; Nilsson et al., 2006; Wennerberg et al., 1998). In contrast, alanine substitution of this particular threonine motif severely compromises integrin function leading to impaired integrin activation and abrogation of cell-matrix adhesion (Fagerholm et al., 2005; Hibbs et al., 1991; Nilsson et al., 2006; Wennerberg et al., 1998). These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown.*"

In addition to these prior studies our results presented in the two manuscripts clearly demonstrate that the phosphorylation status of the integrin β1 TT motiv correlates with integrin activity and integrin-mediated cell adhesion. This is demonstrated in several independent cell models in **Manuscript A** and **Manuscript B**, where deletion or depletion of PPM1F leads to enhanced TT phosphorylation. Phosphorylation of the TT motiv is accompanied by increased cell adhesion and elevated integrin activity levels, which both are reverted upon re-expression of wildtype PPM1F, but not PPM1F D360A.
Together, the published data on the role of T788 (pseudo-)phosphorylation in integrin β1 and our results in independent cell models with constitutive T788/T789 phosphorylation strongly support the idea that phosphorylation of this motif leads to increased integrin activity and enhanced matrix adhesion.

5. How does direct phosphorylation of the beta1-integrin tail impact filamin, talin, kindlin and 14-3-3 binding (eg rather than charge-mimicking DD mutations)? This can be studied with phosphorylated peptides. Please confirm the data (filamin/talin binding) with phosphorylated and nonphosphorylated beta1-integrin peptides.

Following the valuable suggestion by the reviewer, we have performed additional pull-down experiments with biotinylated phospho-T788/T789 and unphosphorylated synthetic integrin β1 peptides (AA762-798) using recombinant purified talin-head, full-length kindlin-2, and the filaminA integrin binding region (Ig19-21). These novel results are included in additional panels in Figure 1D and in Figure 7C and G of **Manuscript A**.
In agreement with our prior data using the recombinant wildtype and pseudo-phosphorylated (TT/DD) versions of the integrin β1 cytoplasmic domain, we again find that talin binds independently of TT phosphorylation, while both kindlin2 and filaminA only interact with the wildtype integrin form, while they do not bind on their own to phosphorylated T788/T789 (Fig. 1D, Fig. 7C).
Most importantly, we could observe a co-operative binding of talin and kindlin in the case of phosphorylated integrin β1, which excludes filamin binding (Fig. 7G). These novel data not only validate the binding behavior of talin, kindlin and filamin as observed before with charge-mimicking (TT/DD) proteins, but also provide a detailed explanation as to how the PPM1F-controlled phospho-switch at T788/T789 can orchestrate the binding of positive (talin/kindlin) and negative (filaminA) regulators of integrin activity.
6. Why is there no filamin recruitment to the wt integrin construct in cells (Fig 2)? The integrin is certainly not phosphorylated to 100% stoichiometry in cells. Is filamin binding to integrin beta-tails an in vitro artefact?

These are interesting questions raised by the reviewer, which we also considered. First, we do not think that filamin binding to integrin beta-tails is an *in vitro* artefact. Indeed, there are not only numerous biochemical studies by different groups on filaminA-integrin β tail interaction *in vitro* (including also quantitative NMR or SPR data and crystal structures confirming the ability of filaminA to bind to different integrin β tails), but also *in vivo* pull-down data, which indicate a close association of the full-length proteins in intact cells. Importantly, these biochemical data are supported by functional data, which indicate a suppressive role for filaminA due to the stabilization of the low affinity integrin conformation (Loo et al., 1998; Sharma et al., 1995; Calderwood et al., 2001; Takala et al., 2008; Kiema et al., 2006; Truong et al., 2015; Chatterjee et al., 2018). Indeed, site-directed mutagenesis of the integrin binding site in Ig21 of filaminA, expression of integrin non-binding versions of filaminA in cells, or displacing filaminA from integrins by migfillin or by filaminA S2152 phosphorylation directly leads to increased cell adhesion and elevated integrin activity (Liu et al., 2015; Waldt et al., 2018; Das et al., 2011; Ithychanda et al., 2009; Lad et al., 2008). Therefore, it is very unlikely that the filamin-integrin β interaction is an *in vitro* artefact.

With regard to the stoichiometry of integrin phosphorylation, which might be responsible for the lack of recruitment of filaminA to the wildtype β1cytoplasmic tail in our experiments, we can only offer our current hypothesis, but have no quantitative data. Based on the impressive quantitative work by by Springer and colleagues (Li et al., 2017), only a minor fraction of integrin heterodimers on a given cell might be in the extended-open “active” conformation and would contain p-T788/p-T789. Conversely, one would expect most integrin heterodimers to be in a non-phosphorylated state and, therefore, accessible to filaminA binding. However, one has to consider that our experiments with integrin β1 cytoplasmic tails (OPTIC assays) are conducted with CEACAM-integrin fusion proteins, where the β subunit is expressed in the absence of a corresponding α subunit (Baade et al., 2019). By binding to the multi-valent bacteria, these chimeric integrins proteins cluster around the attachment site and thus mimic early focal adhesion assembly. Therefore, this situation rather reflects a constitutive active, unclasped conformation, where the β subunit is not in contact with the α subunit and freely available. We believe that in this situation, the isolated β subunit in the wildtype form is mainly phosphorylated and therefore, filaminA is only recruited to the TT/AA mutant, but not the wildtype integrin β1 tail (Fig. 2B). Evidence for this idea is provided by the results presented in Figure 9B, where in the same experimental setting the overexpression of wildtype PPM1F, but not the overexpression of phosphatase inactive PPM1F D360A, suddenly leads to recruitment of filaminA to the wildtype integrin β1 tail in intact cells (Fig. 9B). This observation is in line with the idea that this fusion protein of the integrin β1 cytoplasmic tail is mainly

phosphorylated, but upon overexpression of PPM1F becomes de-phosphorylated by the protein phosphatase allowing filaminA binding. We now include a synopsis of these thoughts in the discussion section of our **Manuscript A** on page 18, line 518ff:

"*When we express the isolated wildtype integrin β1 tail in intact cells, a situation mimicking the unclasped integrin, we do not observe filaminA recruitment. However, upon overexpression of PPM1F, but not PPM1F D360A, filaminA accumulates at the wildtype integrin β1 cytoplasmic tail. This finding could indicate that the threonine motif is mainly phosphorylated, when the integrin heterodimer is in the unclasped conformation and the β subunit is separated from the α subunit. Phosphorylation of the conserved threonine motif under these circumstances would not only displace the negative regulator filamin, but it would also prohibit kindlin2 from driving integrin inside-out signaling in the absence of talin. This scenario is in line with the observation that kindlin overexpression does not lead to integrin inside out activation (Ma et al., 2008; Harburger et al., 2009; Ye et al., 2010; Li et al., 2017).*"

7. Phosphatases such as PP2A has been previously implicated in dephosphorylation of the threonines of the beta1-integrin (Kim et al, JBC 2004). Please include as a control here.

The reviewer makes a valid point in that Kim et al. previously investigated PP2A in E63 skeletal myoblasts in the context of integrin β1 phosphorylation and Ca^{2+} signaling (Kim et al., 2004). The authors co-immunoprecipitate PP2A with integrin β1 and detect *in vitro* activity of PP2A towards phospho-T788/T789 integrin peptides. In myoblasts, they show that after 4 hours of treatment with okadaic acid (OA), the integrin β1 phospho-T788/T789 levels are increased. Clearly, OA is also a known inhibitor of PP1 and the reported phenotype could be indirectly caused, since both PP1 and PP2A are ubiquitously expressed versatile holo-enzymes involved in several pathways with numerous substrates (Janssens et al., 2001; Virshup, 2000; Wlodarchak et al., 2016; Clark and Ohlmeyer, 2019). Genetic experiments directed towards PP2A (knock-down/knock-out/overexpression) were not attempted by the authors. Therefore, conclusions about the relevance of PP2A-mediated dephosphorylation of integrin β1 pT788/pT789 in myoblasts, but even more so about the role of this enzyme for integrin dephosphorylation in other cell types might be preliminary. In contrast, we demonstrate that depletion of PPM1F in several distinct cell types (A172 glioblastoma cells, human embryonic kidney 293T cells, normal human dermal fibroblasts, murine embryonic fibroblasts) results in the same phenotype of elevated integrin β1 T788/T789 phosphorylation and/or integrin function, which can be reverted by re-expression of the wildtype enzyme.

In this context it is also interesting to consider that PPM1F was not found to be expressed in striated muscle and heart (**Manuscript B**; Fig. 1) indicating that other phosphatases could take over the role of PPM1F in these tissues and PP2A might be one of the possible candidates.

To remind the readers of the prior findings of Kim et al. with regard to PP2A, we have now included the following statement in the discussion section in **Manuscript A** on page 17, line 468ff.:

"*It is important to note that PPM1F, but not the related PPM family member ILKAP, readily dephosphorylated the phospho-T788/T789 motif. In myoblasts, the phosphatase PP2A has been shown to associate with and dephosphorylate the T788/T789 motif on integrin β1 (Kim et al., 2004 #8506}. Interestingly, striated muscle is one of the few tissues that shows negligible expression of PPM1F (see accompanying paper by Dierdorf et al./Manuscript B) and muscle cells require stable, long-term adhesion for their physiological function. Therefore, PPM1F-mediated functions might not be required to the same extent in striated muscle, where PP2A could be involved in integrin regulation*."

8. What is the general effect of PPM1F deletion on Ser/Thr phosphorylation in cells/of proteins in focal adhesions? Is the increased threonine phosphorylation of the beta1-integrin unique? Please examine experimentally.

This is an important point raised by the reviewer, which we want to follow up in the future by performing phospho-proteomic investigations using our A172 wildtype and PPM1F knock-out cells seeded onto an integrin-dependent matrix or keeping cells in suspension. However, we feel that this will be the start of an additional, completely new study, which will produce large amounts of novel data and novel hypotheses, and therefore deserves a separate investigation.

With regard to additional PPM1F substrates, we believe that it is highly likely that PPM1F also regulates other focal adhesion factors to fine-tune integrin-mediated events. Indeed, it has been reported before, that PPM1F de-phosphorylates PAK (Koh et al., 2002; Susila et al., 2010) and that PPM1F controls CaMKII activity (Harvey et al., 2004; Ishida et al., 1998) and we have cited the respective studies already in our initial submission. Interestingly, CaMKII is one of the potential kinases targeting the conserved threonine motif in integrin β subunits (Takahashi, 2001; Suzuki and Takahashi, 2003; Rehberg et al., 2014) and we find strong in vitro phosphorylation of the integrin β1 tail by CaMKII (**Manuscript A**; Fig. 6E; Fig. S5 F)

Therefore, we have included a paragraph in the discussion section of **Manuscript A**, page 19, line 555:

"*It is tempting to speculate that PPM1F is ideally suited to serve as key control for the T788/T789 phospho-switch, as it not only dephosphorylates the integrin β cytoplasmic domain, but this phosphatase is also able to reverse the auto-phosphorylation of CaMKII at Thr286 (Harvey et al., 2004; Ishida et al., 1998). Thus, PPM1F could shift the balance towards the unphosphorylated, inactive integrin by acting on both an integrin-directed serine/threonine kinase as well as on the integrin T788/T789 motif itself.*"

A further potential substrate of PPM1F might be filaminA itself. Indeed, phosphorylation of filaminA at S2152, a site located between filaminA Ig20 and Ig21 close to the integrin binding site, was reported to prohibit filaminA - integrin β2 binding and, thus, promote integrin activity in T cells (Waldt et al., 2018).

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Reviewer #2 (Comments to the Authors (Required)):

References cited in our response to Reviewer 2 can be found at the end of this section (page 29)

1) Figure 1B. The authors may want to consider validation their "pseudophosphorylation mutant integrin tail data" (Figure 1B,C) with biotinylated recombinant peptides that are phosphorylated at these sites. These are commercially available (and have been used by the authors in figure 6c) and would directly show that role of phosphorylation (rather than negative charge) in regulating the binding.

