Ubiquitous Membrane-bound DNase Activity in Podosomes and Invadopodia

Kaushik Pal, Yuanchang Zhao, Yongliang Wang, and Xuefeng Wang

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September 5, 2020

Re: JCB manuscript #202008079

Prof. Xuefeng Wang
Iowa State University
12 Physics Hall
Ames, IA 50011

Dear Dr. Wang,

Thank you for submitting your Article manuscript entitled "Ubiquitous Membrane-bound DNase Activity in Podosomes and Invadopodia" to Journal of Cell Biology. As part of our normal reviewing procedure, your paper has been evaluated by at least two editors and an editorial statement is provided below. You will see that, in the consensus opinion of our editors, although we are interested in the concepts presented in this study, the manuscript is too preliminary for external review. We have thus decided not to subject your manuscript to a lengthy review process. We would be willing to consider a revised manuscript containing data addressing the detailed editorial comments below, assuming the novelty of the findings has not been compromised in the interim.

Because Journal of Cell Biology addresses a wide and diverse audience of cell biologists, we must give priority to manuscripts that provide a substantial advance of broad appeal to the cell biology community, even though many others also present interesting and important advances for researchers in a particular field.

I am sorry that our answer on this occasion is not more positive, and I hope that this outcome will not dissuade you from submitting other manuscripts to us in the future.

Thank you for your interest in Journal of Cell Biology.

With kind regards,

Anna Huttenlocher
Senior Editor
Journal of Cell Biology

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Editorial Statement:

In this study Pal et al. demonstrate that several human and mouse cells lines have podosomes and invadopodia with DNase activity. They further show that this activity is likely mediated by the GPI-anchored DNase X, requires actin polymerization, and begins rapidly after actin nucleation initiation. These findings are intriguing but the work is mainly descriptive and does not provide significant novel mechanistic insight into invadosome DNase activity. Thus, despite the high quality of this study, the biological advance is not sufficiently developed in its current state to be competitive in review as an Article in JCB. The study is also not a good fit in the Report format because it does not identify a functional role for the observed DNase activity. However, we would be open to re-
considering a substantially improved version of this work that addressed these issues, particularly the identification of a functional role for invadosome DNase activity.
Dear Prof. Wang,

Thank you for submitting your manuscript entitled "Ubiquitous Membrane-bound DNase Activity in Podosomes and Invadopodia." Thank you also for your patience with the peer review process. Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that reviewers find your study interesting but also raise substantial concerns that need to be fully addressed in a revised version in order to be suitable for JCB. Specifically, they ask for additional data to validate that the observed DNase X containing structures are indeed invadosomes, confirm DNase X knockdown, and further characterize the time dynamics of actin assembly, DNase accumulation, and matrix degradation.

Reviewer #3 raises concerns regarding technical rigor and statistical analysis which must be addressed. The numbers of experiments, replicates, and statistical tests & analyses should be included in all figure legends. In this regard we strongly recommend that you follow the guidelines for statistical representation and analysis laid out by Lord et al 2020 (https://doi.org/10.1083/jcb.202001064). Images of multiple representative cells should be shown. Please also note the following JCB policies for 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 also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

Additionally, when revising your manuscript please include full method details for cytochalasin and blebbistatin treatments; when were these added, how long were cells treated, and whether this was done before or after cell spreading on SNS. Finally, please adjust the colors of fluorescence images in Figure 4 to be compatible for color blind scientists.

Please let us know if you are able to address the major issues outlined above and wish to submit a...
revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000; a full Research 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: A Report may include up to 5 main text figures; a full Research Article may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures; a full Research Article 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.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Anna Huttenlocher, MD
Monitoring Editor
Journal of Cell Biology
Reviewer #1 (Comments to the Authors (Required)):

