



A kindlin-3-leupaxin-paxillin signaling pathway regulates podosome stability

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April 15, 2019

Re: JCB manuscript #201903109

Dr. Markus Moser
Max-Planck-Institute of Biochemistry
Am Klopferspitz 18
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Dear Dr. Moser,

Thank you for submitting your manuscript entitled "A kindlin-3-leupaxin-paxillin signaling pathway regulates podosome stability". 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.

You will see that the reviewers are all interested in the new insight provided into the regulation of Kindlin3 and podosome stability by leupaxin and paxillin. However, the reviewers provide some recommendations for additional work to bolster the main claims that we agree are necessary for resubmission, particularly those raised by Reviewer #1 and #2 about the quality of imaging, statistical analysis and confirmation of a functional outcome of altered podosome stability. Although we agree that more detail about the changes to podosome structure suggested by Reviewer #3 would be interesting, the live imaging approaches suggested are likely beyond the scope of the current study and changes to degradative activity could perhaps be assayed by other methods.

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.

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Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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

Sincerely,

Anna Huttenlocher, M.D.
Editor

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

Journal of Cell Biology

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

Podosomes are specialized adhesion structures composed of an actin core and a rim of many of the same adhesion components that are found in focal adhesions. These dynamic structures mediate matrix degradation and invasion among other functions. In the submitted study the Moser-lab has investigated the role of the hematopoietic specific integrin activator, kindlin-3, in assembly and dynamics of podosomes. They provide convincing biochemistry to show that kindlin-3 associates with a paxillin family member leupaxillin. The interaction requires the M3 cluster of the F0 domain of kindlin-3 and an intact Zn-coordinated LIM3 domain of leupaxillin. In addition, the find that kindlin recruited leupaxillin regulates the phosphatase activity of PTP-PEST, a known interactor of leupaxillin and paxillin, and thereby controls PTP-PEST mediated paxillin phosphorylation and podosome turnover.

The biochemistry is convincing and the authors nicely take advantage of all the established mutants described for the various components studied. In contrast, the imaging is not of sufficient quality for JCB. The images are often overexposed, the magnification is too small to make out clearly individual podosome rings, the actin displayed is rather challenging to see, the dotted lines in the figures are not explained. Many of the key conclusions in the manuscript are based on imaging so this is a major limitation of this study.

- The podosome life time experiments lack statistics 1F, 4D,4E,4H, 7F

- Figure 4. The pPaxillin data based on the imaging is not the most convincing. Why was paxliin

phosphorylation not studied by western blot (as in figure 8) to support these claims? PY118 site should be investigated as well.

- The text mentions that vanadate decreases podosome lifetime to a similar extent as in K3n/- cells (Figure H), but this figure does not include those cells.

- Figure 5, from the image quality in 5F it is hard to say where the PTP-PEST mutants localize. Is this identical to the wt PTP-PEST?

- on page 10 the authors indicate that both kindling-3 and M3 mutant rescue podosome formation. This is not clear from the images (Fig. 6) and the "podosome rescue" was not quantified. The line scans in 6D looks nice by they are hard to relate to the images shown. It is also hard to imagine how it is possible to define "podosomes with distinct kindling-3 localisation" from the images (Figure 6E).

- The usage of the statistics seems inappropriate. T-test is not suitable for small sample sizes such as 4 or 5 as it is not possible to test these for normal distribution. For example - Mann-Whitney U-test should be used. When t-test is used for multiple comparisons the p-value (In Fig. 5G must be corrected for multiple comparisons (by multiplying p with the n of comparisons). In addition, also here the n-number is too low for a t-test.

- Could the authors investigate the functional significance/outcome of the podosome stability regulated by the complex they define here? This would increase the cell biology relevance of their work greatly.

Minor:

- Figure 2 would benefit from inclusion of a cartoon of the kindlin constructs/domains as well

- typos in y-axis word "fluorescence"

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

The study done by Sarah Klapproth et al. investigated how kindlin-3 regulates podosome stability and revealed that kindlin-3 is essential for podosome formation and regulates the turnover and lifetime of podosomes in myeloid cells by recruiting leupaxin to the adhesion complex to control paxillin phosphorylation. It is an interesting story. The work is in generally well-done and provides sufficient data to support the conclusion. However, some modifications should be completed before publication.

1. The authors state that the direct binding of Leupaxin and Kindlin-3 is shown in Fig 2B. But Co-IP assay cannot prove the direct binding. Leupaxin and Kindlin-3 proteins should be expressed and purified from *E. coli*, and pull-down assays should be performed to detect the direct binding of the two proteins in vitro. This is an important experiment.

2. From the gel images in Fig 2E, besides the F0 domain interacts with leupaxin, F1 and F2 also bound leupaxin weakly. Furthermore, F0+1 and F0-2 display much stronger binding compare to F0 domain alone, which suggests that F1 and F2 are required for F0 domain interaction with leupaxin. This should be described and discussed in the results. It is might be better that the domain structure of kindlin-3 is also shown in Fig 2A for easy referring.

3. In Fig 3, a slight reduction of Leupaxin in total cellular leupaxin levels to approximately 70% was observed in K3n/-cells. However, the level of leupaxin did not change at all in all analyzed clones of Kindlin-3-deficient RAW cells. Why? Does kindlin-3 harbors a role on the regulation of leupaxin level?

4. Please show the fluorescence imaging data of p-Paxillin Y118.

5. All of the data of p-Paxillin are shown in the form of fluorescence imaging. P-Paxillin expression needs to be determined by Western blot using anti-p-Paxillin antibody in some key experiments.

