JCB

The Septin Cytoskeleton Regulates Natural Killer Cell Lytic Granule Release

Prasad Phatarpekar, Brittany Overlee, Alex Leehan, Katelynn Wilton, Hyoungjun Ham, and Daniel Billadeau

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Review Timeline:

Submission Date:	2020-02-25
Editorial Decision:	2020-03-25
Revision Received:	2020-06-26
Editorial Decision:	2020-07-21
Revision Received:	2020-07-25

Monitoring Editor: Ana-María Lennon-Dumenil

Scientific Editor: Andrea Marat

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202002145

March 25, 2020

Re: JCB manuscript #202002145

Dr. Daniel D Billadeau
Mayo Clinic
Mayo Clinic 200 First Street SW
Rochester, MN 55905

Dear Dr. Billadeau,

Thank you for submitting your manuscript entitled "The Septin Cytoskeleton Regulates Natural Killer Cell Lytic Granule Release". 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 interested in the new information provided about how the septin cytoskeleton contributes to immune cell function by controlling lytic granule release by NK cells. Most of the points raised by the referees correspond to control experiments (or to clarifications about methods and data presented) and should thus be addressed for resubmission with new experimentation where necessary, with the exception of point #3 of referee #1 (analyzing Septin phosphorylation), which should only be discussed. Given the exceptional circumstances due to COVID pandemia, we recommend that you propose a reasonable revision plan for feedback at an early stage to avoid spending time on experimental revisions that may not be necessary or feasible at the moment. Since many laboratories are closed at present, an extension to the standard revision period is also doable.

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.

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. 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,

Ana-María Lennon-Dumenil, Ph.D.
Monitoring Editor

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

Journal of Cell Biology

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

Septins are GTP-binding proteins which hetero-oligomerize to form filaments that serve the dual function of participating in the cell cytoskeleton to control cell shape and motility, and acting as adaptors to recruit proteins at specific subcellular localizations. While septins have been implicated in immune cell function, to date these studies have been limited to phagocytes and T cells. In this manuscript Phatarpekar and colleagues contribute to fill this gap by investigating the role of the septin cytoskeleton in lytic granule release by NK cells and addressing the underlying mechanisms. Using pharmacological and reverse genetics approaches, they show that septin filament dynamics is essential for NK cell-target cell conjugate formation and NK-mediated killing. Intriguingly, septin depletion did not affect conjugate formation or lytic granule polarization to the lytic synapse, but impaired the process of fusion of lytic granules with the plasma membrane. Biochemical analyses revealed that this results from the ability of septins to localize to lytic granules and recruit components of the synaptic machinery, including syntaxin-11 and its binding partner STXBP2, which is required for the fusion step. Collectively, these results provide important new insights into the processes regulated by septins in immune cells.

Major points

1. The authors show that septins accumulate largely at the NK cell cortex, while the synaptic area

is largely cleared of septins but for limited accumulation of puncta (Fig.1, S2). This would suggest that, due to their ability to provide rigidity to the cell, septin filaments would hinder the profound rearrangements that occur at the plasma membrane on NK interaction with its cognate target. Hence the synaptic area would need to be cleared of septins, similar to what occurs with F-actin, to allow the synapse to form and the lytic granules to be released. This is indeed what the experiments carried out on cells treated with FCF, which stabilizes septin filaments, suggest (Fig.2). I find the results obtained on septin-depleted NK cells, while interesting, less convincing in their interpretation. The authors show that in the absence of septins NK cell-mediated killing is impaired, but conjugate formation, centrosome and lytic granule polarization, as well as signaling, occur normally (Fig. 3, 4, 6, S3). They conclude that that septin filament dynamics, but not septins themselves, are required for these processes. Since septin 7 depletion leads to a downregulation of other septins and to the polymerization of abnormal filaments that likely do not support cell shape and motility (Fig.S5), would this not be expected to impair immune synapse formation upstream of lytic granule release?

2. A second major consideration, related to the previous one, is the regulation of lytic granule exocytosis by the minor pool of septins that appear as punta at the lytic synapse. The fractionation experiments show that indeed lysosomes/lytic granules contain septins 1, 2 and 7. However, large amounts are associated with the post-nuclear and post-mitochondrial fractions, which are relatively poor in LAMP-1, while the crude lysosomal fraction in highly enriched in LAMP-1 and relatively poor in septins (Fig.5). Additionally, the co-localization experiments show septin+ and syntaxin+ dots not only at the synapse, but along the whole cortex (Fig.7B). Also, PLA spots showing syntaxin 11-septin 7 interactions can be seen along the cortex more than at the synapse. Could this mean that septins are implicated in basic vesicular cell processes at the cell cortex, where they are much more abundant than at the synapse? Do the syntaxin 11-septin 7 interactions increase following NK cell activation?

3. Septins are know to be regulated by phosphorylation as well as sumoylation events. This could account for the regulation of lytic granule fusion at the synapse by the minor pool of synapse-associated septins. This would deserve to be investigated.

Specific points

1. Figure 5. Panel B: The localization of septin 1 in proximity to lytic granules is not very clear. A more representative image should be shown. Panel D: An immunoblot of perforin should be added. The presence of STXBP2 and STX11 should also be assessed (see also point 3 below).

2. Figure 7. Panel A: the impact of NK cell activation on the interaction of syntaxin 11 with syntaxin 7 should be assessed. Panel B: Why is the septin 7 staining punctate also along the cortex, at variance with figure 1 where it appears very compact? Is this compatible with the structures formed by septin filaments?

3. Figure 8I. The amount of STXBP2 co-precipitating with STX11 should be quantified over multiple experiments, also following NK cell activation. Also, a staining with a lytic granule marker would be useful to support the notion that PLA spots in panel B correspond to lytic granules.

4. Figure S3. To rule out a defect in signaling other markers shold be used beside Vav1 and Erk phosphorylation. Blots should be quantified over multiple experiments (with stats).

5. Figure S5. Panel B: please provide higher magnifications of the cells to better visualize the

filament abnormalities. Panel C: please specify how "abnormal" was evaluated for the quantification.

6. Since septins have been implicated in the regulation of both the actin and microtubule cytoskeletons, F-actin accumulation at the synapse should be quantified and microtubules stained both in cells treated with FCF and in septin-depleted cells.