This is a puzzling story describing the presence of DNAse activity in punctate structures known as podosomes and invadopodia forming at the ventral cell surface of several cell types including macrophage- and tumor-derived cell lines. DNase enzymatic activity in association with the invadosomal structures is convincingly visualized in situ by fluorescence loss by plating cells on a substratum consisting of immobilized fluorescently-labeled double-stranded DNA, or by fluorescence activation of immobilized dsDNA labeled with a dye-quencher pair. The invadosomal nature of the identified punctate DNAse-containing structures is demonstrated by several convergent observations including the co-occurrence of cortactin and F-actin, two classical podosome and invadopodia markers and by the presence of dual matrix- and DNA-degradative activities in association with invadosomes. In addition, the invadosome-associated DNAse activity is shown to be sensitive to the actin polymerization and invadosome inhibitor, cytochalasin-D. The biological relevance of these structures is corroborated by the observations that DNase-positive invadosomes can form at contact sites between the plasma membrane of THP-1 macrophages and immobilized E. coli and represent sites of pericellular bacterial DNA degradation. Overall, the experiments reported in this study are well performed and even though it is mostly descriptive in nature, the identification of a DNAse activity associated with long known, well-studied podosomes and invadopodia is puzzling and worth reporting in this condensed report format.

Some points would require some further clarification.

1- The invadosomal DNAse is opportunistically identified as being the GPI-linked DNAse X. The authors should clarify how they precisely identified DNase X among other DNases. In addition, at least, they should comment on how a GPI-linked protein may be accumulated and retained in punctate invadosomal structures and whether these features are conserved among other GPI-linked proteins or are specific to DNase X?

2- The dynamics of actin polymerization and DNase activity are monitored by live-cell imaging and it was concluded that there is a 1-minute difference between the visualization of actin accumulation and DNase I activity (Figure 3). The authors should clarify whether this lag-time is required for the accumulation of detectable SNS fluorescence or whether it represents an actual time shift between actin assembly and DNase accumulation. Would it be possible to image simultaneously fluorescently-labeled DNase X or any GPI-linked reported protein (see Point 1) and actin assembly to clarify this point.

3- The signaling events that trigger invadosome assembly are relatively well established. The recruitment and activation of the tyrosine-kinase, c-Src, seems to be a common requirement during the multistep mechanisms leading to podosome and invadopodia formation. It would be interesting to assess whether c-Src is similarly required for the formation of DNA-degrading invadosomes that form at contact sites between macrophages and E. coli.

Reviewer #3 (Comments to the Authors (Required)):
This is an interesting and well written paper describing a novel observation that invadosomes do not just act as delivery processes for matrix degradation but also deliver localized dsDNA degradation. I am delighted to see the identification of DNAse X as this really adds to the story. I would like to see a bit more information about the proportion of the population of cells with dsDNA degradative activity not just the stats for individual cells. I would also like some further validation of invadosomes presence in some of the later experiments as described below. I have some concerns about the number of cells quantified as described below.

Specific points
Figure 1: would like to see a magnified image of 2-3 puncta with the co-localisation of F-actin, vinculin and dsDNS/SNS image included. I would also like a TKS5 stain to confirm the invadopodia status.
Figure 2: what about population statistics - how many cells in the different populations exhibit dsDNA degradative activity - is it comparable with matrix degradation activity?
Page 5 please insert a reference for the invadosomes negative status of CHO-K1 cells
Figure 4: please provide the experimental data to confirm the extent of DNaseX knockdown
Please clarify the biological replication. 20 cells for each data point. Is that 20 cells over 3 separate experiments or 20 cells from one experimental data set? 20 cells is a low number for this kind of analysis. Were the authors surprised at the DNAseX signal - it seems to be almost exclusively in the podosome. Is this the same for all of the cell lines tested?
Figure 5: I am concerned that the SNS signal is assumed to be an invadosomes signal in this experiment. Can this experiment be repeated with the LifeAct expressing cell line?
I am concerned about the interpretation of experiment in 5e. What happens if you leave the MTC cells longer on the coverslip - do the authors know that these cells have matrix degrading activity in this system? Have they tried other substrates such as gelatin? MBA-MB-231 cells can degrade matrix within 3 hours of plating - can this experiment be repeated with these cells to validate the assumption that matrix degradation is indeed delayed with respect to dsDNA degradation in the invadopodia setting.
Figure 6: it is not clear to me why there is so much SNS signal in panel a? For b&c how do we know we are looking at invadosomes? Please can an F-actin stained fixed image be added as is shown in (d)
Figure S1 - this is very important data can it be added to Figure 1 (especially the line scans)
Re: Ms. Ref. No.: 202008079R-A

