

Peer Review File

Manuscript Title: Blebs Promote Cell Survival by Assembling Oncogenic Signaling Hubs

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In this interesting and very relevant study the authors develop and try to prove an exciting idea that blebs are the cause of anchorage independent cell survival via septin-mediated signaling hubs as way for detached cancer cells to escape anoikis. A central mechanistic theme that authors try to address is that the positive membrane curvature at the neck of blebs recruit curvature sensing septins that assemble into signaling platforms that include oncoproteins that promote survival signaling such as originating from mutant NRas . The two complementary approaches to affect bleb formation based on glycocalyx cross linking by WGA or VitroGel coffins with integrin binding RGD domains are original and very well suited to explore how bleb perturbation affects signaling and phenotypic outcome. The data provided to show a relation between bleb formation, septin assembly and survival signaling in detached cells is convincing and important for cancer biology. However, the evidence provided that the positive membrane curvature at the neck of the bleb is the cause of the septin mediated assembly of signaling hubs is not consistently convincing. The question whether the septin assembly is mediated by septin-cortical actin interactions instead of solely membrane interactions, was also not addressed. The major point for which there is a lack of evidence is that survival signaling originates from septin platforms at the boundary of blebs. However, in my opinion this could be addressed by the authors with further experimentation, specially using the approaches described in the last part of the ms on bleb dynamics and septin recruitment.

Concluding, the study contains very relevant and innovative ideas in relation to morphodynamics and signaling that need more work in order to univocally nail the mechanism. From a personal view, I would not start the manuscript with a scheme that explains the mechanism before solid proof is provided by the experiments. Such a scheme works better as a last figure.

I will try to discuss the individual parts below highlighting the strong and less strong points from my perspective.

Relation between blebbing of detached cells and survival

Strong points

Inhibition of blebbing by WGA induces strong additional cell death in detached Nras driven MV3 cells and to a lesser extend in BRasV600E driven M498. This is interesting because BRasV600E could provide survival signals from the cytoplasm. This effect is also seen for MV3 cells in vitrogel where

anchorage is induced by the integrin binding domain RGD. These two completely independent measures of the effect of bleb perturbation makes this very strong in my opinion.

Slightly weaker point

Only 'prestressed' A375 cells show strong additional cell death when detached. This points at an additional factor (dynamic state of actin cytoskeleton?) that has a strong influence on survival of detached cells because these cells exhibit blebs (Fig.3A)

The relation between membrane curvature and septin recruitment

Strong point:

Lower fraction of peripheral cell surface that recruits septins upon bleb inhibition (WGA, VitroGel).

Weaker points:

The data points at blebs excluding Septins, not necessarily recruiting at positive curvature. For negative curvature in the blebs there is a precipitous drop in recruitment whereas area with low curvature (close to -0.1) recruit almost equally to positive curvature area near bleb (Fig.2C,E). The problem is that septin recruitment does not follow a similar pattern as curvature (Fig.1E). This is also a bit misleading in figure 1C where the recruitment between -0.1 and 0.4 drops as little as for a curvature between 0.4-2. The problem is that the overall curvature of the bleb-free areas of the cells should be indicated to provide a reference point to not confuse with the negative curvature within the blebs.

Same applies to figure 1H, where the same overall recruitment is observed for the three cases above and below 0.4 μm^{-1} curvature. Also correlation coefficients should be provided for each case in these graphs.

Both WGA and vitroGel coffins affect overall septin recruitment. Is there an effect of these perturbations on the dynamical structure of the cortical actin cytoskeleton that affects septin recruitment instead of membrane curvature? What is the relation of septin recruitment to cortical actin structures? The reason I ask this is because maybe it is the cortical actin dynamics that cause septin recruitment that correlates with bleb formation. There could be a strong interrelation between dynamic cortical actin structure, septin recruitment and bleb formation here that is difficult to disentangle.

One thing that points in this direction is that Ezrin inhibition induces large scale cortex membrane detachment that inhibits septin recruitment (Fig. 2F). I presume that ROCK inhibition also affects actin dynamics.

I therefore would recommend to perform imaging of the actin cytoskeleton in relation to septin localization for the different perturbations.

The Septin structures look also quite different in MV3 and M498 cells, being more fibrillous in the latter case (Fig.3A). This could also point at different actin mesh configurations. The relation between the blebs and the septin structures is also not very apparent. Interestingly the non-stressed A375 cells still exhibit enrichment of septins (albeit more diffuse) at the cell cortex. The contrast could be optimized in Fig.3A.

Survival signaling from septin platforms

Strong

Reduced Erk activation upon septin inhibition by FCF or dominant negative SEPT2(33-306) as well as vitrogel coffins in detached cells (and not in adherent cells). This shows a clear relation between blebs, septins and enhanced (survival) signaling. It however does not show that this enhanced signaling originates from septin-NRas scaffolds. See also the following point.

Weaker

Inspection of Figure 4A reveals that the detailed distribution of septins is rather different than that of NRas. For example, NRas tends to be inside the blebs whereas septins are not. The data related to this figure therefore needs a careful and quantitative colocalization analysis. The same holds for the PH domain distribution in figure 4H. The reason why this could be important is that the septins could affect the morphology of the membrane (and the blebs) that could not only change the amount of NRas on the membrane (for example by a change in surface/volume ratio) but also change the interaction of NRas with effectors in septin free areas (for example by trapping of effectors such as PI3K within the blebs). This would mean that septins change the membrane morphology in a way that affect Ras signaling from the plasma membrane without generating septin based signaling hub complexes that contain Ras. Data as shown in figure S3 could also provide information on this effector engagement in specific areas by ratiometric representation of the NRas/PH domain images. For this ratiometric analyses the signal/noise of the images has to be improved however.

Bleb dynamics in relation to cortical septin structures

The last part of the manuscript is from my perspective the most interesting part, because it relates bleb dynamics to the nucleation of septins. The idea that the dynamically instable blebs generate a transient positive curvature that initiates the nucleation of septins that can then stabilize after reaching a sufficiently high threshold would explain why larger septin oligomers are not confined to the neck of the bleb as seen in the static data. It might also explain an interrelation between bleb and septin formation and a possible role of the dynamic actin cortex. Unfortunately the data presented is very sparse and qualitative, merely showing some examples. Quantification is needed. More importantly, this approach could be used to relate bleb dynamics and septin assembly to signaling by time-lapse co-imaging of septins, Ras and the PH domain. This could prove or disprove the septin signaling scaffold idea for survival signaling or reveal another septin related mechanism in my opinion.

Philippe Bastiaens

Referee #2 (Remarks to the Author):

This is a very interesting and exciting study, which reports a novel morphodynamic mechanism for the pro-survival signalling of non-adherent cancer cells that evade anoikis. The paper shows septins assemble and form pro-survival signalling hubs on the positive curvature domains of cell membrane blebs. Conceptually, this discovery introduces a novel signalling paradigm and indicates a novel oncogenic function for septin GTPases that stems from their membrane curvature-binding properties.

The authors combine cutting edge microscopy (e.g., lattice light sheet) and tools to manipulate cell adhesion (e.g., hydrogels), innovative computational image analyses, as well as pharmacological and dominant negative approaches and signalling assays to demonstrate that membrane blebs and septins promote the survival of non-adherent cancer cells. In total, the work is both a technical and conceptual tour de force.

Overall, the novelty of the findings and the exciting implications for cancer cell biology make this manuscript highly suitable for publication in Nature. However, the manuscript requires revisions to strengthen the key results on septins and to improve conceptually the function of septins and their contribution in cancer cell escape of anoikis.

In particular, the specificity of septin-targeting approaches requires improvements, and more evidence and conceptual clarity is necessary for the localization and function of endogenous septins, and their oncogenic potential through the reported morphodynamic mechanism.

Major concerns/issues:

1) The FCF compound along with a dominant negative SEPT2 construct are used as a means of disrupting SEPT6 function. These approaches are not very septin and SEPT6-specific.

First, it is unclear why the authors focused on SEPT6. A better justification could be provided based on emerging knowledge of a C-terminal amphipathic helix that recognizes membrane curvature, and is conserved between the yeast septin Cdc12 and SEPT6 (Cannon et al, J Cell Biol 218:1128, 2019). In that regard, use of a SEPT6 C-terminal mutant would be more appropriate and specific for disrupting SEPT6 function. Of note, a SEPT6 C-terminal truncation functioned as dominant negative in a recent study (Hamdan et al, Nature Communications 11: 100, 2020) and a SEPT6 construct lacking the amphipathic helix is being used by some labs. These constructs can be used in conjunction with SEPT6 knock-down in a gene replacement-like approach (expression from bicistronic plasmid that expresses septin shRNA).

Second, although the FCF compound binds and affects septins directly, it is thought to have more targets than septins. This reduces confidence in the interpretation of the data obtained with FCF. It is also conceptually difficult to understand the nature of the effects. FCF stabilizes septin assemblies, so one can potentially envisage that FCF would amplify membrane-associated septins stabilizing membrane blebs and enhancing signalling. It is unclear how FCF impacts septin localization on the membrane, and the dynamics of membrane blebbing. At the very least, it is critical to demonstrate

that SEPT6 loss-of-function approaches impact septin localization to blebs and/or membrane blebbing. At a basic level, testing whether SEPT6 knock-down and over-expression reduce and enhance blebbing, respectively, are necessary experiments, which would also provide clarity in whether septins play a role in blebbing in addition to forming a signalling scaffold/hub (see below; point #3).

2) All observations with septins have been conducted with an ectopically expressed SEPT6-GFP. To ensure validity of observations, it is critical to demonstrate that endogenous septins localize similarly at the base of membrane blebs and show the overall localization/distribution of endogenous SEPT6 in the cell types used.

SEPT6 has been posited to contain a nuclear localization signal, and shuttling of SEPT6 between the nucleus and membrane might be of functional relevance to the signalling aspects of this work, and the interpretation of the findings. Have the authors looked at endogenous SEPT6 in the cancer cell lines of this work and is there any indication of SEPT6 presence in the nucleus in adherent and non-adherent conditions? The interaction with nucleolin hints on the possibility of SEPT6 localizing in the nucleolus as it's also seen for SEPT9 in some cell lines.

3) Figure 5 presents a very interesting finding and the idea of septins as a spatiotemporal integrator and filter of membrane blebbing events is conceptually novel. The finding is much more relevant and suitable to Figure 2, which provides the initial septin characterization with respect to membrane blebs. As a stand-alone figure 5, this finding comes across a bit as an afterthought and not as substantive as the rest of the figures of a Nature publication.

Additionally, the figure highlights an obvious question about the role of septins with respect to membrane blebbing. Are septins (SEPT6) required for membrane blebbing to take place? Or do they merely accumulate at curvature domains to scaffold pro-survival signalling?

This is also an important question with respect to the concern above about endogenous septins. It is plausible that septin over-expression enhances membrane blebbing, which in turn promotes the formation of septin-based membrane curvature pro-survival signalling hubs. Ectopic expression of SEPT6-GFP expression would amplify this effect, as a result of artefactual over-expression, but in the context of endogenous septin over-expression might be highly significant for cancer pathophysiology. Especially as septins are known to be over-expressed in cancer. Therefore, septin over-expression may drive a positive feedback loop between blebbing and signalling in cancer cells.

It would greatly enhance the physiological relevance of the paper's findings if the authors can provide any data that demonstrate a correlation between septin over-expression and anoikis resistance in the cell line panel of their study, which can be done by looking for number of SEPT6 gene copies using FISH and levels of SEPT6 mRNA. For example, is there a SEPT6 upregulation in the pre-stressed A375 cells? Additional data might be able to be derived from looking at septin levels of expression in cancer databases (COSMIC, TCGA, GENT2) for melanomas which are known to evade anoikis. This would further substantiate the septin function as an oncogenic mechanism, strengthening the implication of the septin findings for cancer.

4) How do the authors envisage septins (SEPT6) function in activating N-Ras at membrane domains of curvature beyond just recruiting or clustering it? Although this question goes beyond the scope of the current study, it's important to provide some clarity on the potential mechanism of signalling pathway activation. Do septins form scaffolds for N-Ras activation by GEFs? How do they function in the case of PI3K signalling at the cell membrane? Some pointers in the text would improve the conceptual framework of the findings.

Minor issues:

1) Comparisons in Figures 1B, 3D, 4D-F require tests of statistical significance.

2) In Figure 2H, the linear fits are not clear. In general, this graph is difficult to comprehend. It is also unclear what the shaded colored surface contours that accompany the plot points represent and how they were derived.

