nature research

Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: ECM mechanotransduction licences an antioxidant response by regulating mitochondrial morphology and NRF2 activity
Corresponding author name(s): Sirio Dupont

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Professor Dupont,

Your manuscript, "ECM mechanotransduction licences an antioxidant response by regulating mitochondrial morphology and NRF2 activity", has now been seen by 3 referees, who are experts in redox signalling (referee 1); mitochondria morphology (referee 2); and cell mechanics (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A) Assess tissue tension in primary and secondary sites and compare ROS/NRF2/DRP1 levels in situ in each setting (reviewer #1)

B) Distinguish between oxidative stress causing toxicity and signaling associated with redox biology (reviewer #1)

C) Investigate the role of ER-induced ROS (reviewer #2 and #3)

D) Provide mechanistic insight into softness-induced mitochondrial fission

E) Provide in vivo relevance for the proposed mechanism (reviewer #3)

I should stress that the referees' concerns point to a premature dataset and these concerns would need to be addressed with experiments and data, and reconsideration of the study for this journal and re-engagement of referees would depend on strength of these revisions

F) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes, should also be addressed.

G) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and https://www.nature.com/nature/for-authors).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here https://www.nature.com/documents/nr-reporting-summary.pdf). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.

-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Nature Cell Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

[REDACTED]

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Ana Mateus

Ana Mateus
Editor
Nature Cell Biology

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
ECM mechano-transduction licenses an antioxidant response by regulating mitochondrial morphology and NRF2 activity

Tensional homeostasis is known to be disrupted in malignancies where tissue stiffness is increased due to increased ECM deposition. As the mechanisms contributing to the transmission of mechanical forces to biochemical signaling pathways remain relatively poorly understood, the observations from the current study could be of great interest to the field. Specifically, the authors delineated the connection between ECM stiffness and cancer cell redox response through F-actin induced mitochondrial recruitment of DRP1 and subsequent mitochondrial fission. The authors suggest that sustained elevation of NRF2 dependent antioxidant response as a consequence of ROS induction when cells grow in a soft matrix renders cancer cells more resistant to chemotherapeutics. This conclusion is conceptually confusing for the following reasons and should be clarified experimentally:

Major concerns:
1. Most cancer cells reside in a TME that is more stiff than normal tissue due to enhanced deposition of ECM. This is a hallmark feature of solid tumors such as breast cancer, and is true in both primary and metastatic sites. In fact, for breast cancer, increased fibronectin expression in secondary sites has been reported to prime the development of a pre-metastatic niche (reviewed in detail here: PMID 18814043). Thus, the authors' conclusion that metastatic breast cancer cells reside in a soft TME and as a result are more resistant to chemotherapy is not supported by our current knowledge. To support this conclusion, the authors must perform measurements of tissue tension in primary and secondary sites and compare ROS/NRF2/DRP1 levels in situ in each setting.
2. How does the tension of "soft matrix" compared to normal tissue? As described in the second last paragraph of "Results" and in Figure 6A-B, physiological lung ECM stiffness is equivalent to soft ECM conditions used throughout the study. If this is true, and increased 8OHG, 4HE, and glutathione oxidation is induced in cells grown under soft ECM conditions, are the authors suggesting that untransformed cells residing in normal, soft tissue would exhibit chronic lipid oxidation and activation of the NRF2 antioxidant pathway? It is important to note that NRF2 is known to be expressed at very low levels in normal cells due to KEAP1 dependent turnover and is induced only when ROS oxidizes KEAP1.
3. The authors report that matrix detachment and growth in soft matrix both result in elevated ROS followed by a chronic, sustained induction of NRF2. While the authors attribute ROS induction in cells residing in soft matrix to be due to DRP1-dependent mitochondrial changes, they did not consider the possibility that growth in soft matrix reduces glucose uptake and therefore NAPDH production through PPP, a mechanism previously reported to regulate redox metabolism of breast cancer cells upon matrix detachment (PMID 19693011). It is important to examine the glucose-PPP axis before concluding distinct redox mechanisms downstream of soft matrix growth vs matrix detachment.
4. The authors argue that the ROS induced in cells grown in soft matrix is "hometic" and therefore not toxic, supported by the lack of nuclear DNA damage (Fig 1M). Intriguingly, the authors also report a very robust induction of lipid oxidation when cells are grown in soft matrix (Supp Fig 1N). It is known that indiscriminate oxidation of lipids and nuclear DNA both occur through hydroxyl radicals generated from H2O2 through the fenton reaction. It is surprising that there is selective oxidation of lipids but not DNA. The authors should examine different parameters to assess DNA damage more carefully. It

is also important to note, that the changes in H2O2 required for signaling (hometic) do not cause significant changes in intracellular ratio of oxidized glutathione (GSSG)/reduced glutathione (GSH) or NADPH/NADP+ (PMID 21964034). In fact, large changes in these parameters (as shown in Fig1) are usually a sign of oxidative stress causing toxicity rather than signaling associated with redox biology (PMID 21954972).

Other concerns
1. Does growth in soft matrix reduce overall mitochondrial numbers? Figure 4B seems to suggest so.
2. Does DRP1 overall expression change?
3. Does growth in soft ECM alter PI3K signaling does that contribute to DRP1 and/or ROS/NRF2 regulation (PMID1993152).
4. Timepoints of some experiments not indicated in either the methods or the figure legend, eg C11 Bodipy
5. Fig 6I-J: shNRF2 cells are more sensitive to cisplatin is an established phenomenon in cell 2D cell culture—this is independent of matrix tension and should be described carefully to avoid confusion.

Reviewer #2:
Remarks to the Author:
This manuscript from Romani etal. investigates the relationship between matrix stiffness and the antioxidant response in breast cancer cells. The authors demonstrate that stiffness induces mitochondrial remodeling which in turn promotes the generation of mitochondrial ROS, activation of NRF2 and subsequent upregulation of glutathione synthesis. This is an intriguing and novel paper and the manuscript is, for the most part, very thorough and convincing. However, there are a few aspects of their model which could use additional support. In particular, there is not much data supporting their model of actin's involvement in Drp1 recruitment or in whether mitochondrial fission itself is inducing ROS. Specific comments are listed below:

Figure 3 – The authors see significant effects on ROS using TUDCA, yet don't really address whether ER stress is playing a role. I'm curious whether the ER may be contributing to the changes in mitochondrial morphology through ER-mediated mitochondrial division under the different matrix conditions or whether this is a red herring. The authors should comment on the potential role of ER, if any, given this result.

Figure 3 – The authors dismiss defects at the level of oxidative phosphorylation as the source of ROS, yet this conclusion seems premature at this point in the manuscript. While the changes in basal respiration they measure are modest, the decrease in maximal respiration is very large. This is actually consistent with the increased fragmentation they observe in the next figure and consistent with a model in which fragmentation leads to ROS generation through decreased Ox-Phos efficiency.

Figure 4C – The number of Drp1 puncta is consistent with increased Drp1 recruitment, but should really be backed up by biochemical fractionation and immunoblotting of Drp1. It would also be informative to determine whether there are changes in the levels of Drp1 or of its phosphorylation on S616 and S637.

Figures 4-5 – Knockdowns (Drp1, Nrf2) should be confirmed by immunoblot.

Figure 4 – The authors should comment on the specificity of Mdivi-1. It has well publicized off-target effects, including on Complex I, making it difficult to interpret results from these experiments.

Figure 5 – The link between actin and Drp1 recruitment is very speculative and not well supported by the data provided. The fact that actin is recruited to mitochondria under conditions under which fission is being induced is expected, as actin filaments are known to be present at sites of fission. While this is formally consistent with their model that these actin contacts are "causing enhanced Drp1 recruitment", they do not provide any data supporting this direct causality. Further, the authors note this similar pattern of actin puncta and Drp1 puncta, but offer no evidence that these two overlap.

Figure 6 – The authors should note that Drp1 has been shown to promote tumorigenic growth in a number of systems and that it is possible that the effects of Drp1 knockdown on tumor burden they observe can also result from functions of mitochondrial shape independent of the glutathione synthesis mechanism they propose.

Reviewer #3:
Remarks to the Author:
Romani and coworkers report that human Ha-Ras-transduced MCF710A as well as mouse D2.0R breast cancer cells treated with ROCK and MLCK inhibitors or seeded on soft ECM (Matrigel or fibronectin) induce F-actin accumulation in the proximity of mitochondria, an elevation of Drp1 activity and mitochondria fission followed by mtROS and ER-ROS production. The high mtROS levels stabilize Nrf2, which leads to nuclear translocation of Nrf2, transcription of an antioxidant gene program including the gene encoding SLC7A11 that in turn provokes the uptake of extracellular cysteine and the production and oxidation of glutathione. Finally, the authors demonstrate that loss of Nrf2- or Drp1-expression in D2.0R breast cancer cells curbs metastatic tumor growth in lungs when injected into the tail vein of Cisplatin-treated syngeneic mice.
The paper is difficult to read since the findings are not logically stringed together and the results are often superficially described. The major problem, however, is the limited novelty and conceptual advance of the paper. It is well known in the field of mechanobiology that human adipose tissue-derived mesenchymal stem cells (MSCs) seeded on a soft substrate induce intracellular ROS levels leading to nuclear Nrf2 translocation and the transcription of an anti-oxidant program (as well as the nuclear translocation of HIF1alpha and the production of a pro-angiogenic secretome; DOI: 10.1002/adhm.201900929). Furthermore, it has also been reported that polymerized actin is required to induce Drp1-mediated mitochondrial fission, that mitochondrial fission increases ROS and that increased levels of ROS stabilize Nrf and upregulate expression of 'antioxidant genes' including the glutamate/cysteine antiporter SLC7A11 that imports extracellular cysteine. Whereas publications reporting the latter findings are, at least partially, mentioned in the manuscript, the key report showing that substrate softness induces ROS, Nrf2 and an antioxidant program is for unexplainable reasons neither mentioned nor discussed.