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

This manuscript examines the interesting question of the role of septins in NK cell secretion. Using a combination of functional assays, biochemical and genetic approaches and imaging they show that depletion of septin 7, required for the formation of septin complexes, leads to a reduction in NK cell killing. They go on to examine the molecular basis for this, suggesting that septin 7 interacts with syntaxin 11, a key component in the SNARE machinery required for secretion in NK and CTL.

The manuscript is well written and easy to follow. There are some areas that are not clear (that I outline below) and I think that some of the conclusions need to be toned down, the reasons for which I also outline below.

Figure 1: Examines the localization of septin 7 across the synapse. In the text the authors describe a reduction in septin 7 staining across the cytotoxic synapse (CS). Does this depletion occur at the center of the CS and vary according to the plane. Please add a description of panel C in the figure legend.

Figures 2 and 3 examine the role of septins in killing. Figure 2 shows a relatively modest reduction in killing upon treatment with FCF and I wonder how much this data adds to the manuscript given the off-target effects of FCF (see below). I do not really see the added value of panels B and D as they show the data for one point of the graphs in A and C.

Figure 3 shows a more impressive reduction of killing upon siSept7 treatment, particularly for the primary cells. Once again, I do not think that F and H add to the information in this panel, and it is not at all clear why the authors show the single point for an E:T ratio of 1:1.25 in this figure, but 1:5 in Figure 2. As the primary NK data is more compelling it would be good to present this first.

Figure 4G shows conjugate formation is unaffected and is well documented. Panel G refers to "IS" rather "CS".

Figure 5A shows Airyscan imaging of septin 7. I am curious as to why the septin staining appears so punctate and differs from the images in Figure 1. I wonder whether this is fixation dependent. Or is it because the limit of resolution has been reached. Do they have an explanation?This figure provides a relatively small Figure A and an enormous western blot (D). I would suggest that panel A is enlarged and the western blot reduced in size.

I was not convinced by the conclusion drawn from 5D that "septin filaments in NK can be co-purified with the lysosomal fraction" as there are bands for septins 1,2, and 7 in all of the fractions and they appear reduced in the crude lysosomal fraction. This contrasts with known lysosomal markers, LAMP1 and granzyme B that are enriched in the crude lysosomal fraction relative to the band in the post-nuclear fraction. The only protein that does not appear to be enriched in the CLF is GAPDH. Were there any others that could be added to support the idea that septins are specifically recruited to this fraction?

Figure 6 looks at the effect of septins on degranulation. The figure is very tiny and difficult to read the numbers in the FACS plots. Also, the figure legend does not specify where the data represented in I and J comes from. Although the authors describe a "substantially reduced degranulation" upon siSept7-1 treatment, this is not evident in the data shown in panel H, where there is the tiniest shift in MFI. In addition, there is some curious signal high in the SSC. Could this be from doublets?

Figure 7 and 8 suggest an interaction between septin 7 and syntaxin 11, making it important to have some validation of the syntaxin 11 antibody. Is this available?
Figure 7 A is confusing as septin 7 should have a molecular weight of ~ 51kD and syntaxin 11 ~33kD and this does not appear to be the case in 7A. As mentioned above, two molecular weight markers per western strip would allow readers to work out the band size, but I suspect that they mislabelled this panel. The staining of syntaxin 11 looks quite different from previous reports on cytotoxic synapses using antibodies validated against patient cells lacking syntaxin 11 (eg Traffic 16:1330). Why is the appearance of syntaxin 11 so punctate here? Is it fixation dependent? How well is the specificity of the antibody being used, validated? Validation of this antibody is critical to making a convincing case for an interaction between STX11 and Septin 7.

In Figure 8 the authors return to using the inhibitor FCF rather than the more convincing siSept7 to show a decrease in signal from the proximity ligation assay for syntaxin 11 and STXBP2 (also referred to as Munc18-2). It would be good to use siRNA rather than the drug. It would also be important to include the individual stains of each of the antibodies used for PLA in order to demonstrate the localization as detected by these antibodies. Again, the source and specificity demonstration of these antibodies needs to be included. This data is used to support a model that septins play a role in strengthening the interaction between STX11 and STXBP-2. As this interaction is required for secretion, it is curious why septin localization is thought to decrease across the synapse where secretion occurs. Another outstanding question is whether this interaction might be direct or indirect. Did the authors manage to show a direct interaction with purified proteins? A clearer explanation of the model would be helpful.

Additional comments:
The authors include some data in the supplementary figures that might be much better included in the main text. For example S7 supports a close association with lytic granules.

Materials and Methods section:
Please state where all reagents come from. In particular where does "forchlorfenuron" (FCF) come from and how confident are the authors about the specificity of this plant growth inhibitor for septins, which has been challenged (doi: 10.1128/EC.00191-14). This needs to be discussed so that readers can focus more on the siRNA studies or at least be aware of the caveats.
Another important omission from the materials section is the source, and validation, of the antibody against syntaxin 11. This is particularly important for the data shown in Figures 7 and 8.
It is not clear which detergent is used for permeabilization from the SurfactAmps range from ThermoFisher (p12). Please specify.

For all figures please state:
(i) whether images are single planes or projections;
(ii) include at least 2 molecular weight markers per western blot slice so that the reader can judge the molecular weight of the band shown.