3) The mechanisms and signalling pathways that mediate anoikis resistance should be more explicitly stated in the introduction of the manuscript. It is unclear whether the signalling factors and pathways (N-RAS, MAPK, PI3K), which are linked to septins, have been established as anti-anoikis pathways and mechanistically how they prevent cell death during anoikis. This should be more explicitly addressed through text revisions.

Elias Spiliotis

Referee #3 (Remarks to the Author):

The manuscript "Blebs Promote Cell Survival by Assembling Oncogenic Signalling Hubs" by Weems et al reports on a novel mechanism allowing the survival of cells whose cell-substrate adhesion is compromised. The authors propose that blebbing activity leads to the formation of hubs for signalling proteins that protect melanoma cells from death when detached from the substrate. The model the authors present is novel, is potentially relevant for a range of other cell types in different physiological and pathological contexts and as such is in principle of interest for the readership of Nature. The results are mostly very convincing and the paper reads very well.

I have two general suggestions the authors must have thought of, but mention them here, just in case. The first would make the paper of relevance for a broader audience, and the second would increase the confidence in the proposed model.

A. To try to identify unrelated cell type/s, where bleb-dependant anoikis-resistance occurs, thereby making the proposed model relevant beyond the melanoma field. A possible way to screen for such cases would be to induce blebbing in cells that depend on cell adhesion for their survival and check for resistance to anoikis.

B. Another line of experiments that would strongly support the model suggested by the authors would be to conduct gain of function experiments to activate/enhance the resistance, rather than “just” reducing it as they do. For example, if enhancing blebbing activity or the level of relevant signalling increases the resistance of melanoma cells to anoikis, the model would be more convincing. e.g. add activated Ras / Remove PTEN / increase contractility in melanoma cells and determine the resistance to anoikis. As it is, the results of the experiments support the model the authors propose, but at the same time, could stem from adding more stress to cells that are already stressed by the detachment.

I am listing below some more specific comments in order of appearance in the paper that I hope help improving it.

1. Define “PI3K” the first time it is mentioned.

2. “to be concentrated in the vicinity of melanoma blebs, including KRAS and active PI3K 14,31,32 “. - Is reference Nr 32 wrong here?

3. “To investigate putative bleb function as a driver of anchorage-independent cell survival we designed assays that consistently minimized both cell-substrate and cell-cell attachment over a period of 24 hours. We accomplished this goal by seeding cells at very low density (approx. 250 cells/ml) in non-adherent culture dishes under constant gentle agitation.”

– I guess there is a simple explanation to that, but if the point is to present the mechanisms allowing cell survival under non-adherent condition, why not do it without the agitation? The way it is presented, one could argue that the phenomenon studied is resistance to agitation, not to lack of adhesion. This should be made clear to readers not familiar with the experimental system.

4. “Both adhered and detached M498 cells die upon WGA treatment, but detached cells experience significantly more death, both in aggregate and in individual matched experiments (Fig 1B, Fig S1).” - In Fig 1B indicate on the X-axes the amounts of WGA also for M498 and A375.

5. “..at first sight indicating that bleb inhibition is not a universal mechanism for establishing anoikis resistance.”

-The basis for the resistance of unstressed A375 is not clear. The confidence in the result would be increased if a similar TREND is observed in the other cell types. i.e. does pre-stressing makes cells more bleb-dependent in the other cell types.

6. “these results indicate that dynamic blebbing contributes to anoikis resistance both in NRAS- and in BRAF-mutated melanoma, suggesting that this cell morphological program is a broadly adopted survival strategy in melanoma cells.”

-In the spirit of “broadly”, as mentioned above, identifying more cell type/s that show the bleb-dependent resistance would make the results relevant for a much broader audience. Can anoikis resistance be conferred to sensitive cells by inducing blebs (by enhancing contractility).

7. In Fig 2F, in the WGA treatment, it looks like the amount of Septin scaffolds is even higher than in untreated cells, although those are not as evenly distributed. If the role of the Septin scaffold is just

to bring together the signalling molecules, what is the basis for the WGA effect on the resistance to detachment? Based on the presented data, it is not clear why WGA should have an effect.

8. In the Figures the authors use the term “Unstressed A375” and in the text “Non-stressed A375”. Better to use the same term.

9. “Taken together, these data suggest that the mechanism allowing blebs to confer anoikis resistance upon melanoma cells depends on bleb adjacent cortical septin structures.”.

-Is there an effect for the drug treatment on bleb formation?

10. “Importantly, reexamination of our previous pulldown data revealed that NRAS interacted with SEPT6 in MV3 cells, but not in unstressed A375..”

– Does it interact in pre-stressed A375 cells?

11. “..and our previous work has shown that bleb inhibition alters PI3K activity¹⁴”.

- Doesn't the previous work mainly show that PI3K enhances blebbing and not vice versa?

12. “we found that high PI3K activity co-localizes with both septins and NRAS in rounded, poorly-adhered MV3 cells embedded in soft collagen, in support of septin-scaffolded NRAS signaling through PI3K (Fig 4G, Fig S3). “

-This point is not very clear in Fig 4G. Most of the PI3K activity is found in positions where Septin is not observed.

13. “our high-speed time lapse data showed stable septin structures emerging only when several pulses occurred in close proximity, resulting in their coalescence into bright, long lived structures whose intensity does not seem to rely on local curvature profiles”

-This point is not obvious from the movie. Perhaps running it slower and marking which blebs to look at would help. As it is, it is not clear why Septin polymerization occurs at the specific point shown.

Referee #4 (Remarks to the Author):

Comments to the authors:

The paper reports the accumulation of septin at the curved base of plasma-membrane blebs that actively form in non-attached cells. These septins in turn recruit active mutant NRAS and effectors, themselves associated with upregulation of ERK and PI3K and their signaling cascades. While blebs of detached cells recruit the mutant NRAS, they fail to efficiently recruit wt NRAS and don't activate ERK/PI3K signaling, resulting in cell death. These and other clever cell biological experiments demonstrate an unforeseen relationship between membrane blebbing and cell survival. These observations are particularly interesting and timely, given that most earlier studies connecting membrane blebs and signaling have been done in the context of cell motility and not in relation to cell survival. This topic is of relevance to cell survival and metastasis, especially in the context of

minimal cell adherence.

This study includes well performed and properly described cell biological experiments such as effect of relevant mutants, force perturbation of cell morphology, proximity labeling and mass spectrometry and so forth. Key for the investigation was live cell visualization that capitalized on the virtues of diagonally scanned light sheet microscopy implemented by the Danuser group; this approach provides thin plane illumination and detection, 3D visualization and low photo toxicity.

Septin polymers act as large-scale membrane curvature sensors. In addition to previous elegant studies from the Danuser group, others have also contributed in significant ways, see for example Beber et al, Nat Commun. 2019 Jan 24;10(1):420. Membrane reshaping by micrometric curvature sensitive septin filaments. Their work should also be mentioned.

Several studies have noted appearance of blebs during mitosis. While the Weems et al study refers to Sedzinski, J. et al. Polar actomyosin contractility destabilizes the position of the cytokinetic furrow. Nature 476, 462–468 (2011), a more relevant reference would be E. Boucrot, T. Kirchhausen PNAS 2007, 104:7939. Endosomal recycling controls plasma membrane area during mitosis.

The authors used a home-built microscope with stated raw resolution of $\sim 400 \times 400 \times 400$ nm. The size of the point spread function would also be around this range. I am therefore unable to follow what they mean by an optical section of 160 nm. Perhaps they mean a step movement of 160 nm along the z-axis? Please clarify.

Missing in the study by Weems et al is a detailed description of how the software was used in the analysis of the images. The authors state "All code/algorithms/software central to the findings of this study are available from the corresponding author upon reasonable request. Most (italics added) can be found at <https://github.com/DanuserLab>". Similarly, the authors state that "Cell morphology and septin localization were analyzed principally via u-shape3D". What were the analyses that were done with other algorithms or other software? It is essential that published manuscripts should uphold to current standards and provide enough details so that the study can be fully reproduced; this applies not only to experimental aspects but also extend to the use of analytical tools. In particular, all the code/algorithms/software should be deposited in the github directory cited; "reasonable request" is inappropriately vague.

Author Rebuttals to Initial Comments:

We thank the referees for their exceptional engagement with our previous manuscript and for the consistently useful critiques and suggestions. Based on these reviews, we have performed several new experiments and analyses that significantly strengthen the paper, increasing its impact, breadth, and quality. Following are our point by point responses to the questions raised.

We sincerely appreciate the time and effort for a second round of feedback,

The Authors

Referee #1 (Remarks to the Author):

In this interesting and very relevant study the authors develop and try to prove an exciting idea that blebs are the cause of anchorage independent cell survival via septin-mediated signaling hubs as way for detached cancer cells to escape anoikis. A central mechanistic theme that authors try to address is that the positive membrane curvature at the neck of blebs recruit curvature sensing septins that assemble into signaling platforms that include oncoproteins that promote survival signaling such as originating from mutant NRas . The two complementary approaches to affect bleb formation based on glycocalyx cross linking by WGA or VitroGel coflins with integrin binding RGD domains are original and very well suited to explore how bleb perturbation affects signaling and phenotypic outcome. The data provided to show a relation between bleb formation, septin assembly and survival signaling in detached cells is convincing and important for cancer biology. However, the evidence provided that the positive membrane curvature at the neck of the bleb is the cause of the septin mediated assembly of signaling hubs is not consistently convincing. The question whether the septin assembly is mediated by septin-cortical actin interactions instead of solely membrane interactions, was also not addressed. The major point for which there is a lack of evidence is that survival signaling originates from septin platforms at the boundary of blebs. However, in my opinion this could be addressed by the authors with further experimentation, specially using the approaches described in the last part of the ms on bleb dynamics and septin recruitment.

Concluding, the study contains very relevant and innovative ideas in relation to morphodynamics and signaling that need more work in order to univocally nail the mechanism. From a personal view, I would not start the manuscript with a scheme that explains the mechanism before solid proof is provided by the experiments. Such a scheme works better as a last figure.

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Strong points

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Slightly weaker point

Only 'prestressed' A375 cells show strong additional cell death when detached. This points at an additional factor (dynamic state of actin cytoskeleton?) that has a strong influence on survival of detached cells because these cells exhibit blebs (Fig.3A)

This is a very valid point and has triggered significant further experiments and thinking on our behalf. Indeed, as the referee points out, several factors play into the process of cortical septin assembly. One necessary factor for cortical septin assembly is blebbing, as shown already with the original manuscript. However, in certain conditions blebbing is not sufficient for this assembly, as evidenced by the scenario in A375 cells. At first sight, it seems logical that the anoikis-resistance of normal A375 cells is insensitive to bleb inhibition because of constitutively active MAPK-signaling downstream of cytoplasmic BRAFV600E (Fig. 1B). It is then puzzling that A375 cells that are cultured in overconfluency for extended periods (the “pre-stressed” condition described in the manuscript) become sensitive to bleb inhibition although they are still expressing the same BRAFV600E mutation. In Fig. 3A we partially resolve the puzzle by showing that regular and pre-stressed A375 cells differ in the absence of cortical septin assembly in the former – despite their blebbing activity (!) – versus the presence of septin assembly in the latter. The exact reason behind the absence of septin structures in regular A375 cells remains to be determined. We are in the process of developing and testing hypotheses to explain this particularity of septin biochemistry (including the effects of cortical actin state), but no immediate answers are in sight. However, this should not detract from our core message that cell blebbing confers survival signaling/anoikis-resistance via promotion of septin scaffold assembly.

We have, however, tested other stress conditions in A375 to better understand conditions that promote septin assembly. Intriguingly, one such stressor is co-treatment with the MAPK inhibitors Dabrafenib and Trametinib, a drug combination used against BRAFV600E-harboring melanoma in clinical settings. MAPK inhibition both drives the formation of septin structures in A375 cells and sensitizes these cells to bleb and septin inhibition as seen in “pre-stressed” A375. This suggests bleb/septin-mediated signaling could be acting as an important non-genetic drug resistance mechanism in melanoma.

An important paper by the Sanz-Moreno lab in 2020¹ shows that melanoma drug resistance (including in A375 cells specifically) requires blebby amoeboid morphologies, and that blocking this morphology via ROCK inhibition (coincidentally, using the same ROCK inhibitor we show ablates blebs and septin structures in figure 2) causes cell death and restores drug sensitivity *in vitro* and *in vivo*. The authors further show that the morphology is correlated with drug resistance in human patients. However, the paper offers no mechanism for how blebby morphologies translate into melanoma survival. Our data now suggests that this drug-resistance phenomenon is driven by bleb/septin signaling. While this does little to explain how stress activates septin structure formation in A375 cells, it adds important context to the result and greatly improves the relevance of the paper.