Additional comments:
(1) Why has Nrf1 not been analyzed? A combinatorial loss of Nrf1 and Nrf2 could have an even more profound effect on metastatic tumor growth?
(2) The F-actin staining suggest that the tagged G-actin leads to actin clumping/aggregation rather than polymerisation into filaments or networks. This issue needs experimental clarification.
(3) Why has the ER-induced ROS not been further analyzed? Does ER-ROS also contribute to Drp1-

mediated mitochondrial fission?
(4) Mechanism underlying softness-induced mitochondrial fission is not investigated. The mechanism is not known and hence, examining this mechnaism would give big novelty to the paper.
(5) Why is lung metastasis of D2.0R breast cancer cells curbed upon Nrf2- or Drp1-deletion in Cisplatin-treated mice? The literature would point to either cell survival (cell autonomous effect) or tumor angiogenesis (cell non-autonomous effect)? Or both?
(6) An in vivo metastasis model into 'soft' (lung) versus 'stiff' (bone) tissues would have highlighted the cancer cell-mechanical influence on survival, proliferation, etc.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

MANUSCRIPT FORMAT – please follow the guidelines listed in our Guide to Authors regarding manuscript formats at Nature Cell Biology.

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary.

Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see https://www.nature.com/licenceforms/nrg/competing-interests.pdf.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled “Statistics and Reproducibility”.

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement as a separate section after Methods but before references, under the heading "Data Availability”. . For Springer Nature policies on data availability see http://www.nature.com/authors/policies/availability.html; for more information on this particular policy see http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf. The Data availability statement should include:

• Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here http://www.nature.com/authors/policies/availability.html#data.

• Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details http://www.nature.com/sdata/data-policies/repositories).

• At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

• If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs $600 for the first, and $300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and

green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.

- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the

manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the "unprocessed scans" Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – We are trying to improve the quality of methods and statistics reporting in our papers. To that end, we are now asking authors to complete a reporting summary that collects information on experimental design and reagents. The Reporting Summary can be found here https://www.nature.com/documents/nr-reporting-summary.pdf)If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test

used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

--------- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements ---------

Author Rebuttal to Initial comments

Reviewer #1 - expert in redox signalling

Remarks to the Author:
ECM mechano-transduction licenses an antioxidant response by regulating mitochondrial morphology and NRF2 activity

Tensional homeostasis is known to be disrupted in malignancies where tissue stiffness is increased due to increased ECM deposition. As the mechanisms contributing to the transmission of mechanical forces to biochemical signaling pathways remain relatively poorly understood, the observations from the current study could be of great interest to the field.
Specifically, the authors delineated the connection between ECM stiffness and cancer cell redox response through F-actin induced mitochondrial recruitment of DRP1 and subsequent mitochondrial fission. The authors suggest that sustained elevation of NRF2 dependent antioxidant response as a consequence of ROS induction when cells grow in a soft matrix renders cancer cells more resistant to chemotherapeutics.

Our response to the Reviewer:
We thank the reviewer for appreciating the potential relevance of our findings. As detailed below in our point-to-point revision, we worked to reinforce our observations and to clarify multiple points of the study along the lines indicated by the Reviewer.

This conclusion is conceptually confusing for the following reasons and should be clarified experimentally:

Major concerns:
1. Most cancer cells reside in a TME that is more stiff than normal tissue due to enhanced deposition of ECM. This is a hallmark feature of solid tumors such as breast cancer, and is true in both primary and metastatic sites. In fact, for breast cancer, increased fibronectin expression in secondary sites has been reported to prime the development of a pre-metastatic niche (reviewed in detail here: PMID 18814043). Thus, the authors' conclusion that metastatic breast cancer cells reside in a soft TME and as a result are more resistant to chemotherapy is not supported by our current knowledge. To support this conclusion, the authors must perform measurements of tissue tension in primary and secondary sites and compare ROS/NRF2/DRP1 levels in situ in each setting.

We think there are two questions here:

1A) whether the metastatic tumor-microenvironment of the lung is soft

For this point we joined forces with colleagues of the Department of Engineering to measure tissue stiffness by Atomic Force Microscopy. We first compared the stiffness of the pristine mouse mammary gland with that of a primary tumour induced by orthotopic injection of D2.0R cells into the mammary fat pad. As shown in Fig. 7d we found that a primary breast cancer displays increased stiffness.This data provides evidence that our cell model behaves as expected from the literature, forming primary breast cancer tumors with a stiffer micro-environment (TME) compared to the normal tissue (e.g. Katara Mol Onc 2017; Peuhu EMBO J 2017; Lopez Integr Biol 2011; Plodinec Nat Nanotech 2012; Acerbi Integr Biol 2015).

As for the stiffness of the metastatic TME, this remains a poorly investigated topic (Kai Dev Cell 2019; Gensbittel Dev Cell 2020). We measured the physiological stiffness of the lung, which is in the soft range, and comparable to that of the normal mammary gland (Fig. 7d and ED Fig 7b), in agreement with several reports (e.g. Liu Am J Physiol Lung Cell Mol Physiol 2010, Polio Plos ONE 2018, Chauduri Nature 2020, Guimaraes Nat Materials 2020). Moreover, tissue stiffness was not changed in the presence of early disseminated cells, while it was much stiffer in late metastatic outgrowths (Fig. 7h). This indicates that (i) metastatic spreading to the lungs occurs towards a softer tissue, and that (ii) the lung metastatic

niche remains a soft one during the time-window which is relevant for chemotherapy treatment in our experiments.

The concept of a soft metastatic niche also applies to the bone marrow, as recently discussed by Dr. Egeblad, an expert in breast cancer metastasis, who published on how neutrophils regulate bone marrow metastatic niche stiffness to awaken disseminated cells (Albrengues Science 2018): "Furthermore, D2.0R and MCF-7 cells are dormant in soft environments in vitro-such as on matrigel, laminin-111 gel, and 0.2-kPa hydrogel (which mimics the stiffness of the lungs)-but these cells proliferate on stiff 2D cell culture dishes. In vivo, dormant, disseminated tumor cells are preferentially located within the soft niches of stiff tissues such as bones-e.g., in the bone marrow."

The Reviewer also mentioned the possibility of mechanical metastatic TME preconditioning, i.e. that cancer cells from the primary tumour can precondition the metastatic TME to facilitate dissemination. So far, two main mechanisms of preconditioning have been observed: recruitment of immune cells (and, thus, changes of the cytokine microenvironment), and increased deposition of some ECM proteins (and, thus, changes in the type of integrin engagement). There is only one paper that provided specific support to the idea of "mechanical" preconditioning, showing that LOXL2 secreted from the primary tumour increased collagen cross-linking and stiffness at the pre-metastatic niche (Erler Nature 2006). However, this article has been recently retracted (https://www.nature.com/articles/s41586-020-2112-4), so that the review indicated above in support of this idea, Erler and Weaver 2009, is no longer valid.

Please also note in support of this that Weaver herself, in a more updated review (Kai Dev Cell 2019), clearly states how preconditioning of the mechanical properties of the metastatic TME remains so far unexplored: "The role of altered ECM mechanics in the formation of the pre- metastatic niche is unclear. For instance, bleomycin-induced lung injury that is characterized by fibrosis favors the metastasis of intravenously injected tumor cells (Orr et al., 1986). Moreover, experimental lung metastases contain more fibrillar linearized type I collagen (Mayorca-Guiliani et al., 2017). Whether these fibrotic changes stiffen the lung parenchyma and if this stiffening occurs prior to and fosters metastatic colonization remains an open question."

Finally, the Reviewer refers to a potential role of fibronectin in the metastatic TME. Disseminated cancer cells can secrete fibronectin when they home at the metastatic niche, which is required to reinforce attachment and survival signaling cues during the early phase of quiescence (Mohammadi &

Sahai NCB 2018, Montagner NCB 2020). However, fibronectin secretion is different from ECM stiffness. Adhesion to a soft fibronectin ECM provides survival cues, while adhesion to a stiff fibronectin ECM induces cell proliferation. Indeed disseminated cancer cells still display inhibition of the YAP/TAZ mechanosensitive factors during the early phase of quiescence (Montagner NCB 2020 and our new data in Fig. 7i), indicating that the sole presence of this adhesive protein cannot account for mechano-sensitive phenotypes

In sum, we think our idea of the lung metastatic niche as a soft TME is well rooted in the literature and confirmed by new data.

1B) whether the pathway that we describe in vitro is also regulated by stiffness in vivo

In this case, we built on previous work that indicated how D2.0R cells plated on a soft ECM are initially sensitive to softness, which induces their proliferative quiescence, but over longer periods of time they are able to secrete Collagen-I and stiffen the ECM, enabling cell proliferation (Barkan Cancer Res 2008, Barkan Cancer Res 2010, Barkan Clin Cancer Res 2011). This likely explains why D2.0R cells initially lay quiescent in the lung metastatic niche (where the ECM is soft), but eventually resume growth (when the lung TME becomes stiffer, thanks to cancer cell-mediated ECM remodeling). This also explains why D2.0R cells seeded into a lung made stiffer by fibrosis do not enter quiescence but rapidly originate a metastatic outgrowth (Vera-Ramirez Nat Commun 2018).