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

This work addresses the role of the septin cytoskeleton in regulating the exocytosis of lytic granules at the cytolytic synapse established between NK cells and target cells. The authors show that stabilization of septins, triggered by pharmacological treatments or silencing particular septins (2 or 7) decreases degranulation and impairs killing by NK cells. Interestingly the authors find that septins co-purify with lytic granules and interact with components of the SNARE fusion machinery, such as syntaxin 11. In other cell types, septins are known to regulate membrane rigidity as well as interact with snare components, however their role in NK cells remains less understood. Thus, this work advances our knowledge on cellular mechanisms that regulate NK cytotoxicity.
1. Main points:
i-Septins interact with Dock8 in NK cells: the immunoprecipitation displayed as a supplementary figure is very clear and supports their point.
ii-Septin filaments do not appreciably accumulate at the NK-target cytotoxic synapse: Figures 1 and Sup figure 2 support the claim that septin 7 mainly accumulates at cortex. However, it remains unclear if this cytoskeletal component is actively cleared from this platform. This point could be strengthened by evaluating the dynamics of septin 7 distribution in NK cells upon forming a synapse. The authors could perform live cell imaging of NK cells expressing a fluorescently tagged version of septin 7 and track its distribution upon encounter with a target cell. If this tool is not available, the authors should stain for septin 7 localization in NK cells forming conjugates at early and late time points. Importantly, the time points used to evaluate conjugates in not specified.
iii-Septins are required for NK cell-mediated killing: The authors used a plant cytokinin (FCF), which enhances septin stability and show that in NK cells this drug impairs cytolytic functions. Similar results are shown when septin 2 or 7 are depleted. The killing assays are clear and although the defects are not very strong, especially considering the scales of the graphs, they seem to be consistent. The authors should show the effect of FCF in NK cells in terms of septin cytoskeleton. Are septin bundles observed under the experimental conditions that were used? Staining of the septin cytoskeleton should be shown (a similar figure to sup 5B should be included).
iv-Role of septins in NK cells conjugate formation: The authors show an impaired spreading response in FCF-treated NK cells treated seeded on fibronectin as well in the formation of conjugates, which was not observed in septin depleted cells. The authors explain why there could be a functional difference between both conditions in terms of conjugate formation. Given this result, shouldn't FCF treatment have a stronger effect on NK cell-mediated killing? This point also be addressed.
v-Loss of septin 7 in lytic granule/MTOC convergence to the CS: The results displayed seem to indicate no difference in the recruitment of lytic granules or MTOC to the synaptic membrane, however it is still possible that this process could be slowed down in septin-silenced cells. The authors should indicate time points at which conjugates were analyzed and determine whether there is a delayed recruitment of lytic granules, which could account for the defects observed in killing.
vi-Septins localize with lytic granules: Indeed, this is an interesting observation, which is supported by biochemical assays. However, the images displayed do not seem to show that both are significantly localized together at the cs, particularly Septin 1. The authors should show quantitative data of the images to better highlight this point.
vii-Septin depletion or stabilization impair NK cell degranulation: The degranulation assays in NK cells under different conditions, clearly support a role for septins and their stabilization in the process of degranulation. However, the conclusion stating that NK cells must "dynamically reorganize their septin filaments to degranulate" is not formally demonstrated. This point should be evaluated further. Images of lytic granules in FCF-treated cells should be shown and the localization

of lytic granules evaluated. As stated above (ii), the dynamic re-organization of the septin cytoskeleton should be directly evaluated.
viii-Septins interact with lytic granule machinery: the data presented are very supportive. The Co-IP between septin 7 and syntaxin 11 suggests that both proteins interact. However, this interaction seems to occur in resting conditions. The authors should comment on this observation.
It is important to point out that the results shown in this section seem rather contradictory with the initial findings/conclusions of the authors, where they state that "septin filaments do not appreciably accumulate at the NK/target cytotoxic synapse" (figure 1). Indeed, this point is confusing, and the authors should clarify how they view the association of septin 7 with the cytolytic synapse and particularly with lytic granules within this area. Additionally, PLA spots (used to monitor septin-syntaxin 11 interactions) were detected throughout the plasma membrane. This observation would suggest that septins are not specifically required for degranulation within the cytolytic synapse. This important point must be discussed (see next point).
viii-Septin stabilization/depletion impair the interaction between STX11 and STXBP2: the biochemical and imaging data support a role for septins in the interaction between snare components. This point is interesting however, functional implications of this interaction are lacking. This could be clarified by measuring degranulation events at the synaptic membrane to provide a clearer functional link between the role for septins in bringing the snare machinery to lytic granules and their degranulation at the cytolytic membrane.
Additional issues:
Overall, the figure legends should better describe the experiments (include time points and statistical analysis). A scheme depicting the mechanism proposed by the authors on the role of the septin cytoskeleton in lytic granule release should be included.

Point – by – Point Response:

We would like to thank all three reviewers for the critical review of our manuscript. We feel that because of your comments and suggestions, we have been able to produce a significantly improved revised manuscript that further supports our data showing a role for septins in lytic granule release. Due to Covid-19 restrictions, we have been unable to perform some of the requested experiments, but feel that the revised manuscript is sufficiently improved and we trust you will see the significance of this study as it is the first demonstration of the role of the septin cytoskeleton in NK cell-mediated killing.

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

Septins are GTP-binding proteins which hetero-oligomerize to form filaments that serve the dual function of participating in the cell cytoskeleton to control cell shape and motility, and acting as adaptors to recruit proteins at specific subcellular localizations. While septins have been implicated in immune cell function, to date these studies have been limited to phagocytes and T cells. In this manuscript Phatarpekar and colleagues contribute to fill this gap by investigating the role of the septin cytoskeleton in lytic granule release by NK cells and addressing the underlying mechanisms. Using pharmacological and reverse genetics approaches, they show that septin filament dynamics is essential for NK cell-target cell conjugate formation and NK-mediated killing. Intriguingly, septin depletion did not affect conjugate formation or lytic granule polarization to the lytic synapse, but impaired the process of fusion of lytic granules with the plasma membrane. Biochemical analyses revealed that this results from the ability of septins to localize to lytic granules and recruit components of the synaptic machinery, including syntaxin-11 and its binding partner STXBP2, which is required for the fusion step. Collectively, these results provide important new insights into the processes regulated by septins in immune cells.

We thank the reviewer for appreciating the novelty of the study and providing us with a constructive critique of the work.