A foreword to our point-by-point rebuttal of this section:

The reviewer makes the point that septins are not only known to bind membrane, but also actin. Because we lack the resolution to definitively observe whether septins are binding the plasma membrane or actomyosin cortex, the reviewer offers the alternative hypothesis that bleb-related actin changes might be recruiting septins instead of membrane curvature as we suggest. He goes on to develop this hypothesis in his interpretation of our data. We sincerely appreciate the intellectual engagement with our work that such an analysis requires. And while our comments below will show that we do not believe this hypothesis is well-supported, we would like to acknowledge, as the reviewer did in his comments to us, that such relationships are very difficult to disentangle. We agree that the cortical septin structures we observe are most likely interacting with both cortical actin and the plasma membrane. We would even go so far as to suggest that some cortical states likely affect these septin structures in a manner that influences their formation (or lack thereof). However, although septins might interact with actin after being recruited to the surface, our data (now expanded with new experiments and analyses) strongly supports bleb-induced membrane curvature as necessary for triggering this recruitment.

Strong point:

Lower fraction of peripheral cell surface that recruits septins upon bleb inhibition (WGA, VitroGel).

Weaker points:

The data points at blebs excluding Septins, not necessarily recruiting at positive curvature. For negative curvature in the blebs there is a precipitous drop in recruitment whereas area with low curvature (close to -0.1) recruit almost equally to positive curvature area near bleb (Fig2C,E). The problem is that septin recruitment does not follow a similar pattern as curvature (Fig.1E).

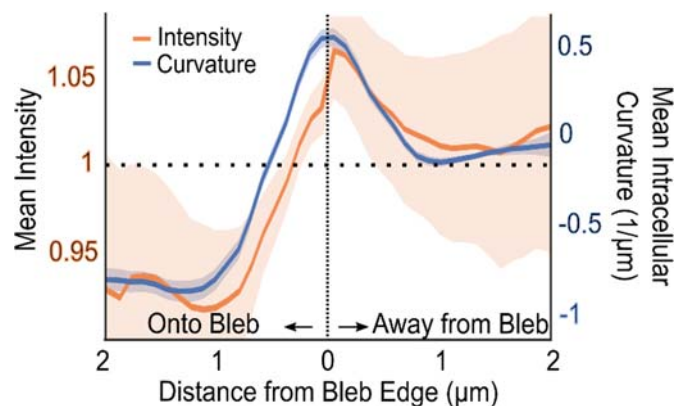
Importantly, our model argues that only a subset of the septins localized to the surface of these cells are being *actively* recruited by curvature: bleb-generated curvature consistently recruits septins to the plasma membrane, and those septins tend to lose membrane attachment once the bleb retracts and curvature is reduced. We refer to these events as septin “pulses” in the manuscript. If this was the whole of our model, then we would agree with the reviewer that the data do not fit our hypothesis. However, our model further suggests (in revised figures 2J-N, S7C, S8, movie 4, and associated text) that during some blebbing events curvature-driven local septin concentration reaches levels high enough to promote efficient septin polymerization, which stabilizes membrane binding such that it no longer relies on local curvature²⁻⁴. This leads to the formation of stable, long-lived septin structures whose localization to the cell surface is less dependent upon local curvature profiles.

The data in figure 2E are compatible with this model. The dataset is an aggregate of septin pulses (which are highly correlated with local curvature) and stable septin structures (which sample a wider range of curvature profiles). The figure shows high mean intensity of the SEPT6-GFP probe at the curvy bases of blebs, where the signals are consistently enriched

due to septin pulses, and a reduction in mean septin intensity as one moves away from the bleb edge towards less curved, non-bleb surfaces, where bright signals from stable septin structures are sometimes found. Because of the presence of the heterogeneous distribution of these polymerized structures across the non-bleb cell surface, the relation between SEPT6-GFP intensity and curvature is not linear. The drop is more precipitous as one moves towards the center of the bleb. This is probably affected by two mechanisms: i) reduced septin affinity for high negative curvature and ii) membrane/cortex crosslinking by polymerized septins⁵ biases them against localization to on-bleb membrane that has detached from the cortex during bleb formation.

Based on the reviewer's comments we performed additional experiments and analyses to more thoroughly explore the nature of septin exclusion from blebs and to corroborate curvature sensing as the primary mechanism of septin recruitment to the surface. First, as suggested also by Reviewer 2, we designed a septin mutant with disabled curvature sensing. It has been demonstrated that septins' increased affinity for positively curved membranes is due to the activity of an amphipathic helix located near the C-terminus of SEPT6, the removal of which destroys their ability to "sense" micron-scale positive curvature while preserving their ability to bind membrane⁴. Though much of this work has been performed using *in vitro* biochemical techniques, a recent paper by the Mostowy lab employs a SEPT6 mutant lacking this 18aa helical domain in human cells, showing that it efficiently integrates into septin complexes and blocks their ability to sense membrane curvature⁶. We recreated this mutation in our SEPT6-GFP construct and found that it completely blocks septin recruitment to the surface, supporting the idea that whatever other cellular conditions might influence septin localization in these cells, septin curvature sensing is absolutely required for bleb-driven surface localization (revised figures 2I and S6).

Next, to test our assertion that the lack of linearity in the relationship between SEPT6-GFP signal and curvature local to bleb edges is due to an admixture of curvature-dependent septin pulses and curvature-independent polymerized stable septin structures, we examined cells in which septin polymerization is blocked by a dominant negative mutant. We recreated the plot shown in 2E, but now using cells expressing SEPT2(33-306), a mutant allele that dominantly blocks inter-oligomer septin polymerization. We reasoned that if the stable structures present in flatter membrane regions require polymerization, we should see an increase in the rate of signal depletion moving away from the bleb edge, while enrichment at the bleb edge is preserved due to the continuation of curvature-dependent septin pulses. This trend is clearly evident in the new plot (revised figures 2N and S8), which shows that expression of the polymerization mutant effectively linearizes the relationship between septin localization and local curvature. This is especially apparent when the y-axis of the mean intensity plot is rescaled, as shown on the right.



We thank the reviewer for these comments, as the resulting experiments and analyses greatly improve confidence in the "membrane curvature as septin recruiter" model. Alongside our

new time series analysis of septin dynamics (described below) they give a more complete understanding of the interplay between septin recruitment via curvature sensing and septin stabilization via inter-oligomer polymerization.

This is also a bit misleading in figure 1C where the recruitment between -0.1 and 0.4 drops as little as for a curvature between 0.4-2. The problem is that the overall curvature of the bleb-free areas of the cells should be indicated to provide a reference point to not confuse with the negative curvature within the blebs.

This is a good point, and we agree that curvature/intensity relationships are more informative when shown for both “on-bleb” and “off-bleb” portions of cell surfaces. We have altered the figure as suggested, now superimposing curves showing the septin/curvature relationship for on-bleb faces, off-bleb faces, and all faces. Notably, these groups produce similar curves, suggesting membrane curvature similarly influences septin localization regardless of whether that membrane is part of a bleb.

Same applies to figure 1H, where the same overall recruitment is observed for the three cases above and below 0.4 μm^{-1} curvature. Also correlation coefficients should be provided for each case in these graphs.

Figure 2H shows cortical septin levels expressed as a function of cell surface fraction containing different positive intracellular curvature profiles, either above the threshold previously shown to recruit septins ($>0.4 \mu\text{m}^{-1}$) or below it (between 0 and $0.4 \mu\text{m}^{-1}$). Because bleb surfaces possess almost exclusively negative intracellular curvature, on-bleb curvature data is already largely excluded from this dataset. Furthermore, because revised Fig 2C shows that on-bleb curvature domains are almost identically influenced by positive intracellular curvature as off-bleb surfaces, we feel it is appropriate to include whatever small amount of surface that is on-bleb and $>0.4 \mu\text{m}^{-1}$ within the dataset. We agree that correlation coefficients improve the figure, and they have been added.

Both WGA and vitrogel coffins affect overall septin recruitment. Is there an effect of these perturbations on the dynamical structure of the cortical actin cytoskeleton that affects septin recruitment instead of membrane curvature? What is the relation of septin recruitment to cortical actin structures? The reason I ask this is because maybe it is the cortical actin dynamics that cause septin recruitment that correlates with bleb formation. There could be a strong interrelation between dynamic cortical actin structure, septin recruitment and bleb formation here that is difficult to disentangle. One thing that points in this direction is that Ezrin inhibition induces large scale cortex membrane detachment that inhibits septin recruitment (Fig. 2F). I presume that ROCK inhibition also affects actin dynamics.

This is an interesting question, but we do not think it is likely. We chose to perturb blebbing using 4 orthogonal methods to control against the exact possibility that the changes we see in septin levels are caused an off target effect (such as affecting actin dynamics). Given that their mechanisms of altering curvature are entirely unrelated, it is improbable that they would

each produce the same effect. In all 4 cases we find that septin levels at the surface are greatly reduced, and that remaining surface septins are found at high curvature areas (even high curvature areas apparently created by non-bleb membrane morphologies, as seen in ROCK inhibition). We then use the 2 methods that most consistently reduce positive intracellular curvature (WGA and VitroGel) to quantitatively show that they also reduce surface septin levels, and that they do so in direct proportion to the amount of septin-recruiting curvature individual cells possess, regardless of treatment group. Considering the results of these perturbation experiments, along with the timelapse data in Figures 2K, 2L, and Movie 3 directly showing septin signal increasing at the curvy bases of blebs after bleb formation and fading as blebs retract and curvature is reduced, we think our data strongly support blebbing as the cause of septin structure formation. With this in mind, it is possible that some condition of cortical actin might be an intermediary in this causal chain, with blebbing causing an actin state that recruits septins to the cortex. And though Figure 2K and Movie 3 show septin recruitment occurring exclusively at the bleb base where positive intracellular curvature is generated, it is possible that the proposed actin recruiting event might also occur specifically at these locations. If so, it would also have to begin only after bleb formation and cease immediately after blebs are resolved in order to match our data. In short, it would have to be entirely simultaneous with and immediately adjacent to curvature profiles that have been shown to specifically recruit septins in multiple high quality publications (notably, these data include cell-free biochemical experiments that lack actin)^{3,4}.

With the addition of the new SEPT6(Δ AH)-GFP experiments (described above) showing surface recruitment is completely blocked when curvature sensing is mutationally disrupted, we hope the reviewer agrees that these results show the necessity of septins' ability to sense positive membrane curvature for the formation and accumulation of the septin structures described in this paper.

I therefore would recommend to perform imaging of the actin cytoskeleton in relation to septin localization for the different perturbations.

As outlined in our response above, we do not feel the data supports prioritizing pursuit of the "actin as septin recruiter" hypothesis within the scope of this paper. The proposed analysis is also significantly complicated by a lack of prediction for what specific actin characteristics might recruit septins. Would this actin state be observable using gross f-actin localization on the spatial scale of the microscopy performed in the rest of the paper? Would it be apparent in static datasets, or could it be a dynamic characteristic that would require timelapse microscopy and complex timeseries analysis of cortical actin dynamics? We feel that given the strength of the arguments laid out above, and because the central message of the paper, i.e. blebby morphologies drive anoikis resistance and cancer cell survival, would ultimately not be affected by the possibility that cortical actin conditions are intermediary players in the curvature-to-septin causal chain, such technically demanding and open-ended experiments are not justified.

The Septin structures look also quite different in MV3 and M498 cells, being more fibrillous in the latter case (Fig.3A). This could also point at different actin mesh configurations. The relation between the blebs and the septin structures is also not very apparent. Interestingly the non-stressed A375 cells still exhibit enrichment of septins (albeit more diffuse) at the cell cortex. The contrast could be optimized in Fig.3A.

The apparent differences in septin structures between the MV3 and M498 cells pictured in figure 3A are largely due to variance of optical resolution due to light scattering by the hydrogels these cells are embedded in. Regardless, we feel the important takeaway from this figure is the stark difference between the “septin positive” MV3/M498/prestressed A375/MAPKi+A375 cells and “septin negative” A375 cells, and the fact that sensitivity to both bleb and septin inhibition seems to be so binary in its correlation with the presence of cortical septins.

Survival signaling from septin platforms

Strong

Reduced Erk activation upon septin inhibition by FCF or dominant negative SEPT2(33-306) as well as vitrogel coffins in detached cells (and not in adherent cells). This shows a clear relation between blebs, septins and enhanced (survival) signaling. It however does not show that this enhanced signaling originates from septin-NRas scaffolds. See also the following point.