Prompted by the Reviewer comment, we carried out the following experiments:

(i) we compared lung tissue stiffness in wild-type mice, in mice with early D2.0R cancer cell dissemination, and in mice displaying metastasis relapse at late time-points. In Fig. 7h we now show that the lung remains soft in the presence of early disseminated cancer cells (i.e. during the quiescence phase), while the tissue becomes stiffer when cancer cells grow into overt metastases, at late time points (i.e. when cells wake up and relapse).

(ii) we performed stainings on metastatic cells disseminated at the lung at early and at late time points. We found that the mechanosensitive YAP/TAZ factors are initially cytoplasmic and then become nuclear, following increased Collagen-I deposition surrounding cancer cells (Fig. 7i), and

following increased tissue stiffness. Strikingly, also the 8OHG, 4HNE and GFP-Grx1 redox and markers follow tissue stiffness (Fig. 7j,k).

(iii) we provide new evidence in support of the idea that D2.0R cells in vitro are initially sensitive to ECM softness imposed by a BM ECM hydrogel, and actively remodel it into a stiffer one after two weeks in culture. Also here, YAP/TAZ were cytoplasmic (i.e. inactive) at early time points, while we could observe a clear late increase in nuclear (i.e. active) YAP/TAZ (Fig. 7a). This was paralleled by increased pericellular Collagen-I deposition at late time-points, an indication of ECM remodelling (Fig. 7a). Strikingly, at these late time-points we also observed reduced redox markers (Fig. 7b), and a corresponding increased sensitivity of cells to Cisplatin chemotherapy (Fig. 7c).

(iv) we included new experiments where we compare cells cultured on lung decellularized ECM from normal mice and from mice with lung fibrosis, which is known to stiffen the ECM and to reawaken D2.0R cells from quiescence (Vera-Ramirez Nat Commun 2018). Also in this case, as with Ribose-mediated stiffening, redox markers are lowered, indicating that a physiological tissue stiffening is sufficient to modulate ROS production (Fig. 7e,f). Moreover, we also infiltrated cells into lung decellularized ECM from normal mice and from mice with lung fibrosis, and we found that NRF2 target genes are downregulated upon fibrosis (Fig. 7g), while YAP/TAZ target genes are upregulated as expected (ED Fig. 7e).

Overall this data supports the view that differential metastatic TME stiffness regulates novel (ROS/NRF2) and established (YAP/TAZ) mechanotransduction pathways in vitro, ex vivo and in vivo.

2. How does the tension of "soft matrix" compared to normal tissue? As described in the second last paragraph of "Results" and in Figure 6A-B, physiological lung ECM stiffness is equivalent to soft ECM conditions used throughout the study. If this is true, and increased 8OHG, 4HE, and glutathione oxidation is induced in cells grown under soft ECM conditions, are the authors suggesting that untransformed cells residing in normal, soft tissue would exhibit chronic lipid oxidation and activation of the NRF2 antioxidant pathway? It is important to note that NRF2 is known to be expressed at very low levels in normal cells due to KEAP1 dependent turnover and is induced only when ROS oxidizes KEAP1.

The Reviewer is right, the soft ECM hydrogels that we use experimentally are in the range of stiffness of the lung and mammary gland (see response to point 1).

We understand this comment incorporates different questions:

2A) whether non-transformed cells also display the same response to softness, or whether this is specific for cancer cells

We performed experiments with immortalized non-transformed MCF10A cells and we provide evidence that:

- ROCK/MLCK inhibition induces ROS production (Extended Data Fig. 1k,m);
- ROCK/MLCK inhibition and a soft ECM induce NRF2 target genes (ED Fig. 2b,g);
- ROCK/MLCK inhibition induces cystine uptake (Fig. 1e);
- a soft ECM provides protection from cumene hydroperoxide, which depends on NRF2 (Fig. 2q). This data now provides experimental validation for correlative GSEA analyses provided at the time of the first submission (now in ED Fig. 3a), which already suggested conservation of the NRF2 response to softness in non-transformed (MCF10A) as well as in fully metastatic breast cancer cells (D2.0R, MDA-MB-345, MMTV-PyMT). So, we believe this response is a general one, at least in the mammary lineage, irrespective of oncogene transformation and of the cancer subtype (TNBC vs. hormone-positive).

2B) whether the response that we describe represents a chronic oxidative stress, or whether this represents a beneficial amount of ROS

We may define this response as "chronic" or "sustained" as we do observe long-term enhancement of ROS production in cells on a soft ECM. However, we do not believe this represents oxidative stress. Oxidative stress is generally defined as an imbalance between ROS production and the cell's antioxidant defenses, to such an extent that it results in decreased cell fitness or survival. In our experiments, enhanced ROS on a soft ECM are not toxic for cells, not even in absence of the NRF2 antioxidant response (Fig. 2o,p,q). So, it is legitimate to conclude that on a soft ECM elevated ROS stay within a physiological range, and are actually beneficial because they help cells build stronger antioxidant defences.
To reinforce this notion experimentally, we explored our phenotypes in light of lipid peroxidation and ferroptosis. Also in this case a soft ECM empowers resistance to ferroptosis (Fig. 2r,s and ED Fig. 4g,h). This occurs despite elevated basal levels of lipid peroxides, indicating these levels are below the threshold triggering oxidative damage to cells. Of note, while studying the relevant literature we noted how the accumulation of glutamyl-amino acids observed in cells cultured on a soft ECM (Fig. 1c) has been described as a specific response to NRF2 activation, and represents an additional mechanism by which NRF2 prevents ferroptosis induction, in addition to stimulation of glutathione synthesis (Kang Cell Metab 2021).
Finally, please see below our response to point 4 where we compare the levels of ROS production in different conditions and address concerns on DNA damage, to further reinforce the view that a soft ECM induces low levels of ROS that are not comparable to cytotoxic oxidative stress.

2C) whether it is legitimate to expect basal ROS and NRF2 activity in normal cells of normal tissues, given that most tissues are in the soft range.

Please let us stress that the idea that "untransformed cells residing in normal, soft tissue would exhibit chronic lipid oxidation and activation of the NRF2 antioxidant pathway" is in principle a logical consequence of our findings, but is based on the assumption that ECM softness is the only parameter regulating these phenotypes in a tissue, which is clearly not the case. For example, lipid peroxidation levels greatly depend on the activity of the GPX4 protein, which is a selenium-dependent enzyme, and the level of selenium is very low in common cell culture media compared to the plasma (and, thus, by approximation, in tissue interstitial fluids) (Vande Voorde Sci Adv 2019), so that the efficiency of the GPX4 enzyme is expected to be greater in vivo, and the levels of lipid peroxides lower. Moreover, the local availability of oxygen and of nutrients can profoundly alter metabolic pathways that influence ROS production and scavenging (glycolysis, oxidative phosphorylation, the PPP, cytoplasmic glutaminolysis, the availability of cysteine and methionine etc.). As a consequence, it is complicated to directly compare in vitro/in vivo levels. However, we are not making claims on absolute levels, which we realize would be a slippery argument. What we propose is that ECM elasticity can promote ROS production and NRF2 activity relative to the basal levels determined by other inputs.
Finally, we believe that even if it is true that NRF2 is known to be expressed at very low levels in normal cells, there can still be some NRF2 activity in normal tissues. For example, we discuss data in mice which indicates a key function of basal ROS at maintaining basal NRF2 activity to keep the homeostatic equilibrium between self-renewal and commitment to differentiation in neural stem cells during mouse brain development (Khacho Cell Stem Cell 2016), indicating a physiological ROS/NRF2 activity.

2D) whether this level of ROS is sufficient to regulate KEAP1 function.

We now show that cells with decreased actomyosin contractility display decreased interaction between NRF2 and its inhibitor KEAP1, at the endogenous protein level (Fig. 2h). This nicely complements previous data on increased nuclear NRF2 (now in Fig. 2f,g). Moreover, for consistency we also show that KEAP1 knockdown in 2D plastic cell culture also protects cells from oxidative stress (ED Fig. 4b,c,d), as expected. This now provides a mechanistic basis for NRF2 activation on a soft ECM (expected, but yet to be demonstrated), whereby ROS relieve the inhibitory function of KEAP1.

3. The authors report that matrix detachment and growth in soft matrix both result in elevated ROS followed by a chronic, sustained induction of NRF2. While the authors attribute ROS induction in cells residing in soft matrix to be due to DRP1-dependent mitochondrial changes, they did

not consider the possibility that growth in soft matrix reduces glucose uptake and therefore NAPDH production through PPP, a mechanism previously reported to regulate redox metabolism of breast cancer cells upon matrix detachment (PMID 19693011). It is important to examine the glucose-PPP axis before concluding distinct redox mechanisms downstream of soft matrix growth vs matrix detachment.

We thank the Reviewer for this suggestion. As discussed above, matrix detachment and growth in soft matrix bear both important similarities and important differences. Several publications indicate that matrix detachment causes an impairment in glucose uptake and in NADPH production by the PPP pathway, and/or in the ability of cells to regenerate the cytoplasmic vs. mitochondrial NADPH pools (PMID 19693011, i.e. Schafer Nature 2009, but also Grassian G&D 2011, Jiang Nature 2016, Hawk NCB 2018, Takahashi Mol Cell 2020). Whether the same applies in soft matrix has not been directly addressed so far. Data in bronchial epithelial cells does not indicate reduced glucose uptake on a soft ECM, but rather reduced usage in "lower" glycolysis because PFK is inhibited (Park Nature 2020), which would in principle provide continued or even increased supply of G6P to the PPP. So, it is not straightforward to make predictions.

