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Structural centrosome aberrations promote non-cell-autonomous invasiveness

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Pre-decision consultation - editor 15 December 2017

Thank you for submitting your manuscript EMBOJ-2017-98576, "Structural centrosome aberrations promote non-cell-autonomous invasiveness", to our journal. We have now received a complete set of reviews from all referees, which I am enclosing below for your information. As you will see, referees 1-3 appreciate the novelty and potential significance of your findings and would in principle support publication pending some modifications and clarifications. However, referee 4 who has assessed the study particularly as an expert on AFM measurements raises several major criticisms regarding the cell stiffness measurements in the paper, which in our view have the potential to seriously affect key conclusions of the study. In this light, it would be important to hear how you might be able to address these key concerns and substantiate the conclusions if given the opportunity to revise the manuscript. I would therefore like to invite you to discuss the attached reports with your coworkers, and to draft a tentative point-by-point response detailing how you would envision addressing the referees' comments, so that we could take this into account when making our final decision on this manuscript. I would appreciate if you could send us such a tentative response letter (parts of which we may choose to share and discuss with the referees) ideally by mid of the coming week. Should you have any further questions in this regard, of course please do not hesitate to let me know.

REFEREE REPORTS

Referee #1

(Report for Author)
Centrosomes are the major microtubule organizing centers of mammalian cells and are important for forming the bipolar spindle in mitosis to allow for the accurate segregation of chromosomes into each daughter cell. Both structural and numerical alterations of centrosomes are frequently observed in human tumors. However, while the role of numerical centrosome aberration in tumorigenesis has been extensively studied, the role of structural aberrations has received relatively little attention.

Examining whether and how structural alterations in centrosomes contribute to tumorigenesis is timely and of considerable interest to the field.

In this manuscript, Ganier, Schnerch et al., use Ninein-like protein (NLP) overexpression to generate alterations that are reminiscent of structural centrosome aberrations observed in human tumors. The authors examine the effect of NLP overexpression on integrity of epithelial cells grown in 3D spheroid cultures. They find that NLP overexpression triggers a non-cell autonomous "budding" of cells from the epithelial layer. This cellular extrusion is based on two properties caused by NLP-induced structural aberrations: 1) an impairment in the remodeling of E-cadherin junctions between neighboring cells in mitosis, and 2) an increased stiffness of NLP overexpressing cells. Together, these properties lead to the extrusion of mitotic cells from the 3D epithelial culture. The authors propose that epithelial "budding" could contribute to the dissemination of cancer cells. This offers a new model for how centrosome aberrations could contribute to invasiveness in a non-cell autonomous manner, wherein the features which contribute to dissemination need not be present in the established tumor.

The work presented in this manuscript describes a new mechanism through which cells with centrosome aberrations could contribute to malignancy in cancer. The manuscript is well-written and the experiments are carefully executed. The findings are novel and exciting and will be of considerable interest to the readers of The EMBO Journal. Thus, in my opinion, this is a very important study and I would recommend publication with high priority.

I have only a few suggestions below that the authors may choose to ignore if they wish.

Minor Comments:
• The authors may consider referencing the recent JCB paper from Godinho's lab that was published while this manuscript was under review;
http://jcb.rupress.org/content/early/2017/11/10/jcb.201704102/tab-figures-data

• I feel it would be helpful to plot the fraction of cells that undergo budding that are GFP-NLP negative. At present the authors state that "... less than 50% of all budding cells expressed detectable GFP-NLP", but unless I missed something, they don't provide the actual numbers.

• "Nocodazol" should read "Nocodazole"

• In Figure 5D-E did the authors confirm that all the mitotic cells analyzed were overexpressing GFP-NLP?

Referee #2

(Report for Author)
The article of O. Ganier, entitled: "Structural centrosome aberrations promote non-cell-autonomous invasiveness" investigates the consequneces of Nlp over-expression in MCF10A-derived acini 3D culture models. They find that a subset of cells exit the main cell mass and move away. The further characterise the "leaving" cells and found that they normally undergo mitosis before disseminating away. The most interesting, original and rather unexpected finding reported in this article is that the cells that leave the main cell mass, do not have to be the ones that over-express Nlp. This indeed suggests a non-cell autonomous effect that can explain why certain tumours contain high levels of centrosome abnormalities if these are normally considered to inhibit survival. Overall this study is well done and very easy to follow. I only have a few comments that might be worth taking into account within a context of a minor revision.

1) For non matrigel and collagen I aficionados, why the addition of both favours budding in the Nlp cultures?
2) The cell exiting the mamosphere in fig 1C that contains Nlp expression, is it really possible to interpret its origin? Unlike in other examples, appears quite far from the main cell mass.
3) Still related with this figure, why do control spheres show apoptosis, why Nlp spheres do not? I would imagine that a certain degree of apoptosis might b einherent to the system, although I am not an expert, but is it possible that Nlp is involved in apoptotic inhibition? I do not think this will

radically change the interpretation of the data, but would support another layer of complicity in maintaining budding cells alive.
4) I am not sure to fully understand the rationale behind the defects in E-Cadherin remodelling. The authors suggest it is through increased MT nucleation and their stabilization. But in the pictures shown in EV5, there are small cells with NLP-GFP signals without increased detyrosinated tubulin. Maybe it would be possible for the authors to quantify the levels of tubulin, in general and investigate whether these correlate with increased de-tyrosinated tubulin levels and with Nlp levels? Will Plk4 over-expression, which is also leading to increased MT nucleation according to Godinho et al, 2014 have the same effect on de-tyrosinated tubulin levels?
5) The picture provided for membrane blebbing is difficult to see. Can the authors provide with zoom regions? Do the authors think that these result from decreased E-cadherin levels? Since it is not directly related with NLP expression, as it can be observed in the budding cells that are non Nlp, can it be related with extension of mitotic timing?

Referee #3

(Report for Author)
In this manuscript, Olivier Ganier, Dominik Schnerch and their colleagues studied the impact of the over-expression of ninein-like protein (NLP). In a previous paper, published in 2015 in Oncogene, Dominik Schnerch and Erich Nigg showed that NLP over-expression induces the assembly of abnormaly large centrosomes, which boosted microtubule nucleation, impaired the establishment of cell polarity, stimulated cell proliferation and strikingly perturbed mammary acini architecture in 3D ECM gels.
In this work, the authors investigated further the origin of this architectural disorder. They found that initially spherical acini formed outward buds which, unexpectedly, were less rich in abnormal centrosomes than the cells of the acinus. This data set led the authors to convincingly conclude that cells with abnormal centrosome were responsible for the bulging out of cells with normal centrosome. This conclusion is innovant and very interesting for several reasons. Firstly it shows that cells with abnormal centrosomes can affect normal cells which are thus forced to be involved in invasive-like phenotype. Secondly it accounts for the unexplained dilema that abnormal centrosomes are toxic for cells yet present in most advanced and metastatic tumours.
Then authors used a myriads of techniques to quantitatively describe the structural consequences of aberrant centrosome on cell morphology and to shine some light on the physical mechanism responsible for the misorganisation of acini architectures.
Firstly, they showed that NLP over-expression induces E-cadherin delocalization from the junction to the cytoplasm. More precisely, they found that NLP-expressing cells had difficulty to reassemble E-cadherin-based junctions following their disassembly.
Secondly, authors found that extruded cells were most often mitotic cells, the spindle of which was misoriented. In addition, these extruded cells in mitosis formed numerous blebs.
Thirdly, authors measured cell stiffness with Atomic Force Microscopy. Authors found that NLP over-expression increased cell stiffness in interphase (4x) and in mitosis (1,5x). They also found that microtubule stabilization with Taxol increased interphase cell stiffness (1.4x) whereas nocodazole had no effect. Actin depolymerization abolished all these effects, suggesting that the increase of stiffness was due to the remodelling of the actin network by the extra-microtubules, which resulted from the aberant centrosome shape following NLP over-expression.
These three sets of data led the authors to conclude that the increased stiffness in NLP-expressing cells pushed outward the normal cells as they entered mitosis. These extruded cells failed to reestablish proper junctions with their neighbours after mitosis and thus could not reintegrate the epithelium.
As a whole I found this manuscript very interesting and very well written. The data are novel and thought-provoking. The most important result is the « non-autonomous » induction of invasiveness, ie the observation that NLP-expressing cells push normal cells out of the epithelium. This result contrasts with classical believes in the field and is fully demonstrated. This conclusion by itself is sufficient to justify publication of this manuscript in a high-standard journal such as EMBO Journal. The identification of three interesting parameters which may be involved in this mechanism (loss of E-cadherin from junctions, specific extrusion of mitotic cells, increase of stiffness) makes the overall study even more exciting. I was fully convinced by each data set and the conclusions drawn by the authors. Their direct contribution to the budding mechanism is not demonstrated (E-cadherin has not been restored, mitosis prevented or stiffness reduced to prevent the budding) but I agree

these parameters (adhesion, proliferation and stiffness) are likely to be involved in tissue disorganization and worth being described. As discussed below, the data on stiffness measurements were the most surprising. However they should be careful checked by an external AFM expert as they contrasted with previous observations. Apart from this I found all results very convincing and interesting. Therefore I fully support the publication.
The final scheme, in which the three parameters were integrated in a consistent scenario, is not fully clear to me. I guess some confusion also remains in authors mind and explains why the scheme is in supplementary data. I would suggest to move it to the main figures anyway. This would promote further discussion in the field, accounting that demonstrated and speculated elements are made clearly distinct.

Here are few points which may deserve to be addressed to clarify the conclusions.