Title: Ubiquitous Membrane-bound DNase Activity in Podosomes and Invadopodia

Dear editor,

Thank you for considering our manuscript and obtaining detailed reviews. We’d also like to thank both reviewers for the careful and generally positive review which indeed improved the state of the manuscript. We have revised the manuscript by addressing their concerns, comments and suggestions. Revision suggestion from the editor was also implemented to the revised manuscript. Text revision was edited in red color in the manuscript. Point-to-point responses to editor and reviewers are included in this letter in blue. The general guidelines for the manuscript format were also followed. Because both the number of main figures and the number of supplementary figures exceed the limit of a JCB Report, we’d like to ask the editor to consider this manuscript as an Article. If the editor feels otherwise, we are also open for further format revision to fit the manuscript to a Report by, e.g. figure merging.

Editor’s suggestion:

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 also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that “Data distribution was assumed to be normal but this was not formally tested.”

We added all requested information regarding the statistics into the corresponding locations in figure legends. A paragraph about statistical methods was added into Materials and Methods section. The statement of “assumption of data normal distribution” was also included in the Methods section.

Additionally, when revising your manuscript please include full method details for cytochalasin and blebbistatin treatments; when were these added, how long were cells treated, and whether this was done before or after cell spreading on SNS. Finally, please adjust the colors of fluorescence images in Figure 4 to be compatible for color blind scientists.

Cytochalasin and blebbistatin were added in cell suspension solution prior to cell plating on the SNS surface and remain in the solution throughout the entire assays. This information was added
to the method section. Colors of fluorescence images were re-adjusted in Fig. 4. Images with pseudo color have been converted to grayscale except the overlay (merged) images. Readers with color vision deficiency should be able to compare SNS, F-actin and DNase X distributions in the images.

Reviewer #1: This is a puzzling story describing the presence of DNAse activity in punctate structures known as podosomes and invadopodia forming at the ventral cell surface of several cell types including macrophage- and tumor-derived cell lines. DNase enzymatic activity in association with the invadosomal structures is convincingly visualized in situ by fluorescence loss by plating cells on a substratum consisting of immobilized fluorescently-labeled double-stranded DNA, or by fluorescence activation of immobilized dsDNA labeled with a dye-quencher pair. The invadosomal nature of the identified punctate DNAse-containing structures is demonstrated by several convergent observations including the co-occurrence of cortactin and F-actin, two classical podosome and invadopodia markers and by the presence of dual matrix- and DNA-degradative activities in association with invadosomes. In addition, the invadosome-associated DNAse activity is shown to be sensitive to the actin polymerization and invadosome inhibitor, cytochalasin-D. The biological relevance of these structures is corroborated by the observations that DNAse-positive invadosomes can form at contact sites between the plasma membrane of THP-1 macrophages and immobilized E. coli and represent sites of pericellular bacterial DNA degradation. Overall, the experiments reported in this study are well performed and even though it is mostly descriptive in nature, the identification of a DNAse activity associated with long known, well-studied podosomes and invadopodia is puzzling and worth reporting in this condensed report format.

We thank the reviewer for the careful and generally positive review. We have revised the manuscript according to the suggestion and comments.

1. The invadosomal DNAse is opportunistically identified as being the GPI-linked DNAse X. The authors should clarify how they precisely identified DNAse X among other DNases. In addition, at least, they should comment on how a GPI-linked protein may be accumulated and retained in punctate invadosomal structures and whether these features are conserved among other GPI-linked proteins or are specific to DNAse X?

It is actually fairly straightforward to identify the DNAse in invadosomes as GPI-anchored DNAse X. This is because DNAse X (also known as DNAse-1-like 1) is the only DNAse known to be membrane-bound and GPI-anchored (D. Shiokawa et al. J. Biol. Chem., 2007, 282, 17132-17140). Since we observed DNAse activity in podosomes on the cell membrane, this motivated us to identify the podosomal DNAse by checking whether such DNAse is GPI-anchored. It turns out that the GPI-anchor cleaving reagent PI-PLC did abolish all DNAse activity in podosomes, demonstrating that podosomal DNAse is GPI-anchored and therefore most likely is DNAse X. To further confirm its identity, we have performed the immunostaining with anti-DNAse X antibody and also the DNAse X siRNA gene-knock down assay. Both assays suggested that the podosomal DNAse is indeed DNAse X. With these three lines of evidence, we identified the podosomal
DNase as DNase X. To increase the readability, we have made language revision in the corresponding paragraph to follow above logic better.