6. To support the statement that low kindlin-3 expression results in impaired leupaxin podosomal targeting and the increased paxillin phosphorylation, rescue experiments need be performed. Leupaxin should be transfected into K3n/-cells and then paxillin phosphorylation is detected.

7. To support PTP-PEST, Paxillin, Leupaxin and Kindlin-3 are in a complex, the results in Figure 5A are not enough. The reciprocal interaction experiments need to be performed by using different anti-PTP-PEST, Paxillin, Leupaxin separately. In fact, sequential co-IP for these proteins is necessary to show them in a molecular complex.
8. It is interesting that whether kindlin-2 is involved in this molecular complex?
9. Whether the cell motility is altered in the K3n^{-/-} cells, leupaxin null cells, paxillin null cells and leupaxin double knockout cells?
10. The authors stated that low expression of kindlin-3 causes a decrease in the number of podosome, and that the role of Kindlin-3 is mainly exerted by leupaxin in the regulation of leupaxin. However, in Figure 4 A, B, why low expression of leupaxin does not cause a change in podosome?
11. In Figure 5 E, it is interesting to know that whether loss of leupaxin affect the level of PTP-PEST?

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

The manuscript of Klapporth et al. investigates the signaling pathways downstream of the integrin regulator Kindlin-3 in podosomes of pre-OCL cells (OsteoCLast, myeloid lineage). Through the use of knock-out mouse models, hypomorph mouse models and numerous CRISPR models in the well-established RAW cell line, the authors identified that kindlin 3 binds leupaxin, a paxillin family member, that regulates the activity of the phosphatase PTP-PEST in order to control phosphorylation of its binding partner paxillin. These data described that leupaxin is controlling a negative feedback loop downstream of kindlin 3.

The manuscript is extremely interesting, high quality and highlight the complex roles of kindlin3 and the paxillin family members in podosomes from myeloid lineage. The data quality is really impressive and support strongly the proposed models. However, some functional evaluations of podosome activity are missing and some points should be precise in order to reinforce the message of the authors.

Major points:

1-I am quite surprise by the following sentence « We have previously shown that kindlin-3 deficient cells fail to assemble podosomes because of their inability to activate, cluster and recruit integrins (Schmidt et al., 2011) » since this paper clearly shows that podosomes (especially cores) are clearly formed in kindlin3^{-/-} OCLs. Moreover, kindlin3^{-/-} OCLs in Fig.6 are clearly forming podosome clusters. Only metaorganization and level of actin cloud of podosome is affected. I did not find any data measuring podosome life-span in Kindlin3^{-/-} OCLs in order to compare it to the hypomorph mouse model used in this manuscript.

2-Fig.1: The manuscript will have greatly benefit of analyzing degradation activity of the observed podosomes in kindling3 n^{-/-} and RAW paxillin^{-/-}/leupaxin^{-/-} cells. Moreover, it will have been also extremely interesting to quantify the average diameter of podosome cores (as in Fig.7). It seems to me that kindlin3 n^{-/-} model present a clear decrease of the podosome core without changing intensity of surrounding cloud (as indicated by the identical surface of podosomes clusters). This probably indicates a defect in mechanical activity of podosomes. In order to answer this point without using delicate technic such as protrusion microscopy or tension FRET probes, could the authors analyze podosome cores oscillations in kindlin3 ^{+/+}, n^{-/-} cells and RAW paxillin^{-/-}/leupaxin^{-/-} ?

3-Fig.2: The large increase in leupaxin binding of Kindlin F0-2 mutant is poorly explained in the result section. This is a very important data that should be more comment. Is it possible to imagine

another inhibitory mechanism through its PH domains? Does it mean that there is a competition between integrins and leupaxin for kindlin3 binding?

3- Fig.3: I have the feeling that there is decrease of paxillin recruitment in kindlin3 n/- cells (Fig.3B). This is not consistent with author's comments: «A striking observation of our study was that in contrast to leupaxin, paxillin is normally targeted to podosomes at very low kindlin-3 levels.»

4- Fig.5E: Could the authors comment if they think that there is a competition between paxillin and leupaxin for PTP-PEST? This will not be consistent with results in Fig.7E and G. This is especially important since the manuscript rather suppose a direct activation of PTP-PEST through presence of leupaxin. This mechanism is poorly comment in the discussion and could involve other kinases binding to leupaxin.

5- The fact that Hic-5 expression seems not affected by western-blot is extremely interesting. To reinforce this point, could the authors follow Hic-5 mRNA by qPCR and see how it is affected by both paxillin and leupaxin loss? Moreover, it will be extremely interesting to express GFP-Hic-5 in RAW paxillin-/-/leupaxin-/- cells and test its ability to rescue podosome phenotypes. This will help to understand the differential sensitivity of SRC-dependent invadosome and invadopodia to paxillin and Hic-5 (Pignatelli et al., 2012; Petropoulos et al., 2016).

6-Fig.8B: I am quite surprised by the comment of the authors on the absence of phenotype on podosome structures in RAW paxillin-/-/leupaxin-/- cells. The image clearly indicates a strong reduction of the actin cloud surrounding the core. Numerous data suggest that leupaxin and paxillin regulates the equilibrium between podosome cores and actin cloud. This could indicate a change of mechanical properties of podosomes in this condition. Moreover, it will be extremely interesting to have a measurement of the podosome life-span in these doubleKo cells.

7-Fig.8G: The differential sensitivity between FAK and Pyk2 autophosphorylation in absence of leupaxin could suggest that leupaxin could be involved in calcium response. Indeed, autophosphorylation of Pyk2 was reported as calcium sensitive, not for FAK. The relationship between leupaxin and calcium response could then lead to consider its relationship with calpains. Could the authors rescue leupaxin-/- or kindlin3-/- phenotypes on podosome life-span by calpains inhibitors (Calle et al., 2006)?