Major points

1. The authors show that septins accumulate largely at the NK cell cortex, while the synaptic area is largely cleared of septins but for limited accumulation of puncta (Fig.1, S2). This would suggest that, due to their ability to provide rigidity to the cell, septin filaments would hinder the profound rearrangements that occur at the plasma membrane on NK interaction with its cognate target. Hence the synaptic area would need to be cleared of septins, similar to what occurs with F-actin, to allow the synapse to form and the lytic granules to be released. This is indeed what the experiments carried out on cells treated with FCF, which stabilizes septin filaments, suggest (Fig.2). I find the results obtained on septin-depleted NK cells, while interesting, less convincing in their interpretation. The authors show that in the absence of septins NK cell-mediated killing is impaired, but conjugate formation, centrosome and lytic granule polarization, as well as signaling, occur normally (Fig. 3, 4, 6, S3). They conclude that that septin

filament dynamics, but not septins themselves, are required for these processes. Since septin 7 depletion leads to a downregulation of other septins and to the polymerization of abnormal filaments that likely do not support cell shape and motility (Fig.S5), would this not be expected to impair immune synapse formation upstream of lytic granule release?

The observation that i) septin-depleted NK cells form conjugates with target cells indicates that the polymerization of F-actin at the CS is intact, otherwise the cells would not be able to promote firm adhesion through their integrins; ii) also since the MTOC and lytic granules polarize to the CS is indicative that all upstream signaling pathways are intact in the absence of septin filaments and that there is no defect in either microtubule-dependent motors moving lytic granules or processes that winch the MTOC to the CS. New results looking at the kinetic of MTOC polarization and lytic granule movement comparing control cells to Septin 7 KO cells further supports the lack of defective microtubule dynamics.

2. A second major consideration, related to the previous one, is the regulation of lytic granule exocytosis by the minor pool of septins that appear as punta at the lytic synapse. The fractionation experiments show that indeed lysosomes/lytic granules contain septins 1, 2 and 7. However, large amounts are associated with the post-nuclear and post-mitochondrial fractions, which are relatively poor in LAMP-1, while the crude lysosomal fraction in highly enriched in LAMP-1 and relatively poor in septins (Fig.5). Additionally, the co-localization experiments show septin+ and syntaxin+ dots not only at the synapse, but along the whole cortex (Fig.7B). Also, PLA spots showing syntaxin 11-septin 7 interactions can be seen along the cortex more than at the synapse. Could this mean that septins are implicated in basic vesicular cell processes at the cell cortex, where they are much more abundant than at the synapse? Do the syntaxin 11-septin 7 interactions increase following NK cell activation?

One cannot compare a component of the lytic granule such as Granzyme B or Lamp1 to something that might be dynamically recruited to the lytic granule such as septins, microtubule moters, etc... The notion that there should be more septin in the CLF compared to the other fractions is unreasonable since septins reside throughout the cytoplasm and are performing functions throughout the cell, not just at the lytic granule. In fact, the amount of septin protein co-purifying with the lytic granule fraction is similar to that observed for components of the dynein-dynactin motor complex that promotes the minus-end directed movement of lytic granules to the MTOC (10.4049/jimmunol.1402897 ; 10.4049/jimmunol.0804337)

We have examined whether the interaction of Septin7 and STX11 increases with stimulation, however we did not see any appreciable increase in this interaction following PMA+iono or NKG2D/2B4 receptor ligation. These data are shown in Figure 7A.

As pointed out by the reviewer, we see interactions of STX11 and Septin 7 along the membrane throughout the cell, and as indicated by the reviewer this interaction might

be functioning at sites of vesicle fusion similar to what we are showing for lytic granule fusion. We have added a comment regarding this in the Discussion.

3. Septins are know to be regulated by phosphorylation as well as sumoylation events. This could account for the regulation of lytic granule fusion at the synapse by the minor pool of synapse-associated septins. This would deserve to be investigated.

We have added a comment regarding this in the Discussion.

Specific points

1. Figure 5. Panel B: The localization of septin 1 in proximity to lytic granules is not very clear. A more representative image should be shown. Panel D: An immunoblot of perforin should be added. The presence of STXBP2 and STX11 should also be assessed (see also point 3 below).

We have replaced the image with a better one as suggested. We have immunoblotted for STX11 and STXBP2 and added this to the data shown in Figure 5D. We don't see a reason for blotting for perforin since we are already showing the purity of the CLF by blotting for the lytic granule component Granzyme B.

2. Figure 7. Panel A: the impact of NK cell activation on the interaction of syntaxin 11 with syntaxin 7 should be assessed. Panel B: Why is the septin 7 staining punctate also along the cortex, at variance with figure 1 where it appears very compact? Is this compatible with the structures formed by septin filaments?

We added this to Figure 7A as indicated above.

The reason for the difference in septin 7 staining is that the images shown in Figure 1A-C are confocal, and the images shown in Figure 5 and 7 were captured using an LSM800 with Airyscan which provides a consistent 2x resolution improvement and 4- to 8-fold signal-to-noise benefit over conventional confocal detection. This information is provided in both the Materials and Methods section as well as in the text when we switch from conventional confocal microscopy to confocal microscopy with Airyscan.

3. Figure 8I. The amount of STXBP2 co-precipitating with STX11 should be quantified over multiple experiments, also following NK cell activation. Also, a staining with a lytic granule marker would be useful to support the notion that PLA spots in panel B correspond to lytic granules.

We have quantified the PLA spots for STX11-STXBP2 interaction in septin 7 KO cells and added that data in Figure 8D and 8E. We have removed the immunoblot since the PLA is much more quantitative.

The staining with perforin is not possible given the limitation of the PLA system. Also, we weren't measuring PLA spots with lytic granules, but simply showing that the

stabilization of the septin filament system affects the interaction of STXBP2 with STX11. Interestingly, since there is a whole cell loss of this interaction as measured by the PLA technique, it might also suggest that septins and the STX11/STXBP2 interaction is operative well beyond what that happening with lytic granules.

4. Figure S3. To rule out a defect in signaling other markers should be used beside Vav1 and Erk phosphorylation. Blots should be quantified over multiple experiments (with stats).

We have quantified all of the blots as requested and added the data in Supplemental Figure S3G and 3I. The reason we picked Vav1 and Erk, is that they are well known to be involved in the killing process as well as granule polarization and adhesion. Thus, the demonstration that signaling to these key molecules downstream of NKG2D/2B4 ligation is not affected by FCF treatment indicates that signaling is occurring normally. Immunoblotting for other proximal signaling molecules will again not add any new information, other than to say, yes it looks normal too.

5. Figure S5. Panel B: please provide higher magnifications of the cells to better visualize the filament abnormalities. Panel C: please specify how "abnormal" was evaluated for the quantification.