To sort out this issue experimentally, we monitored the redox state of NADP+/NADPH in response to cytoskeletal tension by using the iNap1 ratiometric sensor (Tao Nat Methods 2017). We initially validated the iNap1 sensor in our cells by combined Diamide and DHEA treatments (i.e. treatment with a general oxidant together with an inhibitor of G6PD to prevent metabolic compensation), inducing strong and durable NADP oxidation (ED Fig. 1h) as in (Tao Nat Methods 2017). As a negative control, and to exclude potential influence of the pH, we used the iNapC NADP-insensitive probe (Tao Nat Methods 2017). We then compared iNap1 in cells treated with DMSO or with ROCK/MLCK inhibitors for 3 hours (namely, in the early time-frame where we observe ROS production). As shown in ED Fig. 1h the steady-state NADP+/NADPH ratio is not altered, and this also holds true after 24 hours (not shown). So, we concluded that cells on a soft ECM do not display major defects in NADP regeneration. This makes it unlikely that unbalanced NADP oxidation is a primary cause for ROS accumulation.

We also reinforced the link between ECM mechanics, DRP1 and ROS induction, thus providing additional support to our model. These experiments were done in response to Reviewers 2 and 3, and now show that:

- DRP1 is required for mtROS production, NRF2 activation and chemotherapy resistance by using an additional and more specific small molecule inhibitor, Driptor1a, potentially amenable to in vivo use (Wu FASEB J 2020) - see multiple panels in Fig. 4, 5 and 6 ;
- the fission factors and DRP1 adaptors MIEF1/2 are required for mtROS production, while the Fis1 and MFF factors are not (Fig. 4i and ED Fig 5j-n);
- peri-mitochondrial F-actin is instrumental for enhanced DRP1 recruitment (Fig. 5d,e);

- the Spire1C mitochondrial-associated F-actin nucleation factor is required for mtROS production, while the ER-associated INF2 nucleation factor is not (Fig. 5f,g and ED Fig. 5s-u), thus indicating a previously unappreciated specificity of these factors for mitochondrial fission;
- cells on a soft ECM display less ER-mt contacts, a phenotype previously associated with increased DRP1 activity (Cieri CDD 2018), and that DRP1 is required for this phenotype (ED Fig. 5f). This now provides an additional DRP1 read-out that supports our model, independent of ROS measurements.
- a significant portion of the genes regulated by ECM stiffness in D20R cells is coherently regulated by DRP1 and by NRF2, by performing RNAseq analyses (Fig. 4l). This expands our initial qPCR analyses and shows how ECM stiffness controls a whole set of genes by the mechanism we here propose. Interestingly, this analysis also unveiled a set of genes which is regulated by ECM stiffness and DRP1, but not by NRF2 (not shown). We believe this is consistent with our model where DRP1 acts upstream of NRF2 regulation, and potentially regulates multiple phenotypes besides NRF2.

Collectively, we believe that our data now convincingly supports the view that regulation of ROS in response to ECM stiffness is mainly related to DRP1-dependent mitochondrial remodeling, at least in the mammary cell lines that we tested.

4. The authors argue that the ROS induced in cells grown in soft matrix is "hormetic" and therefore not toxic, supported by the lack of nuclear DNA damage (Fig 1M). Intriguingly, the authors also report a very robust induction of lipid oxidation when cells are grown in soft matrix (Supp Fig 1N). It is known that indiscriminate oxidation of lipids and nuclear DNA both occur through hydroxyl radicals generated from H2O2 through the fenton reaction. It is surprising that there is selective oxidation of lipids but not DNA. The authors should examine different parameters to assess DNA damage more carefully. It is also important to note, that the changes in H2O2 required for signaling (hormetic) do not cause significant changes in intracellular ratio of oxidized glutathione (GSSG)/reduced glutathione (GSH) or NADPH/NADP+ (PMID 21964034). In fact, large changes in these parameters (as shown in Fig1) are usually a sign of oxidative stress causing toxicity rather than signaling associated with redox biology (PMID 21954972).

We more carefully examined DNA damage. We now show that culturing cells on a soft ECM does not cause any DNA damage compared to cytotoxic doses of Cisplatin, As2O3 and Doxorubicin treatments, based on RNase-resistant nuclear 8OHdG staining (Fig. 1o) and on the specific DNA double-strand break

damage marker gammaH2AX (Fig. 1p). Thus, the increase in ROS observed on a soft ECM is not associated with DNA toxicity.
We also performed functional experiments with Doxorubicin, an intercalating agent that kills cells by inducing DNA damage. In this case, neither treatment with the N-acetyl-cysteine antioxidant, nor plating cells on a soft ECM protected cells (ED Fig. 6e,f). So, a soft ECM specifically protects cells from drugs inducing oxidative stress.
As for the magnitude of lipid peroxidation, we now provide a comparison between cells cultured on a soft ECM and cells treated with RSL3, inducing much higher doses of toxic lipid peroxides (ED Fig. 1q-s). This is in line with new functional data indicating that cells on a soft ECM are more resistant to ferroptosis induction by Erastin and RSL3 (Fig. 2r,s and ED Fig. 4g,h). So, increased lipid peroxidation on a soft ECM does not represent an indiscriminate oxidation of lipids, but rather a shift in their levels within a non-toxic window.

Other concerns

5. Does growth in soft matrix reduce overall mitochondrial numbers? Figure 4B seems to suggest so.

The purpose of these pictures was to provide representative examples of mitochondrial morphology. These are now in Fig. 4a with a new color-blind-friendly palette. Cells on a stiff substratum are extremely flat, while cells on a soft ECM take on a more rounded/3D shape. As a consequence, a single confocal section may provide the false impression of more mitochondria on a stiff matrix.
To tackle the issue of mitochondrial numbers independent of cell geometry, we measured total mitotracker fluorescence by flow cytometry (this stain is taken up by mitochondria independent of mitochondrial membrane potential, and is thus an accepted read-out of total mitochondrial mass). As an additional and technically independent read-out, we performed qPCR on multiple mtDNA loci vs. nuclear DNA. Results shown in ED Fig. 5b,c indicate that mitochondrial mass and mtDNA content remain the same.
Please also note that, thanks to an ongoing collaboration with Dr. Archer, we now used additional methods to quantify the morphology of mitochondrial networks in an unbiased manner, including an automated system based on machine-learning, which confirmed our previous non-blind quantification (Fig. 4c,g and ED Fig. 5a).

2. Does DRP1 overall expression change?

We checked DRP1 mRNA levels by qPCR and both total DRP1 and phospho-DRP1 protein levels by immunoblotting. We did not detect significant differences, as now shown in ED Fig. 5d,e.

6. Does growth in soft ECM alter PI3K signaling does that contribute to DRP1 and/or ROS/NRF2 regulation (PMID1993152).

In response to this concern, we performed an experiment with the Wortmannin PI3K inhibitor. As shown in Fig. 3a Wortmannin does not induce ROS on its own, and it is unable to counteract early ROS induced by ROCK/MLCK inhibition. So, we think that PI3K signaling is not a major input here.

7. Timepoints of some experiments not indicated in either the methods or the figure legend, eg C11 Bodipy

The timepoints were similar to experiments with other ROS probes. This information can be now found in the Figure legends.

8. Fig 6I-J: shNRF2 cells are more sensitive to cisplatin is an established phenomenon in cell 2D cell culture-this is independent of matrix tension and should be described carefully to avoid confusion.

The reviewer is right. Of course we are not claiming NRF2 is active ONLY on a soft matrix, as there is plenty of NRF2 activity on tissue culture plastics (as seen by downregulation of target genes in cells with NRF2 knockdown see Fig. 2). So, NRF2 plays a role also in 2D plastic cell culture (i.e. stiff condition), as seen by others. It may seem that NRF2 knockdown could not sensitize MCF10A-RAS cells on plastics, but this depends on the fact that we choose drug doses having strong effects on plastics, to show these were reduced on a soft ECM. We now included an explicit statement to avoid confusion: "Some chemotherapeutics work by inducing oxidative stress, so that the antioxidant response triggered by NRF2 is known to favor therapy resistance in multiple conditions, including on plastics... " (page 10-11)

Finally, please note that in response to other Reviewers' comments, we now show:

- that the protective response induced on a soft ECM depends on NRF2 but not on NRF1 (ED Fig. 4e,f);
- that the altered oxidative phosphorylation observed on a soft ECM in MCF10A-RAS cells is also observed in D2.0R cells, indicating a conserved phenomenon (Fig. 3f,g). However, we also show that this metabolic trait is not a simple consequence of DRP1 activation (ED Fig. 5o-r), likely suggesting that production of mtROS is not a linear consequence of altered respiration;
- independent technical evidence on the regulation of peri-mitochondrial F-actin (Fig. 5b), and new data suggesting that formation of DRP1 puncta depends on the balance between two opposing F-actin pools, i.e. contractile actomyosin (dependent on ROCK and NMII) and peri-mitochondrial F-actin (dependent on Arp2/3 and on Spire1C) (Fig. 5);

- that the suppressive effect of NRF2 inactivation together with cisplatin treatment correlates with increased cell death of disseminated metastatic cells, indicating a cell-autonomous effect also in vivo (ED Fig. 7i).

In sum, we think that the experiments proposed by the Reviewer provided a more precise description of our findings, and at the same time reinforced our claims. We hope that the Reviewer will appreciate our efforts, and now support publication.