8-Fig.9: Could the authors change their model to show different integrins that bind differently paxillin and leupaxin in order to reinforce the fact that leupaxin binds kindlin3 not attached to integrins.

9-Discussion: Could the authors comments the possible stimuli and signaling pathways that control kindlin3-leupaxin binding? Is it a constitutive binding or is modulated by specific microenvironments?

Minor points:

Fig.2B: could the authors comment the switch of kindlin3 size in the total lysates versus IP. Is it an indication of cleavage or dephosphorylation?

Fig.4F: the level of phosphorylated-paxillin seems quite high in comparison to the change quantified in the associated graph. Could the authors comment this point?

Fig.5F: Could the authors quantify phosphorylated-paxillin level in Fig.5F?

1st Revision - Authors' Response to Reviewers: July 8, 2019

Dear Dr. Huttenlocher,

Thank you for reviewing our manuscript entitled, "A kindlin-3-leupaxin-paxillin signaling pathway regulates podosome stability" (Ms# 201903109) and for being willing to re-consider a revised manuscript. We would also like to thank the reviewers for their positive evaluation and constructive criticism. We carried out a series of additional experiments and were able to address all points raised by the reviewers. The additional experiments have substantially improved the manuscript.

Please find a point-by-point response to the criticism raised by the three reviewers below.

We hope that our manuscript is now acceptable for publication in *Journal of Cell Biology* and look forward to hearing from you.

Sincerely,

Markus Moser

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

Podosomes are specialized adhesion structures composed of an actin core and a rim of many of the same adhesion components that are found in focal adhesions. These dynamic structures mediate matrix degradation and invasion among other functions. In the submitted study the Moser-lab has investigated the role of the hematopoietic specific integrin activator, kindlin-3, in assembly and dynamics of podosomes. They provide convincing biochemistry to show that kindlin-3 associates with a paxillin family member leupaxillin. The interaction requires the M3 cluster of the F0 domain of kindlin-3 and an intact Zn-coordinated LIM3 domain of leupaxillin. In addition, they find that kindlin recruited leupaxillin regulates the phosphatase activity of PTP-PEST, a known interactor of leupaxillin and paxillin, and thereby controls PTP-PEST mediated paxillin phosphorylation and podosome turnover.

The biochemistry is convincing and the authors nicely take advantage of all the established mutants described for the various components studied. In contrast, the imaging is not of sufficient quality for JCB. The images are often overexposed, the magnification is too small to make out clearly individual podosome rings, the actin displayed is rather challenging to see, the dotted lines in the figures are not explained. Many of the key conclusions in the manuscript are based on imaging so this is a major limitation of this study.

It is true that many of the conclusions made are based on imaging, thus we carefully selected those images, which represent our findings. Since some of the images may not show sufficient magnifications, we now show higher magnifications for Figures 4A, 5B and 7B. We hope that the individual substructures of podosomes are better visible now. The reviewer is also right in terms of the actin signal, which is shown in blue and often only weakly visible on a black background. We therefore show the actin channel in “white” now and leave actin in “blue” in the merged images. We also apologize that we indeed forgot to mention the meaning of the dotted lines. They mark the cell borders, which is now also mentioned in the figure legends. However, we disagree with the statement that images are often overexposed. All images, which are directly compared, were taken with the same microscopical settings and processing. Consequently, some pictures are brighter than others, showing biological variance. Measurements were done on unmodified raw pictures. In Figure 6A we changed the modifications to get less bright pictures.

- The podosome life time experiments lack statistics 1F, 4D, 4E, 4H, 7F

Statistics for the podosome life times are now included. We determined the time, at which 50% of the observed podosomes had disappeared. A mean value was determined for all analysed cells within one cell culture dish and considered as one data point. These values from multiple plates were subjected to statistical analyses, assuming normal distribution. An unpaired Student's *t* test was applied to compare two data sets. A One-way ANOVA followed by a Sidak's multiple comparison test was performed to compare three or more data sets.

- Figure 4. The pPaxillin data based on the imaging is not the most convincing. Why was paxillin phosphorylation not studied by western blot (as in figure 8) to support these claims? PY118 site should be investigated as well.

We now provide immunofluorescence stainings for phospho-Paxillin Y118, which also show increased signals in K3n^{-/-} cells and Leupaxin^{-/-} RAW cells. These data are shown in new Supplementary Figure S2A and C.

In addition, we performed adhesion signaling experiments with leupaxin^{-/-} cells, equivalent to the ones shown in Figure 8H and analysed pPaxillin Y31 and Y118 levels by Western blotting. These experiments are in line with the immunofluorescence stainings and show that phosphorylation of both tyrosines is strongly increased in mutant cells as shown now in Supplementary Figure S2B.

Although individual podosome clusters of K3n^{-/-} cells show increased paxillin phosphorylation (Figure 4F; Supplemental Figure S2C), Western blot analyses of total cell lysates do not show increased paxillin phosphorylation, because only a reduced number of cells form adhesion structures due to reduced integrin signaling (data not shown).

- The text mentions that vanadate decreases podosome lifetime to a similar extent as in K3n^{-/-} cells (Figure H), but this figure does not include those cells.

The lifetime measurements of vanadate treated wild-type cells were done in the same series of experiments, in which we measured the podosome lifetime of K3n^{-/-} and wildtype control cells (w/o paxillin wt/2YF expression). We initially added the lifetime measurements of vanadate treated wildtype cells to Figure 4H, since expression of wt paxillin did not change the podosome lifetime in K3n^{-/-} cells. We now show it in Figure 1G, which is more correct.