We are thankful for this advice by the reviewer (see also Major Comment 5 by reviewer 1).
Following the suggestion by the reviewers, we have conducted pull-down experiments with biotinylated phospho-T788/T789 and unphosphorylated synthetic integrin β1 peptides (AA762-798) using recombinant purified talin-head, full-length kindlin2, and the filaminA integrin binding region (Ig19-21). These novel results are included in additional panels in Figure 1D and in Figure 7C and G of **Manuscript A**.

In agreement with our prior data using the recombinant wildtype and pseudo-phosphorylated (TT/DD) versions of the integrin β1 cytoplasmic domain, we again find that talin binds independently of TT phosphorylation, while both kindlin2 and filaminA only interact with the wildtype integrin form, while they do not bind on their own to phosphorylated T788/T789 (Fig. 1D, Fig. 7C).
Most importantly, we could observe a co-operative binding of talin and kindlin in the case of phosphorylated integrin β1, which excludes filamin binding (Fig. 7G). These novel data not only validate the binding behavior of talin, kindlin and filamin as observed before with charge-mimicking (TT/DD) proteins, but also provide a detailed explanation as to how the PPM1F-controlled phospho-switch at T788/T789 can orchestrate the binding of positive (talin/kindlin) and negative (filaminA) regulators of integrin activity.

2) (Figure 3 and 4) The authors should consider strengthening their data on integrin activation upon PPM1F silencing/knock-out by employing additional b1-integrin activation epitope antibodies (such as 12G10) and using golden-standard integrin activation assay where binding of labelled FN fragments to integrins are investigated with flow cytometry. The extent of activation should be compared to the maximum activation achieved with Mn2+. Possible off-target effects need to be controlled for by using at least a second independent shRNA or with rescue experiments.

For integrin activity assays it is common practice to use either a fluorescence-labeled ligand (such as fibronectin) or to use a conformation-specific monoclonal antibody (such as 9EG7 for active integrin β1) to detect the active receptor. In parallel, samples are with an antibody detecting total integrin levels (such as clone AIIB2 for total integrin) to allow normalization of the signal with regard to total integrin expression levels (Chen et al., 2006; Li et al., 2017; Nilsson et al., 2006; Gao et al., 2019; Tadokoro et al., 2003; Moser et al., 2008; Kiema et al., 2006). When making use of knock-out and re-constituted cell lines, it is also instrumental to first assure similar total integrin expression levels on the used cells, which we have done in all instances (see **Manuscript A**; Figure S2 and S3; **Manuscript B**; Fig. S2). Our integrin activation assay is based on the above mentioned “gold-standard” in combining a non-labeled FN fragment as a ligand for integrin α5β1 and detecting the active integrin β1 with 9EG7 antibody. We always normalize data to the total integrin β1 surface levels detected by monoclonal antibody AIIB2 in the case of human cells or monoclonal antibody Hmβ1-1 in the case of murine cells. Moreover, we always refer in individual assays to the mean fluorescence intensity obtained for the wildtype cells. Therefore, we believe that we perform these integrin activity measurements according to current standards.

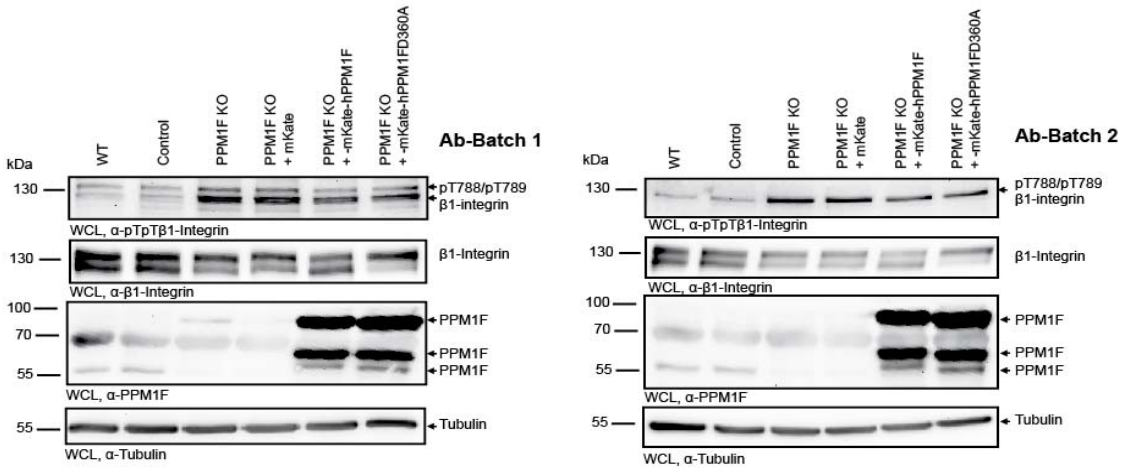
Importantly, we observe consistent results in multiple cell models and can also demonstrate that the integrin activity phenotype of knock-out cells can be resuced upon re-expression of the wildtype enzyme. These rescue experiments with regard to integrin activity have been presented for A172 PPM1F KO cells in the initial mansucript and are now presented in **Manuscript A**, Fig. 5E.

We completely agree with the reviewer, that genetic knock-out combined with complementation (rescue experiments) is the most convincing way to validate the role of a given protein. Therefore, we have followed the suggestions by the reviewer and have conducted a second set of integrin activity measurements, in this case with PPM1F-/- fibroblasts isolated from mouse embryos and the derived reconstituted cells re-expressing either the wildtype phosphatase or the inactive PPM1F D360A. The results of these experiments are now presented in **Manuscript B**, Fig. 3E, where we again use the monoclonal antibody 9EG7 to detect active integrin β1 and employ the monoclonal antibody Hmβ1-1 to detect the total murine integrin β1 levels.

The results strongly support our hypothesis that integrin β1 activity is negatively regulated by PPM1F. Moreover, the reversion of the PPM1F knock-out phenotype by re-expression of the wildtype enzyme verifies that alterations seen in the PPM1F knock-out cells are a primary and direct effect of the lack of this phosphatase.

3) Figure 5. The data with increased integrin phosphorylation upon PPM1F are rather convincing. However, it seems that the TTpp positive band corresponds to the lower MW migration beta1 band. This is considered in the field to correspond to the immature/ER resident form of beta1. Is PPM1F regulating phosphorylation of the immature b1-integrin ? How would this be linked to integrin activity on the cell surface/in adhesion regulation?

The reviewer is perfectly right in pointing out the different sizes of the integrin reactive bands, both for the general anti-integrin β1 antibody used to detect human integrin β1 (clone D2E5, monoclonal rabbit anti-human integrin β1, Cell Signaling; 1:1000 WB) or the general anti-integrin β1 antibody used to detect murine integrin β1 (M-106, polyclonal rabbit anti-human integrin β1, cross-reactive with murine integrin β1, Santa Cruz Biotechnology; 1:500 WB) as well as for the phospho-specific pT788/pT789 antibody (44-872G, polyclonal rabbit anti-human, Thermo Scientific; 1:1000 WB) raised against human integrin β1 phospho-peptide. In all these cases, we observe a band with lower (~135 kDa) and a band with higher (~120 kDa) mobility upon SDS-PAGE, which appear in whole cell lysates of different human and murine cells. The same pattern has been reported from various other human cell types and has been shown to reflect two differentially glycosylated forms of integrin β1, which are both exposed on the surface of the cell (Meng et al., 2005). Therefore, we do not assume that the lower band corresponds to an immature, ER-localized non-glycosylated form, as a deglycosylated integrin β1 has an even higher mobility in SDS-PAGE and runs around 90 kDa (Meng et al., 2005).
Interestingly, the reviewer has correctly observed that the main reactivity of the phospho-specific pT788/pT789 antibody is directed towards the higher mobility integrin band at ~120 kDa. However, there seems to be some batch variation in the commercial preparations of this polyclonal antibody, as the first batch of phospho-specific pT788/pT789, which we purchased in 2015, always yielded two bands in Western Blots corresponding to the two integrin β1 bands (Review Figure 1; left side), while a second batch, which we received in 2018, had a preference for the faster mobility, ~120 kDa band (Review Figure 1; right side). We do currently not have an explanation for this, but we have controlled the specificity of both phospho-antibody preparations with our recombinant integrin β1 cytoplasmic domains, emlyoing them in the wildtype unphosphorylated and in the phosphorylated form as well as emplyoing the non-phosphorylatable TT/AA versions. Importantly, the phospho-specific pT788/pT789 antibody in each case only recognized the phosphorylated integrin β1 peptide, but not the unphosphorylated peptide or the TT/AA version (see **Manuscript A**, Supplemental Fig. S5, panel F for details).



Review Figure 1: Comparison of the Western Blot band pattern observed with the 1st and the 2nd batch of polyclonal phospho-specific pT788/pT789 integrin β1 antibody (44-872G, polyclonal rabbit anti-human, Thermo Scientific; 1:1000 WB). The identical samples are run on parallel gels and probed with the 1st antibody batch (upper panel, left side) or the second antibody batch (upper panel, right side). The other antibodies used to detect total integrin β1 (second panels), PPM1F (thirs panels), and tubulin (lowest panels) were identical for both blots.

Therefore, we do not think the protein detected corresponds to the immature, non-glycosylated integrin, but rather that both surface exposed gylcoforms of integrin β1 can get phosphorylated, but depending on the antibody preparation the phospho-specific antibody preferentially reacts with one isoform.

4) Why is the phenotype of the shPPM1F cells (Fig G) and the ppm1f-/- cells (Increased active b1 positive FAs) and the phenotype of PPM1F KO A172 cells (poor spreading and integrin/talin ring) so different if the mechanism is the same? Is the cell spreading phenotype of A172 PPM1FKO cells sensitive to the ECM ligand density such that on very low ECM ligand density these cells would spread better than the ctrl cells? Can the phenotype be reverted by titrating in low amounts of b1-integrin antagonists?

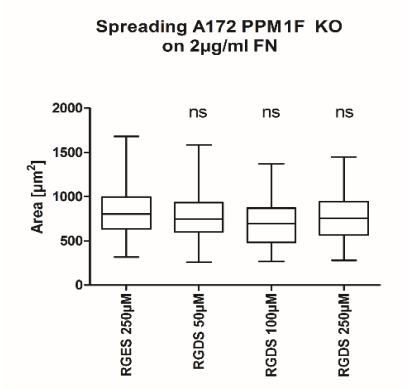
The reviewer brings to our attention a point, where we seem to have failed to correctly explain and stress the slightly different experimental set-ups. Whenever we analysed the spreading of the cells, we focussed on the initial 30-40 minutes of cell attachment to an extracellular matrix ligand (initial spreading). The cells used in these assays have been serum-starved, kept in suspension,

and then replated in the absence of serum on the respective ligand coated at the indicated concentrations (low/high). Under these conditions, the A172 knock-out cells, but also the murine embryonic fibroblast PPM1F-/- cells and the respective cells re-constituted with the inactive phosphatase show a retarded spreading and their phenotypes are comparable (see **Manuscript A**, Fig. 4; **Manuscript B**; Fig. 3G).

When the cells are allowed to attach for longer on the extracellular matrix substrate (2 h), then they still show enhanced accumulation of active integrin in the periphery, but whereas the A172 PPM1F KO cells are still smaller and less well spread (see **Manuscript A**, Fig. 4D), the MEF PPM1F-/- cells are able to catch up with the wildtype MEF and show then also a comparable spreading (**Manuscript B**, Fig. 3C). Similarly, the PPM1F shRNA knock-down Normal Human Dermal Fibroblasts (NHDF) depicted in Figure 3F have been allowed to spread for prolonged time and then, similar to murine MEF PPM1F-/- cells, show comparable spreading as the wildtype cells over extended time points. Therefore, there are differences with regard to the spreading kinetics seen in all PPM1F-deficient cell types (and we refer to this throughout the manuscript now as “initial spreading”), while there are some cells (murine and human fibroblasts, which upon prolonged attachment are able to extend as far as the respective wildtype cells.

The differences in the ability of PPM1F-deficient A172 cells and PPM1F-deficient fibroblasts to fully spread even after prolonged periods of time might be due to differences in the expression of integrin subunits in these distinct cell types. E.g. A172 cells express low levels of β3 integrin, while β1 seems to be the predominant integrin subunit (**Manuscript A**, Figure S3). In contrast, murine fibroblasts show high expression of both α5β1 and αvβ3 integrin (**Manuscript B**; Figure S1).