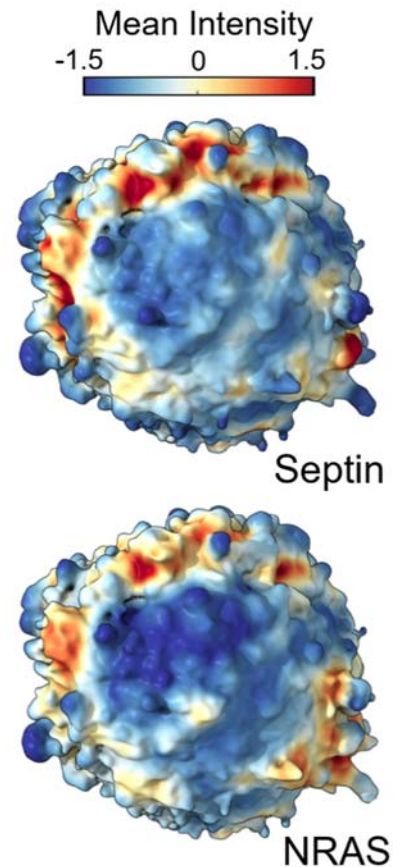
Weaker

Inspection of Figure 4A reveals that the detailed distribution of septins is rather different than that of NRas. For example, NrAs tends to be inside the blebs whereas septins are not. The data related to this figure therefore needs a careful and quantitative colocalization analysis. The same holds for the PH domain distribution in figure 4H. The reason why this could be important is that the septins could affect the morphology of the membrane (and the blebs) that could not only change the amount of NRas on the membrane (for example by a change in surface/volume ratio) but also change the interaction of NRas with effectors in septin free areas (for example by trapping of effectors such as PI3K within the blebs). This would mean that septins change the membrane morphology in a way that affect Ras signaling from the plasma membrane without generating septin based signaling hub complexes that contain Ras. Data as shown in figure S3 could also provide information on this effector engagement in specific areas by ratiometric representation of the NRas/PH domain images. For this ratiometric analyses the signal/noise of the images has to be improved however.

Our proposal that septins are serving as protein scaffolds is not based solely on colocalization data, but rather on their well-described performance of this role in various signaling pathways^{7,8,17–19,9–16}, paired with our proteomic data that shows physical protein-protein interactions occurring between both septins and NRAS and septins and NRAS effectors (shown in multiple experiments using protein crosslinking and proximity labeling of septin interactors). The colocalization of activity biosensors for this pathway with septins and the observation that inhibition of septins reduces signaling in the pathway provides further support. However, in addition to serving as protein scaffolds septins have been

demonstrated to also act as membrane diffusion barriers, meaning they are almost certainly concentrating membrane-bound NRAS in blebby regions of the cell, thereby increasing its avidity with effectors. With this in mind, we do not believe that septins scaffolding NRAS and creating kinetic traps for NRAS are mutually exclusive. We envision that plasma membrane-bound NRAS proteins, which temporarily lose contact with septins, tend to remain in close proximity due to septin-enforced diffusion traps. We propose that this is the reason why NRAS signals appear to be brightest where they co-localize with septins, while still being highly-concentrated in nearby non-septin-decorated areas of the plasma membrane (see figure to the right, which shows a surface diagram of septin and NRAS signals from the cell seen in figure 4A of the original manuscript).

In our original manuscript we did not quantify co-localization because we judged the effect strong enough to be apparent without this analysis. Upon consideration of the comments made by several of the reviewers we now developed new software that allows for the measurement of colocalization between two different signal distributions over the same cell surface by first calculating Spearman correlation between the two signals on mesh representations of the cell surfaces, followed by significance analysis using Costes' randomization. We performed this analysis on Septin/NRAS, NRAS/AktPH, and Septin/AktPH datasets, finding strong colocalization between all, and also on a control dataset of unrelated signals (septins and extracellular collagen hydrogel) which gave a negative result (see revised figures 4A and 4G). We are certain the reviewers appreciate our developing novel software to enable such an analysis on a rugged cell surface.



Bleb dynamics in relation to cortical septin structures

The last part of the manuscript is from my perspective the most interesting part, because it relates bleb dynamics to the nucleation of septins. The idea that the dynamically unstable blebs generate a transient positive curvature that initiates the nucleation of septins that can then stabilize after reaching a sufficiently high threshold would explain why larger septin oligomers are not confined to the neck of the bleb as seen in the static data. It might also explain an interrelation between bleb and septin formation and a possible role of the dynamic actin cortex. Unfortunately the data presented is very sparse and qualitative, merely showing some examples. Quantification is needed. More importantly, this approach could be used to relate bleb dynamics and septin assembly to signaling by time-lapse co-imaging of septins, Ras and the PH domain. This could prove or disprove the septin signaling scaffold idea for survival signaling or reveal another septin related mechanism in my opinion.

We keenly share the reviewer's interest in performing multi-color, high-speed, and high resolution volumetric timelapse imaging on cells embedded deep within biomimetic gels, and

agree that such data would answer many questions about the dynamics between blebbing, septins, NRAS, and AktPH. Unfortunately, the microscopy systems required to generate such data in isotropic 3D volumes collected at speeds of ~1 Hz are simply beyond our current capabilities. We are, however, now able to provide quantitative analysis of our septin timelapse data.

As suggested, we have performed timeseries analysis on these high-speed septin timelapse data, now presented in figures 2L and S7. Again, we are certain the reviewers appreciate that this analysis in three dimensions was a significant undertaking requiring nearly a year of work, deserving a technical publication on its own. We have decided not to pursue the path of a separate publication for now, but present these unique analytics together with the new cell biological results. Interestingly, they suggest the presence of two pools of bright off-bleb septin signals:

- 1) a dynamic pool that is highly sensitive to local curvature, changes intensity frequently, and does so at fast rates
- 2) a stable pool that is less sensitive to positive curvature, changes intensity less frequently, and when intensity does change it does so at a relatively slow rate

We believe that this supports our model of brief curvature-dependent “pulses” of oligomeric septin complexes co-existing alongside stable higher-order structures that have achieved inter-oligomer polymerization. We continue to work towards quantification of new stable septin structures being formed by repeated high-frequency blebbing, but the rarity of these events (we have observed only 2 across dozens of timelapse experiments) makes this an extremely challenging task, likely requiring the development of new microscopy systems and analytical pipelines that automatically set the acquisition to events of interest. Regardless, our conclusion that pulses lead to polymerized stable structures is supported by the new SEPT6(Δ AH)-GFP experiments, which show that stable structures cannot be formed without the curvature sensing that drives pulsing.

As the reviewer notes, these timelapse data and their associated hypotheses provide important context for results from the static analyses described in figure 2 of our original manuscript. We had initially conceived of a narrative flow that put these dynamic results at the end of the paper to emphasize their importance, but now realize that this structure is not ideal. In the revised manuscript we place these results now immediately after our static analyses in a combined new figure 2, allowing the reader to consider our dynamic results alongside the static data. Moreover, while we appreciate the reviewer’s interest in the septin assembly process (an interest that we enthusiastically share), we also realize that it is only a mechanistic detail underlying a more profound cellular process, i.e. morphological programs like blebbing govern the penetrance of oncogenic signals. Putting the septin assembly dynamics last perhaps detracted from this key message. We are certain that this reviewer will be as excited as we are by the new final figure, which establishes the causal axis from blebbing to anoikis resistance in an untransformed fibroblast. This demonstration of universality shall be the new high-point of the paper.

Referee #2 (Remarks to the Author):

This is a very interesting and exciting study, which reports a novel morphodynamic mechanism for the pro-survival signalling of non-adherent cancer cells that evade anoikis. The paper shows septins assemble and form pro-survival signalling hubs on the positive curvature domains of cell membrane blebs. Conceptually, this discovery introduces a novel signalling paradigm and indicates a novel oncogenic function for septin GTPases that stems from their membrane curvature-binding properties.

The authors combine cutting edge microscopy (e.g., lattice light sheet) and tools to manipulate cell adhesion (e.g., hydrogels), innovative computational image analyses, as well as pharmacological and dominant negative approaches and signalling assays to demonstrate that membrane blebs and septins promote the survival of non-adherent cancer cells. In total, the work is both a technical and conceptual tour de force.

We thank the reviewer for this positive assessment. We just want to clarify one small misunderstanding: we are not using lattice light sheet microscopy for this work. In fact, lattice light sheets are unsuitable for imaging as far away from the cover glass as we do. This work relies on a series of light sheet microscopes that were designed for the purpose of imaging with high-resolution deeper inside model tissues and hydrogels. The imaging set-ups have all been published before and are cited throughout the manuscript. We raise this point to underline that this cell biological investigation is enabled by a culmination of ~8 years of custom imaging and software development in our labs.

Overall, the novelty of the findings and the exciting implications for cancer cell biology make this manuscript highly suitable for publication in Nature. However, the manuscript requires revisions to strengthen the key results on septins and to improve conceptually the function of septins and their contribution in cancer cell escape of anoikis.

In particular, the specificity of septin-targeting approaches requires improvements, and more evidence and conceptual clarity is necessary for the localization and function of endogenous septins, and their oncogenic potential through the reported morphodynamic mechanism.

Major concerns/issues:

1) The FCF compound along with a dominant negative SEPT2 construct are used as a means of disrupting SEPT6 function. These approaches are not very septin and SEPT6-specific.

First, it is unclear why the authors focused on SEPT6. A better justification could be provided based on emerging knowledge of a C-terminal amphipathic helix that recognizes membrane curvature, and is conserved between the yeast septin Cdc12 and SEPT6 (Cannon et al, J Cell Biol 218:1128, 2019). In that regard, use of a SEPT6 C-terminal mutant would be more appropriate and specific for disrupting SEPT6 function. Of note, a SEPT6 C-terminal truncation functioned as dominant negative in a recent study (Hamdan et al, Nature Communications 11: 100, 2020) and a SEPT6 construct lacking the amphipathic helix is being used by some labs. These constructs can be used in conjunction with SEPT6 knock-down in a gene replacement-like approach (expression from bicistronic plasmid that expresses septin shRNA).

While we share the reviewer's interest in the molecular mechanisms governing septin function, we respectfully disagree that a granular examination of the contributions made by a single septin isoform is warranted in this particular paper. We use the SEPT6-GFP construct only for its ability to act as a fluorescent marker of endogenous septin localization within living melanoma cells, and did not intend for its usage to suggest a focus on SEPT6's specific role in the bleb/septin signaling phenomenon. It was chosen based on its characterization in previous work showing that it has minimal effects on septin phenotypes and does not cause the cytoplasmic septin aggregates that can sometimes be seen in cells bearing different fluorescently tagged septins²⁰. Moreover, previous papers have shown that it colocalizes with endogenous septin structures²¹, suggesting successful oligomerization into septin rods. We confirmed this integration into endogenous septin complexes by protein pull-down experiments and mass-spec analysis (Table 2).

With this in mind, the dominant negative SEPT2 construct and FCF were not chosen for their specific effect on SEPT6, but rather because they are orthogonal approaches that each efficiently disrupt the bleb-associated septin cytoskeletal structures seen within these cells. We have confirmed this experimentally for both (see figure to the right). However, we now realize that this experimental confirmation was only explicitly stated and shown in the paper for the dominant negative SEPT2. We have added language describing the effects of FCF treatment on cortical septins in these cells (disruption of enrichment at cell surface), as well as data showing septin localization upon FCF treatment in figure S2 of the revised manuscript. We thank the reviewer for drawing our attention to this accidental omission.

We also thank the reviewer for the idea of generating a SEPT6 mutant that lacks its C-terminal amphipathic helix (AH). We applied the same 18 aa deletion used by the Mostowy lab⁶ to our SEPT6-GFP construct, and found that it caused a complete disruption of septin localization at the cell surface (see detailed discussion above and revised figures 2I and S6), much like FCF and SEPT2(33-306) expression. We feel this result further validates our thesis that septin curvature sensing enables the formation of septin structures at the plasma membrane. It also replicates the results of the Gladfelter and Mostowy labs in a novel biological system, showing that whether the context is hyphal branching in yeast, cell-autonomous immunity in HeLa cells, or bleb-activated survival signaling in melanoma, septin oligomers carrying an AH-lacking isoform in the SEPT6 position are incapable of micron-scale curvature sensing.

Second, although the FCF compound binds and affects septins directly, it is thought to have more targets than septins. This reduces confidence in the interpretation of the data obtained with FCF. It is also conceptually difficult to understand the nature of the effects. FCF stabilizes septin assemblies, so one can potentially envisage that FCF would amplify membrane-associated septins stabilizing membrane blebs and enhancing signalling. It is unclear how FCF impacts septin localization on the membrane, and the dynamics of membrane blebbing. At the very least, it is critical to demonstrate that SEPT6 loss-of-function approaches impact septin localization to blebs and/or membrane



blebbing. At a basic level, testing whether SEPT6 knock-down and over-expression reduce and enhance blebbing, respectively, are necessary experiments, which would also provide clarity in whether whether septins play a role in blebbing in addition to forming a signalling scaffold/hub (see below; point #3).