Reviewer #2 - expert in mitochondrial morphology

Remarks to the Author:
This manuscript from Romani et al. investigates the relationship between matrix stiffness and the antioxidant response in breast cancer cells. The authors demonstrate that stiffness induces mitochondrial remodeling which in turn promotes the generation of mitochondrial ROS, activation of NRF2 and subsequent upregulation of glutathione synthesis. This is an intriguing and novel paper and the manuscript is, for the most part, very thorough and convincing.

Our response to the Reviewer:
We thank the Reviewer for the positive assessment of our work. In these months we worked along the suggestions indicated by the Reviewers, and we think the manuscript is now stronger and more convincing also in the part concerning the role of F-actin, DRP1 and its adaptor molecules in driving fission and ROS production. We hope the Reviewer will appreciate our efforts, and provide support for publication.

However, there are a few aspects of their model which could use additional support. In particular, there is not much data supporting their model of actin's involvement in Drp1 recruitment or in whether mitochondrial fission itself is inducing ROS. Specific comments are listed below:

POINT1 on Figure 3 - The authors see significant effects on ROS using TUDCA, yet don't really address whether ER stress is playing a role. I'm curious whether the ER may be contributing to the changes in mitochondrial morphology through ER-mediated mitochondrial division under the different matrix conditions or whether this is a red herring. The authors should comment on the potential role of ER, if any, given this result.

We did not analyze further ER-induced ROS because we found an interesting lead on mitochondrial ROS and mitochondrial fission that was sufficient to explain the phenotypes we observed. We kept the data with TUDCA for completeness, and to acknowledge complexity. This observation might be important for colleagues working on similar topics, or to leave open the possibility that additional mechanisms, of which we are not aware today, contribute to the phenotypes we observe.

Experimentally, we performed the following experiments:

A) We measured mitochondrial ROS (by using MitoSOX) in cells treated with TUDCA. As now shown in Fig. 3c, TUDCA was not sufficient to reduce mtROS. This excludes the ER as the major source for mtROS in our conditions. We speculate that mtROS might influence, directly or indirectly, ER ROS, and that this amplifies further cytoplasmic ROS.

B) Prompted by this comment, and keeping in mind that F-actin mediated mitochondrial fission has been proposed to occur (also) at ER-mt contact sites, we explored the possibility that ECM mechanical properties change ER-mt contact sites.

We transiently transfected cells with two different split-GFP-based ER-mt contact site sensors (SPLICS), detecting short-range (8-10nm) or long-range (40-50nm) interactions (Cieri CDD 2018; Vallese Nat Commun 2020). We then quantified the number of contacts from 3D rendering of complete z-stacks to measure contacts independent of cell geometry. The data indicates a reduced number of short-range ER-mt interactions on a soft ECM (ED Fig. 5f).

At first, this might seem at odds with increased DRP1 recruitment. However, the ER-localized F-actin polymerizing factor INF2, which can mediate fission at ER-mt contacts, is not involved in our phenotypes (ED Fig. 5s-u). So, our model does not imply increased ER-mt contacts. Instead, we note how a reduced number of ER-mt interactions is expected in cells with high DRP1 activity (Cieri CDD 2018; Vallese Nat Commun 2020). Strikingly, treating cells on a soft ECM with the Driptor1a DRP1 inhibitor rescues the number of ER-mt contact sites (ED Fig. 5f). This suggests that in our systems mitochondrial fission is upstream of ER-mt contacts, and not downstream as proposed from the Higgs group based on data in U2OS cells. More importantly, this provides additional evidence that a soft ECM triggers DRP1 activity, by using an independent read-out besides ROS/NRF2.

POINT 2 on Figure 3 - The authors dismiss defects at the level of oxidative phosphorylation as the source of ROS, yet this conclusion seems premature at this point in the manuscript. While the changes in basal

respiration they measure are modest, the decrease in maximal respiration is very large. This is actually consistent with the increased fragmentation they observe in the next figure and consistent with a model in which fragmentation leads to ROS generation through decreased Ox-Phos efficiency.

We thank the reviewer for pointing out this issue, and for proposing an interesting hypothesis, i.e. that decreased maximal respiration observed on a soft ECM is caused by increased fragmentation and potentially an integral part of the response that we observe.

To explore this possibility we measured Ox-Phos also in D2.0R cells and observed consistent effects with MCF10A-RAS (Fig. 3f,g). This has also been observed by others in dendritic cells cultured on a soft ECM (Chakraborty Cell Reports 2021), indicating it is a widely conserved phenotype, even if still unexplained. We then compared control vs. Drp1-knockdown or Drp1-inhibited cells (ED Fig. 5o-r), which excluded a simple link between ECM stiffness, DRP1 and Ox-Phos. We speculate that the reduced maximal respiration on a soft ECM might be related to altered proline metabolism, as proposed by others (Guo Nat Commun 2019).

Please note that our data indicates the interesting possibility that mitochondrial fragmentation and/or mtROS production might fulfill additional signaling functions in response to a soft ECM, beyond activation of NRF2. For example, modulation of ER-mt contacts (see the response to your point 1), which may have an impact on ER and/or mitochondrial Calcium dynamics. Moreover, we noted in support of this idea a significant number of genes that are upregulated on a soft ECM in a Drp1-dependent, yet NRF2-independent manner (not shown). It will be interesting in future to understand what factor(s) and signalling events mediate this second retrograde signalling response, and what is its functional significance.

POINT 3 on Figure 4C - The number of Drp1 puncta is consistent with increased Drp1 recruitment, but should be backed up by biochemical fractionation and immunoblotting of Drp1.

The proposed experiment involves the lysis of cells and ultracentrifugation for quite a long time (i.e. at least 30min) to precipitate mitochondria. However, in these conditions F-actin will not be conserved, because cell lysis induces the rapid dissociation of microfilaments. So, while ultracentrifugation is a good assay to measure the intrinsic localization of DRP1, it is expected not to capture the contribution of F-actin. Maybe, this is why all the published evidence on a role for F-actin in regulating DRP1 recruitment and mitochondrial fission is only based on imaging techniques, never on ultracentrifugation (see all papers from the Higgs lab and the recent ones from Lippincott-Schwartz and Manley labs in Nature).

As an alternative response to this point, we reinforced the notion of DRP1 recruitment by showing that the DRP1 adaptors and OMM integral proteins MIEF1/2 are specifically required for mtROS production in response to ROCK/MLCK inhibition (Fig. 4i and ED Fig. 5j-n). Moreover, we now show that actomyosin

tension regulates DRP1 puncta in two cell lines (Fig. 4d and 5e), and their dependence on Arp2/3 (Li JCB 2015) (see Fig. 5 and the response to point 7 below for more details). Finally, new data discussed above on ER-mt contacts provides an independent read-out that DRP1 activity is enhanced on a soft ECM. Thus, we believe the data now support, although indirectly, the view that DRP1 recruitment to mitochondria is regulated by ECM mechanical cues.

POINT 4 on Figure 4c - It would also be informative to determine whether there are changes in the levels of Drp1 or of its phosphorylation on S616 and S637.

Thanks for the suggestion. We monitored total and phospho-DRP1 levels by immunoblotting, and we did not detect significant variations upon inhibition of ROCK and MLCK (ED Fig. 5e). We performed phospho-DRP1 immunofluorescence, and detected an increased number of endogenous phospho616-DRP1 puncta upon inhibition of ROCK and MLCK (Fig. 4e). Together, this suggests that inhibition of actomyosin tension induces the formation of more DRP1 puncta, which contain active phospho-DRP1. At the same time, this data fails to support the idea that a soft ECM works by regulating the activity of a DRP1 kinase or phosphatase.

POINT 5 on Figures 4-5 - Knockdowns (Drp1, Nrf2) should be confirmed by immunoblot. We now provide this information in ED Fig. 2i, 4d, 5h.
POINT 6 on Figure 4 - The authors should comment on the specificity of Mdivi-1. It has well publicized off-target effects, including on Complex I, making it difficult to interpret results from these experiments.

We were informed that the MDIVI1 compound can have off-target effects, which is why we wrote in the manuscript that we treated cells with "titrated doses of MDIVI1". Owing to its inhibitory effects on Complex I (Bordt Dev Cell 2017), high doses of MDIVI1 can result in production of mtROS, similar to the Complex I inhibitor Rotenone. As a rule of the thumb, we thus used half the maximal dose of MDIVI1 that was not causing basal enhancement of mtROS in MCF10A cells. We now explicitly comment on this and cite Bordt Dev Cell 2017 on MDIVI1 off-target effects.

In response to this concern, we obtained in collaboration with Prof. Archer another recently developed DRP1 small-molecule inhibitor, Driptor1a, which is active in the nanomolar range, potentially amenable to in vivo use, and highly specific for DRP1 (see the thorough validation in Wu FASEB J 2020). With this compound we repeated or performed key experiments:

- rescue of mtROS production (Fig. 4h)
- rescue of Cystine uptake (Fig. 4m)
- rescue of sensitivity to chemotherapy in both cell lines (Fig. 6g,h and ED Fig. 6h)
- rescue of decreased ER-mt contact sites (ED Fig. 5f)

In conclusion, we overall remain confident that the effects of MDIVI1 were specific and, most importantly, that the functional role of DRP1 and mitochondrial fission is now confirmed by multiple independent reagents and assays.