- Figure 5, from the image quality in 5F it is hard to say where the PTP-PEST mutants localize. Is this identical to the wt PTP-PEST?

We had difficulties to express PTP-PEST in primary pre-osteoclasts and had to use lentiviral instead of retroviral transduction. Still only few cells were transduced and the expression levels were rather low. Nevertheless, expression and localization of wild-type and mutant PTP-PEST EGFP were comparable, as shown in the Figure 1 for the reviewer.

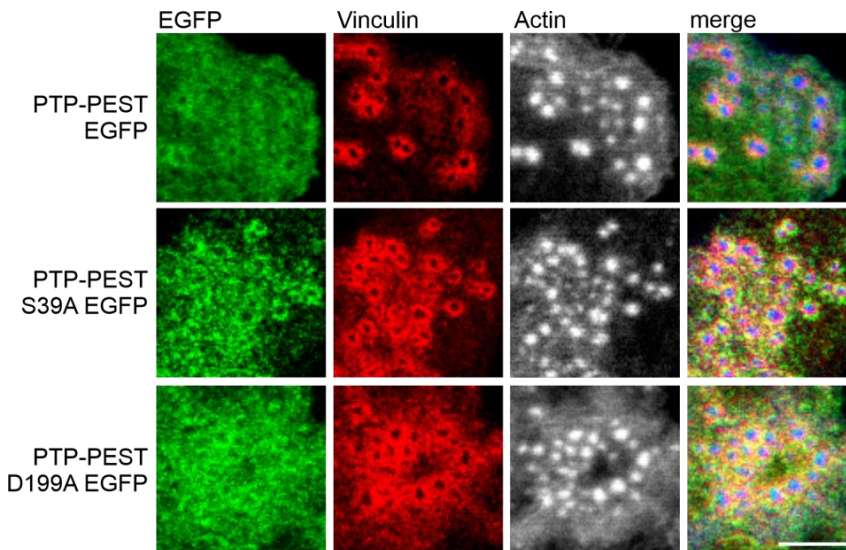


Figure 1 for the reviewer: Wild-type pre-osteoclasts were lentivirally transduced with wild-type PTP-PEST EGFP, PTP-PEST S39A EGFP or PTP-PEST D199A EGFP and subjected to immunofluorescence staining for vinculin (red) and actin (white/ in merge blue). Scale bar 5 μ m.

- on page 10 the authors indicate that both kindlin-3 and M3 mutant rescue podosome formation. This is not clear from the images (Fig. 6) and the "podosome rescue" was not

quantified. The line scans in 6D looks nice by they are hard to relate to the images shown. It is also hard to imagine how it is possible to define "podosomes with distinct kindlin-3 localisation" from the images (Figure 6E).

Thank you for this comment. We now show higher magnifications of podosomal clusters in Figure 6A and quantified "podosome rescue" by measuring podosome core size of K3^{-/-} cells transduced with EGFP, EGFP-K3 and EGFP-K3 M3. These data are shown in new Figure 6B. In addition, we now show high magnifications of the three podosomes next to the fluorescence intensity profiles that were selected for the line scans in Figure 6A in new Figure 6E.

Figure 2 for the reviewer shows two representative examples of cells with "distinct" and "diffuse" EGFP-K3 M3 localization within podosome clusters. The person evaluating the cells was blinded to the kindlin-3 variant and assigned each transduced and podosome forming cell to one of these two categories.

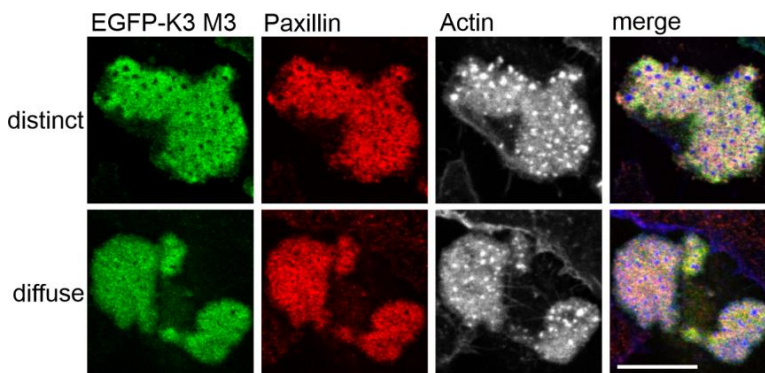


Figure 2 for the reviewer: Immunofluorescence staining and confocal imaging of K3^{-/-} pre-osteoclasts retrovirally transduced with the EGFP-kindlin-3 M3 mutant (green) for paxillin (red), and actin (white/ in merge blue). Scale bars 5 μ m.

- The usage of the statistics seems inappropriate. T-test is not suitable for small sample sizes such as 4 or 5 as it is not possible to test these for normal distribution. For example - Mann-Whitney U-test should be used. When t-test is used for multiple comparisons the p-value (In Fig. 5G must be corrected for multiple comparisons (by multiplying p with the n of comparisons). In addition, also here the n-number is too low for a t-test.

This is an important point to be clarified. We therefore contacted Dr. Tobias Straub, head of the bioinformatics core facility at the Bio Medical Center at the Ludwigs-Maximilian-University in Munich and an expert in statistics (he gives lectures on statistical analyses for scientists). We performed our statistics now according to his recommendations.