- It seems to me these observations are more related to budding or some sort than to « invasion »
About E-cadherin:
- In the case of mosaic acini, was E-cadherin mislocalized in NLP-expressing cells only or in all cells? It was not clear whether E-cadherin was mislocalized in extruded cells and/or in their neighbours. One could imagine that normal cells may have difficulties to assemble proper junctions with NLP-expressing cells in which E-cadherin is mislocalized.
- Did the lack of adhesion contribute to the expulsion of cells? Did it affect the rounding process of mitotic cells? One could think that it promoted cortical contraction since the tension that was no longer applied to junctions was probably redistributed internally in the cortex.
- Did the lack of adhesion impair daughter cells re-incorporation into the epithelium (as suggested by the work of Bergstralh and St Johnston published in Nature Cell Biology in 2017) ?
- Was the lack of E-cadherin responsible for spindle misorientation (as suggested by the work of Den Elzen published in Molecular Biology of the Cell in 2009)? Was spindle misorientation truly involved in the budding? in its amplification?
About mitotic cells contractility:
- Blebbing is known to result from excessive cortical contraction and/or lack of adhesion (see the work of Ewa Paluch). This is consistent with the high stiffness in NLP-expressing mitotic cells. But cells that are described in Figure 4 have been shown not over-express NLP (it is the big point of the paper!). So I am confused. Why do these « normal » cells display increased contraction? Is it due to their lack of adhesion with the epithelium?
About cell stiffness:
- Cell stiffness is related to cell contraction (see the work of Solon and Janmey published in Biophysical Journal in 2007). Nocodazole is known to trigger cell contraction (in spread and in round cells) and therefore cell blebbing. But generally speaking, cell contraction is promoted by the activity of Rho, not Rac. How can Rac inactivation rescue a mechanism based on hyper-stiffness and therefore hyper-contraction? I am confused.
- The effect of taxol on cell contraction is more controversial. Here, how can taxol trigger the increase of cell stiffness? Microtubule polymerization should trigger Rac activation, not Rho. Same in NLP-expressing cells: more polymerized microtubule should increase Rac activation, not Rho.
- The reduction of cortical stiffness when cells enter mitosis in an epithelium is very surprising (and very novel contrary to what the citation of the work of Saw and Ladoux published in Nature in 2017 could suggest). It contrasts with previous works, which all reported RhoA activation during mitotic rounding. It is true that these works were mostly (all?) performed on single cells and that the authors did proper control to show that in their hands stiffness also increased during mitosis in isolated cells. But I am afraid there is something wrong in this set of data. A better expert on AFM should look more carefully at the way AFM has been done (and at the method that was used to normalize measurements).
About the final scheme:
- in the WT case the mitotic cells has a strange shape (is it true that it is not round?). It seems to deform adjacent cells. But authors argued mitotic cells were softer than adjacent cells. How could they deform them? What are the « deformable (soft) cells »?
- In an heterogeneous epithelium, I agree that rounding up (by cortical contraction and volume increase) in a stiff environment should promote cell extrusion. But if the mitotic cells are softer than their surrounding, which is even more the case with normal mitotic cells and NLP-expressing surrounding cells, the mitotic cells should deform rather than pop out. The whole scenario would be more consistent if mitotic cells would actually increase their contraction to round up. Rounding would be feasible within a soft epithelium and impossible in a stiff one, which would thus trigger

mitotic cells extrusion. This is the reason why I think it would be worth double-checking the data on mitotic cell stiffness.

Referee #4

(Report for Author)
Specific comments.

The stiffness measurements are difficult to interpret due to the fundamental problem that cells are not composed of a homogenous material. This means that cells do not have a homogeneous stiffness as used by the authors to compare the apparent stiffnesses measured for the cells. Furthermore, it is not clear how cellular stiffnesses were derived from stiffness maps. It is obvious that stiffness maps of cells having different morphologies (eg interphase vs mitotic cells) must be different because the pyramidal AFM tip interacts differently with different cell shapes. As one consequence among several different models must be applied to estimate stiffnesses from the measurements. Obviously this has not been done. Currently the quality of the presented AFM data does not warrant the strong conclusions made.

The authors did not describe sufficiently how they determined cellular stiffness maps and cell stiffness. The authors refer to some papers previously published. However, these papers applied AFM to estimate the stiffness of different biological systems and rather superficially describe how the data was analyzed. For example to understand the submitted paper essential information is missing on the depth of the AFM tip indenting into the cell surface, which force curves from the force volume data have been selected for analysis, which criteria has been applied to select force curves for analysis, how the force curves were analyzed, it is not described which model has been applied to derive the stiffness values from experiments, it has not been described whether the experimental conditions allow applying the model, it is not clear why a maximal force and not a maximal indentation was chosen to record stiffness maps, and it is also not clear how stiffness ratios were determined.

Certainly, a resistance to deformation is measured by sticking a sharp pyramidal AFM tip presumably 1-5 μm into a cell. Such mechanical measurements could be of some comparative value. However, to interpret which of the multitude of cellular structures contributes how much to resisting the deformation is not possible from the measurements presented by the authors.

The normalization of the stiffness measured for each experiment is suspect. Particularly for the mitotic vs. interphase MDCK cell measurements. For these, I suggest that the authors perform trans-mitosis experiments, in with they follow a cell's stiffness before, during and after mitosis. Histone morphology (chromosome condensation) can be used to detect cells before nuclear envelope brake down. Furthermore stiffness maps of mitotic cells have not been shown.

The authors write that to record stiffness maps of cells required about 20-45 min. However, as the authors also report that the duration of mitoses is about 40-50 min. One thus wonders what the stiffness map represents and from which to which mitotic state it was recorded.

Figure 5 A and B, force/volume map stiffness scales have completely wrong numbering.

Figure 5B. It is not clear ho left and middle image correlate. It is not clear what middle image shows, what are the cells? Not clear how right image correlates to left and middle images.

Figure 5D is key for the paper. However, no stiffness maps are shown. These maps together with height images/topographs should be shown for interphase and mitotic cells. The same counts for expanded Figure 9C. The fluorescence image shown in Figure 9C does not show a nice mitotic spindle.

Expanded Figure 9 A and B. Please also show stiffness maps to connect stiffness histograms with cellular morphology/localization.

The descriptions in the figure legends are insufficient and vague. In general, a description of what is displayed is preferable over an interpretation of the data.

Stiffness and height maps of the MDCK experiments should be provided. Given the impressive imaging system, the images are not impressive.

The ‚close to physiological conditions' under which AFM experiments were done have not been specified.

I do not understand why the stiffness maps show bright as softer and dark as stiffer and not the other way. This is counterintuitive. In addition it would be helpful to provide the height/topographic maps of the cells, as the topography is an important parameter to morphologically interpret stiffness maps.

It is difficult to understand why the authors used very sharp tips having diameters ranging from 10-20 nm to characterize cell stiffness. Stiffness measurements using a sharp tip suffer from the heterogeneous structural and mechanical properties of the cell. Such cellular heterogeneity is also observed by the authors. It would make more sense to take a blunt tip.

Pre-decision consultation - author response 21 December 2017

Thank you very much for the reviewer's feed-back on our manuscript. We are very pleased to see that 3 out of 4 referees express highly favorable opinions, and we are confident that we will be able to address all their minor issues. Regarding referee 4 – the "AFM expert" – we have discussed this person's criticism with our AFM collaborators. Please find our response attached to this mail (note that we also include responses to those points of referee 3 that concern AFM). We also attach a Figure that is currently part of another manuscript (in preparation) but will hopefully help to explain how AFM data were analyzed (note that we do not plan to include this Figure into the Ganier et al., manuscript).

There are just two additional important points that I would like to make about the AFM-related criticism (from a biologists perspective).

First, both referees 3 and 4 raise the issue that our data (showing that mitotic cells within epithelia are softer than neighboring interphase cells) appear to conflict with previous publications reporting that mitotic cells are stiffer than interphase cells. We are fully aware of this apparent discrepancy and have clearly addressed this issue in the manuscript. We are confident that the apparent discrepancy likely reflects the fact that - to the best of our knowledge - we are the first to report measurements on mitotic cells and interphase cells WITHIN AN EPITHELIUM, while all previous studies compared ISOLATED cells. Most importantly, when we carry out AFM stiffness measurements on isolated cells (using our setup), we fully confirm the previous studies: in isolation, mitotic cells are indeed 'stiffer' than interphase cells (see EV9C). We recognize that issues related to cell shape etc. may influence the interpretation of AFM results. Thus, we are in principle prepared to down-tone our conclusions regarding differences between interphase cells and mitotic cells. In any case, these differences are not essential to the main conclusion of our paper: our model only requires that interphase cells harboring (NLP-induced) centrosome aberrations are stiffer than control interphase cells, and that the same is true when comparing mitotic cells with and without aberrations. We feel that both of these conclusions are well supported (see below). This being said, we still think that our comparison of the stiffness of interphase cells and mitotic cells within an epithelium is worth reporting (even if not critical for our model). To the best of our knowledge, this analysis had never been reported before.

Second, regardless of any technical debates amongst 'experts' (regarding the questions of how AFM data should best be acquired and interpreted), we emphasize that our key conclusions do not depend exclusively on our cell stiffness measurements by AFM. Basically, we are saying that in epithelia harboring populations of cells with (NLP-induced) structural centrosome aberrations, mitotic cells are squeezed out through a non-cell-autonomous mechanism (because they come under pressure from their neighbors). In strong support of this view, we note that the 'budding' mitotic cells display exactly those features, prolonged mitosis and extensive membrane blebbing (Figure 4), that have been described by others, using different AFM setups to study mitotic cells under confinement (Cattin et al., 2015, PNAS, op.cit). Moreover, the interphase cells expressing (NLP-induced) structural centrosome aberrations display markedly increased levels of de-tyrosinated tubulin (EV5),

which has previously been shown to correlate with enhanced stiffness (Kerr et al., 2015, Nature Communications, op.cit.; Figure 4 – incidentally, this conclusion was also reached by AFM!). Thus, we have three lines of evidence supporting the conclusion that mechanobiological properties contribute to the 'budding' of mitotic cells; only one of these involves our AFM setup and methodology, while the other two lines of evidence involve AFM done by other laboratories.

Thus, in conclusion, we are highly confident that we can address all the issues raised by the referees, and we hope that EMBO J continues to be interested in publishing this paper. If so, we will be happy (in the new year) to provide all the requested additional AFM data, and we will better explain some of the apparently confusing aspects during revision of manuscript and Figures.

Referee #3

About cell stiffness:

1. - Cell stiffness is related to cell contraction (see the work of Solon and Janmey published in Biophysical Journal in 2007).

Response: It is true that the actin cortex regulates cell contraction (as shown by Solon and Janmey, but also many others). However, cell stiffness does not equal cell contraction. Cell stiffness depends on the specific contributions of the entire cytoarchitecture including actin, intermediate filaments (https://www.ncbi.nlm.nih.gov/pubmed/21426942 http://www.pnas.org/content/110/46/18507.full.pdf) and the microtubule network (see for example: http://iopscience.iop.org/article/10.1088/1367-2630/aa7658/pdf, http://www.sciencedirect.com/science/article/pii/S0021929013005721#)! As illustrated by these articles, the role of different cytoskeleton components in cell stiffness is not dependent on the method used to assess stiffness.

2. Nocodazole is known to trigger cell contraction (in spread and in round cells) and therefore cell blebbing. But generally speaking, cell contraction is promoted by the activity of Rho, not Rac. How can Rac inactivation rescue a mechanism based on hyper-stiffness and therefore hyper-contraction? I am confused.