Higher magnification insets are provided for these figures, as well as an explanation as to how the normal and abnormal were scored is provided in the Materials and Methods section.

6. Since septins have been implicated in the regulation of both the actin and microtubule cytoskeletons, F-actin accumulation at the synapse should be quantified and microtubules stained both in cells treated with FCF and in septin-depleted cells.

Since FCF-treated cells don't form conjugates this experiment will not work, and the examination of cells not in conjugates treated with FCF is not informative. Also, the observation that 1) septin-depleted NK cells form conjugates indicates that they can form a functional CS that allows stable integrin-mediated adhesion, which is F-actin dependent; additionally, since the lytic granules converge to the MTOC and polarizes to the CS, again indicates that there is no defect in microtubule motors or microtubule dynamics/stability.

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

This manuscript examines the interesting question of the role of septins in NK cell secretion. Using a combination of functional assays, biochemical and genetic approaches and imaging they show that depletion of septin 7, required for the formation of septin complexes, leads to a reduction in NK cell killing. They go on to examine the molecular basis for this, suggesting that septin 7 interacts with syntaxin 11, a key component in the SNARE machinery required for secretion in NK and CTL.

The manuscript is well written and easy to follow. There are some areas that are not clear (that I outline below) and I think that some of the conclusions need to be toned down, the reasons for which I also outline below.

We appreciate the reviewer's comments and their interest in this work.

Figure 1: Examines the localization of septin 7 across the synapse. In the text the authors describe a reduction in septin 7 staining across the cytotoxic synapse (CS). Does this depletion occur at the center of the CS and vary according to the plane. Please add a description of panel C in the figure legend.

A description to the Figure legend for panel C (now panel B) has been provided. We have also provided an enface z-stack rendition of the image in C (now panel B) so as to show the localization of septin 7 across the synapse. We have also included a movie – new Supplemental Movie S1.

Figures 2 and 3 examine the role of septins in killing. Figure 2 shows a relatively modest reduction in killing upon treatment with FCF and I wonder how much this data adds to the manuscript given the off-target effects of FCF (see below). I do not really see the added value of panels B and D as they show the data for one point of the graphs in A and C.

We have moved the FCF killing data to Supplemental Figure 2A. Panels B and D show data from multiple experiments. We have utilized this demonstration of the data in multiple publications. It allows us to show the reproducibility of the data from multiple independent experiments instead of just one representative cytotoxicity assay over.

Figure 3 shows a more impressive reduction of killing upon siSept7 treatment, particularly for the primary cells. Once again, I do not think that F and H add to the information in this panel, and it is not at all clear why the authors show the single point for an E:T ratio of 1:1.25 in this figure, but 1:5 in Figure 2. As the primary NK data is more compelling it would be good to present this first.

We have switched the primary NK data with NKL. Again, as indicated above, we have utilized this demonstration of the data in multiple publications. It allows us to show the reproducibility of the data from multiple independent experiments instead of just one representative cytotoxicity assay over.

Figure 4G shows conjugate formation is unaffected and is well documented. Panel G refers to "IS" rather "CS".

We have changed the y-axis in Figure 4G to 'CS'

Figure 5A shows Airyscan imaging of septin 7. I am curious as to why the septin staining appears so punctate and differs from the images in Figure 1. I wonder whether this is fixation dependent. Or is it because the limit of resolution has been reached. Do

they have an explanation? This figure provides a relatively small Figure A and an enormous western blot (D). I would suggest that panel A is enlarged and the western blot reduced in size.

The difference between Figure 1 images and that shown in this Figure is the type of imaging being performed – Confocal vs Airyscan. We have made the 'A' image larger and the blots shown in 'D' smaller. Sew have also added the staining of septin 1 and septin 2 with perforin from a previous Supplemental Figure.

I was not convinced by the conclusion drawn from 5D that "septin filaments in NK can be co-purified with the lysosomal fraction" as there are bands for septins 1,2, and 7 in all of the fractions and they appear reduced in the crude lysosomal fraction. This contrasts with known lysosomal markers, LAMP1 and granzyme B that are enriched in the crude lysosomal fraction relative to the band in the post-nuclear fraction. The only protein that does not appear to be enriched in the CLF is GAPDH. Were there any others that could be added to support the idea that septins are specifically recruited to this fraction?

One cannot compare a component of the lytic granule such as Granzyme B or Lamp1 to something that might be dynamically recruited to the lytic granule such as septins. The notion that there should be more septin in the CLF compared to the other fractions is unreasonable since septins reside throughout the cytoplasm and are performing functions throughout the cell. In fact, the amount of septin protein co-purifying with the lytic granule fraction is similar to that observed for components of the dynein-dynactin motor complex that promotes the minus-end directed movement of lytic granules to the MTOC, myosin IIa, HkRP3 (10.4049/jimmunol.1402897 ; 10.4049/jimmunol.0804337).

Figure 6 looks at the effect of septins on degranulation. The figure is very tiny and difficult to read the numbers in the FACS plots. Also, the figure legend does not specify where the data represented in I and J comes from. Although the authors describe a "substantially reduced degranulation" upon siSept7-1 treatment, this is not evident in the data shown in panel H, where there is the tiniest shift in MFI. In addition, there is some curious signal high in the SSC. Could this be from doublets?

This figure has been edited to make it more legible. We have used a different set of flow data that lacks the high SSC signal, which was not seen in any of the other analyses.

Figure 7 and 8 suggest an interaction between septin 7 and syntaxin 11, making it important to have some validation of the syntaxin 11 antibody. Is this available?

This is a valid point. During the submission of this manuscript we had just got the CRISP/Cas9 system working for knockout in NK cell lines and primary NK cells. Therefore, we generated knockouts of STX11, STBP2 and Septin 7 in order to validate the staining and PLA assays as indicated here and below, but also to carry out some experiments suggested by the reviewers. As shown in the three figures below, we have generated STX11 and STXBP2 KO in NKL cells and validated the antibodies by IF for each of these in Figure 1 and Figure 2, respectively. We have additionally performed a

PLA assay for STX11 and STXBP2 in the control and SXT11 KO NKL cells as shown in Figure 3. Please note the decreased signals in each of the KO cells, as well as the loss of PLA dots in Figure 3. Thus, we believe that the antibodies in question are specific.