POINT 7 on Figure 5 - The link between actin and Drp1 recruitment is very speculative and not well supported by the data provided. The fact that actin is recruited to mitochondria under conditions under which fission is being induced is expected, as actin filaments are known to be present at sites of fission. While this is formally consistent with their model that these actin contacts are "causing enhanced Drp1 recruitment", they do not provide any data supporting this direct causality. Further, the authors note this similar pattern of actin puncta and Drp1 puncta, but offer no evidence that these two overlap.

We were disheartened by the Reviewer's statement that mitochondrial actin recruitment in response to a physiological input such as actomyosin tension is expected. Data on the link between mitochondrial fission and F-actin is almost solely based on U2OS osteosarcoma cells (see all papers from the Higgs and Lippincott-Schwatz groups). Moreover, it remains so far unknown what portion of mitochondrial fission events in a cell depend on peri-mitochondrial F-actin and on its known players, INF2 and Spire1C. For example, the "mechanical" mitochondrial fission induced by local compression or by Shigella does not depend on INF2 and on actin polymerization at all, but requires the MFF adaptor (Helle eLIFE 2017). Another example is the recently described mitochondrial actin comet tails that promote mitochondria fission to spatially shuffle mitochondria, which depend on Arp2/3 and Spire1C, but not on INF2 (Moore PNAS 2016, Nature 2021). Finally, it has been just published that mitochondrial fission can occur by two functionally and mechanistically distinct types of fission in the same cell, at the periphery of mitochondria to induce mitophagy of damaged mitochondria, which depends on the Fis1 adaptor, vs. at the midzone to induce mitochondrial proliferation, which depends on INF2 and on the MFF adaptor, while MIEF1/2 do not play specific roles in this context (Kleele Nature 2021). So, while it is true that the link between fission and F-actin is not new, the picture appears more complicated than previously expected, and deserves more studies.

In response to this concern, we now show:

- colocalization of DRP1 puncta with F-actin/mitochondrial contacts (Fig. 5c). So, we think we are measuring the same entity, which contains both DRP1 and F-actin. Of note, in response to a concern of Reviewer #3, we now show results on F-actin/mitochondrial contacts performed with an independent technique, which cannot suffer from overexpression artifacts noted by Rev. #3 (Fig. 5b).

- that DRP1 puncta and F-actin/mitochondrial contacts induced in conditions of decreased cytoskeletal tension depend on F-actin (by latrunculinA and Arp2/3 inhibitor treatments), both in MCF10A-RAS and in D2.0R, thus providing a causal relationship between reduced actomyosin tension, increased peri-mitochondrial F-actin and increased DRP1 recruitment (Fig. 5a,b,d,e).
- that INF2 knockdown (either as total INF2, or by targeting the CAAX mitochondrial splice isoform specifically) had no effect on mitochondrial morphology and ROS (ED Fig. 5s-u). Transfection of dominant-negative Spire1C (which is the only way to interfere specifically with the mitochondrial splice isoform of Spire1) instead caused elongation of mitochondria and rescued ROS production on a soft ECM (Fig. 5f,g). This indicates that ER-promoted F-actin fission is likely not involved, while F-actin polymerization at the mitochondrial surface does play an important role.

We also discuss our results on MIEF1/2 requirement by pointing out the fact that such factors had been previously shown to bear a broad potential for F-actin interaction, thus perhaps indicating these factors as relevant molecular interfaces in response to ECM mechanical cues.

Overall we think this now better supports a link between F-actin and DRP1.

POINT 8 on Figure 6 - The authors should note that Drp1 has been shown to promote tumorigenic growth in a number of systems and that it is possible that the effects of Drp1 knockdown on tumor burden they observe can also result from functions of mitochondrial shape independent of the glutathione synthesis mechanism they propose.

The Reviewer is right, DRP1 contributes to cancer cell growth by regulating multiple parallel events besides glutathione synthesis. We now show this ourselves as a corollary to data on OXPHOS (see response to point 2). We now comment on this and provide a reference to a recent publication from our collaborator Dr. Archer, where it is shown that DRP1 inhibition has general effects on cancer cell survival in mice (Wu FASEB J 2020).

Finally, please note that, in response to other Reviewer's comments:
- we provide a complete new set of data to better support the idea that the lung metastatic microenvironment is a soft one, and that lung ECM softness is a relevant parameter to control redox homeostasis also in vivo (see the new Fig. 7a-k).
- we show that ROS induced on a soft ECM are not sufficient to cause genotoxic stress, as compared with positive controls and based on gamma-H2AX stainings (Fig. 1o,p).

- we show that cells on a soft ECM are also protected from ferroptosis, a form of cell death specifically linked to glutathione metabolism (Fig. 2r,s and ED Fig. 4g,h), thus reinforcing the notion that a soft ECM empowers resistance to oxidative stress.
- we controlled that culturing cells on a soft ECM does not reduce overall mitochondrial content, as measured by two independent techniques (ED Fig. 5b,c)

In sum, we think that the experiments proposed by the Reviewer provided a more precise description of our findings, and at the same time reinforced our claims. We hope that the Reviewer will appreciate our efforts, and keep supporting publication.

Reviewer #3 - expert in cell mechanics

Remarks to the Author:
Romani and coworkers report that human Ha-Ras-transduced MCF710A as well as mouse D2.0R breast cancer cells treated with ROCK and MLCK inhibitors or seeded on soft ECM (Matrigel or fibronectin) induce F-actin accumulation in the proximity of mitochondria, an elevation of Drp1 activity and mitochondria fission followed by mtROS and ER-ROS production. The high mtROS levels stabilize Nrf2, which leads to nuclear translocation of Nrf2, transcription of an antioxidant gene program including the gene encoding SLC7A11 that in turn provokes the uptake of extracellular cysteine and the production and oxidation of glutathione. Finally, the authors demonstrate that loss of Nrf2- or Drp1-expression in D2.0R breast cancer cells curbs metastatic tumor growth in lungs when injected into the tail vein of Cisplatin-treated syngeneic mice.
The paper is difficult to read since the findings are not logically stringed together and the results are often superficially described.

Our response to the Reviewer:
We realize it is not simple to follow the story, also because there is a non-trivial loop between ROS production and antioxidant activity, and because we touch upon very different topics. It took us time to put all the pieces of the puzzle together. We now hope the revised version of the manuscript and the new experimental results will help clarify the story, and that we now provide a more in depth description of the phenotypes.

The major problem, however, is the limited novelty and conceptual advance of the paper. It is well known in the field of mechanobiology that human adipose tissue-derived mesenchymal stem cells (MSCs) seeded on a soft substrate induce intracellular ROS levels leading to nuclear Nrf2 translocation and the transcription of an anti-oxidant program (as well as the nuclear translocation of HIF1alpha and the production of a pro-angiogenic secretome; DOI: 10.1002/adhm.201900929).
Furthermore, it has also been reported that polymerized actin is required to induce Drp1-mediated mitochondrial fission, that mitochondrial fission increases ROS and that increased levels of ROS stabilize Nrf and upregulate expression of 'antioxidant genes' including the glutamate/cysteine antiporter SLC7A11 that imports extracellular cysteine. Whereas publications reporting the latter findings are, at least partially, mentioned in the manuscript, the key report showing that substrate softness induces ROS, Nrf2 and an antioxidant program is for unexplainable reasons neither mentioned nor discussed.

We thank the Reviewer for bringing about this report, which we now reference as requested. In this short report it is shown that MSCs on soft polyacrylamide hydrogels display enhancement of generic ROS, accompanied by enhanced expression of NRF2 target genes. However, there is no information on how ROS are regulated, on whether there is any cause/effect relationship between ROS and these target genes, on whether these genes are regulated by NRF2 in this context, and, most importantly, on the functional significance of these events. Moreover, please note that MSCs undergo differentiation in response to mechanical cues (Engler Cell 2006), which could add unexpected complexity to the regulation of mitochondrial dynamics, ROS and NRF2 activity (see for example Quinn Sci Rep 2013; Forni Stem Cells 2016; Zhang PLOS One 2013). In addition, please note that another equally relevant study on fibroblasts cultured in vitro on soft vs. stiff polyacrylamide hydrogels indicates enhanced accessibility of NRF2-binding sites on a stiff ECM by ATAC sequencing (Jones JCB 2021), which leads to the opposite prediction - i.e. NRF2 is active on a stiff ECM. In principle, I would rather trust the latter data, coming from the group of Daniel Tschumperlin who has studied fibrosis and fibroblast mechanotransduction for a long time. However, none of these papers provide any general functional relevance for such observations, nor they show such response is important for cancer chemotherapy.

Additional comments:
(1) Why has Nrf1 not been analyzed? A combinatorial loss of Nrf1 and Nrf2 could have an even more profound effect on metastatic tumor growth?

The NFE2L family of transcription factors includes NRF1, NRF2 and NRF3. All these factors share the ability to bind common DNA-binding elements (ARE, antioxidant response elements) in the promoter of antioxidant genes, and potentially contribute to the antioxidant tone of cells. NRF3 has a very restricted

and tissue-specific pattern of expression, while NRF1 and NRF2 are widely expressed in many tissues. While some data point to partially different roles of NRF1 and NRF2 (Ohtsuji JBC 2008, Zhang Biochem J 2016), genetic data in mice provide clear evidence that NRF1 and NRF2 are redundantly required in vivo

for tissue homeostasis (Leung JBC 2003). This indicates that basal NRF2 levels (i.e. in absence of overt oxidative stress) are absolutely required, in addition to NRF1 levels, for tissue development. However, only NRF2 is known to be directly regulated by ROS, so that we focused on NRF2 only.