The idea of the reviewer, to revert the retarded spreading of the A172 PPM1F KO cells by titrating in an integrin β1 antagonist is really fascinating and we have immediately performed the respective experiment.
However, over a wide range of concentrations (50 – 250 µM) of the RGDS peptide, we did not see any effect on the spreading phenotype of the PPM1F-deficient cells (Review Fig. 3). Higher concentrations of the RGDS peptide led to rounding and detachment of the cells (not shown).



Review Figure 3: Spreading of A172 PPM1F KO cells on 2 µg/ml fibronectin in the presence of the integrin blocking peptide RGDS (50 – 250 µM) or the RGES control peptide. n=2, ≥ 53 cells per sample, one-way ANOVA with Bonferroni post-hoc test; ns = not significant)

To conclude, the enhanced integrin β1 T788/T789 phosphorylation and the resulting increase in integrin activity, which would be the cellular phenotypes most directly connected to the activity of the protein phosphatase PPM1F, are strikingly similar in all the different cell types examined. Furthermore, the short-term consequences of this integrin-directed action of PPM1F (matrix adhesion and initial spreading) are also highly consistent between the different PPM1F-depleted or –deficient cell types.
However, the more downstream consequences, such as cell spreading on extracellular matrices over prolonged times (which requires complex interaction between formation and release of cell adhesions, regulation of the cytoskeleton and secretion/deposition of matrix proteins and therefore depends on the interplay of multiple additional factors), show cell-type specific differences. We do not expect that these more downstream consequences can be solely

explained by the effect of PPM1F on integrin β1, as they might be influenced by the presence of particular adhesion receptors or cytoskeleton-associated proteins in the different cell types and by the action of PPM1F on additional phospho-proteins (see our answer to point 5 of reviewer 3 below).

5) "Enhanced integrin activity in the absence of PPM1F correlated with impaired spreading (Fig. 9F)" The enhanced integrin activity in the mouse -/- cells needs to be demonstrated directly, not just through increased cell adhesion.

The reviewer is perfectly right and therefore we performed these additional experiments. Besides the wildtype and PPM1F -/- murine fibroblasts, as suggested by the reviewer, we also employed the reconstituted MEF cells re-expressing either the wildtype phosphatase or the inactive PPM1F D360A as already detailed in our answer to comment 2 (rescue experiments). The results of these experiments are now presented in **Manuscript B**, Fig. 3E, where we use the monoclonal antibody 9EG7 to detect active integrin β1 and employ the monoclonal antibody Hmβ1-1 to detect the total murine integrin β1 levels.

The novel results are in line with the previous statement that the absence of PPM1F leads to increased integrin activity as well as impaired spreading and strongly support our hypothesis that integrin β1 activity is negatively regulated by PPM1F. Moreover, the reversion of the PPM1F knock-out phenotype by re-expression of the wildtype enzyme verifies that alterations seen in the PPM1F knock-out cells are a primary and direct effect of the lack of this phosphatase.

6) Is the integrin b1TTpp signal increased in the ppm1f-/- cells?

We thank the reviewer for bringing up this point, as we had omitted these results due to space limitations in the initial manuscript. We have now added these data on T788/T789 phosphorylation in MEF cells upon cell adhesion into the results section of **Manuscript B** (page 8, lines 232):

"*Most importantly, phosphorylation of the threonine motif in the integrin β1 cytoplasmic tail was dramatically elevated in the MEF PPM1F-/- cells as well as the MEFs reconstituted with the inactive enzyme, while re-expression of active, human PPM1F reduced integrin phosphorylation to levels below those found in wildtype cells (Fig. 3D).*"

The data are presented in a novel panel in **Manuscript B**, Figure 3D. Importantly, PPM1F -/- MEFs and PPM1F -/- MEFs re-expressing the phosphatase-dead mutant of PPM1F show strongly elevated integrin β1

T788/T789 phosphorylation levels compared to wildtype MEFs and PPM1F -/- MEFs re-expressing active PPM1F. These results nicely corroborate our original findings in various human cells illustrating the broad relevance of PPM1F in controlling integrin β1 T788/T789 phosphorylation levels and in fine-tuning integrin activity in multiple cell types.

Minor points:

7) The last paragraph of the introduction seems to be repeating the abstract and could be shortened/modified

Following the suggestion of the reviewer the final paragraph in the introduction has been shortened significantly and now reads in **Manuscript A** (page 5, lines 102ff.):

"*Here we report that phosphorylation of the conserved threonine motif in the cytoplasmic tail of the integrin β1 subunit dissociates filaminA to allow access of talin to its canonical NPxY binding site. Using a focused genetic screen, we identify the serine/threonine phosphatase PPM1F as the critical enzyme responsible for dephosphorylating the threonine motif. Our results uncover the mechanistic details of integrin activity regulation by this conserved phospho-switch and identify the underlying enzymatic machinery, thereby providing a novel access point to modulate integrin activity.*"

8) The authors should mention in the introduction that the role of the double TT motif in integrin activity regulation and filamin binding is not entirely new and has been demonstrated for b2-integrin earlier.

Indeed, the role of the conserved TT motif has been addressed in several prior studies by different groups (see also Major Comment 4) by reviewer 1). Due to the limited space, we had not cited these prior studies adequately in our initial submission and we have to apologize for that omission.

We have now included a complete paragraph in the introduction of **Manuscript A** starting on page 5, line 86:

"*Interestingly, an evolutionary conserved threonine motif within the context of the filaminA and talin core binding sites is located in the cytoplasmic tails of most integrin β subunits (T788/T789 in the human integrin β1, Fig. 1A, Fig.*

S1A) (Garcia-Alvarez et al., 2003; Gingras et al., 2009; Kiema et al., 2006; Liu et al., 2015; Wegener et al., 2007). Upon cell stimulation, these threonine residues are phosphorylated (Buyon et al., 1990; Chatila et al., 1989; Craig et al., 2009; Hibbs et al., 1991; Hilden et al., 2003) and mutations mimicking Ser/Thr phosphorylation lead to enhanced integrin activity and integrin-based cell adhesion in vitro (Craig et al., 2009; Nilsson et al., 2006; Wennerberg et al., 1998). In contrast, alanine substitution of this particular threonine motif severely compromises integrin function leading to impaired integrin activation and abrogation of cell-matrix adhesion (Fagerholm et al., 2005; Hibbs et al., 1991; Nilsson et al., 2006; Wennerberg et al., 1998). These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown."

We now also detail the multiple prior studies that have addressed TT phosphoryation in integrin β2 and included this in the in **Manuscript A**, page 18, line 532:

"*Phosphorylation of the conserved integrin threonine motif has been most intensely studied for the integrin β2 subunit, where T758/T759 form part of the Kx2TTTV motif in the cytoplasmic tail. In this case, stimulation of G-protein coupled receptors or the T-cell receptor leads to phosphorylation of T758 in integrin β2 and enhanced integrin-mediated cell-attachment (Chatila et al., 1989; Fagerholm et al., 2005; Takala et al., 2008; Uotila et al., 2014; Valmu et al., 1991). Application of PKC inhibitors abrogates T758 phosphorylation, and the corresponding synthetic peptides of the integrin cytoplasmic domain are phosphorylated in vitro by conventional and unconventional PKC enzymes (Fagerholm et al., 2002). However, additional kinases such as Ca2+-Calmodulin-dependent kinase II (CaMKII) have been shown to associate with the integrin β1 subunit in breast tumor cells (Takahashi, 2001) and inhibitors of CaMKII prevent the increase in T789 phosphorylation driven by constitutive active Ndr1 kinase, an abundant kinase in differentiating neurons (Rehberg et al., 2014). Our kinase assays with the purified integrin β1 cytoplasmic domain now confirm that CaMKII is a bona fide integrin kinase. These studies indicate that multiple serine/threonine kinases can relay signaling inputs, eventually originating from different extracellular and/or intracellular cues, towards the integrin β1 cytoplasmic domain.*"

9) Filamin binding to β2 integrins is also inhibited by 14-3-3 proteins that bind to β2 integrin tails phosphorylated on Thr758 ((Takala, H. et al. β2 integrin

phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. Blood 112, 1853-1862 (2008).)

This is an interesting point brought up by the reviewer. Indeed, we have cited the respective study by Takala et al. in the introduction section to refer to the known role of phosphorylation of the conserved threonine motif (the T758 residue in integrin b2 in the study by Takala et al.) for regulating filaminA binding and the suggestion that this threonine motif functions as a phospho-switch. See **Manuscript A**, page 4, line 79ff.:

"Besides talin and kindlin as positive regulators of integrin function, several negative regulators of integrin activity such as filaminA, Dok1, Sharpin, or ICAP-1 have been described (Bouvard et al., 2003; Kiema et al., 2006; Liu et al., 2015; Oxley et al., 2008; Rantala et al., 2011). These non-enzymatic proteins are thought to act by competitive binding to the integrin β subunit, where they displace positive regulators of integrin activity. For example, filaminA and talin have overlapping binding sites in the leukocyte-specific integrin subunits β2 and β7, which they occupy in a mutually exclusive manner (Kiema et al., 2006; Takala et al., 2008)...[…] These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown."

However, binding of 14-3-3 proteins (of which 14-3-3ζ was investigated by Takala et al.) has been experimentally studied mainly in the context of phosphorylated T758 contained within the TTT motif of integrin β2, a subunit exclusively expressed in hematopoietic cells (Fagerholm et al., 2002; Fagerholm et al., 2005). Though Takala et al. discuss potential binding of 14-3-3 proteins to other integrin β subunits, this has not been addressed experimentally. Due to amino acid sequence differences between the 14-3-3 binding consensus and the respective amino acid motifs in different integrins β subunits, an extrapolation from integrin β2 to other integrins is not easily possible.

As also reviewer 1 asked for the consequences of kindlin and 14-3-3 binding to the conserved threonine motif in integrin β subunits, we have concentrated on kindlin2 for the following reasons:

i) Kindlin2 also competes with filaminA for the same overlapping binding site;
ii) kindlin is involved in integrin activity regulation; and
iii) kindlin2 is known to interact with integrin β1.

The results of these comprehensive experiments are now presented in several additional figures and are summarized also in our response to reviewer 1, point 3:

We were well aware of the potential role of TT-phosphorylation for regulating access of several proteins to the integrin β1 tail. Therefore, we are thankful for the reviewer to bring this point up, even though these interconnected questions demanded a large amount of additional experimentation. We have concentrated on kindlin2 and investigated its binding behaviour *in vitro* to pseudophosphorylated as well as directly phosphorylated integrin β1 tails and also analysed the competition between filaminA and kindlin. Finally, we also investigated the binding behaviour, when the four critical components, talin, kindlin, filamin and the phosphorylated or unphosphorylated integrin β1 tail, are brought together. We believe that our unexpected results are highly interesting and might help to resolve some of the open questions with regard to the talin-kindlin cooperation in integrin inside-out signaling.

These results are now presented in novel Figures 7 and 8 of **Manuscript A**, and summarized in the novel scheme in Fig. 10. They are described in the results section starting from page 12 to page 14 of **Manuscript A**.
In short, our data demonstrate that kindlin2 on its own does not bind to the integrin β1 tail, when the TT motiv is in the phosphorylated form, as integrin tails harbouring the phosphorylation mimicking TT/DD mutations as well as a phosphorylated synthetic integrin β1 tail peptide do not support kindlin2 binding (Fig. 7A-C). Only in the unphosphorylated form, the integrin β1 tail associates with kindlin2, but under these circumstances, kindlin2 is outcompeted by filaminA (Fig. 7D). However, if the TT motiv in the integrin β1 tail is phosphorylated, the presence of the talin head allows kindlin to associate with its binding site at the distal NPxY motif and in this constellation, filaminA can not displace kindlin from the integrin tail (Fig. 7F and G).