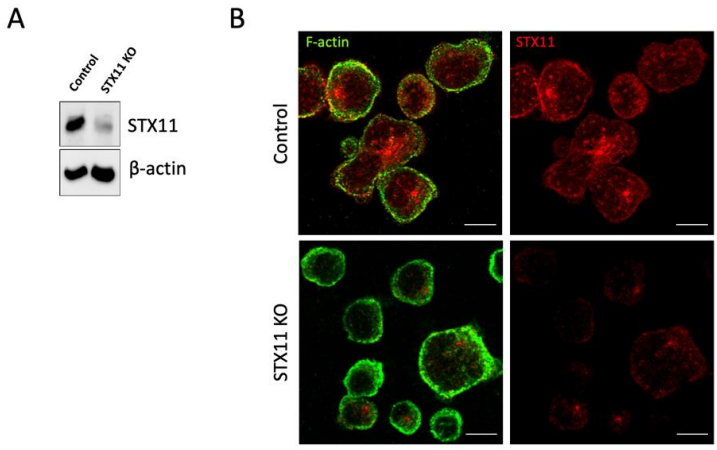

Figure 1. Testing of anti-STX11 antibody in STX11 KO NKL cells. (A) Immunoblot of lysates from the bulk polyclonal control KO and STX11 NKL KO cells with anti-STX11 and beta-actin. (B) Immunofluorescence using anti-STX11 at 1:500 dilution in polyclonal control KO and STX11 NKL KO cells. Note that the majority of STX11 signal (red) is gone in the STX11 KO cells. The remaining centrosome staining is likely non-specific, since this is where the majority of the staining remains in this population.

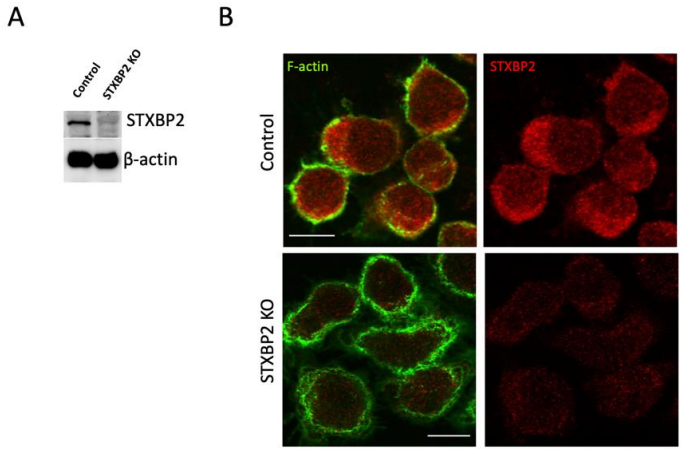

Figure 2. Testing of anti-STXBP2 antibody in STXBP2 KO NKL cells. (A) Immunoblot of lysates from the bulk polyclonal control KO and STXBP2 NKL KO cells with anti-STXBP2 and beta-actin. (B) Immunofluorescence using anti-STXBP2 at 1:500 dilution in polyclonal control KO and STXBP2 NKL KO cells. Note that the majority of STXBP2 signal (red) is gone in the STXBP2 KO cells compared to the KO control cells.

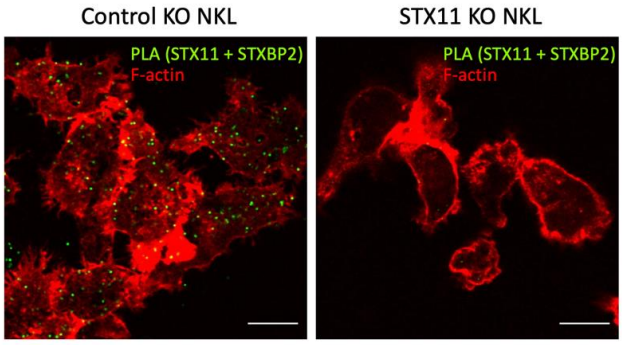

Figure 3. Validation of PLA using STX11 KO NKL cells. Control and STX11 NKL KO bulk populations were subjected to the PLA assay using antibodies for STX11 and STXBP2 under the same conditions as those in the revised manuscript. As can be seen the STX11 KO NKL cells show a near complete loss of PLA positive spots in the STX11 KO cells.

Figure 7 A is confusing as septin 7 should have a molecular weight of ~ 51kD and syntaxin 11 ~33kD and this does not appear to be the case in 7A. As mentioned above, two molecular weight markers per western strip would allow readers to work out the band size, but I suspect that they mislabelled this panel.

Yes, the blots were mislabeled in regard to the molecular weight marker. This has now been corrected in the revision. Additionally, all blots now show a larger area containing at least two molecular weight markers, which are identified.

The staining of syntaxin 11 looks quite different from previous reports on cytotoxic synapses using antibodies validated against patient cells lacking syntaxin 11 (eg Traffic 16:1330). Why is the appearance of syntaxin 11 so punctate here? Is it fixation dependent? How well is the specificity of the antibody being used, validated? Validation of this antibody is critical to making a convincing case for an interaction between STX11 and Septin 7.

The staining for STX11 is likely different because we are using Airyscan confocal imaging, which has much better resolution and signal-to-noise than what is seen in Traffic paper, which is using conventional confocal imaging. As indicated above, we now validated the anti-STX11 antibody in the STX11 NKL KO cell line.

In Figure 8 the authors return to using the inhibitor FCF rather than the more convincing siSept7 to show a decrease in signal from the proximity ligation assay for syntaxin 11 and STXBP2 (also referred to as Munc18-2). It would be good to use siRNA rather than the drug. It would also be important to include the individual stains of each of the antibodies used for PLA in order to demonstrate the localization as detected by these antibodies. Again, the source and specificity demonstration of these antibodies needs to be included.

We agree with the reviewer, but instead of using septin 7 siRNA depleted cells, we have used septin 7 KO NKL cells to address this question. As shown in Figure 8D and E, septin 7 KO cells show diminished STX11-STXBP2 PLA spots. This suggests that either stabilization of septins with FCF or loss of septin filaments through KO of septin 7 lead to impaired STX11-STXBP2 interaction.