Response: Again, it is not contraction i.e. the actin cortex alone, that contributes to cell stiffness. In Figures 3E and 3G, we present data showing that partial inhibition of Rac1 can restore E-Cadherin defects induced by NLP overexpression, and can prevent budding from GFP-NLP+ cysts. This led us to conclude that GFP-NLP overexpression can interfere with E-Cadherin junctions in a Rac1-Arp2/3 dependent pathway. Our data therefore suggest that mitotic budding involves two features: E-Cadherin loss and increased stiffness. Considered individually, these events are necessary but not sufficient to trigger budding of mitotic cells.

- The effect of taxol on cell contraction is more controversial. Here, how can taxol trigger the increase of cell stiffness? Microtubule polymerization should trigger Rac activation, not Rho. Same in NLP-expressing cells: more polymerized microtubule should increase Rac activation, not Rho.

Response: As we explained above, changes in microtubule polymerization and architecture can lead to changes in cell stiffness. Indeed, we fully agree with this referee's point about the effect of microtubules on the overactivation of Rac1: it has clearly been shown that enhanced numbers of microtubules can trigger Rac1 over-activation (see Akhtar & Hotchin, 2001; Chu et al., 2004; Godinho et al., 2014; Waterman-Storer et al., 1999; Xue et al., 2013). Additionally, we have confirmed previous observations showing that Taxol increases cellular stiffness (Kerr, 2015). None of this data contradicts an involvement of RhoA in cellular stiffness. Deregulated microtubules impact on the actin network via the Rac1-Arp2/3 pathway (Waterman-Storer et al., 1999), in line with the notion that both Rac1 and RhoA can mediate effects through actin (Sepp KJ, Development 2003).

3. - The reduction of cortical stiffness when cells enter mitosis in an epithelium is very surprising (and very novel contrary to what the citation of the work of Saw and Ladoux published in Nature in 2017 could suggest). It contrasts with previous works, which all reported RhoA activation during mitotic rounding. It is true that these works were mostly (all?) performed on single cells and that the

authors did proper control to show that in their hands stiffness also increased during mitosis in isolated cells.
But I am afraid there is something wrong in this set of data. A better expert on AFM should look more carefully at the way AFM has been done (and at the method that was used to normalize measurements).

Response: We recognize that our results seem to differ from previously published data. However, we emphasize that we observed cell softening during mitosis in confluent epithelia, and not in single cells undergoing mitosis. As recognized by the referee, our data on single cells are in full agreement with previously published work. To the best of our knowledge, the stiffness of cells in mitosis has not previously been addressed in the context of a confluent epithelium. (The work of Saw and Ladoux published in Nature in 2017 does not seem to address mitotic cells?).

4. About the final scheme:
- in the WT case the mitotic cells has a strange shape (is it true that it is not round?). It seems to deform adjacent cells. But authors argued mitotic cells were softer than adjacent cells. How could they deform them? What are the « deformable (soft) cells »?

- In an heterogeneous epithelium, I agree that rounding up (by cortical contraction and volume increase) in a stiff environment should promote cell extrusion. But if the mitotic cells are softer than their surrounding, which is even more the case with normal mitotic cells and NLP-expressing surrounding cells, the mitotic cells should deform rather than pop out. The whole scenario would be more consistent if mitotic cells would actually increase their contraction to round up. Rounding would be feasible within a soft epithelium and impossible in a stiff one, which would thus trigger mitotic cells extrusion. This is the reason why I think it would be worth double-checking the data on mitotic cell stiffness.

Response: We will carefully consider this comment, and revise manuscript text and Figures as appropriate. Indeed, we found that mitotic cells in confluent epithelia are not actually truly round but tend to elongate. Individual mitotic cells on the other hand clearly round up.

Referee #4

(Report for Author)

Specific comments.

5. The stiffness measurements are difficult to interpret due to the fundamental problem that cells are not composed of a homogenous material. This means that cells do not have a homogeneous stiffness as used by the authors to compare the apparent stiffnesses measured for the cells.

Response: We fully agree with the referee that cells are not homogeneous material and thus do not exhibit homogeneous stiffness properties. This is precisely the reason why we employ the sharp AFM tip that is able to detect local stiffness heterogeneities. This allows us to clearly differentiate the cell boundaries (junctions), which are excluded from the analysis, and it also allows us to assess the stiffness of nuclear and perinuclear regions. The presentation of data in box plots reflects these local heterogeneities by the range of standard deviations (shown in each Figure).

6. Furthermore, it is not clear how cellular stiffnesses were derived from stiffness maps.

Response: We thank the reviewer for raising this point. We will provide additional supplementary material to describing the stiffness analysis workflow in detail (in the meantime, please see the attached Figure).

7. It is obvious that stiffness maps of cells having different morphologies (eg interphase vs mitotic cells) must be different because the pyramidal AFM tip interacts differently with different cell shapes. As one consequence among several different models must be applied to estimate stiffnesses from the measurements. Obviously this has not been done.

Response: We agree that this would be the case for larger blunt tips, where the interfaces between interphase cells and mitotic cells would play a role and could not be discriminated from the stiffness maps, due to the larger diameter of the probe. When a sharp 20 nm probe is used, one can very well discriminate cell boundaries from the rest of the cell. Moreover, in case of mitotic cells, only force curves on the elevated parts of the mitotic cells were used to generate E-modulus values per cell. To avoid the issues raised by the reviewer, force curves recorded on the steep transitions between mitotic (high) and neighboring interphase cells (shallow) were not considered. To better illustrate these points we will amend the manuscript text and provide additional Figures.

8. Currently the quality of the presented AFM data does not warrant the strong conclusions made.

Response: For the reasons explained above and below, we strongly disagree with this statement.

9. The authors did not describe sufficiently how they determined cellular stiffness maps and cell stiffness.

Response: We accept this point and will provide additional detailed technical information on how cellular stiffness was determined. In the meantime, we provide here an extract from the Methods section of one of our previous papers (Plodinec et al. 2011, please see below).

10. The authors refer to some papers previously published. However, these papers applied AFM to estimate the stiffness of different biological systems and rather superficially describe how the data was analyzed.

Response: With all due respect, we disagree with this statement. We cite several previous studies, including our own previous work (Plodinec et al., 2011) that describes the model applied and data analysis in quite some detail. To illustrate the point, a relevant passage from Plodinec et al., 2011 is copied here:

2.5. Analysis of indentation data

For the analysis of AFM force curves, a method described in Loparic et al. was modified (Loparic et al., 2010). Briefly, we developed software in LABWIEV (National Instruments, USA) for the automated analysis of the force–volume data. The contact point was determined by applying a polynomial fit to raw force curves according to a published algorithm (Lin et al., 2007). Force-indentation data were obtained by the indentation h, which corresponds to the difference between piezo displacement and cantilever deflection, and by multiplying cantilever deflection d with the spring constant k to obtain load F. The slope of each data point is calculated by performing a linear fit to the upper 50% of the unloading force curve. Slope values were spatially plotted to yield a stiffness map using Igor Pro (Wavemetrics, LakeOswego, USA). Gaussian distributions were computed from the stiffness values of individual cells. The mean slope value from the Gaussian fit was then used for calculating elastic modulus (E_s) for each cell type according to the Oliver and Pharr theory (Oliver and Pharr, 1992) applied by Loparic and coworkers (Stolz et al., 2004; Loparic

et al., 2010). In the Eq. (1) where E_r represents the measured relative dynamic elastic modulus, E_s is the elastic modulus of the sample and E_i of the indenter

$$\frac{1}{E_r} = \frac{1-\upsilon_s^2}{E_s} + \frac{1-\upsilon_i^2}{E_i} \tag{1}$$

where υ_s and υ_i are Poisson's ratios of sample and indenter. Assuming that the elastic modulus of biological specimens is orders of magnitude smaller than that of the indenter ($E_i >> E_s$), Eq. (1) reduces to

$$\frac{1}{E_r} \cong \frac{1-\upsilon_s^2}{E_s} \tag{2}$$

The Poisson's ratio of cells is assumed to be $\upsilon_s = 0.5$ (45). The stiffness of the sample S is defined as the linear fit between the maximum load $F_2 = 1.8$ nN and the minimum load $F_1 = 0.9$ nN (3). The indentation depth is denoted by h.

$$S = \left[\frac{\Delta p}{\Delta h}\right]_{F_1}^{F_2} = \left[\frac{\Delta d \times k}{\Delta h}\right]_{d_1}^{d_2} \tag{3}$$

Subsequently, the relative elastic modulus E_r is defined by the following equation:

$$E_r = \frac{\sqrt{\pi}}{2}\frac{S}{\sqrt{A_c(h_c)}} \tag{4}$$

where $A_c(h_c)$ is the projection of the contact area as a function of the tip angle (Θ) and the contact indentation depth h_c. Assuming h_c to correspond to the total depth of indentation h yields the following relation for a 4-sided pyramidal tip

$$A_c(h_c) = (2h\tan\Theta)^2 \tag{5}$$

By combining Eqs. (2)–(5) the elastic modulus of the sample E_s can be calculated:

$$E_s = \frac{\sqrt{\pi}}{2}(1-\upsilon_s^2)\frac{1}{\sqrt{A_c(h_c)}}\left[\frac{\Delta d \times k}{\Delta h}\right]_{d_1}^{d_2} \tag{6}$$

2.6. Statistical analysis

11. For example to understand the submitted paper essential information is missing on the depth of the AFM tip indenting into the cell surface, which force curves from the force volume data have been selected for analysis, which criteria has been applied to select force curves for analysis, how the force curves were analyzed, it is not described which model has been applied to derive the stiffness values from experiments, it has not been described whether the experimental conditions allow applying the model, it is not clear why a maximal force and not a maximal indentation was chosen to record stiffness maps, and it is also not clear how stiffness ratios were determined.

Response: We recognize that our description of methodology may have been aimed more at a readership of cell and molecular biologists than AFM experts. Thus, we thank the referee for insisting on this issue. Although we have previously described elements of our analyses (see above), it is true that previous work did not apply to epithelial MDCK cells. Hence, in the revised manuscript we will include more detailed descriptions of recording and analysis methods.

12. information is missing on the depth of the AFM tip indenting into the cell surface,

Response: We will revise the text accordingly: the indentations were typically 1-2 micrometers. The cell areas (i.e. curves in the FV map) used for analysis refer to regions inside the perimeters of the junctions (but not including the junctions), for reasons described above (pt. 7).

13. which criteria has been applied to select force curves for analysis, how the force curves were analysed

Response: A set of commonly used criteria (signal-to-noise ratio, forward and backward curve tilt, hydrodynamic drag between forward and backward curves) was examined to select the maps suitable for analysis. This has been described previously (Plodinec and Lim, 2015) and we will include this reference in the manuscript.