To answer this question we tested the effects of NRF1 knockdown on our phenotypes. As shown in ED Fig. 4e,f, NRF1 knockdown had no effects on survival to cumene hydroperoxide either in stiff or soft conditions. We also excluded compensatory upregulation of NRF2 transcripts, which may have masked NRF1 requirements (ED Fig. 4f). To reinforce the idea that NRF2 is the main player, we also performed KEAP1 knockdown (because KEAP1 controls NRF2 but not NRF1 activity). As shown in ED Fig. 4b-d KEAP1 knockdown protected cells on plastics (stiff) from cumene hydroperoxide, as expected, and similar to a soft ECM. Moreover, we now also show that endogenous KEAP1/NRF2 interaction is reduced (i.e. NRF2 is activated) in conditions of reduced tension (Fig. 2h). This now provides a mechanistic basis for NRF2 activation on a soft ECM, whereby ROS relieve the inhibitory function of KEAP1.

Our data do not exclude that combinatorial loss of NRF1 and NRF2 could have even more profound effects. However, we believe such a genetic approach could be hardly translated into a therapeutic approach, given the strong effects of the combined NRF1 and NRF2 inactivation on normal tissue survival discussed above, and because no inhibitor has been developed that is able to inhibit both factors. Instead, we think that using the Driptor1a DRP1 small molecule inhibitor, which we showed is able to modulate the whole pathway in vitro in response to a concern from Reviewer #2, might be a more interesting approach to disable metastatic tumor growth, also in light of the specificity, potency (Driptor1a acts in the nanomolar range) and in vivo activity of the compound (see Wu FASEB J 2020).

(2) The F-actin stainings suggest that the tagged G-actin leads to actin clumping/aggregation rather than polymerisation into filaments or networks. This issue needs experimental clarification.

We performed new Proximity Ligation Assays (PLA) between F-actin and mitochondria by detecting endogenous F-actin with biotin-phalloidin. In this case, phalloidin is used after cell fixation, and there can be no overexpression artefacts. This provides the same results we had previously obtained with tagged WT Actin, so that our results are now supported by two independent techniques, shown back to back in Fig. 5a,b. In response to Reviewer #2, we also provide co-localization of these F-actin/mitochondria PLA "contacts" with DRP1 (Fig. 5c), indicating that our PLA assays recognize the relevant type of F-actin.

(3) Why has the ER-induced ROS not been further analyzed? Does ER-ROS also contribute to Drp1-mediated mitochondrial fission?

In response to this request we performed the following experiments:

A) We measured mitochondrial ROS (by using MitoSOX) in cells treated with TUDCA. As shown in Fig. 3c, TUDCA was not sufficient to reduce mtROS. This excludes the ER as the major source for mtROS in our conditions. We speculate that mtROS might influence, directly or indirectly, ER ROS, and that this functions as an amplification of cytoplasmic ROS.

B) Prompted by this comment, and keeping in mind that F-actin mediated mitochondrial fission has been proposed to occur (also) at ER-mt contact sites, we explored the possibility that ECM mechanical properties change ER-mt contact sites.
We transiently transfected cells with two different split-GFP-based ER-mt contact site sensors (SPLICS), detecting short-range (8-10nm) or long-range (40-50nm) interactions (Cieri CDD 2018; Vallese Nat Commun 2020). We then quantified the number of contacts from 3D rendering of complete z-stacks to measure contacts independent of cell geometry. The data indicates a reduced number of short-range ER-mt interactions on a soft ECM (ED Fig. 5f).
At first, this might seem at odds with increased DRP1 recruitment. However, the ER-localized F-actin polymerizing factor INF2, which can mediate fission at ER-mt contacts, is not involved in our phenotypes (ED Fig. 5s-u). So, our model does not imply increased ER-mt

contacts. Instead, we note how a reduced number of ER-mt interactions is expected in cells with high DRP1 activity (Cieri CDD 2018). Strikingly, treating cells on a soft ECM with the Driptor1a DRP1 inhibitor rescues the number of ER-mt contact sites (ED Fig. 5f). This suggests that in our systems mitochondrial fission is upstream of ER-mt contacts, and not downstream as observed in U2OS cells. More importantly, this provides additional evidence that a soft ECM triggers DRP1 activity, by using an independent read-out besides ROS/NRF2.

(4) Mechanism underlying softness-induced mitochondrial fission is not investigated. The mechanism is not known and hence, examining this mechanism would give big novelty to the paper.

Following the Reviewer's suggestion, we deepened our understanding of the mechanism by which a soft ECM induces mitochondrial fission:

A) We tested a role for the INF2 inverted formin family member, based on its role as promoter of mitochondrial constriction, DRP1 recruitment and mitochondrial fission (mainly, work from the Higgs lab). Knockdown of total INF2 (i.e. both the cytoplasmic and the ER-tethered isoform specifically involved in mitochondrial fission) or only of CAAX-INF2 (i.e. only the ER-tethered isoform) has no effects on ROS production in response to a soft ECM (ED Fig. 5s-u), and no effects on mitochondria elongation (we only comment this in the main text). This may seem unexpected, but it must be considered that all papers from the group of Dr. Higgs on the function of INF2 for mitochondrial

fission have been performed with U2OS cells uniquely, and it remains unclear how general this function is in other cells and tissues. Recent studies by other groups actually showed there are fission events that do not depend on INF2 (see the references in the final discussion on this point here below). So, while we cannot exclude a role for INF2 in other contexts or cell lines, this makes unlikely a major role for INF2 in our cells.

B) We tested a role for the isoform C of the Spire1 actin polymerising factor, which is specifically localized at the mitochondrial outer membrane, and which plays a role in mitochondrial fission by promoting DRP1 activity (Manor eLife 2015). In this case we used published dominant-negative constructs that specifically interfere with the mitochondrial pool of the protein, which we show are able to elongate mitochondria and to prevent induction of mtROS on a soft ECM (Fig. 5f,g). Current knowledge on the regulation of Spire1C activity is lacking, but it will be interesting in future to better understand whether Spire1C activity is regulated by ECM stiffness, and how. Please also note on this point that even in other papers on INF2 and Spire1C there is no mechanistic clue as to how peri-mitochondrial actin polymerization actually promotes DRP1 function. The current model predicts that F-actin promotes the local deformation of mitochondria, which favors the assembly or circling of DRP1 oligomers, but this awaits future validation.

C) We explored the involvement of other known mitochondrial fission factors that operate as DRP1 adaptors (MFF, Fis1, MIEF1 and MIEF2). For this we tested and selected independent pairs of siRNAs causing efficient downregulation of their target mRNAs (ED Fig. 5k-n). Then, we challenged mtROS production and we found that only the combined knockdown of MIEF1 and MIEF2 (also known as MiD49 and MiD51) prevents mtROS induction upon treatment with ROCK/MLCK inhibitors (Fig. 4i and ED Fig. 5j). This finding is in line with the known redundant function of MIEF1/2 (Atkins Clin Sci 2016). More importantly, this provides independent experimental evidence that mitochondrial fission plays a key role to drive the response to a soft ECM. Moreover, we discuss published data indicating a wide potential for interaction of these receptors with the filamentous actin cytoskeleton (Osellame JCS 2016, Palmer EMBO Rep 2011), thus perhaps indicating MIEF1/2 as the relevant molecular interface between peri-mitochondrial F-actin and DRP1 recruitment in response to ECM mechanics.

D) To show that peri-mitochondrial F-actin remodelling is causal in the response to ECM stiffness and to cell tension, we measured F-actin/mitochondrial contacts and endogenous DRP1 puncta by including the combined treatment with ROCK/MLCK inhibitors (YM) together with latrunculinA, a general F-actin inhibitor, or together with the CK666 Arp2/3 inhibitor, based on the notion that peri-mitochondrial F-actin depends on Arp2/3 (Li JCS 2015; Moore NCOMMS 2016; Moore Nature 2021). As these treatments abolish the formation of F-actin/mitochondrial

contacts and endogenous DRP1 puncta (Fig. 5a,b,d,e), this indicates a causal relationship between decreased actomyosin tension, enhanced F-actin/mitochondrial contacts and enhanced DRP1 recruitment at mitochondria.

Collectively, these experiments advance our understanding of the mechanism underlying softness-induced mitochondrial fission, even if the ultimate molecular link between ECM mechanics, actomyosin tension and peri-mt F-actin still escapes us, requiring dedicated studies. In future, it will be interesting to deepen this model by testing the role of other actin-binding molecules, and by studying how the activity of Spire1C and MIEF1/2 is regulated in this context. Moreover, it could be interesting to explore what other potential fission factors (Cretin EMBO Mol Med 2021, Basu JCB 2021) regulate mitochondrial shortening in response to a soft ECM.

Our data contribute to the notion that different peri-mitochondrial F-actin regulators and different DRP1 adaptors play different roles. For example, the "mechanical" mitochondrial fission induced by local compression or by Shigella does not depend on INF2 and on actin polymerization at all, but entails the function of the MFF adaptor to recruit DRP1 (Helle eLIFE 2017). In contrast, the recently described mitochondrial actin comet tails that promote fission, and that spatially shuffle mitochondria, depend on Arp2/3 and Spire1C, but not on INF2 (Moore PNAS 2016, Nature 2021), which is more similar to our findings. In future, it will be interesting to better understand which actin nucleators and DRP1 adaptors mediate fission in different conditions, how these are regulated, and the underlying logic.