While it is true that FCF is most often thought of as a stabilizer of septin polymers, it has been shown to produce a variety of different effects on septin localization across diverse experimental systems. And while many of those effects involve the inappropriate assembly of higher-order structures such as filaments and aggregates, several papers have described FCF producing the opposite effect, resulting in the disruption of pre-existing structures and creating more homogenous cytoplasmic distributions^{11,17,22}. Based on our experiments, detached blebby melanoma cells fall into this latter group, with FCF treatment resulting in the elimination of cortical septin structures in favor of a more diffuse cytoplasmic localization. Furthermore, because we found that expression of the dominant negative SEPT2(33-306) mutant produces similar disruption of septin structures, we chose to employ it as an orthogonal approach to septin inhibition by FCF. We believe that this approach effectively controls for the off-target effects the reviewer notes FCF is thought to have.

Regarding the effect of SEPT6-GFP expression and septin inhibition on blebbing, when we began performing these experiments we qualitatively examined cells and perceived no difference in blebbiness by eye. However, we agree with the reviewer that this argument deserves quantification, which can now be found in revised figure S2B. These new analyses show that SEPT6-GFP expression, FCF treatment, and SEPT2(33-306) expression have no appreciable effect on cell blebbiness (compare to figure 1A, showing the same analysis for WGA treatment).

It is worth noting that the vast majority of our experiments were performed in cells not expressing the SEPT6-GFP construct. The SEPT6-GFP probe was used only to investigate septin localization in figures 2, 3A, 4A, 4G, and 5A of the revised manuscript. Even if this probe produces some cryptic effects that escape our analysis, none of the experiments measuring viability, NRAS localization, or downstream signaling would be affected by it.

2) All observations with septins have been conducted with an ectopically expressed SEPT6-GFP. To ensure validity of observations, it is critical to demonstrate that endogenous septins localize similarly at the base of membrane blebs and show the overall localization/distribution of endogenous SEPT6 in the cell types used.

We agree with this criticism, and have included new 3D immunofluorescence (IF) data in figure S5 of the revised manuscript. We found that the IF signal quality was significantly worse than that seen with SEPT6-GFP, producing discontinuous punctate signals and significantly reduced SNR (fig S5A and S5B). Nevertheless, these signals were sufficient to demonstrate bleb-adjacent septin localization nearly identical to that seen with SEPT6-GFP (fig S5D), as well as strong enrichment of septins near the surface in cells lacking SEPT6-GFP expression (fig S5B and S5E). Importantly, overexpression of SEPT6-GFP did not affect this IF signal enrichment, indicating that SEPT6-GFP has no effect on the gross localization of endogenous septins (fig S5E).

SEPT6 has been posited to contain a nuclear localization signal, and shuttling of SEPT6 between the nucleus and membrane might be of functional relevance to the signalling aspects of this work, and the interpretation of the findings. Have the authors looked at endogenous SEPT6 in the cancer cell lines of this work and is there any indication of SEPT6 presence in the nucleus in adherent and non-adherent conditions? The interaction with nucleolin hints on the possibility of SEPT6 localizing in the nucleolus as it's also seen for SEPT9 in some cell lines.

While we agree that this is interesting and worthy of future study, as explained above we do not feel that investigations of SEPT6 function are warranted for this particular paper. We hope the reviewer agrees that the acute and specific effects we can introduce to cell viability and survival signaling by various orthogonal manipulations of blebbing and septin scaffold assembly provide strong evidence for the proposed link between morphodynamics and anoikis resistance.

3) Figure 5 presents a very interesting finding and the idea of septins as a spatiotemporal integrator and filter of membrane blebbing events is conceptually novel. The finding is much more relevant and suitable to Figure 2, which provides the initial septin characterization with respect to membrane blebs. As a stand-alone figure 5, this finding comes across a bit as an afterthought and not as substantive as the rest of the figures of a Nature publication.

We agree with this observation and have edited the manuscript accordingly (see above). We think this change greatly improves the narrative flow of the paper and allows the reader to more easily compare our static septin localization datasets to the dynamic datasets (which have been enhanced with quantitative timeseries analysis). We sincerely thank the reviewer for suggesting this improvement.

Additionally, the figure highlights an obvious question about the role of septins with respect to membrane blebbing. Are septins (SEPT6) required for membrane blebbing to take place? Or do they merely accumulate at curvature domains to scaffold pro-survival signalling?

This is also an important question with respect to the concern above about endogenous septins. It is plausible that septin over-expression enhances membrane blebbing, which in turn promotes the formation of septin-based membrane curvature pro-survival signalling hubs. Ectopic expression of SEPT6-GFP expression would amplify this effect, as a result of artefactual over-expression, but in the context of endogenous septin over-expression might be highly significant for cancer pathophysiology. Especially as septins are known to be over-expressed in cancer. Therefore, septin over-expression may drive a positive feedback loop between blebbing and signalling in cancer cells.

As discussed above, we have performed new analyses that show SEPT6-GFP overexpression and septin inhibition have no effect upon blebbiness in these cells. These results, considered alongside figure S5E, which shows that SEPT6-GFP overexpression does not seem to affect endogenous septin localization, suggests that the existence of such a positive feedback loop is unlikely (at least in the context of these cell types in these conditions).

It would greatly enhance the physiological relevance of the paper's findings if the authors can provide any data that demonstrate a correlation between septin over-expression and anoikis resistance in the cell line panel of their study, which can be done by looking for number of SEPT6 gene copies using FISH and levels of SEPT6 mRNA. For example, is there a SEPT6 upregulation in the pre-stressed A375 cells? Additional data might be able to be derived from looking at septin levels of expression in cancer databases (COSMIC, TCGA, GENT2) for melanomas which are known to evade anoikis. This would further substantiate the septin function as an oncogenic mechanism, strengthening the implication of the septin findings for cancer.

Our proteomic data does not suggest major shifts in septin expression between MV3, A375, and pre-stressed A375 cells. All cells express the same 8 septins at roughly the same ratio to one another, and at roughly the same percentage of total protein expression. However, because this approach might miss smaller changes in expression levels we performed Western blots and densitometry analysis of SEPT2, SEPT6, SEPT7, and SEPT9 (the 4 septin proteins with highest expression according to mass spec of lysate) in a dose response study of MAPKi in A375 cells (fig S9). Across 3 replicates we find little difference in septin expression, with any expression changes consistently falling below 2-fold differences (and often changing in different directions). Moreover, we saw no appearance of new bands, which would suggest the expression of alternate isoforms. We find these negative results very interesting, as they suggest that the “septin activation” seen in A375 cells is not due to alternate expression, but a currently unknown post-translational mechanism that triggers/inhibits the formation of septin structures at the membrane. Of potential interest to the reviewer, we find that SEPT6 does not pull down BORG family proteins in MV3, unstressed A375, or prestressed A375, suggesting that septin activation is not regulated by these well-known septin effectors.

4) How do the authors envisage septins (SEPT6) function in activating N-Ras at membrane domains of curvature beyond just recruiting or clustering it? Although this question goes beyond the scope of the current study, it's important to provide some clarity on the potential mechanism of signalling pathway activation. Do septins form scaffolds for N-Ras activation by GEFs? How do they function in the case of PI3K signalling at the cell membrane? Some pointers in the text would improve the conceptual framework of the findings.

We avoided going into great detail on this topic, as it quickly becomes very speculative given our data. The novelty of our data is the evidence of bleb-mediated septin scaffolding being necessary for Ras-promoted anoikis resistance. In response to the reviewer's specific questions, because MV3 carries a constitutively-active NRAS mutant, NRAS activation by an upstream signal is not required. However, constitutive activation of NRAS seems not strictly necessary for scaffolding to have an effect on signal amplitude. This is shown in our experiments with A375 cells that harbor a Braf instead of a Ras mutation, and in our new experiments with untransformed fibroblasts that show the same anoikis evasion under sustained blebbing (see below). We cannot exclude that in these two latter cases the septin-scaffolds aide wildtype Ras activation by recruitment of a GEF. As the reviewer points out, there are numerous secondary questions that will hopefully addressed in follow-up studies. As all reviewers emphasize in their comments, this study is opening new lines of research rather than closing a field.

Minor issues:

1) Comparisons in Figures 1B, 3D, 4D-F require tests of statistical significance.

We disagree that significance testing is appropriate given the small sample sizes of these datasets, but appreciate the reviewer's point that statistical analysis would increase confidence in the result. Accordingly, we have added Gardner-Altman estimation plots²³ using bootstrapped distributions of paired mean differences for the figures in question. These plots can be found in revised figure S2.

2) In Figure 2H, the linear fits are not clear. In general, this graph is difficult to comprehend. It is also unclear what the shaded colored surface contours that accompany the plot points represent and how they were derived.

The shaded bands are 95% confidence intervals – this information was mistakenly left out of the legend and has been added. We appreciate the correction. We have also added R^2 values for each experimental group.

3) The mechanisms and signalling pathways that mediate anoikis resistance should be more explicitly stated in the introduction of the manuscript. It is unclear whether the signalling factors and pathways (N-RAS, MAPK, PI3K), which are linked to septins, have been established as anti-anoikis pathways and mechanistically how they prevent cell death during anoikis. This should be more explicitly addressed through text revisions.

We have changed the introduction to now explicitly note that the signaling pathways we previously found associated with blebs are known to be involved in anoikis resistance.

Elias Spiliotis

Referee #3 (Remarks to the Author):

The manuscript "Blebs Promote Cell Survival by Assembling Oncogenic Signalling Hubs" by Weems et al reports on a novel mechanism allowing the survival of cells whose cell-substrate adhesion is compromised. The authors propose that blebbing activity leads to the formation of hubs for signalling proteins that protect melanoma cells from death when detached from the substrate. The model the authors present is novel, is potentially relevant for a range of other cell types in different physiological and pathological contexts and as such is in principle of interest for the readership of Nature. The results are mostly very convincing and the paper reads very well.

I have two general suggestions the authors must have thought of, but mention them here, just in case. The first would make the paper of relevance for a broader audience, and the second would increase the confidence in the proposed model.

A. To try to identify unrelated cell type/s, where bleb-dependant anoikis-resistance occurs, thereby making the proposed model relevant beyond the melanoma field. A possible way to screen for such cases would be to induce blebbing in cells that depend on cell adhesion for their survival and check for resistance to anoikis.

We would like to thank the reviewer for this suggestion, as it motivated us to attempt an experiment that we had long considered but judged as too high risk. Taking the reviewer's suggestion perhaps even one step further, we had indeed wondered whether the formation of blebs would be sufficient to drive the cancer hallmark of anchorage-free survival in a cell without oncogenic mutations. This experiment resembles the strategy some of the pioneers of molecular cancer biology pursued when they tested the effect of early oncogenes, including Ras, in normal cells like fibroblasts. We are very excited to report that the experiment was successful. As illustrated in a new figure 5 we now show that fibroblasts without substrate-attachment evade anoikis as long as they are enabled to form blebs, and this anchorage-independent survival can be impeded by inhibition of bleb formation or septin assembly – just as in melanoma. This result underlines the fundamental role cell morphological programs like blebbing seem to play in cell signaling.

B. Another line of experiments that would strongly support the model suggested by the authors would be to conduct gain of function experiments to activate/enhance the resistance, rather than "just" reducing it as they do. For example, if enhancing blebbing activity or the level of relevant signalling increases the resistance of melanoma cells to anoikis, the model would be more convincing. e.g. add activated Ras / Remove PTEN / increase contractility in melanoma cells and determine the resistance to anoikis. As it is, the results of the experiments support the model the authors propose, but at the same time, could stem from adding more stress to cells that are already stressed by the detachment.

Though such an approach is conceptually sound in theory, these melanoma cells have very little room for improvement regarding anoikis resistance. Indeed, MV3 cells already carry activated Ras and inactivated PTEN as the reviewer suggested, not to mention at least 124 other cancer-associated mutations according to our deep-sequencing results. Given the complexity of this oncogenic genetic landscape, candidate alleles that might improve anoikis

resistance are not immediately apparent to us, nor would we predict such manipulations to necessarily produce the results expected (take for example the mutual exclusivity of oncogenic NRAS and BRAF mutations²⁴). For these reasons, we do not feel such an experiment is advisable.