14. it is not clear why a maximal force and not a maximal indentation was chosen to record stiffness maps, and it is also not clear how stiffness ratios were determined.
Response: Pioneering work using AFM to measure the stiffness of living cells clearly demonstrates the necessity to use constant force (article (https://ac.els-cdn.com/S0006349598778683/1-s2.0-S0006349598778683-main.pdf?_tid=dd30ba8a-e5d3-11e7-957d-00000aab0f01&acdnat=1513808557_1bb91dbcd0cb8d5d5966cd9d08b936ae, http://onlinelibrary.wiley.com/doi/10.1002/sca.1998.4950200504/epdf) rather than indentation depth in order to derive comparable E-moduli. Indentation depth assessment induces significant errors in the analysis of soft viscoelastic samples, which is why the actual geometry of the probe needs to be considered (http://biomechanical.asmedigitalcollection.asme.org/article.aspx?articleid=140209c). This is precisely what we have done for our analysis. For details see point 10 and Plodinec et al., 2011. Moreover, due to errors associated with the indentation depth some groups are developing models that enable determining indentation modulus without including indentation depth (Gaylord Guillonneau, Guillaume Kermouche, Sandrine Bec, Jean-Luc Loubet. Determination of mechanical properties by nanoindentation independently of indentation depth measurement. Journal of Materials Research, Cambridge University Press (CUP), 2012, 27, pp.2551-2560. <10.1557/jmr.2012.261>. <hal-00826308>).

15. Certainly, a resistance to deformation is measured by sticking a sharp pyramidal AFM tip presumably 1-5 μm into a cell. Such mechanical measurements could be of some comparative value. However, to interpret which of the multitude of cellular structures contributes how much to resisting the deformation is not possible from the measurements presented by the authors.

Response: We fully agree with the referee on this point. However, we would like to emphasize the importance of normalization of stiffness values. It is precisely this normalization, i.e. the comparison of NLP overexpressing cells (or mitotic cells) with their surrounding neighbouring cells (controls) within the same sample, that enables us to interpret the impact of NLP overexpression on cellular stiffness (in spite of the multitude of cellular structures that likely contribute to deformation). We will amend the manuscript text to clarify this point better.

16. The normalization of the stiffness measured for each experiment is suspect. Particularly for the mitotic vs. interphase MDCK cell measurements. For these, I suggest that the authors perform trans-

mitosis experiments, in with they follow a cell's stiffness before, during and after mitosis. Furthermore stiffness maps of mitotic cells have not been shown.

Response: For the reasons just described above, we disagree with the referee and certainly do not consider our stiffness measurements 'suspect'. Also, we do not consider it necessary to carry out trans-mitosis experiments, because we have correlative optical data (mCardinal-histone H1) that clearly show which cells are in mitosis or interphase.

17. Histone morphology (chromosome condensation) can be used to detect cells before nuclear envelope brake down.

Response: Indeed, we have routinely identified mitotic cells by using an mCardinal-tagged histone marker. For details please see Figure 5D.

18. Furthermore stiffness maps of mitotic cells have not been shown.

Response: We agree that the stiffness maps should be included and will amend the Figures accordingly.

19. The authors write that to record stiffness maps of cells required about 20-45 min. However, as the authors also report that the duration of mitoses is about 40-50 min. One thus wonders what the stiffness map represents and from which to which mitotic state it was recorded.

Response: As explained above, we have optical controls for cells undergoing mitosis. Indeed, because the time is of essence (as correctly pointed out by the referee), all experiments on mitotic cells were done within 20 minutes. To make this possible, we have used lower resolutions of 32x32 pixels. We will amend the text to clarify this point.

20. Figure 5 A and B, force/volume map stiffness scales have completely wrong numbering.

Response: This is not the case – the numberings are correct. Figure 5A depicts cells in 2D and Figure 5B shows 3D cysts. The absolute stiffness values are in a different range for 2D versus 3D, as illustrated by different stiffness scales.

21. Figure 5B. It is not clear ho left and middle image correlate. It is not clear what middle image shows, what are the cells? Not clear how right image correlates to left and middle images.

Response: In Figure 5B, the left image shows the AFM tip over an 3D MDCK cyst, the middle image shows the stiffness map of cells recorded in the central part of the corresponding cyst. We would have thought this should be clear from the descriptions provided, but will reconsider carefully how we might possibly improve the presentation of this Figure for increased clarity.

22. Figure 5D is key for the paper. However, no stiffness maps are shown. These maps together with height images/topographs should be shown for interphase and mitotic cells. The same counts for expanded Figure 9C.

Response: We accept this point and will include topography/stiffness maps into revised figures 5D and 9C.

23. The fluorescence image shown in Figure 9C does not show a nice mitotic spindle.

Response: This is correct, because Figure 9C does not show mitotic spindle but condensed (chromatin mCardinal histone H1), as clearly indicated in the Figure.

24. Expanded Figure 9 A and B. Please also show stiffness maps to connect stiffness histograms with cellular morphology/localization.

Response: We will include maps and revise the Figure accordingly.

25. The descriptions in the figure legends are insufficient and vague. In general, a description of what is displayed is preferable over an interpretation of the data.

Response: We accept this criticism and will revise the Figure legends accordingly.

26. Stiffness and height maps of the MDCK experiments should be provided. Given the impressive imaging system, the images are not impressive. :

Response: As stated above (pt. 22), we will provide topography/stiffness maps. Regarding our 'impressive imaging system' not producing 'impressive images'. it is not clear to us which specific data the referee refers to. We may be biased, but we do consider our images to be sufficiently impressive to convey the intended messages.

27. The ,close to physiological conditions' under which AFM experiments were done have not been specified.

Response: This is a good point and we apologize for not having provided more specific information. We will amend the Methods text to explain that "all AFM experiments were carried out at temperatures close to 37°C. During the measurements, culture dishes were replenished with fresh cell medium saturated with 5% CO2 to maintain pH and compensate for evaporation. In the course of an experiment, the pH ranged between 7.4 and 7.5."

28. I do not understand why the stiffness maps show bright as softer and dark as stiffer and not the other way. This is counterintuitive. In addition it would be helpful to provide the height/topographic maps of the cells, as the topography is an important parameter to morphologically interpret stiffness maps.

Response: We consider this to be an aesthetic issue, since false-colour scales are completely arbitrary. However, we will be happy to revise the Figures to show images with soft areas in darker tones and stiffer regions in brighter colour. We will also be happy to include topography images.

29. It is difficult to understand why the authors used very sharp tips having diameters ranging from 10-20 nm to characterize cell stiffness. Stiffness measurements using a sharp tip suffer from the heterogeneous structural and mechanical properties of the cell. Such cellular heterogeneity is also observed by the authors. It would make more sense to take a blunt tip.

Response: The rationale behind using sharp tips has been described above (pt. 7). To emphasize this more clearly, we will be happy to prepare an additional Figure that will demonstrate the differences in sensitivity and specificity that arise when using blunt vs. sharp AFM probes to differentiate specific cellular regions (i.e. junctions, perinuclear, nuclear regions).

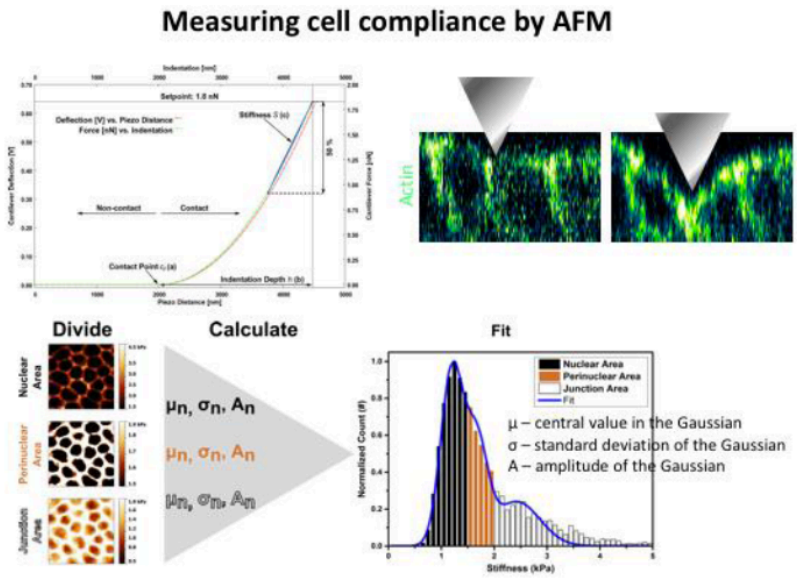


Figure XX. Force curves generated by the AFM stiffness maps are analyzed using a custom-made software in Labview and semi-automatically divided into three areas, cell center (nuclear region), the perinuclear (between nucleus and junction) and the junctional region. Initial guesses for a triple Gaussian fit are extracted from the split histogram and the fit is performed to obtain mean values and standard deviations for each of the three specific areas. The image above the histogram displays an orthogonal projection of cells before loading and under the load. The cantilever tip is drawn to scale.

1st Editorial Decision 21 December 2017

Thank you very much for your detailed responses regarding the main critical points by referees 3 and 4 on your recent manuscript. I appreciate the provided clarifications related to your AFM analyses, and realize (also in light of cross-refereeing comments received in the meantime from some of the other referees) that many of the key conclusions of the study would in any case not be directly affected by these issues. In this light, we shall be happy to consider a revised version of the manuscript further for publication in The EMBO Journal, incorporating the presented responses as well as addressing the various other/more specific points raised in all reports.

I will just add that it is our policy to allow only a single round of revision, making it important to carefully answer all points raised at this point, and that publication of any competing work elsewhere during the revision period will of course have no negative impact on our final assessment of your own study. Detailed information and guidelines on how to prepare a revision can be found below, as well as in our online Guide to Authors.

Thank you again for the opportunity to consider this interesting work, and I look forward to your revision!

1st Revision - authors' response 14 February 2018

Point per point reply to Referees:

Referee #1

The work presented in this manuscript describes a new mechanism through which cells with centrosome aberrations could contribute to malignancy in cancer. The manuscript is well-written and the experiments are carefully executed. The findings are novel and exciting and will be of considerable interest to the readers of The EMBO Journal. Thus, in my opinion, this is a very important study and I would recommend publication with high priority.

Response: We thank this referee for recommending publication of our work with high priority.

Minor Comments:
• The authors may consider referencing the recent JCB paper from Godinho's lab that was published while this manuscript was under review;
http://jcb.rupress.org/content/early/2017/11/10/jcb.201704102/tab-figures-data

Response: We thank the referee for drawing our attention to this recent paper and we now cite this reference in the manuscript.