The exact pathway of the DNase X accumulation and retaining in punctate invadosomal structure is unclear. However, we doubt that GPI-anchor alone is sufficient for DNase X clustering in invadosomes, as there are at least 150 GPI-anchored proteins in human cells (T. Kinoshita, J Lipid Res. 2016 Jan; 57(1): 4–5). It’s unlikely that these proteins would all be recruited to invadosomes. Therefore, GPI-anchor may not guarantee protein accumulation in invadosomes. Here, we only made use of GPI-anchor to identify the DNase, and did not attempt to study the role of GPI-anchor in DNase accumulation in podosomes.

2. The dynamics of actin polymerization and DNase activity are monitored by live-cell imaging and it was concluded that there is a 1-minute difference between the visualization of actin accumulation and DNase I activity (Figure 3). The authors should clarify whether this lag-time is required for the accumulation of detectable SNS fluorescence or whether it represents an actual time shift between actin assembly and DNase accumulation. Would it be possible to image simultaneously fluorescently-labeled DNase X or any GPI-linked reported protein (see Point 1) and actin assembly to clarify this point.

This is really a great point. Experiments shown in figure 3 were conducted to study how quickly podosomes can start degrading external DNA relative to its structural formation. We completely agree with the reviewer that it would be even more accurate if we do the similar experiment with fluorescently labeled actin and DNase X in co-transfected cells. Unfortunately, we do not have the plasmid of FP-fused DNase X. Such plasmid is not commercially available, and we don’t have the expertise to clone it by ourselves. We also feel that DNase X-FP plasmid could be an important resource benefiting our future research of invadosomal DNase activity. However, it is not essentially needed for this current paper as the main conclusions can be drawn without real-time visualization of DNase X. Still, in light of reviewer’s suggestion, we have added sentences to caution readers that the 1-min time delay may not accurately reflect the time gap between actin and DNase X assemblies. We only claim that DNase activity started to appear in about 1 min after F-actin nucleation in podosomes. Such message is still informative and useful to the field.

3. The signaling events that trigger invadosome assembly are relatively well established. The recruitment and activation of the tyrosine-kinase, c-Src, seems to be a common requirement during the multistep mechanisms leading to podosome and invadopodia formation. It would be interesting to assess whether c-Src is similarly required for the formation of DNA-degrading invadosomes that form at contact sites between macrophages and E. coli.

Truly the recruitment of tyrosine-kinase is related to the formation of invadosomes. It was established that the actin rosette formation is impaired when tyrosine-kinase Src is knocked down hence no formation of invadopodia (C. V. Rajaduari et al. J. Cell Sci., 2012, 125, 2940-2953) or podosomes (A. Baruzzi et al. Eur. J. Immunol., 2012, 42, 2720-2726). As we see in figure 3 and S5 that the actin core formation is essential to show any further DNase activity and the activity is well after the actin core formation. Naturally something impairing the actin rosette formation will definitely affect the DNase activity.
We agree that it would be interesting to check if c-src is required for podosome formation in macrophages on E. coli. This is in fact great advice inspiring some direction in our future study related to podosome formation during macrophage combating pathogens. The current manuscript, however, is mainly concerned with the DNase activity in invadosomes. Therefore, we deliberately concentrated our study on the functionality of podosomes, not the structural formation.

Reviewer #3: This is an interesting and well written paper describing a novel observation that invadosomes do not just act as delivery processes for matrix degradation but also deliver localized dsDNA degradation. I am delighted to see the identification of DNAse X as this really adds to the story. I would like to see a bit more information about the proportion of the population of cells with dsDNA degradative activity not just the stats for individual cells. I would also like some further validation of invadosomes presence in some of the later experiments as described below. I have some concerns about the number of cells quantified as described below.

Thanks to the reviewer for the careful and positive review. In our revised version of the manuscript we have incorporated the suggestions and concerned issue.