However, we believe (and are confident that the reviewer will agree) that the above response to suggestion A) outlines an ideal gain-of-function experiment addressing the sufficiency of blebbing as a survival mechanism. As the reviewer correctly points out, the fact that our previous experiments all relied on a readout of reduced viability upon inhibition of blebs/septins made the interpretation of the resulting data somewhat vulnerable to non-specific effects. That the opposite experiment (activation of blebs/septins) also causes the opposite effect (increased viability) greatly raises confidence in the specificity of our interpretations. We thank the reviewer for both suggestions.

I am listing below some more specific comments in order of appearance in the paper that I hope help improving it.

1. Define “PI3K” the first time it is mentioned.

This has been added to the revised manuscript.

2. “to be concentrated in the vicinity of melanoma blebs, including KRAS and active PI3K 14,31,32 “. - Is reference Nr 32 wrong here?

The reference was intended, but the text did not correctly reflect its content. We have corrected this in the revised manuscript.

3. “To investigate putative bleb function as a driver of anchorage-independent cell survival we designed assays that consistently minimized both cell-substrate and cell-cell attachment over a period of 24 hours. We accomplished this goal by seeding cells at very low density (approx. 250 cells/ml) in non-adherent culture dishes under constant gentle agitation.”

– I guess there is a simple explanation to that, but if the point is to present the mechanisms allowing cell survival under non-adherent condition, why not do it without the agitation? The way it is presented, one could argue that the phenomenon studied is resistance to agitation, not to lack of adhesion. This should be made clear to readers not familiar with the experimental system.

We found agitation to be necessary through trial and error. Without it, cells consistently began adhering to substrate ~6-8 hours after beginning incubation, despite our use of culture dishes designed to be “non-adherent”. It is true that this agitation might act as a cryptic variable between the adhered and detached groups, however the orthogonal VitroGel approach effectively controls for it. We have added text to briefly explain the need for agitation.

4. “Both adhered and detached M498 cells die upon WGA treatment, but detached cells experience significantly more death, both in aggregate and in individual matched experiments (Fig 1B, Fig S1).” - In Fig 1B indicate on the X-axes the amounts of WGA also for M498 and A375.

The x-axes are identical in each panel, and were left unlabeled for aesthetic and space-saving reasons. We now note that treatments are identical in the figure legend.

5. *“..at first sight indicating that bleb inhibition is not a universal mechanism for establishing anoikis resistance.”*

-The basis for the resistance of unstressed A375 is not clear. The confidence in the result would be increased if a similar TREND is observed in the other cell types. i.e. does pre-stressing makes cells more bleb-dependent in the other cell types.

Because we do not yet know the mechanism by which pre-stressed A375 cells activate and become reliant upon septin structure formation, we do not believe it is appropriate to predict that stress will affect MV3 or M498 cells in a similar fashion. However, as discussed above we now include data showing that MAPK inhibitors currently used clinically as front-line therapies also sensitize A375 to bleb and septin inhibition, suggesting that the phenomenon of “septin activation” could be involved in melanoma drug resistance. We believe this adds clinical context and increases confidence in the result, especially given the parallel results published by the Sanz-Moreno lab in 2020 (also discussed above).

6. *“these results indicate that dynamic blebbing contributes to anoikis resistance both in NRAS- and in BRAF-mutated melanoma, suggesting that this cell morphological program is a broadly adopted survival strategy in melanoma cells.”*

-In the spirit of “broadly”, as mentioned above, identifying more cell type/s that show the bleb-dependent resistance would make the results relevant for a much broader audience. Can anoikis resistance be conferred to sensitive cells by inducing blebs (by enhancing contractility).

See response to A) above.

7. *In Fig 2F, in the WGA treatment, it looks like the amount of Septin scaffolds is even higher than in untreated cells, although those are not as evenly distributed. If the role of the Septin scaffold is just to bring together the signalling molecules, what is the basis for the WGA effect on the resistance to detachment? Based on the presented data, it is not clear why WGA should have an effect.*

We believe the reviewer is missing the 3-dimensional nature of these data: The images on top are maximum intensity projections, whereas the ones below are single z-slices of the same cell. When examined in this context, one sees that the bright septin structures in the projection on top are in fact large septin aggregates that are grossly mislocalized, having become detached from the plasma membrane and internalized.

8. *In the Figures the authors use the term “Unstressed A375” and in the text “Non-stressed A375”. Better to use the same term.*

Thanks, this has been corrected.

9. *“Taken together, these data suggest that the mechanism allowing blebs to confer anoikis resistance upon melanoma cells depends on bleb adjacent cortical septin structures.”*

-Is there an effect for the drug treatment on bleb formation?

As discussed above, we now include data in figure S4 showing that both FCF and SEPT2(33-306) have no effect on blebbiness.

10. *"Importantly, reexamination of our previous pulldown data revealed that NRAS interacted with SEPT6 in MV3 cells, but not in unstressed A375.."*

– Does it interact in pre-stressed A375 cells?

It does. We now include text stating: "Importantly, reexamination of our previous pulldown data revealed that NRAS interacted with SEPT6 in MV3 and pre-stressed A375, but not in unperturbed A375, indicating that the NRAS/septin interaction depends on the assembly of bleb-nucleated septin structures."

11. *"..and our previous work has shown that bleb inhibition alters PI3K activity¹⁴".*

- Doesn't the previous work mainly show that PI3K enhances blebbing and not vice versa?

The previous paper establishes a positive feedback between PI3K signaling and blebbing. The key result for the line of arguments in the present work is an experiment showing that bleb inhibition by WGA reduces PI3K activity (Figures 5H, I J, and K of our BioRxiv manuscript as of 5/23/22). This is fully in line with the model presented here.

12. *"we found that high PI3K activity co-localizes with both septins and NRAS in rounded, poorly-adhered MV3 cells embedded in soft collagen, in support of septin-scaffolded NRAS signaling through PI3K (Fig 4G, Fig S3). "*

-This point is not very clear in Fig 4G. Most of the PI3K activity is found in positions where Septin is not observed.

We believe this confusion is again stemming from a misunderstanding of the figure, as we described in our response to comment #7. In figure 4G, all of the cortical PI3K activity is occurring in close proximity to septins. Previous work shows that this biosensor adopts cytoplasmic localization when not engaged by enzymatic products of PI3K activity, so we do not believe the cytoplasmic loci reflect active PI3K, but rather excess biosensor. Regardless, we have replaced this figure in the revised manuscript, and now include quantification of this colocalization in figure 4G.

13. *"our high-speed time lapse data showed stable septin structures emerging only when several pulses occurred in close proximity, resulting in their coalescence into bright, long lived structures whose intensity does not seem to rely on local curvature profiles"*

-This point is not obvious from the movie. Perhaps running it slower and marking which blebs to look at would help. As it is, it is not clear why Septin polymerization occurs at the specific point shown.

We have edited the movie as requested.

Referee #4 (Remarks to the Author):

Comments to the authors:

The paper reports the accumulation of septin at the curved base of plasma-membrane blebs that actively form in non-attached cells. These septins in turn recruit active mutant NRAS and effectors, themselves associated with upregulation of ERK and PI3K and their signaling cascades. While blebs of detached cells recruit the mutant NRAS, they fail to efficiently recruit wt NRAS and don't activate ERK/PI3K signaling, resulting in cell death. These and other clever cell biological experiments demonstrate an unforeseen relationship between membrane blebbing and cell survival. These observations are particularly interesting and timely, given that most earlier studies connecting membrane blebs and signaling have been done in the context of cell motility and not in relation to cell survival. This topic is of relevance to cell survival and metastasis, especially in the context of minimal cell adherence.

This study includes well performed and properly described cell biological experiments such as effect of relevant mutants, force perturbation of cell morphology, proximity labeling and mass spectrometry and so forth. Key for the investigation was live cell visualization that capitalized on the virtues of diagonally scanned light sheet microscopy implemented by the Danuser group; this approach provides thin plane illumination and detection, 3D visualization and low photo toxicity.

Septin polymers act as large-scale membrane curvature sensors. In addition to previous elegant studies from the Danuser group, others have also contributed in significant ways, see for example Beber et al, Nat Commun. 2019 Jan 24;10(1):420. Membrane reshaping by micrometric curvature sensitive septin filaments. Their work should also be mentioned.

We have added this reference to the revised manuscript.

Several studies have noted appearance of blebs during mitosis. While the Weems et al study refers to Sedzinski, J. et al. Polar actomyosin contractility destabilizes the position of the cytokinetic furrow. Nature 476, 462–468 (2011), a more relevant reference would be E. Boucrot, T. Kirchhausen PNAS 2007, 104:7939. Endosomal recycling controls plasma membrane area during mitosis.

We have added this reference in the revised manuscript.

The authors used a home-built microscope with stated raw resolution of ~ 400x400x400 nm. The size of the point spread function would also be around this range. I am therefore unable to follow what they mean by an optical section of 160 nm. Perhaps they mean a step movement of 160 nm along the z-axis? Please clarify.

As the reviewer states, we were inappropriately referring to the dimension of the measured voxel, not the optical resolution. This error has been corrected in the revised manuscript.

Missing in the study by Weems et al is a detailed description of how the software was used in the analysis of the images. The authors state "All code/algorithms/software central to the findings of this study are available from the corresponding author upon reasonable request. Most (italics added) can be found at <https://github.com/DanuserLab>.". Similarly, the authors state that "Cell morphology and

septin localization were analyzed principally via u-shape3D". What were the analyses that were done with other algorithms or other software? It is essential that published manuscripts should uphold to current standards and provide enough details so that the study can be fully reproduced; this applies not only to experimental aspects but also extend to the use of analytical tools. In particular, all the code/algorithms/software should be deposited in the github directory cited; "reasonable request" is inappropriately vague.

We fully agree with the reviewer and have added more explicit language in our methods section describing all additional analytical tools. We have also added methods sections describing the new software pipelines we developed for colocalization and time series analyses. As the reviewer may know, our lab has two ways to distribute software. Generalizable, highly-stable and continuously maintained software pipelines are distributed in packages, such as u-shape3D. As indicated, the vast majority of the image analysis was done inside the u-shape3D package. We have then written several dedicated scripts that build on the u-shape3D outputs for the purpose of specific analyses in this study. It is unlikely that these functionalities will be used in a more general format and thus, we will not undertake the (costly) effort of engineering them into a package. However, for the purpose of full transparency we have prepared a bundle of these scripts for download from Github. This will give the interested reader an opportunity to review and reuse our codes.

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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

I thank the authors for their scholarly answers and agree that this manuscript provides a new and highly relevant new concept that relates sustained bleb morphodynamics to survival signaling and anoikis resistance in certain cancer cells. The authors have done substantial new experimentation and analysis as well as restructured the manuscript to make a strong case that the positive curvature at the bleb neck causes septin recruitment and functions as hubs for survival signaling from NRas and PI3K. They have also nicely worked out and provided evidence that the irreversible stable septin polymerization state functions as a memory of pulsatile bleb dynamics.

I shortly list the most relevant new experiments and analysis that sustain the hypothesis of the authors:

Dominant negative effect of polymerization deficient septin mutant SEPT2(33-306) results in a recruitment profile that follows positive curvature.

Bleb Curvature dynamics in relation to reversible-irreversible septin recruitment using cross correlation and the integration of the morphodynamics-septin data in figure 2. This is also consistent with the dominant negative effect of the polymerization deficient SEPT2(33-306) mutant on the septin recruitment profile.

stable septin structures are survival signaling platforms: strongly improved 3D colocalization patterns for NRas-septins, AktPH-septins.

The authors have added evidence that sustaining blebs over longer time yields anoikis resistance, providing an additional orthogonal angle that sustains their hypothesis.

Philippe Bastiaens

Referee #2 (Remarks to the Author):

The authors have performed a number of experiments and textual revisions, which address the original comments of the reviewers. Overall, the manuscript is improved. However, it is concerning that the FCF compound is the only approach for concluding that septins are required for anoikis resistance, and there is also lack of basic controls for the experiments that use the truncated Sept2:

- 1) The conclusion on septin requirement for resistance to anoikis (Figure 3) is based on the use of the FCF compound, which has been reported to have off target effects (Heasley et al, Eukaryotic Cell 2014).

2) Conclusions on the N-ras localization and signaling derive from experiments that express Sept2(33-306) but lack a control such as the full length Sept2 and/or an additional protein as a control for transfection with dual plasmids/constructs. In addition, how do the authors monitor Sept2(3-306) expression in the analyzed cells? Is it a fluorescent construct or tagged with His/FLAG or other? This information should be added to figure legends.

Referee #3 (Remarks to the Author):

The manuscript by Weems et al presents a correlation between blebbing and resistance to anoikis, programmed cell death induced by reduced adhesion. The authors suggest that blebbing enhances the survival of detached cells that would otherwise die. The findings have potentially important implications for the understanding of physiological processes, as well as disease conditions where cells are positioned within environments that do not support adhesion.