As shown in the figures above – NKL STX11 and STXBP2 KO cell lines were generated and the antibodies were validated for both traditional IF, as well as in the PLA assay.

This data is used to support a model that septins play a role in strengthening the interaction between STX11 and STXBP-2. As this interaction is required for secretion, it is curious why septin localization is thought to decrease across the synapse where secretion occurs.

The subcortical staining decreases, but not all of the staining is gone. Also, septin filaments localize with the polarizing lytic granules, thus are brought along with the lytic granule to the CS. We have generated a model figure (Figure 9) to more clearly state the findings in the manuscript.

Another outstanding question is whether this interaction might be direct or indirect. Did the authors manage to show a direct interaction with purified proteins? A clearer explanation of the model would be helpful.

It is well established in the literature that STXBP2 and STX11 interact directly. Performing the requested experiment will not add any new information that is not already known and widely accepted. As requested by Reviewer 3 below, we will generate a 'working model' for inclusion in the final figure.

Additional comments:
The authors include some data in the supplementary figures that might be much better included in the main text. For example S7 supports a close association with lytic granules.

The data that this reviewer is referring to shows unconjugated NK cells spread on fibronectin and stained for septin and perforin. We have now included these data in primary Figure 5a. We were not sure what other supplemental data we could include in the primary figures, but we have now moved old Supplemental Figure 1 to Figure1. We now only have 5 Supplemental Figures as required by the JCB.

Materials and Methods section:
Please state where all reagents come from. In particular where does "forchlorfenuron" (FCF) come from and how confident are the authors about the specificity of this plant growth inhibitor for septins, which has been challenged (doi: 10.1128/EC.00191-14). This needs to be discussed so that readers can focus more on the siRNA studies or at least be aware of the caveats.

The FCF is purchased from Sigma - catalogue #32974. We added a statement in the results section as to the caveats of the drug and that is why we have also done experiments with siRNA. However, it should be pointed out that the cited article was primarily focused in yeast and the effects of FCF treatment on mitochondrial activities.

Another important omission from the materials section is the source, and validation, of the antibody against syntaxin 11. This is particularly important for the data shown in Figures 7 and 8.

The source of the STX11 antibody is provided in Table S1. As indicated above, we have generated NKL STX11 and STXBP2 KO cell lines to validate the antibodies use for IF and PLA. We feel that the centrosomal staining of STX11 that is still observed in the STX11 KO NKL cells represents non-specific accumulation, which can be seen with several antibodies. Importantly, the membranous staining is limited/gone in the majority of cells (this is a polyclonal KO pool not a clone – so we might expect some level of staining if a non-KO cell is in the image view).

It is not clear which detergent is used for permeabilization from the SurfactAmps range from ThermoFisher (p12). Please specify.

Surfactamps was obtained from ThermoFisher and is catalogue #28314. This has been added to the Materials and Methods section.

For all figures please state:
(i) whether images are single planes or projections;

This has been provided in the revision.

(ii) include at least 2 molecular weight markers per western blot slice so that the reader can judge the molecular weight of the band shown.

All blot images are now larger and contain at least 2 molecular weight standards.

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

This work addresses the role of the septin cytoskeleton in regulating the exocytosis of lytic granules at the cytolytic synapse established between NK cells and target cells. The authors show that stabilization of septins, triggered by pharmacological treatments or silencing particular septins (2 or 7) decreases degranulation and impairs killing by NK cells. Interestingly the authors find that septins co-purify with lytic granules and interact with components of the SNARE fusion machinery, such as syntaxin 11. In other cell types, septins are known to regulate membrane rigidity as well as interact with snare components, however their role in NK cells remains less understood. Thus, this work advances our knowledge on cellular mechanisms that regulate NK cytotoxicity.

We thank the reviewer for their appreciation of the work and their concerns that have substantially improved the manuscript.

1. Main points:

i-Septins interact with Dock8 in NK cells: the immunoprecipitation displayed as a supplementary figure is very clear and supports their point.

OK

ii-Septin filaments do not appreciably accumulate at the NK-target cytotoxic synapse: Figures 1 and Sup figure 2 support the claim that septin 7 mainly accumulates at cortex. However, it remains unclear if this cytoskeletal component is actively cleared from this platform. This point could be strengthened by evaluating the dynamics of septin 7 distribution in NK cells upon forming a synapse. The authors could perform live cell imaging of NK cells expressing a fluorescently tagged version of septin 7 and track its distribution upon encounter with a target cell. If this tool is not available, the authors should stain for septin 7 localization in NK cells forming conjugates at early and late time points. Importantly, the time points used to evaluate conjugates in not specified.

Unfortunately, we are not set up to do live cell imaging of conjugates at this time. Thus, as suggested by the reviewer, we have performed a time course experiment and evaluated Septin 7 localization at the contact site. This new data is shown in Figure 2C.

We added the time points used for evaluation of images in the current figures to the Figure Legends.

iii-Septins are required for NK cell-mediated killing: The authors used a plant cytokinin (FCF), which enhances septin stability and show that in NK cells this drug impairs cytolytic functions. Similar results are shown when septin 2 or 7 are depleted. The killing assays are clear and although the defects are not very strong, especially considering the scales of the graphs, they seem to be consistent. The authors should show the effect of FCF in NK cells in terms of septin cytoskeleton. Are septin bundles observed under the experimental conditions that were used? Staining of the septin cytoskeleton should be shown (a similar figure to sup 5B should be included).

We have done this experiment several times and we have not noticed dramatic changes in the septin cytoskeleton in either NK or NKL cells, like that seen in the si-septin 7-treated cells. However, in each experiment that was performed, we tested the ability of the cells to spread on fibronectin and this was still substantially impaired. While we don't have an explanation for this, it is clear that FCF is having an effect on membrane protrusion that would be required for cells to spread, and form effective conjugates.

iv-Role of septins in NK cells conjugate formation: The authors show an impaired spreading response in FCF-treated NK cells treated seeded on fibronectin as well in the formation of conjugates, which was not observed in septin depleted cells. The authors explain why there could be a functional difference between both conditions in terms of

conjugate formation. Given this result, shouldn't FCF treatment have a stronger effect on NK cell-mediated killing? This point also be addressed.