Specific points

Figure 1: would like to see a magnified image of 2-3 puncta with the co-localisation of F-actin, vinculin and dsDNS/SNS image included. I would also like a TKS5 stain to confirm the invadopodia status.

We added enlarged images of podosomes in Fig. 1f and 1g. Line profile graphs were also added here according to the reviewer’s another suggestion. Note that podosomes have sizes around 1 micron. Because of the resolution limit of fluorescence microscope, the enlarged images may not provide new information compared to Fig. 1d and 1e.

We have also performed the TKS5 immunostaining experiment to confirm that the F-actin cores in MDA-MB-231 cells are colocalized with TKS5. Anti-TKS5 was purchased from Millipore (MABT336, Millipore). Combined with cortactin immunofluorescence imaging, it further verified the identity of invadopodia in the cancer cells used in this research. The TKS5 data are included as fig. S4.

Figure 2: what about population statistics - how many cells in the different populations exhibit dsDNA degradative activity - is it comparable with matrix degradation activity?

We went over the dataset presented in Fig. 2 and checked over 50 cells for each cell type. The percentage of podosome-forming cells (macrophages or macrophage-like cells) exhibiting
DNase activity is very high, about 92%. For the few cells that didn’t exhibit DNase activity, they didn’t form F-actin cores either. The percentage of invadopodia-forming cells (MDA and MTC cells) exhibiting DNase activity, is relatively low, about 23%. However, this is a common percentage for cancer cells in forming invadopodia. A previous study shows that 28% of MCF-10A transfected with CD147 formed invadopodia (G. Daniel Grass et al., Regulation of invadopodia formation and activity by CD147. JCS), suggesting that percentage of cancer cells is usually not high in forming invadopodia.

Because this paper studies DNase activity in individual invadosomes. Percentage of cells forming invadosomes is a lesser concern. Therefore, we were focused on the percentage of invadosomes exhibiting DNase activity. The correlation between F-actin core formation and DNase activity in the F-actin cores is nearly 100% at the basis of single cells. If at the basis of single invadosomes, the correlation values are less than 100% (80% for podosomes, and could be down to 40% in invadopodia, as presented in Fig. 2g).

Page 5 please insert a reference for the invadosomes negative status of CHO-K1 cells

In revised version of the manuscript we have incorporated a reference for the invadosome negative status of CHO-K1 cell (T. Uekita et al. J Cell Biol., 2001, 155, 1345-1356).

Figure 4: please provide the experimental data to confirm the extent of DNaseX knockdown
Please clarify the biological replication. 20 cells for each data point. Is that 20 cells over 3 separate experiments or 20 cells from one experimental data set? 20 cells is a low number for this kind of analysis. Were the authors surprised at the DNaseX signal - it seems to be almost exclusively in the podosome. Is this the same for all of the cell lines tested?

We like to thank the reviewer for pointing these things out. We conducted immunofluorescence experiment after DNase X knockdown to demonstrate that the knockdown was effective. The new data are included as fig. S6. Note that fig. S6 was imaged by epi-fluorescence microscopy to monitor DNase X in the whole cells to check its expression level, unlike Fig. 4c which was imaged by TIRF microscopy that only visualizes DNase X at the cell-substrate interface.

20 cells are for each data point. We only used 20 cells because accurately computing the overall SNS signal strength in one cell is not a simple task. We compiled matlab code and use the code to manually select (by clicking the dot centers) all podosomes to calculate SNS signal in each cell. Because DNase X knockdown had a strong effect on DNase activity in podosomes as shown in Fig 4d, 20 cells are adequate to produce the statistical significance of difference between the control and knocked-down samples. For all the assays presented in this paper, we performed at lease twice, and often three times to make sure of the repeatability. Due to this discretion, this project has lasted for 3 years since we observed the invadosomal DNase activity at first. For the presentation of data, however, we usually used cell samples from single assays with essential controls.
We were not surprised to see that DNase X is exclusively present in podosomes. DNase X is supposed to be specifically recruited to podosomes, otherwise we would see SNS signal outside podosomes in the cells. If the reviewer refers to why we didn’t observe the DNase X in cytoplasm or endoplasmic reticulum, this is because total internal reflection microscopy was used for imaging in Fig. 4 and it only illuminates DNase X at cell-substrate interface.