I raised two main issues regarding the previous version of the paper. First, it was not clear if the findings are relevant for other cell types and thus of general interest. More importantly, the conclusions relied on experiments where manipulations that reduced bleb formation could also promote death by mechanisms different from those suggested by the authors. The authors did not provide gain of function data supporting their hypothesis, e.g. confer resistance to anoikis by inducing blebs in cells that would otherwise die.

Both issues were addressed in this version of the paper.

I am listing below specific issues that I hope the authors find useful when revising this version of the paper.

Specific issues in order of appearance in the paper-

1. "This unveils an unanticipated morphological requirement for mutant NRAS to operate as an effective oncoprotein" – put a full stop after "oncoprotein"
2. At the end of the abstract, I would highlight the fibroblast experiments. The "Finally" can be perceived as something related only to the order by which the experiments were conducted. I would use something like "Critically/Importantly", as this is a very important general point. The precise pathways acting downstream of blebs could differ among cells, but the principle of blebs as structures that prevent death could be advertised better.
3. "...such as those forming melanoma, are highly anoikis-resistant and able to indefinitely sustain rounded blebby morphologies in no- and low-attachment environments, often adopting this amoeboid phenotype both in vitro and in vivo – notably at the invasive front of tumors" - as blebs are the major structures here, one should define them better (e.g. actin-free) and not give the impression that blebby = amoeboid.
4. "However, we observed that the confluency of A375 cells prior to these viability experiments influenced outcomes, with cells from more confluent culture conditions experiencing more death upon WGA treatment." - where should one find these results? This sounds like a conclusion from the experiments presented only in the next paragraph.
5. The statement "...which potentially explains their increased susceptibility ..." should be explained,

or (better) removed.

6. "However, we observed that the confluency of A375 cells prior to these viability experiments influenced outcomes, with cells from more confluent culture conditions experiencing more death upon WGA treatment. ", which is a conclusion, the sentence " - this is a conclusion sentence that when mentioned is not supported by data / previous information.

7. "We repeated our bleb inhibition viability assays..". I would exchange "our" with "the".

8. "As expected, far more MV3 cells died..". a style issue, but I would not "expect/predict" results, phrasing that is used a lot in the manuscript.

9. "...shown to successfully integrate into the endogenous septin cytoskeleton with little-to-no effect on septin phenotypes". "function" instead of "phenotypes"?

10. Would be helpful to flip the septin and the curvature surface rendering such that movie 2 and Fig 2A have the same arrangement.

11. It is not clear what the aim of showing the MIPs in Fig 2F is. The only impression one gets from them is that there are very strong differences in the amounts of septin among the different treatments. For example, the MIP would provide an underestimate of the amount of septin in the WGA treatment due to possible overlap among signals at different z planes. Still, there is a much stronger signal in the WGA-treated cells as compared with the other treatments. If the authors do not think the treatments affect septin level, would probably be better not to show the MIPs, or otherwise explain and discuss the basis and implications of the differences.

12. "These data revealed that while "pulses" of septin accumulation can be seen at the curvy bases of blebs, they fade quickly once the bleb is resolved and curvature subsides (Fig 2K, Movie 3)."- this is not clear from the movies. It can be somewhat observed in the marked bleb and in Fig 2K, but even there septin accumulation appears prior to bleb inflation (e.g. in 4.8 sec) and then increases. As the authors state as well, bleb-independent septin clusters can form. The order/mechanism of the events are key aspects of the proposed model and these are not convincingly observed in the movies. As is, the option that domains of high septin levels are devoid of / show less blebbing activity is not excluded (i.e. septin biases the position of the bleb edge and not vice versa). The flow of septin towards forming blebs as part of general cytoplasmic flow towards the inflating bleb (and lack of it when blebbing is inhibited) and exclusion from the forming bleb by actin for example (instead of recognition of curvature) could be the basis for the observations.

13. Using the AH-GFP construct as a control instead of the deletion, which might affect the protein beyond the recognition of curvature would be more convincing.

14. In Figures 4A and 4G, septin appears to be distributed in clusters whose positions are not correlated with the circumference of forming blebs, and NRAS/AktPH appear there as well. Why such bleb-independent clusters do not reduce cell death?

15. The model the authors propose would be strongly supported by increasing the activities of different signaling molecules that function downstream of septin, showing inhibition of anoikis in cells in which blebbing is inhibited.

I appreciate that the suggestions in points 12 and 15 are challenging to follow and could be considered to be "beyond the scope of the paper".

Referee #4 only sent comments to the editor, stating that their concerns have been addressed

Author Rebuttals to First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

I thank the authors for their scholarly answers and agree that this manuscript provides a new and highly relevant new concept that relates sustained bleb morphodynamics to survival signaling and anoikis resistance in certain cancer cells. The authors have done substantial new experimentation and analysis as well as restructured the manuscript to make a strong case that the positive curvature at the bleb neck causes septin recruitment and functions as hubs for survival signaling from NRas and PI3K. The have also nicely worked out and provided evidence that the irreversible stable septin polymerization state functions as a memory of pulsatile bleb dynamics.

I shortly list the most relevant new experiments and analysis that sustain the hypothesis of the authors:

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stable septin structures are survival signaling platforms: strongly improved 3D colocalization patterns for NRas-septins, AktPH-septins.

The authors have added evidence that sustaining blebs over longer time yields anoikis resistance, providing an additional orthogonal angle that sustains their hypothesis.

Philippe Bastiaens

We likewise thank the reviewer for their engagement and effort.

Referee #2 (Remarks to the Author):

The authors have performed a number of experiments and textual revisions, which address the original comments of the reviewers. Overall, the manuscript is improved. However, it is concerning that the FCF compound is the only approach for concluding that septins are required for anoikis resistance, and there is also lack of basic controls for the experiments that use the truncated Sept2:

1) The conclusion on septin requirement for resistance to anoikis (Figure 3) is based the use of the FCF compound, which has been reported to have off target effects (Heasley et al, Eukaryotic Cell 2014).

We wholeheartedly agree with the reviewer's point, especially given the importance of these results to the paper's central thesis and the fact that many other experiments in the paper make use of SEPT2(33-306) expression as a means of perturbing septin function orthogonal to pharmacological inhibition of septin by FCF. We have therefore repeated key experiments in Figure 3 using this dominant negative construct. Like in Figure 1D, we tested the effect of SEPT2(33-306) expression on MV3 and A375 survival in both adhered and detached conditions.

Confirming all our previous data, we found again that genetic inhibition of septins had no effect on A375 cells regardless of adhesion state, while MV3 cells experienced significant death which worsened by detachment (Fig S12). Compared to the FCF-based inhibition, the effect was somewhat lessened by an increased baseline death rate in the MV3 adhered group. This is unsurprising considering that the construct is constitutively expressed. Cells in the adhered group experience septin inhibition while they are detached during transfer to the chamber slides used in the experiment. In FCF experiments, we give such cells an hour to adhere before beginning FCF treatment, a consideration that is impossible in this experimental setup. Moreover, because septins are involved in forming and reinforcing focal adhesions (doi.org/10.1515/BC.2011.088, doi.org/10.1083/jcb.201405050, doi.org/10.1016/j.celrep.2015.11.052) the period of time in which SEPT2(33-306)-expressing cells remain detached during transfer might be prolonged.

Regardless, we hope the reviewer agrees that these results increase confidence in the conclusions of Figure 3, as they match the pattern seen upon FCF treatment, as well as that seen in bleb inhibition via WGA and VitroGel. We thank the reviewer for this suggestion, as we agree that the previous absence of this experiment represented a flaw in the paper.

2) Conclusions on the N-ras localization and signaling derive from experiments that express Sept2(33-306) but lack a control such as the full length Sept2 and/or an additional protein as a control for transfection with dual plasmids/constructs.

It is important to note that none of the experiments described in this manuscript were performed using transient transfection of plasmids. We avoid many of the concerns related to simultaneous

expression of multiple proteins from transfected plasmids (e.g., competition between plasmids for replication machinery or the effects produced by treatment with multiple selection drugs) by creating cell lines that stably express our constructs via genomic integration. Thus, the requested control seems less essential to the support of our conclusions than if the specified NRAS experiments relied upon expression from plasmids as the reviewer suggests.

Nonetheless, we acknowledge that there might be some effect on cells carrying multiple constructs that were genomically-integrated in this way. To control for this, we now include new analyses (Figures S10 and S13) which demonstrate that dual expression does not generate the phenotypes seen upon expression of SEPT2(33-306). As the reviewer requests, we have expressed additional proteins in MV3 cells carrying either NRAS-GFP or SEPT6-GFP constructs (SEPT6-HALO for NRAS-GFP and mCherry for SEPT6-GFP, both using the same pLVX-IRES-Hyg vector as the SEPT2(33-306) construct) and show that the effects upon NRAS and septin localization do not phenocopy the mislocalization caused by SEPT2(33-306). Importantly, confidence in the results of our experiments on NRAS and downstream signaling using SEPT2(33-306) are further increased by the fact that all such experiments were also orthogonally performed using FCF, yielding the same results.

In addition, how do the authors monitor Sept2(33-306) expression in the analyzed cells? Is it a fluorescent construct or tagged with His/FLAG or other? This information should be added to figure legends.

We opted not to monitor expression of the mutant in individual cells due to concerns that fluorophore tagging might alter protein function and thus disrupt the dominance of the mutant. We instead genomically integrated the construct with a lentiviral vector, using hygromycin selection to ensure successful integration. Upon observing the predicted disruption in septin localization we repeated the integration and selection process in different cells to ensure that the effect was not due to the lentiviral vector disrupting endogenous loci, observing the same effect on septins. Confirmation of mutant expression was performed using Western blots against SEPT2, which we now include in figure S9.

Referee #3 (Remarks to the Author):

The manuscript by Weems et al presents a correlation between blebbing and resistance to anoikis, programmed cell death induced by reduced adhesion. The authors suggest that blebbing enhances the survival of detached cells that would otherwise die. The findings have potentially important implications for the understanding of physiological processes, as well as disease conditions where cells are positioned within environments that do not support adhesion.

I raised two main issues regarding the previous version of the paper. First, it was not clear if the findings are relevant for other cell types and thus of general interest. More importantly, the conclusions relied on experiments where manipulations that reduced bleb formation could also promote death by mechanisms different from those suggested by the authors. The authors did not provide gain of function data supporting their hypothesis, e.g. confer resistance to anoikis by inducing blebs in cells that would otherwise die.

Both issues were addressed in this version of the paper.

I am listing below specific issues that I hope the authors find useful when revising this version of the paper.

Specific issues in order of appearance in the paper-

1. "This unveils an unanticipated morphological requirement for mutant NRAS to operate as an effective oncoprotein" – put a full stop after "oncoprotein"

The suggested change has been made.

2. At the end of the abstract, I would highlight the fibroblast experiments. The "Finally" can be perceived as something related only to the order by which the experiments were conducted. I would use something like "Critically/Importantly", as this is a very important general point. The precise pathways acting downstream of blebs could differ among cells, but the principle of blebs as structures that prevent death could be advertised better.

This is a good point. The suggested change has been made.

3. "...such as those forming melanoma, are highly anoikis-resistant and able to indefinitely sustain rounded blebby morphologies in no- and low-attachment environments, often adopting this amoeboid phenotype both in vitro and in vivo – notably at the invasive front of tumors" - as blebs are the major structures here, one should define them better (e.g. actin-free) and not give the impression that blebby = amoeboid.

The language linking rounded, blebby morphologies to the amoeboid phenotype has been softened, as requested. As to defining blebs, we believe this was sufficiently accomplished earlier in the paragraph by the sentence, "Blebs are hemispherical pressure-driven cellular protrusions that occur when portions of the cell membrane decouple from the actomyosin cortex."

4. "However, we observed that the confluency of A375 cells prior to these viability experiments influenced outcomes, with cells from more confluent culture conditions experiencing more death upon WGA treatment." - where should one find these results? This sounds like a conclusion from the experiments presented only in the next paragraph.

These observations were made during early pilot experiments aimed at designing and refining the "adhered vs. detached" viability assay featured in this section. Many experimental variables were in flux during this period, as we worked to design a minimally-invasive assay which consistently kept detached cells from adhering to substrate or one another over a period of 24 hours. The observations were thus suspect, as adhesion state was not a well-controlled or isolated variable at the time. Moreover, these were not rigorously measured results, but rather incidental observations made by the experimenter. Having noted this apparent trend between confluency and viability, we decided to rigorously perform the described experiment using the finalized assay, as described in the manuscript. In short, we do not have results to share in this case, beyond description of the experimenter's observations. The language of this sentence has been amended to succinctly convey this to the reader.