This binding behaviour of kindlin in the presence of talin is also seen in intact cells, where only the wildtype and the pseudophosphorylated integrin tails strongly recruit kindlin2 (Fig. 8A). Moreover, in PPM1F ko cells, where integrin β1 is constitutively phosphorylated at the TT motiv, kindlin shows strong peripheral accumulation together with active integrin, closely mimicking the distribution of talin in these cells (Fig. 8B). The functional cooperation between talin and kindlin does not depend on physical interaction between these two proteins, but talin binding to the membrane proximal NPxY motif seems to reorient the integrin β1 tail in a way, which allows access of kindlin to its membrane distal NPxY binding site despite phosphorylation of the TT motiv (Fig. 7F). Such a re-orientation of the integrin tail by the talinF3 domain, which allows kindlin2 to bind adjacent to talin without noticeably contacting the talinF3

domain itself is completely in line with the results of Bledzka et al (Bledzka et al., 2012). It is important to emphasize that the phosphorylation status of the TT motiv together with the binding behaviour of kindlin2 enforce co-operation between talin and kindlin and explain the different consequences of talin vs. kindlin overexpression on integrin activity (Montanez et al., 2008; Ye et al., 2013). Furthermore, phosphorylation of the TT motif in the integrin tail also can explain why mutations blocking talin binding inhibit both talin- and kindlin-driven integrin activation, while mutations that inhibit kindlin binding still permit talin-mediated activation, although they block the kindlin enhancement effect (Ye et al., 2010).

These data on kindlin-integrin association complement previous studies on talin/kindlin-mediated integrin activation (Montanez et al., 2008; Moser et al., 2009; Moser et al., 2008; Cluzel et al., 2005; He et al., 2011; Ye et al., 2013; Li et al., 2017) and help to explain how kindlin, which is clearly required for full integrin function (Theodosiou et al., 2016), fails to acitvate integrins in intact cells by itself..

The wealth of novel data (including the additional data on FilaminA knock-down; see reviewer 1, comment 2) were compiled into three addditional multi-panel figures and this increase in data made it necessary to split the manuscript into two separate parts:

Manuscript A) an article focussing on the identification of PPM1F and on biochemical elucidation of interactions with the phosphorylated/ unphosphorylated integrin β1 tail.

Manucript B) a report detailing the phenotype of PPM1F deletion in the mouse and the the integrin-dependent phenotype of the primary PPM1F-knock-out cells.

We hope that the reviewer agrees that both aspects, the investigation of the role of TT-phosphorylation for kindlin binding as well as the elucidation of the *in vivo* function of PPM1F in mice, are important aspects, which complement each other and which should be published side-by-side. Therefore, we hope you support our suggestion to split this huge amount of novel data into two separate manuscripts.

10) Page 10, typo "In contrast to wildtpe integrin β1,"

Corrected

References cited in our response to Reviewer 2:

Bledzka K, Liu J, Xu Z, Perera HD, Yadav SP, Bialkowska K, Qin J, Ma YQ, Plow EF (2012) Spatial coordination of kindlin-2 with talin head domain in interaction with integrin beta cytoplasmic tails. *The Journal of biological chemistry* 287: 24585-94

Chen H, Zou Z, Sarratt KL, Zhou D, Zhang M, Sebzda E, Hammer DA, Kahn ML (2006) In vivo beta1 integrin function requires phosphorylation-independent regulation by cytoplasmic tyrosines. *Genes & development* 20: 927-32

Cluzel C, Saltel F, Lussi J, Paulhe F, Imhof BA, Wehrle-Haller B (2005) The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells. *The Journal of cell biology* 171: 383-92

Fagerholm S, Morrice N, Gahmberg CG, Cohen P (2002) Phosphorylation of the cytoplasmic domain of the integrin CD18 chain by protein kinase C isoforms in leukocytes. *The Journal of biological chemistry* 277: 1728-38

Fagerholm SC, Hilden TJ, Nurmi SM, Gahmberg CG (2005) Specific integrin alpha and beta chain phosphorylations regulate LFA-1 activation through affinity-dependent and -independent mechanisms. *The Journal of cell biology* 171: 705-15

Gao J, Bao Y, Ge S, Sun P, Sun J, Liu J, Chen F, Han L, Cao Z, Qin J, White GC, Xu Z, Ma YQ (2019) Sharpin suppresses beta1-integrin activation by complexing with the beta1 tail and kindlin-1. *Cell communication and signaling : CCS* 17: 101

He Y, Esser P, Schacht V, Bruckner-Tuderman L, Has C (2011) Role of kindlin-2 in fibroblast functions: implications for wound healing. *The Journal of investigative dermatology* 131: 245-56

Kiema T, Lad Y, Jiang P, Oxley CL, Baldassarre M, Wegener KL, Campbell ID, Ylanne J, Calderwood DA (2006) The molecular basis of filamin binding to integrins and competition with talin. *Molecular cell* 21: 337-47

Li H, Deng Y, Sun K, Yang H, Liu J, Wang M, Zhang Z, Lin J, Wu C, Wei Z, Yu C (2017) Structural basis of kindlin-mediated integrin recognition and activation. *Proceedings of the National Academy of Sciences of the United States of America* 114: 9349-9354

Meng X, Cheng K, Krohkin O, Mould AP, Humphries MJ, Ens W, Standing K, Wilkins JA (2005) Evidence for the presence of a low-mass beta1 integrin on the cell surface. *Journal of cell science* 118: 4009-16

Montanez E, Ussar S, Schifferer M, Bosl M, Zent R, Moser M, Fassler R (2008) Kindlin-2 controls bidirectional signaling of integrins. *Genes & development* 22: 1325-30

Moser M, Legate KR, Zent R, Fassler R (2009) The tail of integrins, talin, and kindlins. *Science* 324: 895-9

Moser M, Nieswandt B, Ussar S, Pozgajova M, Fassler R (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nature medicine* 14: 325-30

Nilsson S, Kaniowska D, Brakebusch C, Fassler R, Johansson S (2006) Threonine 788 in integrin subunit beta1 regulates integrin activation. *Experimental cell research* 312: 844-53

Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, de Pereda JM, Ginsberg MH, Calderwood DA (2003) Talin binding to integrin beta tails: a final common step in integrin activation. *Science* 302: 103-6

Theodosiou M, Widmaier M, Bottcher RT, Rognoni E, Veelders M, Bharadwaj M, Lambacher A, Austen K, Muller DJ, Zent R, Fassler R (2016) Kindlin-2 cooperates with talin to activate integrins and induces cell spreading by directly binding paxillin. *eLife* 5

Ye F, Hu G, Taylor D, Ratnikov B, Bobkov AA, McLean MA, Sligar SG, Taylor KA, Ginsberg MH (2010) Recreation of the terminal events in physiological integrin activation. *The Journal of cell biology* 188: 157-73

Ye F, Petrich BG, Anekal P, Lefort CT, Kasirer-Friede A, Shattil SJ, Ruppert R, Moser M, Fassler R, Ginsberg MH (2013) The mechanism of kindlin-mediated activation of integrin alphaIIbbeta3. *Current biology : CB* 23: 2288-95

Reviewer #3 (Comments to the Authors (Required)):

References cited in our response to Reviewer 3 can be found at the end of this section (page 48)

1.) While the in vitro biochemistry showing phospho-switching of talin and filamin binding is generally convincing little evidence was presented with the integrin mutants to support the idea that this switching alters adhesion or recruitment at focal adhesions. The effect of removing filamin (or mutating it to prevent integrin binding) is not explored in functional assays.

See also comments 2. and 4. by Reviewer 1.

The reviewer is absolutely correct that while performing experiments with integrin chimeras harbouring different kinds of phospho-mimicking or non-phosphorylatable T-A mutations, we have not conducted cellular assays with full-length integrin mutants. However, there is ample published evidence of the phenotype of cells expressing this type of integrin β1 mutants by different groups in several prior studies. Due to the limited space, we had not cited these prior studies adequately in our initial submission and we have to apologize for that omission. We have now included a complete paragraph in the introduction of **Manuscript A** starting on page 5, line 86:

"*Interestingly, an evolutionary conserved threonine motif within the context of the filaminA and talin core binding sites is located in the cytoplasmic tails of most integrin β subunits (T788/T789 in the human integrin β1, Fig. 1A, Fig. S1A) (Garcia-Alvarez et al., 2003; Gingras et al., 2009; Kiema et al., 2006; Liu et al., 2015; Wegener et al., 2007). Upon cell stimulation, these threonine residues are phosphorylated (Buyon et al., 1990; Chatila et al., 1989; Craig et al., 2009; Hibbs et al., 1991; Hilden et al., 2003) and mutations mimicking Ser/Thr phosphorylation lead to enhanced integrin activity and integrin-based cell adhesion in vitro (Craig et al., 2009; Nilsson et al., 2006; Wennerberg et al., 1998). In contrast, alanine substitution of this particular threonine motif severely compromises integrin function leading to impaired integrin activation and abrogation of cell-matrix adhesion (Fagerholm et al., 2005; Hibbs et al., 1991; Nilsson et al., 2006; Wennerberg et al., 1998). These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown.*"

Moreover, there is also published evidence from filaminA overexpression (Calderwood et al., 2001), filaminA knock-down or from cells expressing filaminA mutants incapable of integrin binding that filamin has a negative effect

on integrin-mediated adhesion (Kim et al., 2010; Kim et al., 2008; Takala et al., 2008; Kiema et al. 2006; Baldassarre et al., 2009; Ithychanda et al., 2009; Das et al., 2011; Waldt et al., 2018; Liu et al., 2015; Hu et al., 2017). Importantly, filaminA knock-down cells also exhibited increased talin-enrichment at active integrins (Kumar et al., 2019), which is in line with our findings in A172 PPM1F KO cells (**Manuscript A**, Figure 3, 4, 9) and PPM1F-/- cells (**Manuscript B**, Figure 3).

However, despite the prior experimental evidence for a role of filaminA in suppressing integrin activity, we have considered the criticism by the reviewer. Thus, we have conducted cell adhesion and integrin β1 activity experiments with A172 cells treated with shRNA directed against human filaminA or a control shRNA to evaluate the consequences of filaminA depletion in this cell type. Furthermore, we have taken advantage of this newly made shRNA-encoding lentivirus to also knock-down filaminA expression in PPM1F-deficient A172 cells. The idea behind this epistasis experiment was to see, if PPM1F and filaminA indeed work together in the same regulatory pathway to control integrin activity.

Importantly, we could confirm a negative regulatory role of filaminA for integrin β1 in A172 cells. In particular, filaminA knock-down strongly resembled the PPM1F knock-out phenotype in terms of reduced cell spreading, elevated integrin-based cell adhesion and increased integrin activity (novel Figure 4 H-J and Figure S4 A-I). Furthermore, the epistasis experiment showed that filaminA knock-down did not further enhance the adhesion and integrin-activation phenotype of PPM1F KO cells, substantiating our hypothesis that PPM1F works via filaminA to regulate integrin β1 activity. Also these results are now contained in novel Figure 4 H-J and Figure S4 A-I of **Manuscript A**.

These novel data are now described on page 10, line 248ff of **Manuscript A**:

"*To further confirm that the increased cell adhesion of PPM1F-deficient cells is connected to filamin-dependent activity regulation of integrins we performed epistasis experiments. Therefore, A172 control cells and PPM1F KO cells received either a control shRNA or shRNA targeting human filaminA (Fig. 4H). Similar to the knock-out of PPM1F and consistent with the known inhibitory role of filaminA (Liu et al., 2015; Takala et al., 2008; Waldt et al., 2018}, shRNA-mediated knock-down of filaminA in A172 control cells increased cell adhesion, reduced cell spreading, and elevated integrin activity (Fig. 4, I and J and Fig. S4, A-D). However, depletion of filaminA in PPM1F KO cells did not further elevate the increased integrin-dependent adhesion or the enhanced integrin activity in these cells, nor did it further reduce cell spreading (Fig. 4, I and J and Fig. S4, A-D). The results of these epistasis experiments highlight the strong*

similarities in the phenotype of PPM1F KO cells and filaminA knock down cells and suggest that PPM1F and filaminA work together in the same pathway controlling integrin activity (Fig. S4 E)."

2.) I appreciate that the manuscript already includes a large body of data but it is very surprising that kindlin binding is not considered here. Modifications of the threonine residues are likely to impact kindlin binding and hence integrin function and cell adhesion. The exclusive focus on talin and filamin seems questionable.