In his opinion, small sample sizes do not exclude t-tests. The t-test requires normal distribution of measurement values in the population, from which the sample has been taken. Therefore, small N samples might well not exhibit normal distribution and still meet t-test assumptions. Accordingly, formal testing of normality on samples with $n \ll 20$ cannot provide a criterion whether or not a t-test can be applied. As most biological experiments deal with very small sample sizes the main decision for a t-test requires prior knowledge on the population. This is given for example in the case of gene expression measurement by fluorescence intensity measurements. Here, a log-normal distribution can be assumed based on the plethora of data collected through the years.

For the comparison of cells that formed podosomes and which was analysed in Figure 1D, a normal distribution was not assumed. Therefore a Mann Whitney test was performed.

Multiple comparisons were done using the software Graphpad Prism. To evaluate three or more datasets, one-way ANOVA was performed followed by a Tukey's multiple comparison test or a Sidak's multiple comparison test, as suggested by the GraphPad PRISM software.

As a simple reference to these claims Dr. Straub suggested the book "Intuitive Biostatistics" by Harvey Motulsky.

- Could the authors investigate the functional significance/outcome of the podosome stability regulated by the complex they define here? This would increase the cell biology relevance of their work greatly.

As podosomes are involved in cell migration, transmigration and matrix degradation we investigated these properties in the various genetically modified RAW cell lines. We measured a stepwise reduction in gelatin degradation of leupaxin, paxillin, double leupaxin/paxillin or kindlin-3 deficient RAW cells. On the contrary, transwell assays revealed increased migration of leupaxin null cells and reduced transmigration of paxillin, leupaxin/paxillin and kindlin-3 deficient cells. These data are now shown in new Figure 9.

Minor:

- Figure 2 would benefit from inclusion of a cartoon of the kindlin constructs/domains as well

Thank you for this suggestion. A cartoon of the kindlin-3 domain structure including the position of some point mutations is now shown in Figure 2D.

- typos in y-axis word "fluorescence"

Thank you – we corrected these spelling mistakes.

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

The study done by Sarah Klapproth et al. investigated how kindlin-3 regulates podosome stability and revealed that kindlin-3 is essential for podosome formation and regulates the turnover and lifetime of podosomes in myeloid cells by recruiting leupaxin to the adhesion complex to control paxillin phosphorylation. It is an interesting story. The work is in generally well-done and provides sufficient data to support the conclusion. However, some modifications should be completed before publication.

1. The authors state that the direct binding of Leupaxin and Kindlin-3 is shown in Fig 2B. But Co-IP assay cannot prove the direct binding. Leupaxin and Kindlin-3 proteins should be expressed and purified from E. coli, and pull-down assays should be performed to detect the direct binding of the two proteins in vitro. This is an important experiment.

Following the reviewer's suggestion, we recombinantly expressed full-length leupaxin as well as the N-terminal LD and C-terminal LIM domains of leupaxin as GST-fusion proteins. The kindlin-3 F0 domain was expressed with a HisSumo-tag. The GST-pulldown experiments, which confirmed direct interaction of the kindlin-3 F0 domain with the C-terminal LIM-domains is shown in new Figure 2J.

2. From the gel images in Fig 2E, besides the F0 domain interacts with leupaxin, F1 and F2 also bound leupaxin weakly. Furthermore, F0+1 and F0-2 display much stronger binding compare to F0 domain alone, which suggests that F1 and F2 are required for F0 domain interaction with leupaxin. This should be described and discussed in the results. It is might be better that the domain structure of kindlin-3 is also shown in Fig 2A for easy referring.

As suggested also by reviewer 1 we show the domain structure of kindlin-3 and specify the point mutants used in this study in Figure 2D.

Indeed, our data suggest that the presence of the F1 and F2 domains support interaction of the F0 domain with leupaxin. We now emphasize and discuss this observation in the results part on page 6.

The reasons why we are convinced that the F1 and F2 domains are not directly involved in leupaxin binding are: (i) the F1, F2, F1F2 and the F1-3 domains interact with leupaxin not above background levels, (ii) the leupaxin homolog paxillin interacts via the F0 domain of kindlin-2 (Boettcher et al., 2017; Gao et al., 2017), and (iv) most importantly the kindlin-3 F0M3 mutation abolishes leupaxin binding completely (see Figure 2G).

3. In Fig 3, a slight reduction of Leupaxin in total cellular leupaxin levels to approximately 70% was observed in K3n/-cells. However, the level of leupaxin did not change at all in all analyzed clones of Kindlin-3-deficient RAW cells. Why? Does kindlin-3 harbors a role on the regulation of leupaxin level?

The reviewer is right. We do not know why primary macrophages from K3n/- mice show reduced leupaxin levels and RAW cell lines, in which we deleted kindlin-3, show normal leupaxin levels. It seems that the two different cell types, primary cells vs. cell line, behave differently. The qPCR studies on RAW cells at least show that leupaxin mRNA levels are not elevated in the absence of kindlin-3. This is shown in new Supplemental Figure S3.

4. Please show the fluorescence imaging data of p-Paxillin Y118.

Immunofluorescence stainings for pPaxillin Y118 in podosomal clusters of K3n/- cells and Leupaxin/- RAW cells are shown in new Supplementary Figure S2A,C.

5. All of the data of p-Paxillin are shown in the form of fluorescence imaging. P-Paxillin expression needs to be determined by Western blot using anti-p-Paxillin antibody in some key experiments.

Please also see the answer to reviewer 1. In the initial manuscript we showed an adhesion signaling experiment in Figure 8H, in which we analysed control, paxillin/-, leupaxin/-, K3/- and paxillin/leupaxin double knockout RAW cells for their phosphorylation status by Western blotting. We repeated the experiment with control and leupaxin/- RAW cells and analysed paxillin phosphorylation at Y31 and Y118 (see new Supplemental Figure S2B).