• I feel it would be helpful to plot the fraction of cells that undergo budding that are GFP-NLP negative. At present the authors state that "... less than 50% of all budding cells expressed detectable GFP-NLP", but unless I missed something, they don't provide the actual numbers.

Response: In response to this suggestion, we now provide a graph that illustrates the striking enrichment of GFP-NLP negative cells amongst the population of budding cells (new Figure 5B).

• "Nocodazol" should read "Nocodazole"

Response: This has been corrected - thanks for spotting this error.

• In Figure 5D-E did the authors confirm that all the mitotic cells analyzed were overexpressing GFP-NLP?

Response: Yes, epifluorescence microscopy was used in all experiments to confirm the GFP-NLP status of cells to be analysed. This is now explicitly stated in the Methods.

Referee #2

The article of O. Ganier, entitled: "Structural centrosome aberrations promote non-cell-autonomous invasiveness" investigates the consequneces of Nlp over-expression in MCF10A-derived acini 3D culture models. They find that a subset of cells exit the main cell mass and move away. The further characterise the "leaving" cells and found that they normally

undergo mitosis before disseminating away. The most interesting, original and rather unexpected finding reported in this article is that the cells that leave the main cell mass, do not have to be the ones that over-express Nlp. This indeed suggests a non-cell autonomous effect that can explain why certain tumours contain high levels of centrosome abnormalities if these are normally considered to inhibit survival. Overall this study is well done and very easy to follow.

Response: We thank this referee for appreciating our work and the importance of its implications.

I only have a few comments that might be worth taking into account within a context of a minor revision.

1) For non matrigel and collagen I aficionados, why the addition of both favours budding in the Nlp cultures?

Response: As we describe in our manuscript, collagen I only has a noticeable impact on invadopodia formation, but not on the main topic of our study, the budding of mitotic cells. The former point is illustrated in EV2 and fully consistent with previous literature (Artym 2016, op. cit.), the latter point is clearly illustrated in Figure 1B.

2) The cell exiting the mamosphere in fig 1C that contains Nlp expression, is it really possible to interpret its origin? Unlike in other examples, appears quite far from the main cell mass.

Response: Having examined many spheres and budding cells, we are highly confident that this image represents a budding event. This being said, the referee is of course correct in that it is formally impossible to draw this conclusion from fixed samples. Hence, we went to greath length throughout this study to complement analyses of fixed samples with live cell imaging (see Figures 2, 3 and 5).

3) Still related with this figure, why do control spheres show apoptosis, why Nlp spheres do not? I would imagine that a certain degree of apoptosis might be inherent to the system, although I am not an expert, but is it possible that Nlp is involved in apoptotic inhibition? I do not think this will radically change the interpretation of the data, but would support another layer of complicity in maintaining budding cells alive.

Response: We are not sure we understand this comment, as both control spheres and spheres expressing GFP-NLP show occasional cells staining positively for cleaved caspase 3 (as illustrated clearly in Figure 1C). Thus, we see no reason to suspect that overexpression of NLP might interfere with apoptosis. (Incidentally, this absence of effect on apoptosis will be fully documented in another context, in a manuscript currently in preparation).

4) I am not sure to fully understand the rationale behind the defects in E-Cadherin remodelling. The authors suggest it is through increased MT nucleation and their stabilization. But in the pictures shown in EV5, there are small cells with NLP-GFP signals without increased detyrosinated tubulin. Maybe it would be possible for the authors to quantify the levels of tubulin, in general and investigate whether these correlate with

increased de-tyrosinated tubulin levels and with Nlp levels? Will Plk4 over-expression, which is also leading to increased MT nucleation according to Godinho et al, 2014 have the same effect on de-tyrosinated tubulin levels?

Response: We thank this referee for raising an important point. That excess NLP causes increased stabilization of microtubules at enlarged centrosomes has been documented previously (Schnerch and Nigg, 2016). Here this is fully confirmed through staining of accumulating microtubule minus ends by antibodies against CAMSAP2 (new Figure 6 in the revised manuscript). Moreover, following the suggestion by this referee we have carried out additional stainings for detyrosinated alpha-tubulin and the results clearly show that this modification accumulates in response to overexpression of NLP but not PLK4 or CEP68 (see new Figure 4). This is indeed an important result and fully supports our conclusions regarding the different effects (on budding) produced by NLP-induced structural centrosome aberrations *versus* PLK4-induced numerical centrosome aberrations. While it is true that PLK4 overexpression also stimulates microtubule nucleation (Godhino et al. 2014), those microtubules are still highly dynamic (as also reported in Godhino et al., 2014), which readily explains why detyrosinated alpha-tubulin accumulates only in response to excess NLP (but not PLK4). In line with this, enhanced stiffness is only triggered by excess NLP but not PLK4. These observations are explained in the revised Results section, notably in the passages that describe the new Figure 4.

5) The picture provided for membrane blebbing is difficult to see. Can the authors provide with zoom regions?

Response: As requested, we now complement former Figure 4 (now Figure 5) with a blow up of the blebbing image. Also, we emphasize that additional examples of blebbing can be seen in other images (notably Figures 2B-C).

Do the authors think that these result from decreased E-cadherin levels?

Response: We do not think that this is the case. First, both NLP and PLK4 overexpressions disorganize E-Cadherin junctions (Figure 3A), and yet, we know that blebbing is observed only in the former case. Second, as shown in Figure 2D, we did not see obvious membrane blebbing upon overexpression of GFP-NLP in 2D cultures, even though E-Cadherin junctions were altered under these conditions (Figure 3C and time lapse experiments EV6).

Since it is not directly related with NLP expression, as it can be observed in the budding cells that are non Nlp, can it be related with extension of mitotic timing?

Response: There is no correlation between membrane blebbing and mitotic timing, indicating that blebbing is not a consequence of increased mitotic timing. In fact, while almost all budding cells display blebs (97%, see Figure 5C), not all budding cells show an increase in mitotic duration (Figure 5D). Yet, as shown previously, both phenotypes are induced by the confinement of mitotic cells (Cattin et al. 2015). Thus, we conclude that budding cells complete mitosis as soon as they evade from acini, thereby escaping the pressure exerted by surrounding epithelial cells (see Figure 2B).

Referee #3

As a whole I found this manuscript very interesting and very well written. The data are novel and thought-provoking. The most important result is the « non-autonomous » induction of invasiveness, ie the observation that NLP-expressing cells push normal cells out of the epithelium. This result contrasts with classical believes in the field and is fully demonstrated. This conclusion by itself is sufficient to justify publication of this manuscript in a high-standard journal such as EMBO Journal.

Response: We thank this referee for his/her praise and for supporting publication of our work in a high-standard journal.

The identification of three interesting parameters which may be involved in this mechanism (loss of E-cadherin from junctions, specific extrusion of mitotic cells, increase of stiffness) makes the overall study even more exciting. I was fully convinced by each data set and the conclusions drawn by the authors. Their direct contribution to the budding mechanism is not demonstrated (E-cadherin has not been restored, mitosis prevented or stiffness reduced to prevent the budding) but I agree these parameters (adhesion, proliferation and stiffness) are likely to be involved in tissue disorganization and worth being described.

Response: While we agree that it is notoriously difficult to "demonstrate" any given mechanism, we emphasize that we did address the points listed here, and our results strongly support our conclusions. First, we show that the defects in E-Cadherin junctions can be prevented by restoring Rac1 or Arp2/3 signaling using NSC2366 or CK-666 (Figure 3E and G), and we also show that these very same compounds prevent budding induced by NLP overexpression (Figure 3H), strongly arguing that weakening of E-Cadherin junctions is required for budding. Second, we show that budding could be prevented by inhibiting mitotic entry through RO-3306 (Figure 2D), indicating that cell cycle progression to mitosis is required for budding. Third, we show treatment of cells with cytochalasin D not only blocks GFP-NLP induced budding (Figure 3H), but also lowers cellular stiffness (Figure 6, formerly Figure 5), supporting the view that increased stiffness is required for budding. Altogether, these observations concur to indicate that loss of E-Cadherin junctions and increased stiffness cooperate to trigger the budding of mitotic cells.

As discussed below, the data on stiffness measurements were the most surprising. However they should be careful checked by an external AFM expert as they contrasted with previous observations.

Response: In the interest of clarity and accessibility to the broad readership of EMBO Journal, as well as length limitations, we had originally omitted some of the technical detail underpinning our AFM measurements. We now recognize that this was unsatisfactory to readers with AFM expertise and thus include more ample documentation in the revised manuscript (see below). However, irrespective of differences in AFM equipment and/or methodologies used in different laboratories, we note that we could readily reproduce previously published AFM data, as illustrated by the stiffness measurement on isolated

mitotic and interphase cells (new Figure 6D, former Figure 5). Moreover, we emphasize that our key conclusions do not depend exclusively on cell stiffness measurements by AFM. Basically, we are saying that in epithelia harboring populations of cells with (NLP-induced) structural centrosome aberrations, mitotic cells are squeezed out through a non-cell-autonomous mechanism (because they come under pressure from their neighbors). In strong support of this view, we note that the 'budding' mitotic cells display exactly those features, prolonged mitosis and extensive membrane blebbing (Figure 5, formerly Figure 4), that have been described by others, using different AFM setups to study mitotic cells under confinement (Cattin et al., 2015, PNAS, op.cit). Moreover, the interphase cells expressing (NLP-induced) structural centrosome aberrations display markedly increased levels of de-tyrosinated tubulin (new Figure 4), which has previously been shown to correlate with enhanced stiffness (Kerr et al., 2015, Nature Communications, op.cit.). Thus, no fewer than three lines of evidence support the conclusion that mechanobiological properties contribute to the 'budding' of mitotic cells; only one of these involves our AFM setup and methodology, while the other two lines of evidence involve purely cell biological assays and/or AFM done by other laboratories.

Apart from this I found all results very convincing and interesting. Therefore I fully support the publication.

Response: Again, we thank this referee for her/his strong support for publication of our study.

The final scheme, in which the three parameters were integrated in a consistent scenario, is not fully clear to me. I guess some confusion also remains in authors mind and explains why the scheme is in supplementary data. I would suggest to move it to the main figures anyway. This would promote further discussion in the field, accounting that demonstrated and speculated elements are made clearly distinct.

Response: Following the suggestion of this referee we now show the final model as a main Figure (new Figure 7). In the accompanying text and legend, we make it clear that this schematic model involves elements of speculation and that it is meant to prompt additional investigation.

Here are few points which may deserve to be addressed to clarify the conclusions.