See also main comment 3 of reviewer 1 and comment 9 of reviewer 2. We would like to stress that we totally agree with the reviewer and that we were also eager to understand the role of TT-motif phosphorylation for the kindlin-integrin interaction. Therefore, we are thankful for the reviewer to bring this point up, even though this suggestion demanded a large amount of additional experimentation.

We have concentrated on kindlin2, as it is the widely expressed family member, and investigated its binding behaviour *in vitro* to pseudo-phosphorylated as well as directly phosphorylated integrin β1 tails and also analysed the competition between filaminA and kindlin. Finally, we also investigated the binding behaviour, when the four critical components (talin, kindlin, filamin and the phosphorylated or unphosphorylated integrin β1 tail) are brought together. We believe that our unexpected results are highly interesting and might help to resolve some of the open questions with regard to the talin-kindlin cooperation in integrin inside-out signaling.

These results are now presented in novel Figures 7 and 8 of **Manuscript A**, and summarized in the novel scheme in Fig. 10. They are described in the results section starting from page 12 to page 14 of **Manuscript A**.

In short, our data demonstrate that kindlin2 on its own does not bind to the integrin β1 tail, when the TT motif is in the phosphorylated form, as integrin tails harbouring the phosphorylation mimicking TT/DD mutations as well as a phosphorylated synthetic integrin β1 tail peptide do not support kindlin2 binding (Fig. 7A-C). Only in the unphosphorylated form, the integrin β1 tail associates with kindlin2, but under these circumstances, kindlin2 is outcompeted by filaminA (Fig. 7D). However, if the TT motiv in the integrin β1 tail is phosphorylated, the presence of the talin head allows kindlin to associate with its binding site at the distal NPxY motif and in this constellation, filaminA can not displace kindlin from the integrin tail (Fig. 7F and G).

This binding behaviour of kindlin in the presence of talin is also seen in intact cells, where only the wildtype and the pseudophosphorylated integrin tails strongly recruit kindlin2 (Fig. 8A). Moreover, in PPM1F ko cells, where integrin β1 is constitutively phosphorylated at the TT motiv, kindlin shows strong peripheral accumulation together with active integrin, closely mimicking the distribution of talin in these cells (Fig. 8B). The functional cooperation between talin and kindlin does not depend on physical interaction between these two proteins, but talin binding to the membrane proximal NPxY motif seems to reorient the integrin β1 tail in a way, which allows access of kindlin to its membrane distal NPxY binding site despite phosphorylation of the TT motiv (Fig. 7F). Such a re-orientation of the integrin tail by the talinF3 domain, which allows kindlin2 to bind adjacent to talin without noticeably contacting the talinF3 domain itself is completely in line with the results of Bledzka et al (Bledzka et al., 2012). It is important to emphasize that the phosphorylation status of the TT motif together with the binding behaviour of kindlin2 enforce co-operation between talin and kindlin and explain the different consequences of talin vs. kindlin overexpression on integrin activity (Montanez et al., 2008; Ye et al., 2013). Furthermore, phosphorylation of the TT motif in the integrin tail also can explain why mutations blocking talin binding inhibit both talin- and kindlin-driven integrin activation, while mutations that inhibit kindlin binding still permit talin-mediated activation, although they block the kindlin enhancement effect (Ye et al., 2010).

These data on kindlin-integrin association complement previous studies on talin/kindlin-mediated integrin activation (Montanez et al., 2008; Moser et al., 2009; Moser et al., 2008; Cluzel et al., 2005; He et al., 2011; Ye et al., 2013; Li et al., 2017) and help to explain how kindlin, which is clearly required for full integrin function (Theodosiou et al., 2016), fails to acitvate integrins in intact cells by itself.

The wealth of novel data (including the additional data on FilaminA knock-down; see point 1 above) were compiled into three addditional multi-panel figures and this increase in data made it necessary to split the manuscript into two separate parts:

Manuscript A) an article focussing on the identification of PPM1F and on biochemical elucidation of interactions with the phosphorylated/ unphosphorylated integrin β1 tail.

Manucript B) a report detailing the phenotype of PPM1F deletion in the mouse and the the integrin-dependent phenotype of the primary PPM1F-knock-out cells.

We hope that the reviewer agrees that both aspects, the investigation of the role of TT-phosphorylation for kindlin binding as well as the elucidation of the *in vivo* function of PPM1F in mice, are important aspects, which complement each other and which should be published side-by-side. Therefore, we hope you support our suggestion to split this huge amount of novel data into two separate manuscripts.

3.) The screening assay which led to identification of PPM1F is also poorly controlled. Subsequent validation of the hit proves it to be interesting, so at one level deficiencies in the screen may not be important, but the choice of 293T cells without any validation that the phospho-mimicking/blocking mutations examined in Fig 1 and 2 have any impact on adhesion is highly questionable. Is there any evidence that the increased adhesion seen in the screen is linked to alterations in integrin phosphorylation in 293T cells? Or that loss of filamin can enhance 293T cell adhesion?

The reviewer criticizes our choice of 293T cells for the initial screen and that we did not investigate the phosphorylation of integrin β1 and its consequences in this cell line. First of all, as also detailed in the manuscript, we deliberately chose this cell line for its known weak adhesion to extracellular matrix substrates and to cell culture dishes. Based on the prior literature on the role of integrin T788/T789 phosphorylation (see also our response to comment 1 above) we hypothesized that increasing phosphorylation of this motif (by knock-down of the phosphatase responsible for dephosphorylating these residues) should lead to a gain-of-function with regard to matrix adhesion. Our choice of 293 cells was therefore guided in the first place by the idea that such a gain-of-function should become more obvious in a cell line model that displays low adhesion under regular growth conditions.

To more clearly describe our rationale, we have re-phrased the corresponding paragraph in the results part of **Manuscript A** on page 8, line 183ff. to now read:

"*Based on the observations that a phosphorylated T788/T789-motif impedes filamin binding and that the pseudophosphorylated integrin β1 T788D promotes cell adhesion (Craig et al., 2009; Nilsson et al., 2006; Wennerberg et al., 1998), we hypothesized that an integrin-directed protein phosphatase(s) counteracts integrin activation. Accordingly, deletion of such a putative protein phosphatase should lead to a gain-of-function with regard to integrin-based cell-matrix adhesion. Therefore, we performed a focused genetic knock-down screen with shRNAs individually targeting all protein phosphatases reported in the integrin adhesome (Zaidel-Bar et al., 2007) (Fig. S2 A). As cellular system we*

deliberately chose 293T cells, a human cell line exhibiting weak adhesion to extracellular matrix proteins under tissue culture conditions."

As our screening results demonstrate, this strategic decision was helpful in identifying PPM1F as a candidate for a serine/threonine phosphatase involved in regulating integrin-based cell adhesion. However, we see this basic screen only as a first hint and have strived to explore the role of this protein phosphatase in more meaningful cell line models: primary human fibroblasts (NHDF), murine embryonic fibroblasts (MEFs), and a neuronal cell line (A172 cells), as PPM1F is highly expressed in neuronal tissue.

We completely agree with the reviewer that the questions arising from this initial finding (do the phospho-mimicking/blocking mutations examined in Fig 1 and 2 have any impact on adhesion/ Is there any evidence that the increased adhesion is linked to alterations in integrin phosphorylation or that loss of filamin can enhance cell adhesion) are critically important. However, we are convinced that by answering these questions in multiple, relevant cell models, we have chosen a more appropriate way to put our candidate protein to the test than by a continued use of 293 cells.

Finally, as requested by the reviewer before (see comment 1 above) we have tested the consequences of the loss of filamin for cell adhesion in A172 cells and therefore, can demonstrate that the filaminA knock-down phenocopies the loss of PPM1F in these cells. These results confirm a negative regulatory role of filaminA for integrin β1 in A172 cells and are presented in novel Figure 4 H-J and Figure S4 A-I of **Manuscript A**.
These novel data are now described on page 10, line 248ff of **Manuscript A**:

"*To further confirm that the increased cell adhesion of PPM1F-deficient cells is connected to filamin-dependent activity regulation of integrins we performed epistasis experiments. Therefore, A172 control cells and PPM1F KO cells received either a control shRNA or shRNA targeting human filaminA (Fig. 4H). Similar to the knock-out of PPM1F and consistent with the known inhibitory role of filaminA (Liu et al., 2015; Takala et al., 2008; Waldt et al., 2018), shRNA-mediated knock-down of filaminA in A172 control cells increased cell adhesion, reduced cell spreading, and elevated integrin activity (Fig. 4, I and J and Fig. S4, A-D). However, depletion of filaminA in PPM1F KO cells did not further elevate the increased integrin-dependent adhesion or the enhanced integrin activity in these cells, nor did it further reduce cell spreading (Fig. 4, I and J and Fig. S4, A-D). The results of these epistasis experiments highlight the strong similarities in the phenotype of PPM1F KO cells and filaminA knock down cells and suggest that PPM1F and filaminA work together in the same pathway controlling integrin activity (Fig. S4 E).*"

4.) Loss of PPMF1 does appear to increase cell adhesion and integrin activation (assessed by 9EG7 binding) and the similarity between shRNA and KO results is encouraging but extensive rescue experiments are not well documented. This is a significant weakness.

Adressing the criticism of the reviewer, we have now performed detailed rescue experiments for both genetically deficient cell lines generated in our study: the A172 PPM1F-KO cells produced via CRISPR/Cas-mediated gene disruption and the PPM1F-/- murine embryonic fibroblasts (MEFs) generated by conventional homologous recombination in murine embryonic stem cells. Importantly, in all cases we observe a reversion of the phenotype of the PPM1F-deficient cells with regard to integrin T788/T789 phosphorylation, talin recruitment, integrin activity, cell matrix adhesion, and initial cell spreading upon re-expression of the active, wildtype PPM1F enzyme. In contrast, re-expression of the phosphatase-deficient D360A mutant of PPM1F (PPM1F DA) does not change, and in some cases even aggravates, the phenotype of the PPM1F-deficient cells.

- These novel data strongly support our previous conclusions and substantiate our hypothesis that PPM1F phosphatase activity controls integrin activity and cell adhesive events by regulating the T788/T789 phospho-switch.

These data are now presented for the A172 cells in **Manuscript A**, Figure 5A-G and for the PPM1f-/- MEF cells in **Manuscript B**, Fig. 3.

We adapted the corresponding paragraph in the results section of **Manuscript A** accordingly, which now reads on page 10, line 278ff.:

"*To rigorously demonstrate that this phenotype is due to the lack of PPM1F activity, we complemented the PPM1F KO A172 cells with either wildtype mKate-PPM1F or the phosphatase-dead mutant mKate-PPM1F D360A (Fig. S3 H). As seen before, expression of core focal adhesion proteins or surface expression of integrin subunits were not altered by this genetic manipulation (Fig. S3 A and B). However, expression of PPM1F wildtype, but not expression of PPM1F D360A, reverted the increased phosphorylation of integrin β1 T788/T789 back to levels seen in wildtype A172 cells (Fig. 5 D). The increased integrin T788/T789 phosphorylation seen in the PPM1F KO cells correlated well with the elevated integrin activity and enhanced cell adhesion to integrin ligands, which was also reverted back to basic levels upon re-expression of PPM1F wt, but not PPM1F D360A (Fig. 5, E-G). Together, these findings are consistent with the idea, that PPM1F regulates the phosphorylation state of the T788/T789 motif, thereby controlling association of talin versus filaminA with the cytoplasmic tail of integrin β1 and determining cell-matrix adhesion strength*

(Fig. 5 H). The uniform phenotype of enhanced integrin activity observed upon depletion or disruption of PPM1F in multiple cell types also indicates that PPM1F might act directly on the integrin β1 subunit."

5.) Even if rescue phenotypes are established it is difficult to determine that the effect is directly due to integrin phosphorylation as opposed to via indirect effects (presumably PPMF1 has other substrates). And if it is due to a loss of integrin phosphorylation is filamin part of the story?

The reviewer touches on a sensitive point that pertaines to the whole field of protein phosphorylation and dephosphorylation: How do you determine a direct action, rather than an indirect action of a kinase or a phosphatase on a given substrate.