As explained above, we did not analyse paxillin phosphorylation of K3n/- cells by Western blotting simply because only a fraction of K3n/- cells form adhesion structures with increased phospho-paxillin levels. Consequently, paxillin phosphorylation is not increased in total cell lysates.

6. To support the statement that low kindlin-3 expression results in impaired leupaxin podosomal targeting and the increased paxillin phosphorylation, rescue experiments need be performed. Leupaxin should be transfected into K3n/-cells and then paxillin phosphorylation is detected.

Thank you for this suggestion. We overexpressed leupaxin in K3n/- cells and although we can force leupaxin targeting into podosomes even at low kindlin-3 levels, phosphopaxillin levels did not decrease. This suggests that leupaxin requires kindlin-3 within the adhesion complex to reduce phospho-paxillin levels. These data are now shown as Supplemental Figure S2D,E.

7. To support PTP-PEST, Paxillin, Leupaxin and Kindlin-3 are in a complex, the results in Figure 5A are not enough. The reciprocal interaction experiments need to be performed by using different anti-PTP-PEST, Paxillin, Leupaxin separately. In fact, sequential co-IP for these proteins is necessary to show them in a molecular complex.

We agree with the reviewer that the IPs shown in Figure 5A and 5E with anti-EGFP-Kindlin3 and anti-Paxillin antibodies are no direct proofs for a protein complex. We also performed an anti-EGFP-Leupaxin IP, which confirmed the association of these proteins (see Figure 5F). As suggested we tried a sequential co-IP, which unfortunately did not work. We therefore down-tuned our conclusion from “in complex” to “in association” on page 9.

8. It is interesting that whether kindlin-2 is involved in this molecular complex?

Kindlin-2 is not expressed in the hematopoietic system and is not upregulated upon loss of kindlin-3 expression (Ussar et al., 2006; Schmidt et al 2011). Consistently, Kindlin-2 protein was not detected in the proteome analyses of RAW cells.

9. Whether the cell motility is altered in the K3n/-cells, leupaxin null cells, paxillin null cells and leupaxin double knockout cells?

To address this question, we performed transwell assays with RAW cells. The results are shown in Figure 9. Interestingly, while paxillin deficient cells and kindlin-3 knockout cells exhibited reduced cell migration, leupaxin deficiency resulted in increased motility.

10. The authors stated that low expression of kindlin-3 causes a decrease in the number of podosome, and that the role of Kindlin-3 is mainly exerted by leupaxin in the regulation of leupaxin. However, in Figure 4 A, B, why low expression of leupaxin does not cause a change in podosome?

This is a misunderstanding. We do not claim that the role of kindlin-3 is mainly exerted by leupaxin. Crucial functions of kindlin-3 are to bind, cluster and activate integrins during integrin inside-out signaling. These functions are significantly impaired at low kindlin-3 levels (Klapproth et al., 2015) and are probably the main reasons for the decrease in podosome-forming cells and the reduced podosome core size. So far the role of kindlin-3 in integrin signaling into the cell (integrin outside-in signaling) remained unclear. Our study revealed a new kindlin-3/leupaxin/paxillin signaling pathway, which is involved in regulating the turnover of the adhesion complex, however, we are convinced that kindlin-3 participates also in other signaling pathways, for instance by interacting with the adapter protein ILK (Huet-Calderwood et al., 2014; Fukuda et al., 2014; Kadry et al., 2018).

11. In Figure 5 E, it is interesting to know that whether loss of leupaxin affect the level of PTP-PEST?

Although the IP loading control shown in Figure 5E might suggest a slight reduction in PTP-PEST expression in leupaxin-null cells, Western blot analyses on control and leupaxin deficient RAW cell clones revealed no difference in PTP-PEST expression (see Figure 2H).

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

The manuscript of Klapporth et al. investigates the signaling pathways downstream of the integrin regulator Kindlin-3 in podosomes of pre-OCL cells (OsteoCLast, myeloid lineage). Through the use of knock-out mouse models, hypomorph mouse models and numerous CRISPR models in the well-established RAW cell line, the authors identified that kindlin 3 binds leupaxin, a paxillin family member, that regulates the activity of the phosphatase PTP-PEST in order to control phosphorylation of its binding partner paxillin. These data described that leupaxin is controlling a negative feedback loop downstream of kindlin 3.

The manuscript is extremely interesting, high quality and highlight the complex roles of kindlin3 and the paxillin family members in podosomes from myeloid lineage. The data quality is really impressive and support strongly the proposed models. However, some functional evaluations of podosome activity are missing and some points should be precise in order to reinforce the message of the authors.

Major points:

1-I am quite surprise by the following sentence « We have previously shown that kindlin-3 deficient cells fail to assemble podosomes because of their inability to activate, cluster and recruit integrins (Schmidt et al., 2011) » since this paper clearly shows that podosomes (especially cores) are clearly formed in kindlin3^{-/-} OCLs. Moreover, kindlin3^{-/-} OCLs in Fig.6 are

clearly forming podosome clusters. Only metaorganization and level of actin cloud of podosome is affected. I did not find any data measuring podosome life-span in Kindlin3^{-/-} OCLs in order to compare it to the hypomorph mouse model used in this manuscript.

We apologize for not being clear at this point. It is a matter of definition. Kindlin-3 deficient cells form adhesion patches that contain actin cores and resemble podosome clusters (as shown in Figure 6 and Supplemental Figure 2). However, integrins do not surround these actin cores and we therefore think that these structures are immature and are not properly assembled.

The number of actin core forming K3^{-/-} pre-osteoclasts is very low and therefore quantification of actin core life time is hardly feasible.