- It seems to me these observations are more related to budding or some sort than to « invasion »

Response: We agree with this point and have adapted some text passages accordingly.

About E-cadherin:
- In the case of mosaic acini, was E-cadherin mislocalized in NLP-expressing cells only or in all cells? It was not clear whether E-cadherin was mislocalized in extruded cells and/or in their neighbours. One could imagine that normal cells may have difficulties to assemble proper junctions with NLP-expressing cells in which E-cadherin is mislocalized.

Response: Because E-Cadherin forms homotypic interactions involving multiple cells, the prediction is that any NLP-induced alteration also concerns neighboring cells. This was indeed observed, as can readily be seen in Appendix Supp Fig S1 (see GFP-NLP$^-$ cell in the center of the image).

- Did the lack of adhesion contribute to the expulsion of cells? Did it affect the rounding process of mitotic cells? One could think that it promoted cortical contraction since the tension that was no longer applied to junctions was probably redistributed internally in the cortex.

Response: Concerning the potential impact of alterations in E-Cadherin junctions on cellular stiffness, we note that we carried out stiffness measurements on cells expressing tagged proteins in already confluent monolayers, which should minimize any potential influence. Under these conditions, overexpression of either GFP-NLP or GFP- PLK4 did not interfere with E-Cadherin distribution/localization (Figure 3C, upper panels). This being said, the referee touches on interesting but very broad questions. To address these issues in more detail would required a large amount of additional work. In consideration of the large body of data incorporated into the present manuscript, we believe that additional extensive investigation is beyond the scope of the present study.

- Did the lack of adhesion impair daughter cells re-incorporation into the epithelium (as suggested by the work of Bergstralh and St Johnston published in Nature Cell Biology in 2017) ?

Response: Although we occasionally saw budding cells re-enter acini that they had just escaped from, such re-incorporation events were rare (see Figure EV3 and corresponding movie EV3). It seems plausible, therefore, that re-insertion of a cell harboring NLP-induced centrosome aberrations could impact tissue architecture, as suggested previously (Schnerch and Nigg, 2016). However, in absence of additional data we would not feel comfortable elaborating on this point.

- Was the lack of E-cadherin responsible for spindle misorientation (as suggested by the work of Den Elzen published in Molecular Biology of the Cell in 2009)?

Response: This is exactly what we think (see also our reply to the next point). We apologize for the omission of a reference to Den Elzen et al., in the original version of this paper. Den Elzen et al., (2009) is now cited in the revised manuscript.

Was spindle misorientation truly involved in the budding? in its amplification?

Response: We believe that disorganization of E-Cadherin plays a central role in all budding events (through weakening of cell-cell contacts). As shown previously, E-Cadherin defects favor mitotic spindle rotation (den Elzen et al, 2009) and this is confirmed in our present study (see Figure 3F for example). If this rotation results in a spindle angle approaching 90°,

this will likely favor the dissemination of one daughter cell, with maintenance of the other cell within the sphere. Such a consequence of spindle orientation was seen in about 15% of the recorded cases of budding (illustrated Figure 2C). In the other 85% of cases, the entire mitotic cell escaped the sphere (illustrated Figure 2B). We therefore conclude that although the spindle mis-orientation primarily reflects the weakening of cell-cell contacts, it may also play a direct role in at least some cases of budding.

About mitotic cells contractility:
- Blebbing is known to result from excessive cortical contraction and/or lack of adhesion (see the work of Ewa Paluch). This is consistent with the high stiffness in NLP-expressing mitotic cells. But cells that are described in Figure 4 have been shown not over-express NLP (it is the big point of the paper!). So I am confused. Why do these « normal » cells display increased contraction? Is it due to their lack of adhesion with the epithelium?

Response: Indeed, this is a major point of the paper. All our data concur to support the conclusion that overexpression of NLP within the budding cell is not necessary for this cell to escape from the epithelium. The blebbing results from the fact that this cell is under confinement, because neighboring cells express NLP and hence display increased stiffness. In other words, the membrane blebs associated with the mitotic budding are the consequences of the confinement (see Cattin *et al.* 2015) imposed by the surrounding cells. WT cells or GFP-NLP^{+} cells will both experience this confinement and therefore display blebs.

- Cell stiffness is related to cell contraction (see the work of Solon and Janmey published in Biophysical Journal in 2007).

Response: Solon and Janmey explain that the actin cortex regulates cell contraction. Yet, whether and how this is linked to cell stiffness is less obvious. More recent evidence shows that intermediate filaments (Plodinec et al., 2011), and the microtubule network (Kubitschke et al., New J. Phys.2017; Pachenari et al., Journal of Biomechanics 2014) also play important roles in regulating cell stiffness.

As we write in the revised manuscript, our results support "the notion that NLP-induced centrosomal aberrations increase cellular stiffness by stabilizing microtubules, which in turn influences the actin cytoskeleton."

Nocodazole is known to trigger cell contraction (in spread and in round cells) and therefore cell blebbing. But generally speaking, cell contraction is promoted by the activity of Rho, not Rac. How can Rac inactivation rescue a mechanism based on hyper-stiffness and therefore hyper-contraction? I am confused.

Response: In Figures 3E and 3G, we present data showing that partial inhibition of Rac1 can restore E-Cadherin defects induced by NLP overexpression, and can prevent budding from GFP-NLP+ cysts. This leads us to conclude that GFP-NLP overexpression can interfere with E-Cadherin junctions in a Rac1-Arp2/3 dependent pathway. Our data therefore suggest that

mitotic budding involves two features: E-Cadherin loss and increased stiffness. Considered individually, these events are necessary but not sufficient to trigger budding of mitotic cells (new Expanded View 5).

- The effect of taxol on cell contraction is more controversial. Here, how can taxol trigger the increase of cell stiffness? Microtubule polymerization should trigger Rac activation, not Rho. Same in NLP-expressing cells: more polymerized microtubule should increase Rac activation, not Rho.

Response: As we explained above, changes in microtubule polymerization and architecture can lead to changes in cell stiffness. Indeed, we fully agree with this referee's point about the effect of microtubules on the overactivation of Rac1: it has clearly been shown that enhanced numbers of microtubules can trigger Rac1 over-activation (see Akhtar & Hotchin, 2001; Chu et al., 2004; Godinho et al., 2014; Waterman-Storer et al., 1999; Xue et al., 2013). Additionally, we have confirmed previous observations showing that Taxol increases cellular stiffness (Kerr, 2015). None of this data contradicts an involvement of RhoA in cellular stiffness. Deregulated microtubules impact on the actin network *via* the Rac1-Arp2/3 pathway (Waterman-Storer et al., 1999), in line with the notion that both Rac1 and RhoA can mediate effects through actin (Sepp & Auld, Development 2003).

- The reduction of cortical stiffness when cells enter mitosis in an epithelium is very surprising (and very novel contrary to what the citation of the work of Saw and Ladoux published in Nature in 2017 could suggest). It contrasts with previous works, which all reported RhoA activation during mitotic rounding. It is true that these works were mostly (all?) performed on single cells and that the authors did proper control to show that in their hands stiffness also increased during mitosis in isolated cells.
But I am afraid there is something wrong in this set of data. A better expert on AFM should look more carefully at the way AFM has been done (and at the method that was used to normalize measurements).

Response: We recognize that our results seem to differ from previously published data. However, we emphasize that we observed cell softening during mitosis in ***confluent epithelia***, and not in single cells undergoing mitosis. As recognized by the referee, our data on single cells are in full agreement with previously published work. To the best of our knowledge, the stiffness of cells in mitosis has not previously been addressed in the context of a confluent epithelium. (Note: The work of Saw and Ladoux Nature 2017 does not address mitotic cells).

About the final scheme:
- in the WT case the mitotic cells has a strange shape (is it true that it is not round?). It seems to deform adjacent cells. But authors argued mitotic cells were softer than adjacent cells. How could they deform them? What are the « deformable (soft) cells »?

- In an heterogeneous epithelium, I agree that rounding up (by cortical contraction and volume increase) in a stiff environment should promote cell extrusion. But if the mitotic cells are softer than their surrounding, which is even more the case with normal mitotic cells and NLP-expressing surrounding cells, the mitotic cells should deform rather than pop out. The

whole scenario would be more consistent if mitotic cells would actually increase their contraction to round up. Rounding would be feasible within a soft epithelium and impossible in a stiff one, which would thus trigger mitotic cells extrusion. This is the reason why I think it would be worth double-checking the data on mitotic cell stiffness.

Response: We have slightly redrawn our Figure, which hopefully will clarify our model. As we state in the text, we assume that cellular stiffness within a normal epithelium (regarding both mitotic cells and interphase cells) has been optimized during evolution such as to allow the accommodation of the products of cell division within the epithelial layer. Thus, for the purpose of our model, detailed aspects of the interactions between mitotic cells and neighboring cells within a normal epithelium are not crucial. Instead, what is crucial is that the reduced deformatility of cells harbouring NLP-induced centrosome aberrations (due to increased stiffness) interferes with the rounding of the dividing cells, so that these cell are frequently sequeezed out of the epithelium (regardless of whether or not they harbour centrosome aberrations themselves). Our experimental data suggest that mitotic cells in confluent epithelia are not actually truly round but tend to elongate. Please see Figure 6D (and associated legend) that includes an AFM 3D quasi topography with stiffness overlay, as well as the corresponding 2D quasi topography and stiffness map. In contrast, individual mitotic cells clearly round up (please see EV4 for details).

Referee #4

The stiffness measurements are difficult to interpret due to the fundamental problem that cells are not composed of a homogenous material. This means that cells do not have a homogeneous stiffness as used by the authors to compare the apparent stiffnesses measured for the cells.

Response: We fully agree with the referee that cells are not homogeneous material and thus do not exhibit homogeneous stiffness properties. This is precisely the reason why we employ the sharp AFM tip that is able to detect local stiffness heterogeneities. This allows us to clearly differentiate the cell boundaries (junctions), which are excluded from the analysis, and it also allows us to assess the stiffness of nuclear and perinuclear regions. The presentation of data in box plots reflects these local heterogeneities by the range of standard deviations (shown in each Figure). In the revised manuscript, we also note that these analyses excluded cell junctions based on the cellular morphology obtained by AFM. The stiffness values of transgene-expressing cells and WT cells (non-overexpressing GFP-NLP), were then normalized so as to interpret the impact of NLP overexpression.

Furthermore, it is not clear how cellular stiffnesses were derived from stiffness maps.