Clearly, a combination of in vitro and in cellulo experimentation is required to provide a convincing argument that your enzyme of interest, in our case PPM1F, is directly responsible for the action on a given substrate, in our case pT788/pT789 of integrin β1.

Accordingly, we demonstrate *in vitro* that purified, recombinant PPM1F, in contrast to other related phosphatases (e.g. ILKAP) de-phosphorylates the particular phospho-residues in the integrin β1 cytoplasmic tail in synthetic peptides, but also in a recombinant integrin tail in vitro phosphorylated by CaM kinase. We also demonstrate that the observed dephosphorylation is not due to the action of a co-purified bacterial phosphatase activity, as the recombinant PPM1F D360A is not active in this format. Furthermore, we demonstrate in two independent cell model systems (A172 and MEF) that the genetic deletion of the enzyme results in elevated phosphorylation levels of the substrate, which is reverted by re-expression of the active enzyme (PPM1F WT), but not the inactive phosphatase (PPM1F DA). Furthermore, by using a number of point mutants of the integrin tail as well as synthetic phospho-peptides we show that the action of the phosphatase on this particular residues has consequences for well-described (by X-ray and NMR structures of the respective complexes) protein-protein interactions of the integrin tail. This is done both with purified components and in a model system inside living human cells demonstrating that the action of the phosphatase on the integrin tail results in clear alterations in protein-protein interactions that have known consequences for integrin activity and integrin-mediated adhesion. Such a combination of approaches is as good as it gets, when it comes to delineating a phosphatase-substrate relationship and its direct consequences.

With regard to additional PPM1F substrates (see also our comment to point 8 of reviewer 1), we believe that it is highly likely that PPM1F also regulates other cellular proteins to fine-tune integrin- and actin-mediated events. Indeed, it has been reported before, that PPM1F de-phosphorylates PAK (Koh et al., 2002; Susila et al., 2010) and that PPM1F controls CaMKII activity (Harvey et al., 2004; Ishida et al., 1998) and we have cited the respective studies already in our initial submission. Interestingly, CaMKII is one of the potential kinases targeting the conserved threonine motif in integrin β subunits (Takahashi, 2001; Suzuki and Takahashi, 2003; Rehberg et al., 2014) and we find strong *in vitro* phosphorylation of the integrin β1 tail by CaMKII (**Manuscript A**; Fig. 6E; Fig. S5 F)

Therefore, we have included a paragraph in the discussion section of **Manuscript A**, page 19, line 555ff.:

"*It is tempting to speculate that PPM1F is ideally suited to serve as key control for the T788/T789 phospho-switch, as it not only dephosphorylates the integrin β cytoplasmic domain, but this phosphatase is also able to reverse the auto-phosphorylation of CaMKII at Thr286 (Harvey et al., 2004; Ishida et al., 1998). Thus, PPM1F could shift the balance towards the unphosphorylated, inactive integrin by acting on both an integrin-directed serine/threonine kinase as well as on the integrin T788/T789 motif itself.*"

A further potential substrate of PPM1F might be filaminA itself, which is clearly working together with PPM1F to regulate integrin activity (see our answer to point 3 above). Indeed, phosphorylation of filaminA at S2152, a residue located close to the integrin binding site between filaminA Ig20 and Ig21, was reported to prohibit filaminA - integrin β2 binding and, thus, promote integrin activity in T cells (Waldt et al., 2018).

6.) Loss of PPM1F clearly impairs cell spreading, conceivably due to "intensified integrin-matrix interaction" but the causal link is not definitively established here so care is needed in the discussion of these results.

Based on the criticism by the reviewer, we have rephrased our statement to use more caution in expression and also included an explanation as to why the known regulatory action of PPM1F on other substrates such as PAK or mDia does not seem to be responsible for the observed spreading defect. The novel sentences in the results section of **Manuscript A** on page 9, line 241 now read:

"*In general, PPM1F KO cells did not spread as fast as A172 wildtype cells and, therefore, covered a smaller area (Fig. 4, F and G; Fig. S3 G) suggesting that*

cell spreading might be compromised due to intensified integrin-matrix interaction. This observation also indicates that other PPM1F substrates such as PAK or mDia, which promote actin-based cell protrusions and which are negatively regulated by PPM1F, might not be responsible for the spreading defect of PPM1F KO cells (Parrini et al., 2009; Koh et al., 2002;Xie et al., 2008)."

Specific points requiring clarification/correction:

7.) In Fig 2 use of the OPTIC assay supports the talin-integrin binding data but the results with filamin-integrin are much weaker. Only the phospho-blocking mutant integrins showed filamin recruitment but this was barely above the 2.0 threshold, despite apparently occurring in most cells. The authors should comment on the very weak filamin recruitment in this assay.

Also reviewer 1 brought up a similar point (see our answer to point 6 by reviewer 1). We think the poor recruitment of filaminA in this set-up (OPTIC assay) might be connected to the extent of integrin phosphorylation under these circumstances. Based on the impressive quantitative work by Springer and colleagues {Li, 2017 #8988}, only a minor fraction of integrin heterodimers on a given cell might be in the extended-open "active" conformation and would contain p-T788/p-T789. Conversely, one would expect most integrin heterodimers to be in a non-phosphorylated state and, therefore, accessible to filaminA binding. However, one has to consider that our experiments with integrin β1 cytoplasmic tails (OPTIC assays) are conducted with CEACAM-integrin fusion proteins, where the β subunit is expressed in the absence of a corresponding α subunit. Therefore, this situation rather reflects a constitutive active, unclasped conformation, where the β subunit is not in contact with the α subunit and freely available. We believe that in this situation, the isolated β subunit in the wildtype form is mainly phosphorylated and therefore, filaminA is only recruited to the TT/AA mutant, but not the wildtype integrin β1 tail (Fig. 2B).

Evidence for this idea is provided by the results presented now in Figure 9B, where in the same experimental setting the overexpression of wildtype PPM1F, but not the overexpression of phosphatase inactive PPM1F D360A, suddenly leads to recruitment of filaminA to the wildtype integrin β1 tail in intact cells (Fig. 9B). This observation is in line with the idea that this fusion protein of the wildtype integrin β1 cytoplasmic tail is mainly phosphorylated, but upon overexpression of PPM1F becomes de-phosphorylated by the protein phosphatase allowing filaminA binding. We now include a synopsis of these thoughts in the discussion section of our **Manuscript A** on page 18, line 518ff:

"*When we express the isolated wildtype integrin β1 tail in intact cells, a situation mimicking the unclasped integrin, we do not observe filaminA recruitment. However, upon overexpression of PPM1F, but not PPM1F D360A, filaminA accumulates at the wildtype integrin β1 cytoplasmic tail. This finding could indicate that the threonine motif is mainly phosphorylated, when the integrin heterodimer is in the unclasped conformation and the β subunit is separated from the α subunit. Phosphorylation of the conserved threonine motif under these circumstances would not only displace the negative regulator filamin, but it would also prohibit kindlin2 from driving integrin inside-out signaling in the absence of talin. This scenario is in line with the observation that kindlin overexpression does not lead to integrin inside out activation (Ma et al., 2008; Harburger et al., 2009; Ye et al., 2010; Li et al., 2017).*"

8.) In discussing the results of Fig 1 and 2 on page 6 the authors conclude that "These results demonstrated that the phosphorylation status of the integrin β1 T788/T789 motif dictates the association with integrin activity regulators in intact cells" - this conclusion seems premature as at this stage they have only assessed T/A and T/D mutants, not phosphorylation. Phospho-mimicking mutations are useful, but imperfect, models so maybe a more conservative interpretation is needed here.

The reviewer is completely right with this remark and this point has been brought up by all three reviewers (see also main point 5 of reviewer 1 and main point 1 of reviewer 2).
Following the valuable suggestion by all three reviewers, we have performed additional pull-down experiments with biotinylated phospho-T788/T789 and unphosphorylated synthetic integrin β1 peptides (AA762-798) using recombinant purified talin-head, full-length kindlin-2, and the filaminA integrin binding region (Ig19-21). These novel results are included in additional panels in Figure 1D and in Figure 7C and G of **Manuscript A**.
In agreement with our prior data using the recombinant wildtype and pseudo-phosphorylated (TT/DD) versions of the integrin β1 cytoplasmic domain, we again find that talin binds independently of TT phosphorylation, while both kindlin2 and filaminA only interact with the wildtype integrin form, while they do not bind on their own to phosphorylated T788/T789 (Fig. 1D, Fig. 7C).
Most importantly, we could observe a co-operative binding of talin and kindlin in the case of phosphorylated integrin β1, which excludes filamin binding (Fig. 7G). These novel data not only validate the binding behavior of talin, kindlin and filamin as observed before with charge-mimicking (TT/DD) proteins, but also provide a detailed explanation as to how the PPM1F-controlled phospho-switch at T788/T789 can orchestrate the binding of positive (talin/kindlin) and negative (filaminA) regulators of integrin activity.

9.) In discussing the results from the initial shRNA screen the authors state that "Compared to control cells, depletion of the protein tyrosine phosphatases PTP-1B and PTP-PEST as well as depletion of the serine/threonine phosphatase PPM1F (...) resulted in enhanced cell adhesion to collagen and fibronectin, but not poly- L-lysine (Fig. 3A and B)." However, while it appears that PPM1F and PTP-PEST enhance binding to both collagen and fibronectin (and probably also to poly-L-lysine) PTP-1B seemed to only impact fibronectin binding. RPTP1α also seemed to increase binding to collagen and fibronectin but was not mentioned. This needs to be explained. Better statistical analysis of the results here might help - the legend seems to indicate that the results shown are for a single experiment with averaging over 3 well, are the screening results reproducible and are changes statistically significant?

The reviewer is completely right in that we missed to mention the effect of RPTP1α knock-down in 293T cells appropriately in our results part. We have rephrased the paragraph now accordingly to read in **Manuscript A** on page 8, line 194:

"*Compared to control cells, depletion of the protein tyrosine phosphatases PTP-1B, PTP-PEST and RPTPα as well as depletion of the serine/threonine phosphatase PPM1F (also known as POPX2 (Koh et al., 2002), CaMKP (Ishida et al., 2008), and hFEM2 (Tan et al., 2001)) resulted in enhanced cell adhesion to collagen and/or fibronectin, but not poly-L-lysine (Fig. 3A and B). PTP-1B, PTP-PEST and RPTPα dephosphorylate the focal adhesion proteins paxillin, p130^{CAS} and c-Src, respectively, which could indirectly affect integrin-mediated adhesion (Arias-Salgado et al., 2005; Garton et al., 1996; Shen et al., 2000, Zheng et al., 2000). As PPM1F, a member of the metal-dependent protein phosphatase family (PPM) {Moorhead, 2009 #6949}, dephosphorylates serine/threonine residues and has not been implicated in cell adhesion, we decided to focus on this enzyme*."

Generally, the shRNA screen was meant as a first indicator for phosphatases within the integrin adhesome, which could serve as candidates for further investigation. The experiment was performed in triplicates and done three times with comparable results, that nevertheless did not reach statistical significance. As also detailed in response to major point 3 above, we see this basic screen only as a first hint and have strived to explore the role of PPM1F in more meaningful cell line models than 293T cells: primary human fibroblasts (NHDF), murine embryonic fibroblasts (MEFs), and a neuronal cell line (A172 cells), as PPM1F is highly expressed in neuronal tissue.

10.) The rescue experiments in Fig 5E are very important but the quantitation shown seems to be of a single blot. The experiment should be repeated several times and results of replicates plotted to provide an idea of the variability in the results.

Following this important advice by the reviewer, we have now based our quantification of integrin β1 T788/T789 phosphorylation for different A172 cell lines on three independent experiments. The data are now presented in **Manuscript A** Figure 5 D and show a significant, 4.5-fold increase of integrin pT788/pT789 levels in PPM1F KO cells and a 3.5-fold increase in PPM1F KO – PPM1F D360A re-expressing cells compared to the wildtype A172 cells. Re-expression of wildtype PPM1F in PPM1F KO cells strongly reduces this increased integrin β1 T788/T789 phosphorylation almost back to the levels in wildtype cells.