One cannot compare the killing experiments to the conjugate assay as they are very different in the way they are performed. In the killing assay, cells are spun together and while adhesion is critical, there is probably some level of killing even when adhesion is slightly impaired. In the conjugate assay, cells are spun together, allowed to incubate for various timepoints and then vigorously vortexed prior to being fixed and analyzed. Any weak conjugates, that might have allowed killing would be dissociated in the conjugate assay. We will provide a comment addressing this in the Discussion.

v-Loss of septin 7 in lytic granule/MTOC convergence to the CS: The results displayed seem to indicate no difference in the recruitment of lytic granules or MTOC to the synaptic membrane, however it is still possible that this process could be slowed down in septin-silenced cells. The authors should indicate time points at which conjugates were analyzed and determine whether there is a delayed recruitment of lytic granules, which could account for the defects observed in killing.

We have performed a time course of MTOC localization using septin 7 KO NKL cells. These new data in Figure XX, show that MTOC polarization occurs with the same kinetics in control and KO NKL cells. Also the PMA + Iono suggests that delayed recruitment is not the issue since signaling from the CS (as a result of defective F-actin, signaling pathway activation), but is most likely at the level of lytic granule fusion.

vi-Septins localize with lytic granules: Indeed, this is an interesting observation, which is supported by biochemical assays. However, the images displayed do not seem to show that both are significantly localized together at the cs, particularly Septin 1. The authors should show quantitative data of the images to better highlight this point.

As indicated above in response to Reviewer 1, we have added a better septin 1 image. It is not clear what sort of quantitation one would do here. We are already confirming the microscopy observation of septins being adjacent to lytic granules by biochemical purification of septins with the lytic granule fraction. We would agree that performing quantitation would be important if we were attempting to show that loss of some key lytic granule protein was affecting the association of septins with the lytic granule, but this is not the point of these data.

vii-Septin depletion or stabilization impair NK cell degranulation: The degranulation assays in NK cells under different conditions, clearly support a role for septins and their stabilization in the process of degranulation. However, the conclusion stating that NK cells must "dynamically reorganize their septin filaments to degranulate" is not formally demonstrated. This point should be evaluated further. Images of lytic granules in FCF-treated cells should be shown and the localization of lytic granules evaluated. As stated above (ii), the dynamic re-organization of the septin cytoskeleton should be directly evaluated.

Examining the localization of lytic granules in FCF-treated cells will not be informative, since these cells do not form conjugates, we would merely be looking at lytic granules in unstimulated cells. We have performed the timecourse of Septin 7 at the CS and these new data are shown in Figure 2C as requested.

viii-Septins interact with lytic granule machinery: the data presented are very supportive. The Co-IP between septin 7 and syntaxin 11 suggests that both proteins interact. However, this interaction seems to occur in resting conditions. The authors should comment on this observation.

We will comment on this as requested by reviewer 1 also.

It is important to point out that the results shown in this section seem rather contradictory with the initial findings/conclusions of the authors, where they state that "septin filaments do not appreciably accumulate at the NK/target cytotoxic synapse" (figure 1). Indeed, this point is confusing, and the authors should clarify how they view the association of septin 7 with the cytolytic synapse and particularly with lytic granules within this area.

As indicated above in response to reviewer 2, we believe based on our data that the sub-cortical septin cytoskeleton that provides rigidity to the plasma membrane is 'depolymerized' at the NK-tumor cell contact site, but as indicated in the text, septin 7 staining is not completely absent. Moreover, the observation that septins also co-purify with the lytic granules indicates that the septins involved in lytic granule fusion, might arrive at the synapse with the lytic granules. We will make this clearer in the revision and it will likely be aided by a 'working model' that has been added to the revised manuscript (Figure XX).

Additionally, PLA spots (used to monitor septin-syntaxin 11 interactions) were detected throughout the plasma membrane. This observation would suggest that septins are not specifically required for degranulation within the cytolytic synapse. This important point must be discussed (see next point).

We agree. We added this to the Discussion as requested by both Reviewer 1 and 3.

viii-Septin stabilization/depletion impair the interaction between STX11 and STXBP2: the biochemical and imaging data support a role for septins in the interaction between snare components. This point is interesting however, functional implications of this interaction are lacking. This could be clarified by measuring degranulation events at the synaptic membrane to provide a clearer functional link between the role for septins in bringing the snare machinery to lytic granules and their degranulation at the cytolytic membrane.

We agree that this would be very interesting and further support the hypothesis that septins are involved in lytic granule fusion. However, establishing live cell TIRM imaging using pH-sensitive fluorophore-conjugated Lamp1 in cells that have been knocked out

for septins is not unattainable in the current situation, but is definitely something we will be working toward.

Additional issues:
Overall, the figure legends should better describe the experiments (include time points and statistical analysis). A scheme depicting the mechanism proposed by the authors on the role of the septin cytoskeleton in lytic granule release should be included.

We have made every attempt to add this information to the Figure Legends in the revised manuscript.

July 21, 2020

RE: JCB Manuscript #202002145R

Dr. Daniel D Billadeau
Mayo Clinic
Mayo Clinic 200 First Street SW
Rochester, MN 55905

Dear Dr. Billadeau:

Thank you for submitting your revised manuscript entitled "The Septin Cytoskeleton Regulates Natural Killer Cell Lytic Granule Release". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, please be sure to address reviewer #3's final minor concerns.

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, 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.**

1) Text limits: Character count for Articles 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.

2) Figures limits: Articles may have up to 10 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.

) 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 (either in the figure legend itself or 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 and title: The abstract should be no longer than 160 words and should communicate

the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

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.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) 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.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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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,

Ana-María Lennon-Dumenil, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors have satisfactorily addressed all the main points raised in my previous review

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

The revisions have addressed my queries.

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

In this revised manuscript the authors have addressed most of the issues that I have raised, either experimentally or in the discussion and therefore I have no further comments.
Some minor points to consider:
Figure 2: The 3D projection does not add any additional information and does not need to be included. In the legend it states: "The individual images are shown from slices XX to XX", the values should be indicated.