Response: We thank the reviewer for raising this point. Please see below (Appendix) a Figure "Response_Fig1_Schematic" that provides the workflow of data analysis: (1) how the stiffness is extracted from the raw measurement (force curves), (2) how individual cells are selected an their stiffness measured and (3) how the average stiffness of cells is aggregated into a box plot to compare the stiffness of cells under varying conditions.

We do not think that this Figure should be included in the manuscript, but would be happy to include it in Supplemental Material if the Editor deems it appropriate.

It is obvious that stiffness maps of cells having different morphologies (eg interphase vs mitotic cells) must be different because the pyramidal AFM tip interacts differently with different cell shapes. As one consequence among several different models must be applied to estimate stiffnesses from the measurements. Obviously this has not been done.

Response: Please see response above (about the heterogeneity of the cells) and Appendix below ("Fig. 2_response"). Furthermore, we find that the Oliver-Pharr model is appropriate when 20 nm pyramidal probes are used to indent cells. We prefer to avoid the below models which are inapplicable to the current study because:

i. the Hertz model considers spherical-spherical contacts.
ii. the Sneddon model only considers the probe radius and not the contact area
iii. the Johnson-Kendall-Roberts (JKR) and DMT (Derjaguin, Muller, and Toporov) model adhesive contacts.

Moreover, in case of mitotic cells, only force curves on the elevated parts of the mitotic cells were used to generate E-modulus values per cell. To avoid the issues raised by the reviewer, force curves recorded on the steep transitions between mitotic (high) and neighboring interphase cells (shallow) were not considered. To better illustrate these points we amended the manuscript text and provide additional response in the Appendix (Figures 1 and 2).

Currently the quality of the presented AFM data does not warrant the strong conclusions made.

Response: We respectfully disagree with this statement based on the reasons explained in this Rebuttal.

The authors did not describe sufficiently how they determined cellular stiffness maps and cell stiffness.

Response: Please see above (reply to "it is not clear how cellular stiffnesses were derived from stiffness maps") and Plodinec et al. 2011 (op. cit). Additionally, see revised Materials & Methods section.

The authors refer to some papers previously published. However, these papers applied AFM to estimate the stiffness of different biological systems and rather superficially describe how the data was analyzed.

For example to understand the submitted paper essential information is missing on the depth of the AFM tip indenting into the cell surface, which force curves from the force volume data have been selected for analysis, which criteria has been applied to select force

curves for analysis, how the force curves were analyzed, it is not described which model has been applied to derive the stiffness values from experiments, it has not been described whether the experimental conditions allow applying the model, it is not clear why a maximal force and not a maximal indentation was chosen to record stiffness maps, and it is also not clear how stiffness ratios were determined.

- information is missing on the depth of the AFM tip indenting into the cell surface,

Response: Please see above (reply to "it is not clear how cellular stiffnesses were derived from stiffness maps"). Also, we have included a more detailed description of the stiffness analysis in the Materials and Methods of the revised manuscript, and we have cited Emad A-Hassan et al., 1998 as a reference to explain why a constant force should be used for measuring the elastic properties of cells. Information regarding the range of indentations (1 to 3 μm) is now also included in Materials and Methods. See also Appendix (Figure 1)

- which criteria has been applied to select force curves for analysis, how the force curves were analysed

Response: A set of commonly used criteria (signal-to-noise ratio, forward and backward curve tilt, hydrodynamic drag between forward and backward curves) was examined to select the maps suitable for analysis. This has been described in detail previously (Section 3.4 "Force curve processing" in Plodinec and Lim, 2015) and this reference is now included in the revised manuscript.

- it is not clear why a maximal force and not a maximal indentation was chosen to record stiffness maps, and it is also not clear how stiffness ratios were determined.

Response: Pioneering AFM studies clearly demonstrate the necessity to use constant force (see A-Hassan Biophys. J. 1998, and Wu Scanning et al., 1998) rather than indentation depth in order to derive comparable E-moduli of living cells. Briefly, indentation depth assessments are imprecise and induce significant errors in the analysis of soft viscoelastic samples because of errors in the determination of the contact area.

In the revised manuscript, we write: "Specifically, for individual maps in Fig 6A; the stiffness values of NLP over-expressing cells were first normalized against neighbouring WT cells that did not overexpress centrosomal protein within one force map. For all other graphs, the stiffness values were directly normalized to the values obtained for WT cells in interphase."

Certainly, a resistance to deformation is measured by sticking a sharp pyramidal AFM tip presumably 1-5 μm into a cell. Such mechanical measurements could be of some comparative value. However, to interpret which of the multitude of cellular structures contributes how much to resisting the deformation is not possible from the measurements presented by the authors.

Response: We fully agree with the referee on this point. However, we would like to emphasize the importance of normalization of stiffness values. It is precisely this

normalization, i.e. the comparison of NLP overexpressing cells (or mitotic cells) with their surrounding neighbouring cells (controls) within the same sample, that enables us to interpret the impact of NLP overexpression on cellular stiffness (in spite of the multitude of cellular structures that likely contribute to deformation). We have amended the manuscript text to better explain this point.

The normalization of the stiffness measured for each experiment is suspect. Particularly for the mitotic vs. interphase MDCK cell measurements. For these, I suggest that the authors perform trans-mitosis experiments, in with they follow a cell's stiffness before, during and after mitosis. Furthermore stiffness maps of mitotic cells have not been shown.

Response: For the reasons described above, we disagree with the referee and certainly do not consider our stiffness measurements 'suspect'. Also, we do not consider it necessary to carry out trans-mitosis experiments, because we have correlative optical data (mCardinal-histone H1) that clearly show which cells are in mitosis or interphase.

Histone morphology (chromosome condensation) can be used to detect cells before nuclear envelope brake down.

Response: Indeed, we have routinely identified mitotic cells by using an mCardinal-tagged histone marker. For details please see Figure 6D.

Furthermore stiffness maps of mitotic cells have not been shown.

Response: We have now included stiffness maps of mitotic cells in (i) Fig 6D (confluency) and (ii) EV4C (single cells). The associated Figure legends have also been modified accordingly.

The authors write that to record stiffness maps of cells required about 20-45 min. However, as the authors also report that the duration of mitoses is about 40-50 min. One thus wonders what the stiffness map represents and from which to which mitotic state it was recorded.

Response: As explained above, we have optical controls for cells undergoing mitosis. Indeed, because the time is of essence (as correctly pointed out by the referee), all experiments on mitotic cells were completed within 5 minutes by lowering the stiffness map to 20 x 20 pixels (force measurements). The Materials and Methods section has been revised accordingly.

Figure 5 A and B, force/volume map stiffness scales have completely wrong numbering.

Response: This is not the case – the numberings are correct. Figure 6A (formerly 5A) depicts cells in 2D and Figure 6B (formerly 5B) shows 3D cysts. The absolute stiffness values are in a different range for 2D versus 3D, as illustrated by different stiffness scales. Nevertheless, we have now changed all units in both Fig. 6A and B (to kPa).

Figure 5B. It is not clear ho left and middle image correlate. It is not clear what middle image shows, what are the cells? Not clear how right image correlates to left and middle images.

Response: former Figure 5B (now Figure 6B) has been modified to provide clear connections between the subpanels.

Figure 5D is key for the paper. However, no stiffness maps are shown. These maps together with height images/topographs should be shown for interphase and mitotic cells. The same counts for expanded Figure 9C.

Response: We have now included stiffness maps in Figures 6D and EV4C (new data). The Figure legends have been edited accordingly.

The fluorescence image shown in Figure 9C does not show a nice mitotic spindle.

Response: This is correct, because EV4C (formerly Expanded View 9C) does not show mitotic spindle but condensed chromatin (mCardinal histone H1), as clearly indicated in the Figure.

Expanded Figure 9 A and B. Please also show stiffness maps to connect stiffness histograms with cellular morphology/localization.

Response: Figs. 9A and B (now EV4) have been amended with stiffness maps and histograms.

The descriptions in the figure legends are insufficient and vague. In general, a description of what is displayed is preferable over an interpretation of the data.

Response: We have revised AFM Figure legends accordingly.

Stiffness and height maps of the MDCK experiments should be provided. Given the impressive imaging system, the images are not impressive.

Response: Stiffness and height maps are now provided (please see above).

The ‚close to physiological conditions' under which AFM experiments were done have not been specified.

Response: We have amended the Methods section text to explain this. “All AFM experiments were carried out at temperatures close to 37°C. During the measurements, culture dishes were replenished with fresh cell medium saturated with 5% CO2 to maintain pH at 7.5 and compensate for evaporation.”

I do not understand why the stiffness maps show bright as softer and dark as stiffer and not the other way. This is counterintuitive. In addition it would be helpful to provide the

height/topographic maps of the cells, as the topography is an important parameter to morphologically interpret stiffness maps.

Response: We have revised the Figures to show images with soft areas in darker tones and stiffer regions in brighter colour. We have also included topographic images.

It is difficult to understand why the authors used very sharp tips having diameters ranging from 10-20 nm to characterize cell stiffness. Stiffness measurements using a sharp tip suffer from the heterogeneous structural and mechanical properties of the cell. Such cellular heterogeneity is also observed by the authors. It would make more sense to take a blunt tip.

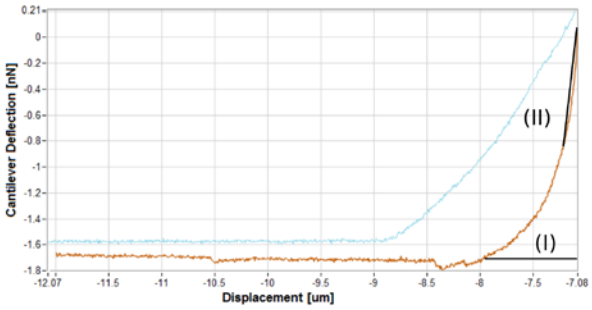
Response: The rationale behind using sharp tips has been described above. Further, please see Appendix below ("Response_Fig2_BluntTipSimulation") where we demonstrate the differences in sensitivity and specificity that arise when using blunt vs. sharp AFM probes to differentiate specific cellular regions (i.e. junctions, perinuclear, nuclear regions). Although this aspect is less fundamental when studying isolated cells, using sharp tips appears mandatory to correctly/accurately delineate the region of interest used to analyse the cellular stiffness of cells within a confluent monolayer (as illustrated in Figure 2_response).

We do not think that this Figure should be included in the manuscript, but would be happy to include it in Supplemental Material if the Editor deems it appropriate.