11.) The experiments in Fig 7 designed to show that PPM1F activity controls integrin tail interaction with talin and filamin A raise a series of questions. What is the basal phosphorylation levels of the chimeric integrin constructs in 293T cells? The large effects observed would seem to require a relatively high stoichiometry of phosphorylation - is there any evidence for this? It is also somewhat unclear why the over-expressed GFP-talin is displaced by PPM1F as talin apparently binds to both the phosphorylated and unphosphorylated tails - presumably the authors invoke competition with the endogenous filamin but are endogenous filamin levels sufficient to compete the over-expressed tagged talin? If filamin is knocked down/out does this prevent the PPM1F effect?

As already discussed above under point 7, we agree with the reviewer that these results might be explained by the stoichiometry of T788/T789 phosphorylation on the chimeric CEACAM3-integrin β1 tail fusion proteins used in the OPTIC assay. We do not have direct evidence for the pT788/pT789 phosphorylation status of these constructs, as we have failed, so far, to isolate (by immunoprecipitation) the bacteria-clustered CEACAM-integrin chimeras in this setup. This might be due to exceptional strong binding of the multivalent, Opa-expressing gonococci to the extracellular CEACAM domain used as a fusion partner for the integrins. Also, the used polyclonal pT788/pT789 antibody was not helpful in immunofluorescence staining approaches with these complex samples, as we observed strong background staining of the attached bacteria. Therefore, we can only offer our current hypothesis (see point 7 above and also point 6 by reviewer 1), but have no quantitative data on the phosphorylation status of the CEACAM-integrin chimeras at this point.

As the reviewer remarks, we indeed think that the effect of PPM1F on talin recruitment in the OPTIC assays is due to competition with filaminA (and presumably other integrin β1-binding partners) present in 293 cells, which have a preference, and probably selective binding capability, towards the unphosphorylated integrin β1 tail. To address this question directly, the idea of the reviewer to deplete filaminA in this context is a splendid suggestion. Indeed, we have tried to use shRNA-mediated depletion of filaminA in 293 cells, which should then have been transfected with integrin chimeras and used in OPTIC assays. However, we have failed in several attempts to obtain a stable filaminA knock-down 293 cell line by our lentiviral shRNA approach. In contrast, the same shRNA-encoding lentivirus allowed us to stably deplete filaminA in the wildtype A172 cells and in the A172 PPM1F KO cells (see our response to major point 1 above). These experiments, shown now in **Manuscript A**, Fig. 4 H-J, do not directly address the phosphorylation state of integrin β1, but they suggest that filaminA and PPM1F work together in the same pathway to enhance integrin activity and strengthen integrin-based cell adhesion (see also our answer to major point 3, above).

12.) The embryonic lethal phenotype of PPM1F knockout mice confirms it importance but it remains to be determined whether this lethality is related to alterations in integrin activity. This should be made clear in the manuscript.

The reviewer makes a valid point in that we cannot be entirely sure that the lethal phenotype in *ppm1f-/-* mice is strictly based on alterations in integrin activity. Clearly, as we also discuss in **Manuscript B** starting from page 11, line 299, there are strong similarities between PPM1F deficiency, filaminA deficiency and mice with expression of non-phosphorylatable TT/AA variants of integrin β1, which support the idea that these proteins are functionally connected.
Not only do these genetic alterations lead to disruption of early embryonic development, but e.g. the compromised control over integrin activity in integrin β1 T788A/T789A expressing mouse embryos or in PPM1F-deficient mouse embryos seems to have common repercussions in the lack of neural progenitor migration and in the structuring of the extracellular matrix, as in both cases laminin deposition in the basement membrane is disorganized.

However, we totally agree that future studies need to clarify, if the embryonic lethality of PPM1F-/- mouse embryos is related to alterations in integrin activity. Therefore, we have included in the discussion section of **Manuscript B**, page 12, line 329 the following paragraph:

"*It has to be noted, that apart from the integrin β1 subunit, PPM1F acts on additional substrates such as kinases, cytoskeletal proteins, and apoptosis regulators (Zhang et al., 2013; Ishida et al., 2018). Therefore, it remains to be determined whether embryonic lethality of PPM1F knock-out mice is a direct consequence of alterations in integrin activity and to which extent deregulation of other PPM1F substrates may play a role. However, the phenotypic similarities upon disruption of genes encoding PPM1F, filaminA, and integrin subunits in mammals and the functional interplay of these proteins in intact cells strongly argue for a critical role of PPM1F-mediated integrin activity regulation in vivo.*"

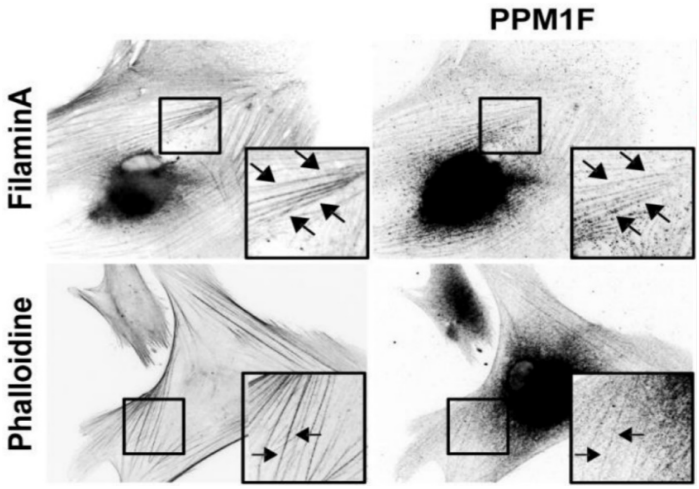
13.) More information on PPM1F, its domain architecture and subcellular localization would help in this manuscript. Does it localize in adhesions in the cells used here?

We agree with the reviewer and we would be happy to elaborate more on the structural aspects of PPM1F. With the massive expansion of data during the revision process it was necessary to subdivide our manuscript into two parts to be able to accommodate all data. We think that a short description of PPM1F is most appropriate for Manuscript B. Therefore, we have now included a novel paragraph in the introduction of **Manuscript B**, page 4, line 100ff, which provides available background information on PPM1F:

"*Indeed, recent data have suggested that the protein phosphatase PPM1F dephosphorylates the T788/T789 motif in integrin β1, thereby supporting filaminA-integrin association and securing the closed, inactive conformation of the receptor (see accompanying paper by Grimm et al.). PPM1F is a member of the PP2C family of Mg^{2+}/Mn^{2+}-dependent protein phosphatases (PPMs), which comprises 18 distinct enzymes in humans (Sacco et al., 2012). PPM1F has been described initially as Calmodulin-dependent kinase phosphatase (CamKP), since it dephosphorylates and inactivates CamKII (Kitani et al., 1999). Furthermore, PPM1F has been termed POPX2 (partner of PIX 2), as it has been found to associate with the Cdc42/Rac1 guanine nucleotide exchange factor PIX and has been shown to dephosphorylate the p21-activated kinase PAK (Koh et al., 2002). Via binding to p95PKL, PIX associates with the core focal adhesion protein paxillin (Turner et al., 1999) providing a potential physical link between PPM1F and integrin-based focal adhesions.*"

We of course were also very keen to localize PPM1F in cells. To our disappointment and despite the reported interaction of PPM1F with the focal adhesion proteins PIX/p95PKL, we did not detect PPM1F in focal adhesion structures in the different cell types used in this manuscript, also under different

plating conditions (minutes to hours after plating on different integrin ligands in low or high concentration). However, PPM1F showed a clear co-distribution together with filaminA along f-actin based stress fibers (Reviewer Figure 5).



Reviewer Figure 5. Filamin and PPM1F co-localize in a pattern paralleling actin stress fibers. Human dermal fibroblasts were seeded onto fibronectin and co-stained for filaminA/PPM1F (upper row) or f-actin/PPM1F (lower row). Fixed samples were imaged using wide field microscopy. The insets show higher magnification and the arrows indicate co-localization of PPM1F with FilaminA or actin stress fibers

This distribution of PPM1F is in line with the idea that PPM1F and filaminA are associated with non-phosphorylated, inactive integrin heterodimers outside of focal adhesions, while active integrins at focal adhesions might be predominantly in a phosphorylated state.

A hypothetical scenario is that filaminA travels with inactive integrins (held in the unphosphorylated, inactive state by the co-occurance of PPM1F) along stress fiber tracks to nascent adhesions in the cell cortex, where integrin activation occurs. To investigate this hypothesis in the future, we are in the process to expand and update our live cell microscopy equipment to conduct more detailed and sensitive microscopic analyses of cells expressing fluorescent-protein-tagged PPM1F in different contexts.

14.) The manuscript by Wennerberg (PMID: 9512507) seems relevant to this manuscript.

We totally agree with reviewer as integrin β1 TT phosphorylation has been analysed in a number of prior studies and, due to the limited space, we had not cited these prior studies adequately in our initial submission. We have to apologize for that omission and have now included a complete paragraph in the introduction of **Manuscript A** starting on page 5, line 86:

"*Interestingly, an evolutionary conserved threonine motif within the context of the filaminA and talin core binding sites is located in the cytoplasmic tails of most integrin β subunits (T788/T789 in the human integrin β1, Fig. 1A, Fig. S1A) (Garcia-Alvarez et al., 2003; Gingras et al., 2009; Kiema et al., 2006; Liu et al., 2015; Wegener et al., 2007). Upon cell stimulation, these threonine residues are phosphorylated (Buyon et al., 1990; Chatila et al., 1989; Craig et al., 2009; Hibbs et al., 1991; Hilden et al., 2003) and mutations mimicking Ser/Thr phosphorylation lead to enhanced integrin activity and integrin-based cell adhesion in vitro (Craig et al., 2009; Nilsson et al., 2006; Wennerberg et al., 1998). In contrast, alanine substitution of this particular threonine motif severely compromises integrin function leading to impaired integrin activation and abrogation of cell-matrix adhesion (Fagerholm et al., 2005; Hibbs et al., 1991; Nilsson et al., 2006; Wennerberg et al., 1998). These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown.*"

References cited in our response to Reviewer 3:

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August 23, 2020

RE: JCB Manuscript #202001057R

Dr. Christof R Hauck
University of Konstanz
Department of Biology
Universitätsstrasse 10
Maildrop X908
Konstanz 78457
Germany

Dear Dr. Hauck,

Thank you for submitting your revised manuscript entitled "PPM1F controls integrin activity via a conserved phospho-switch in the integrin β subunit". You will see that the reviewers are positive about the work and recommend considering the whole study further, pending minor text edits as per Revs#1 and #3. However, the referees strongly feel that the two papers need to be combined for the full story to be clear to readers and to come together.

First, we editorially want to sincerely apologize for the lack of responsiveness you experienced from the office due to confusion following Marie Anne O'Donnell's departure from the JCB staff. We are really sorry. Like the referees, we unfortunately do not feel that the second, new manuscript formatted as a Report can stand alone and be appropriate for publication for JCB. Even for companion submissions and back-to-back papers, our long-standing policy is that each paper must stand on its own merits for publication in JCB. However, if both manuscripts are merged, then the new manuscript will be accepted, subject to Martin checking its content. Please also pay attention to the formatting requirements below and the reviewers' final points. All reviewers felt that the split weakened the story so we feel that this change will make the paper stronger and more appealing to readers. Only essential information from the second manuscript should be included. Please let us know if you have any questions about the final changes needed at this stage.

We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) and to address the changes detailed above.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

JCB Articles can have up to 10 main and 5 supplemental figures, with each figure spanning up to one entire page provided that all panels fit on the page.

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: PPM1F controls integrin activity via a conserved phospho-switch

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to:
main paper: 3B, 3F (magnifications), 4D (mags), 5F, 8B (mags), S2F (mags), S3E (mags), S3D, S4A, S4B (mags)
short, second paper, if the data are moved and included in the final paper: 1C, 1f (magnifications), 2F (mags), 3C (mags), S1D
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: 6B

5) Tables must be formatted as stand-alone tables and provided as editable files (e.g., Word). They should be taken out of the Materials and methods, or if you prefer they could be converted to paragraph form (not a table embedded in text).