2-Fig.1: The manuscript will have greatly benefit of analyzing degradation activity of the observed podosomes in kindlin3^{n/-} and RAW paxillin^{-/-}/leupaxin^{-/-} cells. Moreover, it will have been also extremely interesting to quantify the average diameter of podosome cores (as in Fig.7). It seems to me that kindlin3^{n/-} model present a clear decrease of the podosome core without changing intensity of surrounding cloud (as indicated by the identical surface of podosomes clusters). This probably indicates a defect in mechanical activity of podosomes. In order to answer this point without using delicate technic such as protrusion microscopy or tension FRET probes, could the authors analyze podosome cores oscillations in kindlin3^{+/+}, ^{n/-} cells and RAW paxillin^{-/-}/leupaxin^{-/-}?

As suggested by the reviewer, we performed gelatin degradation assays. The results are shown in Figure 9A,B. In addition, the actin core diameter of paxillin/leupaxin double knockout cells is shown in Figure 8D. We added the actin core diameters of K3n^{-/-} cells in Figure 1C and K3^{-/-} cells in Figure 6B.

We agree that the podosomes of K3n^{-/-} and paxillin/leupaxin double-null RAW cells may have differences in their mechanical activity compared to controls. As said by the reviewer, these measurements are delicate and need to be conducted by absolute experts. We therefore believe that this is beyond the scope of the study.

3-Fig.2: The large increase in leupaxin binding of Kindlin F0-2 mutant is poorly explained in the result section. This is a very important data that should be more comment. Is it possible to imagine another inhibitory mechanism through its PH domains? Does it mean that there is a competition between integrins and leupaxin for kindlin3 binding?

Please see also our response to reviewer 2. We inserted a sentence within the results section, in which we comment on this finding (see page 6). Moreover, we speculate in the discussion that the kindlin-3 F3 domain may exert an inhibitory effect on leupaxin binding by steric hinderance (see page 13).

The kindlin-3 PH domain does probably not interfere with leupaxin binding, as it is also included in the F0-2 mutant. Please see the kindlin-3 domain structure, which we now inserted as Figure 2D.

Our data do not justify an inhibitory effect of leupaxin on kindlin-3/integrin binding. However, our data clearly indicate that the kindlin-3/leupaxin interaction can occur independent of integrins and therefore it is feasible that leupaxin sequesters kindlin-3. In addition, the kindlin-3/leupaxin complex may have other functions than regulating adhesion stability (see page 13 in discussion)

3- Fig.3: I have the feeling that there is decrease of paxillin recruitment in kindlin3^{n/-} cells (Fig.3B). This is not consistent with author's comments: «A striking observation of our study was

that in contrast to leupaxin, paxillin is normally targeted to podosomes at very low kindlin-3 levels.»

We quantified paxillin and vinculin recruitment into podosome clusters of K3n/- cells compared to control cells and found no difference (please see Figure 4G).

4- Fig.5E: Could the authors comment if they think that there is a competition between paxillin and leupaxin for PTP-PEST? This will not be consistent with results in Fig.7E and G. This is especially important since the manuscript rather suppose a direct activation of PTP-PEST through presence of leupaxin. This mechanism is poorly comment in the discussion and could involve other kinases binding to leupaxin.

The facts that more PTP-PEST localizes to podosomes of leupaxin null cells and that PTP-PEST is virtually absent in podosomes of paxillin null cells strongly argues against a competition between paxillin and leupaxin for PTP-PEST. Thus, how leupaxin regulates PTP-PEST activity either by a direct allosteric regulation or indirectly by recruiting a kinase or phosphatase that regulates PTP-PEST activity remains to be shown. We discuss this on page 14 of the discussion section.

5- The fact that Hic-5 expression seems not affected by western-blot is extremely interesting. To reinforce this point, could the authors follow Hic-5 mRNA by qPCR and see how it is affected by both paxillin and leupaxin loss? Moreover, it will be extremely interesting to express GFP-Hic-5 in RAW paxillin-/-/leupaxin-/- cells and test its ability to rescue podosome phenotypes. This will help to understand the differential sensitivity of SRC-dependent invadosome and invadopodia to paxillin and Hic-5 (Pignatelli et al., 2012; Petropoulos et al., 2016).

In Supplemental Figure S4F we examined Hic-5 expression in various RAW cell lines by qPCR and found no induction of its mRNA expression when the other paxillin family members are absent.

As suggested by the reviewer, we also expressed GFP-Hic-5 in paxillin/leupaxin double knockout cells and found that although Hic-5 localizes to the podosomal ring, it does not rescue the actin core size. These data are shown in new Supplemental Figure S4G,H.

6-Fig.8B: I am quite surprised by the comment of the authors on the absence of phenotype on podosome structures in RAW paxillin-/-/leupaxin-/- cells. The image clearly indicates a strong reduction of the actin cloud surrounding the core. Numerous data suggest that leupaxin and paxillin regulates the equilibrium between podosome cores and actin cloud. This could indicate a change of mechanical properties of podosomes in this condition. Moreover, it will be extremely interesting to have a measurement of the podosome life-span in these doubleKo cells.

Like the reviewer we were also very much surprised by the rather mild phenotype of paxillin/leupaxin double knockout cells. However we clearly state that they show smaller actin cores, which we showed in Figure 8D. Nevertheless, the gross morphology of the podosomes are preserved. We thank the reviewer for her/his comment on the equilibrium between podosome core and actin cloud, which seems to be changed in double knockout cells. We discuss this now on page 15.

In addition, we measured the podosome lifetime, which was not further reduced in double knockout compared to paxillin knockout cells and included this data in Figure 8E.