Appendix

“Response_Fig1_Schematic”

1. **Pre-processed Force Curve**



Pre-processing and Contact Mechanics

a) Tilt Force Curve (Lin et al., 2007)

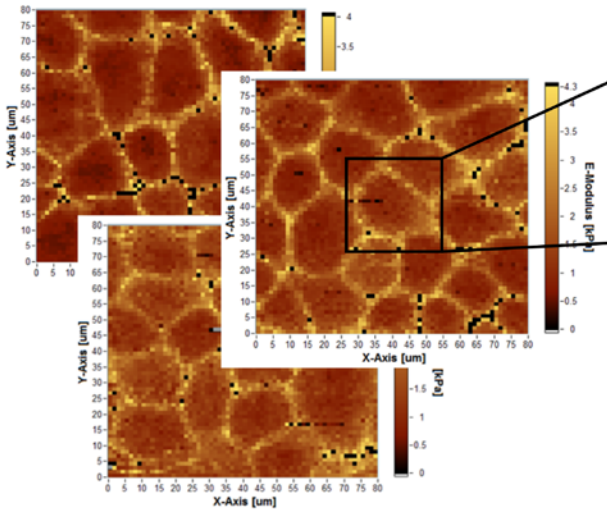
b) Convert piezo-displacement to tip-sample distance

c) Find Contact Point / Calculate Indentation (I, Plodinec et al., 2010)

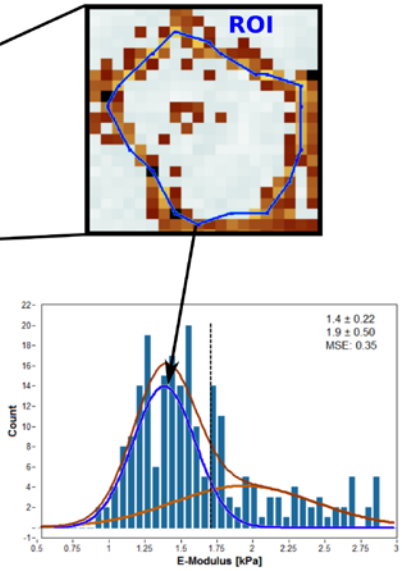
d) Calculate Slope on the top 50 % of the force curve (II, Plodinec et al., 2010)

e) Calculate E-modulus using the Oliver-Pharr model (Oliver & Pharr, 1992)

2. **Multiple Stiffness Map per condition**



Create a ROI per Cell / Extract Mean Value



3. **Compare Cells per Conditions in boxplots (plot taken from Extended Figure 9)**

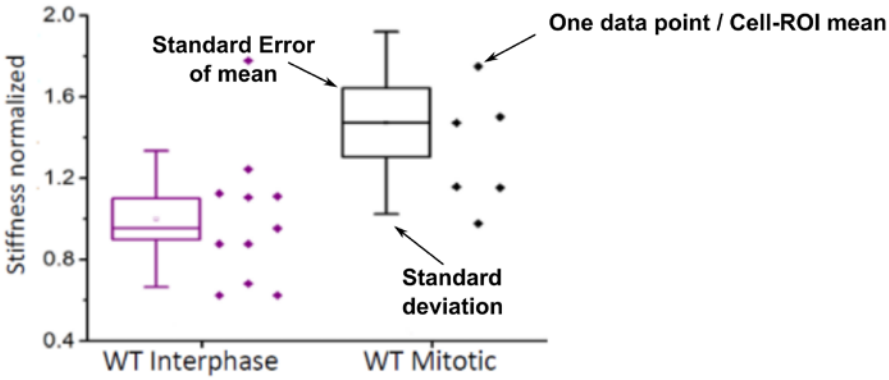


Figure 1_response. Schematics of AFM data analysis of living cells.

1) (left) Typical force curve recorded in the center of an MDCK cell at a maximum load of 1.8 nN. The y-axis of the plotted curve shows the force applied while the x-axis shows the tip-sample distance. (I) denotes the indentation into the sample on the unloading curve (distance between contact point and maximum indentation), and (II)

   denotes the fit of the slope to the upper 50 % of the unloading curve. (right) Sequence of force curve processing from the raw measured AFM force curves up to calculating E-modulus.

2) (left) Multiple stiffness maps are recorded per experimental condition. (right) For each map, a region of interest (ROI) is marked around individual cells and a two peak Gaussian fit is fitted onto the dataset, automatically excluding the junction area i.e. only areas without steep transitions are chosen (for example in case of mitotic cells). The mean value of the first (blue) peak reflects the mean stiffness of the nuclear and perinuclear area for the selected cell. Likewise, for mitotic cells, only areas without steep transitions are chosen.

3) Finally, experimental conditions are compared using boxplots where each shown data point reflects the mean value extracted from a single cell as explained in the step 2. In the boxplots; the whiskers show the standard deviation, the box the standard error of the mean, the bar is the median and the square reflects the mean value for a given condition.

“Response_Fig2_BluntTipSimulation”

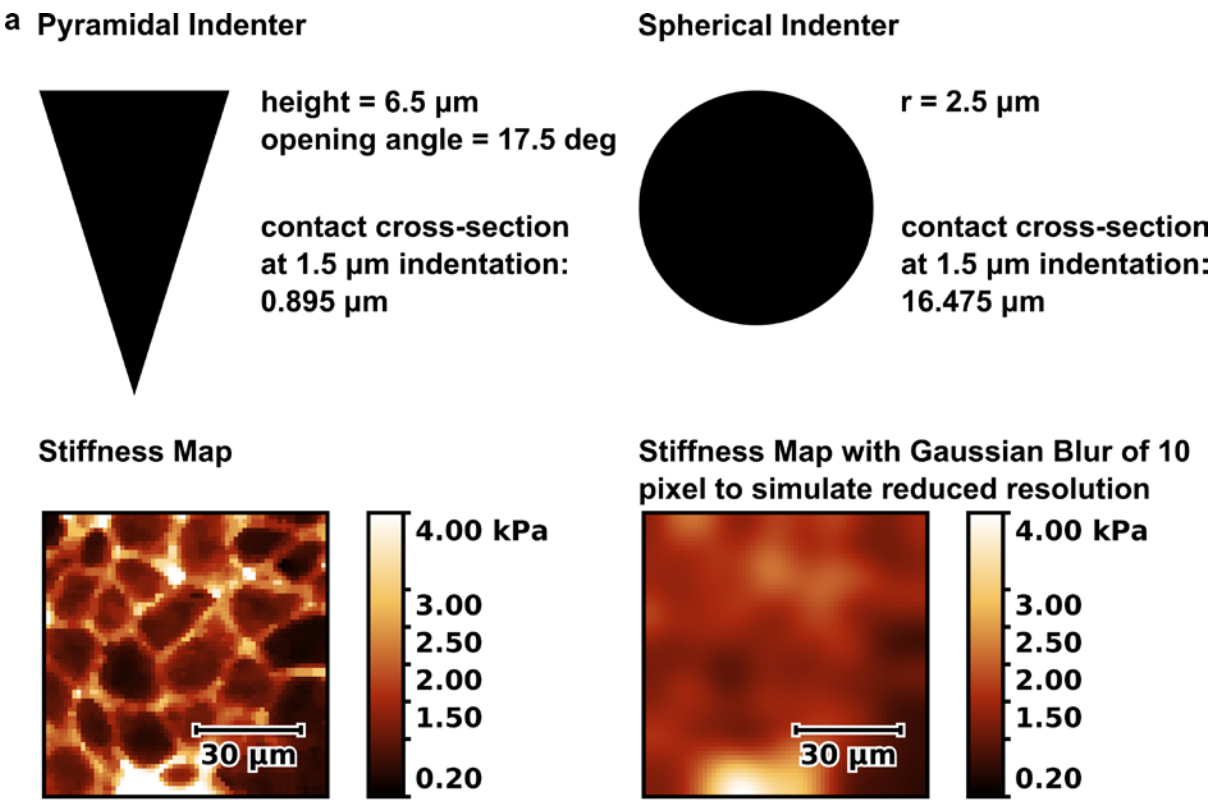


Figure 2_response. Simulation illustrates the resolution of a blunt tip when compared to a sharp tip.

(top) (left) Schematic representation of standard indenters DNP-S10 tip. The contact cross-section corresponding to an indentation of approximately 1.5 µm often depicted in our measurements is 0.895 µm. (right) Drawing depicts a typical 5 µm diameter sphere. The schematic reveals that the contact cross-section of a spherical indenter is at least 10 times larger than for a sharp indenter, i.e. 16.475 µm.

(bottom) Stiffness map recorded with a DNP-S10 sharp tip (left) is blurred with a Gaussian filter (right) exhibiting radius of 10 pixels (corresponding to having at least 10 times larger cross-section). The image clearly demonstrates loss of resolution with a spherical indenter and its inability to differentiate the boundaries of individual cells i.e. junction stiffness versus stiffness of the nuclear and peripheral regions.

2nd Editorial Decision 22 February 2018

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, I would ask you to still take care of several specific editorial issues stemming from our pre-acceptance text and data checks. They are noted in the attached edited/commented text document with activated "Track Changes" option, and I would appreciated if you incorporated the requested final text modifications and answered the Figure legend queries directly in this version, returning the edited main text document via email with changes/additions still highlighted via the "Track changes" option.

With regard to the two referee response figures on AFM techniques, I agree that they do not necessarily need to go into the supplement, given that they would anyway remain accessible to interested readers as part of our Transparent Peer Review Process file.

After clarification/correction of these remaining points, we should be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS

Referee #1 (Report for Author)

The authors have addressed my concerns. I consider this manuscript ready for publication and believe it will be of great interest to the centrosome field.

Referee #3 (Report for Author)

In line with my first comment, I think this manuscript deserve to be published in a high standard journal such as EMBO Journal. I asked for some clarifications and I am satisfied by authors answers.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: E.A. Nigg
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-98576

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to predetermine sample size; the number of samples was determined by the number of events that could be monitored during at least 3 independent experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The investigators were not blinded. For immunofluorescence experiments, fields were imaged randomly within the samples.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were not blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, otherwise stated in the text (Mann-Whitney tests were used)

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Is there an estimate of variation within each group of data?	yes, standard deviation is given for all group of data and when applicable, sem is provided and values ploted if n<5.
Is the variance similar between the groups that are being statistically compared?	yes for t-tests.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	see Material and Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	MCF10A ecoR cells (a gift from Tilman Brummer; University of Freiburg). The MCF10A and MDCK cell lines were tested by their ability to form correct spheroids and cysts upon 3D culture and were routinely tested for the absence of mycoplasma (see Material and Methods section). MDCK II cells (a gift from Inke Naethke, University of Dundee, UK) Phoenix cells (a gift from Stefan Zimmermann , University Medical Center Freiburg) HEK293T cells (provided by Ralph Wäsch; University Medical Center Freiburg)

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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No