5. The statement "...which potentially explains their increased susceptibility ..." should be explained, or (better) removed.

We agree that this statement was poorly explained and not supported by data in the manuscript. We have added text expanding on the observation and its implications for the M498 viability results, as well as a new supplemental figure (S2 in the updated manuscript) showing the morphology of M498 cells when grown in "adhered" conditions. These new data illustrate that M498 are substantially less adherent than MV3 and A375 cells, rendering them susceptible to bleb inhibition even in cases where adherable substrate is provided.

6. "However, we observed that the confluency of A375 cells prior to these viability experiments influenced outcomes, with cells from more confluent culture conditions experiencing more death upon WGA treatment.", which is a conclusion, the sentence " - this is a conclusion sentence that when mentioned is not supported by data / previous information.

See response to #4 above.

7. "We repeated our bleb inhibition viability assays..". I would exchange "our" with "the".

Agreed. The requested change has been made.

8. "As expected, far more MV3 cells died..". a style issue, but I would not "expect/predict" results, phrasing that is used a lot in the manuscript.

We see the reviewer's point, but ultimately disagree. We view prediction and expectation as fundamental components of hypothesis-driven experimentation. Our use of these words is not intended to convey personal beliefs about whether models are true or not (which we agree would be inappropriate), but rather to serve as metrics to determine whether results support hypotheses. Importantly, as we hope the reviewer agrees, our lab has a history of developing assays and tools for automated quantification of data sets with minimal user influence. Thus, we try as much as possible to avoid bias related to predictions/expectations. We hope that the reviewer can agree to our approach to science narration.

9. "...shown to successfully integrate into the endogenous septin cytoskeleton with little-to-no effect on septin phenotypes". "function" instead of "phenotypes"?

We agree, "function" is the better choice here. The requested change has been made.

*10. Would be helpful to flip the septin and the curvature surface rendering such that movie 2 and Fig 2A have the same arrangement.

This is a good point and the suggested change has been made.

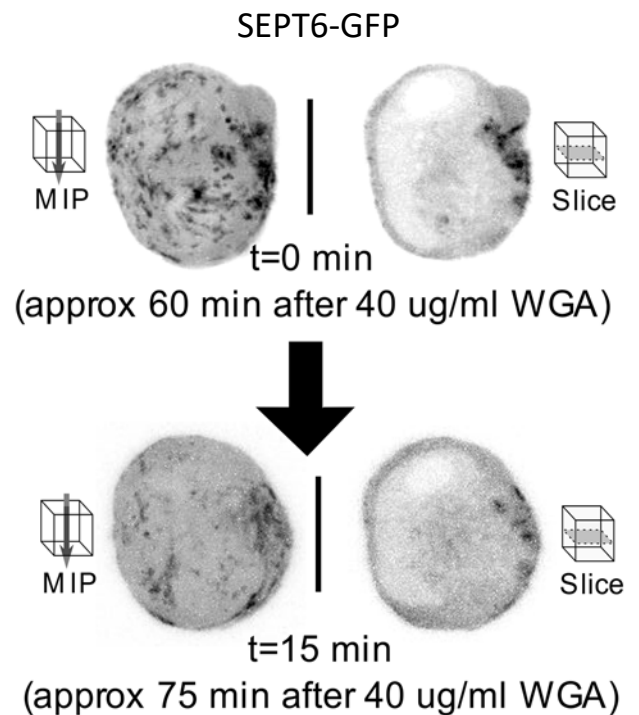
11. It is not clear what the aim of showing the MIPs in Fig 2F is. The only impression one gets from them is that there are very strong differences in the amounts of septin among the different treatments. For example, the MIP would provide an underestimate of the amount of septin in the WGA treatment due to possible overlap among signals at different z planes. Still, there is a much stronger signal in the WGA-treated cells as compared with the other treatments. If the authors do not think the treatments affect septin level, would probably be better not to show the MIPs, or otherwise explain and discuss the basis and implications of the differences.

After some deliberation we arrived at the MIP/slice combination as a consistent means of presenting micrographs throughout the paper, with the MIP showing global

morphology/localization trends and the slice highlighting specific localization relative to the cell surface and its topography. As to the specific case of the micrographs in Fig 2F, we believe the inclusion of MIPs communicates the effects of treatment on overall cell morphology (sphericity) and protrusions ("blebbiness"). They also effectively highlight the gross disruption of the distinct "stippled" septin localization consistently seen in unperturbed cells. Ultimately, however, we agree with the reviewer that such 2D representations are inherently limited when considering 3D datasets and that important information is lost in this visualization medium. It is for this reason that we rely on computer vision approaches such as u-Shape3D to both visualize and, more importantly, quantify such localization. Thus, Figure 2F is a qualitative representation of trends observed upon the various treatments ('seeing is believing') in order to motivate the more rigorous quantitative analysis shown in Fig 2G-H ('measuring is understanding').

The reviewer's comment raises an important point of potential misunderstanding. These perturbations of bleb formation do not appear to alter cellular septin levels, but rather septin localization – specifically, septin recruitment to the cell surface. The goal of figures 2F-I is to demonstrate that septins are recruited to the plasma membrane via bleb-generated plasma membrane curvature: first, by showing that ablation of curvature blocks surface recruitment (2F-G), then by showing that the amount of plasma membrane curvature a cell possesses reliably predicts the amount of septin at the surface for both perturbed and unperturbed cells (2H), and finally by showing that removal of the curvature-sensing domain completely blocks the surface localization of septins (2I and S7). With this in mind, we believe the intensity of intracellular septin aggregates formed as a result of recent bleb inhibition are immaterial to the conclusions supported by this figure.

How such aggregates are formed, why they seem specific to WGA treatment, and to what extent they are capable of serving as signaling platforms when separated from surface-localized components of signaling pathways are all interesting questions, but we believe they are beyond the scope of the paper. However, the reviewer might be interested to know that we have performed pilot work for an unrelated experiment that involved visualizing septin localization in WGA-treated MV3 cells over a period of 15 minutes, during which time these intracellular aggregates were observed to degrade consistently and significantly (see figure to the right, which is representative of eight different experiments). Based on this, we conclude that such structures are a temporary side-effect of WGA treatment and do not persist long enough to affect cell behavior over the 24 hour timescale covered by our viability experiments.



12. "These data revealed that while "pulses" of septin accumulation can be seen at the curvy bases of blebs, they fade quickly once the bleb is resolved and curvature subsides (Fig 2K, Movie 3)." - this is not clear from the movies. It can be somewhat observed in the marked bleb and in Fig 2K, but even there septin accumulation appears prior to bleb inflation (e.g. in 4.8 sec) and then increases. As the authors state as well, bleb-independent septin clusters can form. The order/mechanism of the events are key aspects of the proposed model and these are not convincingly observed in the movies. As is, the option that domains of high septin levels are devoid of / show less blebbing activity is not excluded (i.e. septin biases the position of the bleb edge and not vice versa). The flow of septin towards forming blebs as part of general cytoplasmic flow towards the inflating bleb (and lack of it when blebbing is inhibited) and exclusion from the forming bleb by actin for example (instead of recognition of curvature) could be the basis for the observations.

We respectfully disagree with the reviewer's interpretation of the data in Movie 3 and Figure 2K. To our eyes, the septin signal at the surface before the bleb in 2K and the 5 blebs inset in Movie 3 is well within the range of noise in the micrographs and that strong, consistent signal only emerges after bleb expansion begins. We would suggest that these differing visual analyses, both made in good faith to the best of our abilities, make a strong argument for rigorous quantification. The need for analysis using computer vision is further heightened by the 3D nature of these data – both 2K and Movie 3 are visualizations of a single z-slice in a stack of more than 200. When viewing only the single slice, out of z-frame data (such as blebs in close proximity) cannot be taken into account.

Interpretation is further complicated by the fact that blebbing is spatially recurrent, tending to occur repeatedly in the same general location. This means that septin signal from the previous bleb has often not fully faded before the next bleb is initiated mere seconds later.

To address similar questions by Rev #1 regarding the original version of the manuscript we spent a year developing the software necessary to perform a timeseries analysis capable of disentangling these factors, the results of which are shown in figures 2L and S7. To our knowledge this is the first such analysis of the temporal coupling between a subcellular morphological event and local molecular accumulation at a free form 3D surface. The analyses fully support that septin pulses form only after positive curvature has been generated by blebs: If septin signal increase consistently preceded curvature formation the red/orange/yellow peak would be right-shifted away from zero towards positive lags. This result, combined with 2G (reducing plasma membrane curvature reduces the amount of septin at the surface) and 2I/S7 (septin localization to the surface is blocked when septins lack the ability to sense membrane curvature) all point to bleb-generated membrane curvature being absolutely necessary for septin recruitment to the surface of these cells. The alternative cytoplasmic flow model presented by the reviewer is interesting, but we do not believe it agrees with these data, especially Fig 2I.

As the reviewer states, polymerized septin structures are indeed found at the cell surface absent of proximal bleb-generated curvature, however our data (specifically Figs 2F-I, 2M-N, S7, and S9) suggest that these polymerized structures first require recruitment to the surface by bleb-generated curvature. As stated by Reviewer #1 above, these polymerized structures function as a “memory of pulsatile bleb dynamics.” In this way, none of the septin structures at the surface can be considered truly “bleb-independent,” or else they would be present in bleb-inhibited cells and cells expressing non-curvature-sensing septin mutants.

13. Using the AH-GFP construct as a control instead of the deletion, which might affect the protein beyond the recognition of curvature would be more convincing.

Though we agree that this would, in principle, be a useful experiment, a fluorescently-tagged septin AH domain would likely not behave as predicted. Cellular septins predominantly exist as hexameric or octameric rod-like palindromic complexes, meaning that each complex theoretically contains 2 AH domains which are consistently separated from each other by a distance of ~24 nm. Published work suggests that this distance “tunes” the curvature sensitivity of septins (doi/10.1091/mbc.E20-05-0303), and that individual domains produce alternative curvature affinities. Indeed, just such an experiment as the reviewer recommends was performed by Cannon, et al. in a 2019 paper (doi.org/10.1083/jcb.201807211), finding that the probe possessed high affinity for membrane curvatures far outside of the specific range normally bound by septins. Considering this, we opted to design experiments around the AH deletion mutant instead of an AH-GFP probe.

In response to the reviewer's concern that the mutation might be affecting septin function beyond membrane curvature sensing, we would like to highlight work by the Mostowy lab published last year that characterized this same mutant, showing that its function is preserved along several dimensions (doi.org/10.1038/s41467-021-24721-5). Specifically, they showed that the mutant successfully integrates into endogenous septin complexes and that these complexes localize normally (e.g., decoration of stress fibers) save for enrichment at curved membrane. Work by the Gladfelter lab further shows that a similar mutation of the complimentary gene in yeast causes septins to lose their membrane curvature sensing capabilities while maintaining (or perhaps even increasing) their ability to bind membrane in general, further supporting the specificity of the SEPT6(Δ AH) mutant (doi.org/10.1083/jcb.201807211).

14. In Figures 4A and 4G, septin appears to be distributed in clusters whose positions are not correlated with the circumference of forming blebs, and NRAS/AktPH appear there as well. Why such bleb-independent clusters do not reduce cell death?

As stated in our response to point 12 above, our model holds that these clusters are absolutely producing signaling that reduces cell death, but that their formation depended on previous high-density blebbing that lead to polymerization and thus stable curvature-independent association with the plasma membrane. This model is supported by data in Figures 2, S6, S7, and S8 and is discussed in the associated text of the manuscript where the underlying logic is described. It is further discussed at great length in our previous rebuttal letter, specifically in our response to Reviewer #1.

15. The model the authors propose would be strongly supported by increasing the activities of different signaling molecules that function downstream of septin, showing inhibition of anoikis in cells in which blebbing is inhibited.

We agree with the reviewer's point below that this would fall outside the scope of the paper. We would be further concerned that artificially increasing the activity of individual components of signaling pathways downstream of septins (i.e., MAPK and PI3K) over a period of 24 hours would produce confusing results due to nonlinearity resulting from compensatory effects and the plasticity of these signaling pathways. Indeed, in MV3 cells both of these pathways are basally overactive due to NRAS and PTEN mutations, yet septin function is still required to maintain survival while detached.

I appreciate that the suggestions in points 12 and 15 are challenging to follow and could be considered to be "beyond the scope of the paper".

Referee #4 only sent comments to the editor, stating that their concerns have been addressed

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have addressed the remaining issues and the manuscript is suitable for publication.

Referee #3 (Remarks to the Author):

While I do not fully agree with each and every point in the response, overall the results and especially the methods presented in it would be of interest for the readers of Nature