7-Fig.8G: The differential sensitivity between FAK and Pyk2 autophosphoylation in absence of leupaxin could suggest that leupaxin could be involved in calcium response. Indeed,

autophosphorylation of Pyk2 was reported as calcium sensitive, not for FAK. The relationship between leupaxin and calcium response could then lead to consider its relationship with calpains. Could the authors rescue leupaxin^{-/-} or kindlin3^{-/-} phenotypes on podosome life-span by calpains inhibitors (Calle et al., 2006)?

We followed the suggestion of the reviewer and treated control and leupaxin ko RAW cells with the calpain inhibitor ALLN before measuring the podosome lifetime. Actually, our cells did not tolerate ALLN treatment very well. Incubation of wild-type cells with the reported concentration of 50 μ M did not result in increased podosome lifetime, instead podosomes disappeared faster and the cells died within a few hours. We then tried 5 μ M ALLN, which had a similar but less pronounced effect on podosome lifetime and cell viability (Figure 4 for the reviewer).

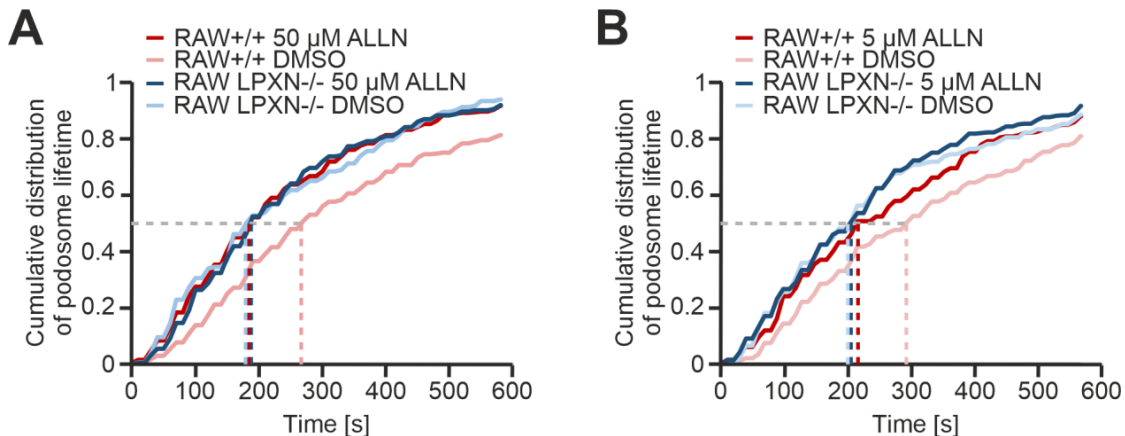


Figure 4 for the reviewer: Podosome lifetime of wild-type and leupaxin^{-/-} RAW cells treated with DMSO, 50 μ M ALLN (A) or 5 μ M ALLN.

8-Fig.9: Could the authors change their model to show different integrins that bind differently paxillin and leupaxin in order to reinforce the fact that leupaxin binds kindlin3 not attached to integrins.

We thank the reviewer for this suggestion and updated our model accordingly. Although our data provide strong evidence for a kindlin-3/leupaxin interaction independent of integrins, we believe that targeting of kindlin-3 and leupaxin to integrin adhesion sites is a prerequisite for controlling paxillin phosphorylation and adhesion site turnover.

9-Discussion: Could the authors comments the possible stimuli and signaling pathways that control kindlin3-leupaxin binding? Is it a constitutive binding or is modulated by specific microenvironments?

This is another very good question. So far, we have no hints, which would support the one or the other possibility. We added a note to the discussion that this will be the aim of further studies.

Minor points:

Fig.2B: could the authors comment the switch of kindlin3 size in the total lysates versus IP. Is it an indication of cleavage or dephosphorylation?

Sorry, the change in size came from incorrect cropping of the image and is not due to cleavage or dephosphorylation. We corrected this mistake.

Fig.4F: the level of phosphorylated-paxillin seems quite high in comparison to the change quantified in the associated graph. Could the authors comment this point?

We share your impression. The fluorescence images suggest much higher differences compared to the fluorescence intensities determined with ImageJ. However, the microscope adjustments were not changed between pictures. The measurements were done on raw images, which were not edited. Picture modifications with Photoshop were performed in the exact same way.

Fig.5F: Could the authors quantify phosphorylated-paxillin level in Fig.5F?

The quantification is shown in Figure 5G.

August 5, 2019

RE: JCB Manuscript #201903109R

Dr. Markus Moser
Max-Planck-Institute of Biochemistry
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Germany

Dear Dr. Moser,

Thank you for contributing your Article entitled "A kindlin-3-leupaxin-paxillin signaling pathway regulates podosome stability". It is a pleasure to let you know that your manuscript is now accepted for publication in Journal of Cell Biology. Congratulations on this interesting work.

Your manuscript will now progress through image editing, copyediting, and proofing. It is journal policy that authors provide original data upon request. You may contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu), with any questions throughout the process.

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Again, congratulations on a very nice paper. I hope you found the review process to be constructive and are pleased with how the manuscript was handled editorially. We look forward to future exciting submissions from your lab.

Anna Huttenlocher, M.D.
Editor

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

Journal of Cell Biology

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

The authors have done a great job in addressing my concerns. I am also grateful to their very informative response regarding the use of statistics.

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

N/A

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

I would thank the authors to have consider and answer my concerns.
Based on their comment of my first concern, I would rather change the sentence "We have previously shown that kindlin-3 deficient cells fail to assemble definitive podosomes" should be replace by "We have previously shown that kindlin-3 deficient cells fail to assemble MATURE podosomes".