Figure 5: I am concerned that the SNS signal is assumed to be an invadosomes signal in this experiment. Can this experiment be repeated with the LifeAct expressing cell line? I am concerned about the interpretation of experiment in 5e. What happens if you leave the MTC cells longer on the coverslip - do the authors know that these cells have matrix degrading activity in this system? Have they tried other substrates such as gelatin? MBA-MB-231 cells can degrade matrix within 3 hours of plating - can this experiment be repeated with these cells to validate the assumption that matrix degradation is indeed delayed with respect to dsDNA degradation in the invadopodia setting.

We understand the concern. We gained the confidence that SNS signal can specifically report invadosome locations, as in our previous assays with fixed cells, punctate SNS signals were always co-localized with stained F-actin in invadosomes. Therefore, here we omitted to visualize F-actin in the live-cell experiments in Fig. 5 because of technical difficulty. LifeAct transfection is a pertinent suggestion. We actually attempted to transfect THP-1 cells with LifeAct and other plasmids many times. However, THP-1 cells were proven to be extremely difficult to transfect, and we didn’t succeed in these attempts. We suspect that this difficulty may be related to the DNase X activity on the cell membrane. Fig. 5e was based on fixed cells and did show that SNS signals are colocalized with F-actin of invadosomes in the degradation assay.

We did confirm that MTC cells and MDA-MB-231 cells can degrade gelatin by invadopodia (data not included in the paper). However, we were unable to immobilize SNS sensor on the soft gelatin surface. Instead, we coated the surfaces with fibronectin that forms a molecular layer, not a gel layer with a thickness of microns. On the fibronectin-coated glass surface, SNS can be co-immobilized. We tried MDA-MB-231 cells on fibronectin surfaces and they didn’t degrade fibronectin either even if given >2 h. It is possible that fibronectin coated glass is too rigid for invadopodia to exhibit the ability of degrading matrix protein. However, DNA degradation can still occur on this surface, and in fact, take place in the timescale of minutes. Therefore, these assays are sufficient to support our point that DNase activity in invadosomes is prompter and more robust than protease activity.

Figure 6: it is not clear to me why there is so much SNS signal in panel a? For b&c how do we know we are looking at invadosomes? Please can an F-actin stained fixed image be added as is shown in (d)

The cell in Figure 6a happened to be migrating and it degraded dsDNA while patrolling on the surface in a punctate pattern (Video 4). That cell started migrating from the top-left corner to the place where it is visible in the bright field and we can see all the dsDNA degradation signal along the path. That is why there is so much signal in panel.
Here we added a F-actin stained image of THP-1 cells interacting with *E. coli* to verify that Fig. 6 b and c were indeed showing DNase activity in podosomes. The image was inserted to Fig. 6 as 6d. The stained image demonstrates good colocalization between SNS signal and F-actin signal. The halo-like pattern around *E. coli* might be a merged cluster of podosomes around the *E. coli*.

Figure S1 - this is very important data can it be added to Figure 1 (especially the line scans)

We removed fig. S1 and added the line scanning data of representative podosomes to the Fig. 1f and 1g.
March 24, 2021

RE: JCB Manuscript #202008079RR

Prof. Xuefeng Wang
Iowa State University
12 Physics Hall
Ames, IA 50011

Dear Prof. Wang,

Thank you for submitting your revised manuscript entitled "Ubiquitous Membrane-bound DNase Activity in Podosomes and Invadopodia." We would be happy to publish your paper in JCB pending final revisions necessary to address a few minor points and to meet our formatting guidelines (see details below).

Your work is best suited for our Report format rather than as an Article so we ask that you reformat the manuscript accordingly with a combined Results & Discussion section and a maximum of 5 main figures. Please see below and our website for full guidelines. We think that figures 2&3 and 6&7 can be combined but ultimately which figures to combine or potentially move to the supplement is up to you. For supplementary figures we can allow you to go over the 3 figure limit.

Additionally, please include P values in all figure legends and provide a reference or link to your Matlab code.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Reports is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Reports may have up to 5 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. You currently do not have scale bars in Figures 1F/G, 3A/B, 4A/magnification in C, 6D.

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 also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract: We suggest toning down the phrase "unambiguously demonstrated" either by removing "unambiguously" or other edits.

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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

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