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Cell lines, plasmids, mouse strains: please include database / catalog IDs (e.g., ATCC, Addgene, etc.) for all or if unavailable please include a brief description of their basic genetic features **even if described in other published work or gifted by other investigators**
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

B. FINAL FILES:

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-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Martin Humphries, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This paper describes a phosphatase that regulates the phosphorylation and function of beta1-integrins. Integrin phosphorylation at two/three conserved threonines has been known for a long time but its functional significance has not been entirely clear. In this paper, the authors describe that beta1-integrin phosphorylation regulates the association of talin/kindlin (two known integrin

activators) versus filamin A (a known integrin negative regulator). Talin/filamin competition, and FilaminA being regulated by phosphorylation are not new findings, but this paper present new evidence for the regulation of these interactions by phosphorylation in cells, and for the functional significance of integrin phosphorylation for integrin activity regulation. In addition, indirect regulation of kindlin-integrin association phosphorylation is an interesting new finding. The main new finding in this paper is that PPMF1 can function as an integrin phosphatase. Because of these new findings, the paper contributes significantly to the field. It is recommended that the PPMF1 knockout mouse should be part of the same paper rather than as a separate paper, as the in vivo evidence significantly supports the conclusions of the main paper. The reviewer's concerns have been adequately addressed in the revised version of the paper, although some minor points remain to be addressed:
Comment 4. Wennerberg 1998 does not look at effect of TT/DD mutations on cell adhesion, please remove citation from this section.
Comment 6. The answer to this comment is highly speculative, and no experimental data is provided to back up the hypothesis that the wt construct would be phosphorylated at a high stoichiometry. It is recommended that the speculative text concerning this (page 18, line 518) is removed from the paper, it would be better to state that it is unclear why filamin does not associate with the wt construct.

Reviewer #2 (Comments to the Authors (Required)):

This is a very interesting study and the authors have addressed nicely all my concerns with explanations and new experimentation. However, it is unclear what prompted the authors to split the paper into two. This made re-reviewing particularly difficult and diminishes the impact. The authors should be encouraged to merge these into one strong JCB paper.

Reviewer #3 (Comments to the Authors (Required)):

The authors have been very responsive to my previous concerns and have greatly improved their manuscript. The addition of data on kindlin is notable and of considerable interest. In addressing the reviewer concerns, an already large manuscript became very large and they therefore chose to split their manuscript into two. Whether this is acceptable for JCB should be an editorial decision. However, the first manuscript (A) reads very well as a stand-alone document and it is difficult to see how the second manuscript can be readily incorporated into the first without loss of some of the detailed and helpful explanation and discussion included in each manuscript.

On its own, the second manuscript (B), while providing a description of the PPM1F knockout phenotype, lacks the mechanistic insight normally associated with JCB. It confirms findings in the first paper and shows that whole animal results are consistent with the conclusions of the first manuscript.

In manuscript B the discussion sections dealing with the T788A/T7809A knock-in mice is a little confusing. The authors note the similar stages of death of this mouse with the PPM1F mouse - but these mice mimic different conditions, the T/A mutant mimics the unphosphorylated form while presumably in the absence of the phosphatase the integrin is shifted towards the phosphorylated form. A T/D mutant mouse might be a more relevant comparison. The extensive discussion of the

neuronal phenotypes and comparison with neuronal phenotypes associated with loss of filamin A is interesting but there are little data directly linking this to effects on integrin binding.

Minor comments:

First manuscript: (A) Lines 280-282 "Quantification of multiple blots demonstrated....(Fig 5D)" Fig 5D did not show this quantification

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The Journal of Cell Biology
The Rockefeller University Press
950 Third Ave., 2nd Floor
New York, NY 10022

10.9.2020

Re-Submission of JCB manuscript #202001057R for publication in *The Journal of Cell Biology*

Dear Dr. Humphries and Dr. Casadio,

Thank you very much for your response and the positive evaluation of our manuscrip(s). We are very happy to hear that the reviewers supported our revised manuscript(s) and that you consider our work for publication in your prestigous journal. Following your recommendations, we have extracted the essential information from the second, report-style manuscript and incorporated this information into the Article-style manuscript. Due to total space and figure number restrictions, we had to omit some results from the second, report-style manuscript (such as the histological analysis of adult and embryonic tissues) to be able to condense the central findings into a single figure.

Furthermore, we have addressed the reviewers further remarks (please find our detailed point-by-point response to the comments of the reviewers (marked in red) from the next page on) and tried to closely adhere to your formatting guidelines. However, as already discussed with you, the additional information requested by the reviewers and the partial merge of two manuscripts resulted in more text. Though we were able to fit everything into 10 packed Main Figures and 5 Suppl. Figures, we were not able to completely comply with your text limits. Overall, we feel that we have now fully addressed the issues raised in the two rounds of review and hope that that our combined, revised manuscript is now be suitable for publication in **Journal of Cell Biology**.
Thank you again for all your efforts.
With best regards from Konstanz

Prof. Dr. Christof R. Hauck

ChemicalBiology
University of Konstanz

Editorial Comments:

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Text limits could not be matched

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Title: PPM1F controls integrin activity via a conserved phospho-switch

Title has been changed

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to:
main paper: 3B, 3F (magnifications), 4D (mags), 5F, 8B (mags), S2F (mags), S3E (mags), S3D, S4A, S4B (mags)
short, second paper, if the data are moved and included in the final paper: 1C, 1f (magnifications), 2F (mags), 3C (mags), S1D
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

We have included scale bars in all microscope images, including the inset magnifications and indicated the size in the Figure legends.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: 6B

The sample size in 6B has been indicated.

5) Tables must be formatted as stand-alone tables and provided as editable files (e.g., Word). They should be taken out of the Materials and methods, or if you prefer they could be converted to paragraph form (not a table embedded in text).

We have listed all generated shRNA constructs and the used oligos in the form of a table (Table 1), which is now provided as an editable Word file. Table 1 is refered to in the Materials and Methods section.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Cell lines, plasmids, mouse strains: please include database / catalog IDs (e.g., ATCC, Addgene, etc.) for all or if unavailable please include a brief description of their basic genetic features **even if described in other published work or gifted by other investigators**
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

We have re-organized the Material & Methods section to provide all information available to us as requested and we have described all relevant procedures in detail irrespective of referencing to previous publications.

Reviewer Comments:

Reviewer #1 (Comments to the Authors (Required)):

This paper describes a phosphatase that regulates the phosphorylation and function of beta1-integrins. Integrin phosphorylation at two/three conserved threonines has been known for a long time but its functional significance has not been entirely clear. In this paper, the authors describe that beta1-integrin phosphorylation regulates the association of talin/kindlin (two known integrin activators) versus filamin A (a known integrin negative regulator). Talin/filamin competition, and FilaminA being regulated by phosphorylation are not new findings, but this paper present new evidence for the regulation of these interactions by phosphorylation in cells, and for the functional significance of integrin phosphorylation for integrin activity regulation. In addition, indirect regulation of kindlin-integrin association phosphorylation is an interesting new finding. The main new finding in this paper is that PPMF1 can function as an integrin phosphatase. Because of these new findings, the paper contributes significantly to the field.

It is recommended that the PPMF1 knockout mouse should be part of the same paper rather than as a separate paper, as the in vivo evidence significantly supports the conclusions of the main paper. The reviewer's concerns have been adequately addressed in the revised version of the paper, although some minor points remain to be addressed:

Following the suggestions of the reviewers and in agreement with the editors, we have incorporated the essential information from the second, report-style manuscript into the main paper.

Comment 4. Wennerberg 1998 does not look at effect of TT/DD mutations on cell adhesion, please remove citation from this section.

The reviewer is completely right. We have removed this citation from the sentence in the introduction on page 5, line 99 and on page 8, line 191 (pertaining to the integrin β1 TT/DD mutation), while we have cited the work by Wennerberg et al. (1998) appropriately, when the integrin β1 TT/AA mutation is mentioned (see e.g. page 5, line 100).

Comment 6. The answer to this comment is highly speculative, and no experimental data is provided to back up the hypothesis that the wt construct would be phosphorylated at a high stoichiometry. It is recommended that the speculative text concerning this (page 18, line 518) is removed from the paper, it would be better to state that it is unclear why filamin does not associate with the wt construct.

As the reviewer correctly states, this is a speculative statement, which we provided as an answer to the reviewers question and which we included in the discussion section of the revised manuscript. However, we believe that our speculation is not completely unfounded. Indeed, overexpression of wildtype PPM1F (but not overexpression of the phosphatase inactive PPM1F D360A) in this context results in filaminA recruitment to the clustered wildtype integrin β1 chimera. Therefore, we would like to keep this potential explanation in the discussion section and have rephrased this paragraph on page 19, line 572 to read:

"Interestingly, clustering of the wildtype integrin β1 chimera in intact cells, a situation mimicking the unclasped integrin, does not lead to filaminA recruitment. This finding could indicate that the threonine motif is mainly phosphorylated, when the integrin β subunit is separated from the integrin α subunit. Intriguingly, upon overexpression of PPM1F, but not PPM1F D360A, filaminA accumulates at the wildtype integrin β1 cytoplasmic tail. This observation suggests that the activity of the overexpressed phosphatase can override a potential default phosphorylation of the threonine motif in the unclasped integrin β subunit to allow filaminA binding. It is interesting to speculate that a default phosphorylation of the conserved threonine motif in the isolated wildtype integrin β1 tail would not only promote displacement of the negative regulator filamin, but it would also prohibit kindlin2 from driving integrin inside-out signaling in the absence of talin. This scenario is in line with the observation that kindlin overexpression does not lead to integrin inside out activation (Ma et al., 2008; Harburger et al., 2009; Ye et al., 2010; Li et al., 2017)."

Reviewer #2 (Comments to the Authors (Required)):
This is a very interesting study and the authors have addressed nicely all my concerns with explanations and new experimentation. However, it is unclear what prompted the authors to split the paper into two. This made re-reviewing particularly difficult and diminishes the impact. The authors should be encouraged to merge these into one strong JCB paper.

Following the suggestions of the reviewers and in agreement with the editors, we have incorporated the essential information from the second, report-style manuscript into the main paper.

Reviewer #3 (Comments to the Authors (Required)):

The authors have been very responsive to my previous concerns and have greatly improved their manuscript. The addition of data on kindlin is notable and of considerable interest. In addressing the reviewer concerns, an already large manuscript became very large and they therefore chose to split their manuscript into two. Whether this is acceptable for JCB should be an editorial decision. However, the first manuscript (A) reads very well as a stand-alone document and it is difficult to see how the second manuscript can be readily incorporated into the first without loss of some of the detailed and helpful explanation and discussion included in each manuscript.

On its own, the second manuscript (B), while providing a description of the PPM1F knockout phenotype, lacks the mechanistic insight normally associated with JCB. It confirms findings in the first paper and shows that whole animal results are consistent with the conclusions of the first manuscript.

In manuscript B the discussion sections dealing with the T788A/T7809A knock-in mice is a little confusing. The authors note the similar stages of death of this mouse with the PPM1F mouse - but these mice mimic different conditions, the T/A mutant mimics the unphosphorylated form while presumably in the absence of the phosphatase the integrin is shifted towards the phosphorylated form. A T/D mutant mouse might be a more relevant comparison. The extensive discussion of the neuronal phenotypes and comparison with neuronal phenotypes associated with loss of filamin A is interesting but there are little data directly linking this to effects on integrin binding.

Following the suggestions of the reviewers and in agreement with the editors, we have incorporated the essential information from the second, report-style manuscript into the main paper. Unfortunately and as indicated by the reviewer, a significant amount of data and text presented in the Report-style manuscript could not be included into the main, Article-style manuscript. We will try to detail these aspects in a separate and more detailed analysis of the PPM1F-knock-out phenotype in a future manuscript.

Minor comments:

First manuscript: (A) Lines 280-282 "Quantification of multiple blots demonstrated....(Fig 5D)" Fig 5D did not show this quantification

The quantification of multiple blots was displayed in Figure 5D as a bar graph below the panel showing a representative Western blot. As this might have been misleading, we have now moved the respective bar graph in Figure 5D to the right hand side of the Western Blot. We hope that this re-arrangement of Figure 5, panel D improves this figure.