We also considered our data in light of a very recently published paper (Tharp Cell Metabolism 2021). The authors propose that ECM stiffness regulates mitochondrial morphology, based on the well-known mechanical regulation of the SLC9A1/NHE1 Na+/H+ transporter (Schwartz PNAS 1989; Schwartz JBC 1990; Schwartz PNAS 1991; Tominaga EMBOJ 1998). Tharp and colleagues show that immortalized MCF10A cells on a stiff hydrogel (60kPa) display toroidal or "donut shaped" mitochondria. Surprisingly however, we realized they only observe this morphology on an (intermediate) stiff hydrogel, but not on (very) stiff plastics, for which they provide no clue. Moreover, they did not provide any quantitative data in support of this phenotype, so that it is impossible for us to compare. Finally, they only worked with non-tumorigenic MCF10A cells, while our study mainly focused on tumorigenic mammary cancer cells (MCF10A-RAS and D2.0R). Trying to reconcile these observations, we induced toroidal mitochondria in our cells by uncoupling of the respiratory chain upon FCCP treatment (Fig. 4a), but this is clearly different from shorter but still filamentous mitochondria that we observe on a soft ECM, and from long filamentous mitochondria that we see on plastics and on a stiff hydrogel, by using both MCF10A-RAS

and D2.0R cells (Fig. 4a,b,c). Moreover, shorter mitochondria in our cells depend on DRP1 and Spire1C, indicating that what we see is increased fission, while a toroidal morphology is known to be DRP1-independent (Miyazono Sci Rep 2018; Liu Cell Death Differ 2011).

(5) Why is lung metastasis of D2.0R breast cancer cells curbed upon Nrf2- or Drp1-deletion in Cisplatin-treated mice? The literature would point to either cell survival (cell autonomous effect) or tumor angiogenesis (cell non-autonomous effect)? Or both?

In response to this point we used automated imaging and quantification tools to explore how the different shRNAs and/or treatments correlated with increased cell death or vicinity to vessels. We performed multiplexed immunofluorescence for activated/cleaved Caspase3 (a marker of apoptotic cell death) and for CD31/PECAM1 (a marker of endothelia) on lung sections harboring disseminated D2.0R cells, followed by spectral imaging with the Mantra Quantitative Pathology Workstation and the inForm Image Analysis software for machine-learning assisted batch analysis. For this we took advantage of small cohorts of mice (n=2 for each condition) that we had harvested after the second Cisplatin treatment in purpose to perform controls, while letting the other mice for long-term intravital quantification (shown in Fig. 7l). We did not detect any significant effect on vicinity to vessels. Perhaps this is expected, because these cells during the early phases are disseminated as single cells in the lung parenchyma (see for example pictures in Fig. 7i or ED Fig. 7g,i), and tumor neo-angiogenesis usually occurs in much larger lesions. Anyway, since we cannot exclude that a more in-depth analysis would have picked some differences, we only discuss this data with the Reviewer. Conversely, we did observe a coherent increase in the number of cancer cells positive for activated/cleaved Caspase3 in the combination shNRF2+Cisplatin treatment (ED Fig. 7i). This now supports the idea of a cell-autonomous effect in vivo, and nicely parallels survival data with Caspase3 in vitro (shown in Fig. 7c).

(6) An in vivo metastasis model into 'soft' (lung) versus 'stiff' (bone) tissues would have highlighted the cancer cell-mechanical influence on survival, proliferation, etc.

We thank the Reviewer for asking us to reinforce the in vivo relevance of our findings. However, the proposed comparison might not be ideal, for the following reasons. The available evidence indicates that bone metastasis seeding occurs in the bone marrow microenvironment, which is a very heterogeneous one, comprising the bulk bone marrow, the perivascular niche, and the endosteal niche that separates the bone marrow from the stiff bone matrix. Bone mets can eventually evolve into matrix-eroding lesions directly contacting the stiff bone ECM, but this usually represents a late event. Recent quantification of the bulk bone marrow physical properties indicated it is a highly visco-elastic tissue, with an elastic modulus (E) ranging from 1 to 100 Pascals (Chen Biophys J 2020), so an extremely soft one. This is also true for bone metastases of classical "bone-homing" breast cancer cells such as MDA-MB-231 (BiorXiv 10.1101/2021.06.01.446539). However, specific niches are expected to display higher stiffness, which could explain the high degree of heterogeneity in bone marrow measurements

(Jansen J Mech Behav Biomed Mater 2015). This question is also complicated by the fact that some techniques measure macroscopic parameters, while cells can become insensitive to a stiff substratum just by an intervening layer of ECM, or by a monolayer of cells such as the endosteum. As a matter of fact, a study addressing systematically what are the different mechanical properties of the bone niches, and whether this is relevant to regulate established mechano-sensitive responses in metastatic cancer cells, is still lacking and would deserve a dedicated study. Thus, the tempting equation bone=stiff in the case of metastatic cells is not supported by experimental evidence, so that we cannot build on this.

This notion is also apparent from the discussion of a recent paper from Dr. Egeblad, an expert in metastasis dormancy who published on how neutrophils regulate bone metastatic niche stiffness (Albrengues Science 2018): "Furthermore, D2.0R and MCF-7 cells are dormant in soft environments in vitro-such as on matrigel, laminin-111 gel, and 0.2-kPa hydrogel (which mimics the stiffness of the lungs)-but these cells proliferate on stiff 2D cell culture dishes. In vivo, dormant, disseminated tumor cells are preferentially located within the soft niches of stiff tissues such as bones-e.g., in the bone marrow."

In addition, please consider that the bone microenvironment is extremely complex, and entails a cocktail of bioactive hemopoietic secreted factors, hypoxia, high calcium concentrations etc. which makes it difficult to untangle in vivo what phenotype is regulated by stiffness as opposed to other parameters, and which makes a comparison with another organ a daunting one.

So, to circumvent these complications, we focused instead on different time-points of the metastatic disease in the same organ, the lung, corresponding to different tissue stiffness due to cell-mediated ECM remodeling. In this case, we built on previous work that indicates how D2.0R cells plated on a soft ECM are initially sensitive to softness, which induces their proliferative quiescence, but over longer periods of time they are able to actively secrete collagen, enabling cell proliferation (Barkan Cancer Res 2008, Barkan Cancer Res 2010, Barkan Clin Cancer Res 2011). This likely explains why D2.0R cells initially lay quiescent in the lung metastatic niche (where the ECM is soft), but eventually resume growth (when the lung TME becomes stiffer, thanks to cancer cell-mediated ECM remodeling). This also explains why D2.0R cells seeded into a lung made stiffer by fibrosis do not enter quiescence but rapidly originate a metastatic outgrowth (Vera-Ramirez Nat Commun 2018). For this we carried out the following experiments:

A) We measured lung tissue stiffness in wild-type mice, in mice with early D2.0R cancer cell dissemination, and in mice displaying metastasis relapse at late time-points. In Fig. 7d,h and ED Fig7b we now show that the normal lung has comparable softness to the normal mammary gland, in line with previous measurements, that this remains the same in the presence of early disseminated cancer cells (i.e. during the quiescence phase), while the tissue becomes much stiffer when cancer cells grow into overt metastases, at late time points (i.e. when cells wake up and relapse).

B) We performed stainings on metastatic cells disseminated at the lung at early time points and at late time points. We found that YAP/TAZ are initially cytoplasmic and then become nuclear, following collagen-I deposition surrounding cancer cells (Fig. 7i), and following tissue stiffness. Strikingly, also the 8OHG, 4HNE and GFP-Grx1 redox markers follow tissue stiffness, being higher at early (softer) time points and lower at late (stiffer) time points (Fig. 7j,k).

C) We provide support to the idea that D2.0R cells are initially sensitive to ECM softness imposed by a basement-membrane ECM hydrogel in vitro, and actively remodel it into a stiffer one after two weeks in culture. YAP/TAZ nuclear localization was cytoplasmic at early time points, while after three weeks in culture we could observe a clear increase in nuclear YAP/TAZ. This was paralleled by increased pericellular Collagen-I deposition at late time-points, and by a corresponding acquisition of a spread cell morphology (Fig. 7a). Altogether this indicates ECM remodelling, and the transition to a state characterized by higher cell-ECM adhesion strength. Strikingly, at these late time-points we also observed reduced redox markers (Fig. 7b), and a corresponding increased sensitivity of cells to Cisplatin chemotherapy (Fig. 7c).

D) we included new experiments where we compare cells cultured on lung decellularized ECM from normal mice and from mice with lung fibrosis, which is known to stiffen the ECM and to reawaken D2.0R cells from quiescence (Vera-Ramirez Nat Commun 2018). Also in this case, as with Ribose-mediated stiffening, redox markers are lowered, indicating that a physiological tissue stiffening is sufficient to modulate ROS production (Fig. 7e,f and ED Fig. 7c,d). Moreover, we also infiltrated cells into lung decellularized ECM from normal mice and from mice with lung fibrosis, and we found that NRF2 target genes are downregulated upon fibrosis, while YAP/TAZ targets are activated (Fig. 7g and ED Fig. 7e). This further extends the generality of our findings by using a third independent experimental set-up.

Overall this data supports the view that differential metastatic TME stiffness regulates novel (ROS/NRF2) and established (YAP/TAZ) mechanotransduction pathways in vitro and in vivo, matching the highest standards in the mechanobiology field.

In sum, we think that the experiments proposed by the Reviewer brought several elements of novelty to the revised manuscript, reinforcing our claims. We hope that the Reviewer will share with us the excitement for these new results, and now support publication.

Decision Letter, first revision:

18th October 2021

Dear Dr. Dupont,

Thank you for submitting your revised manuscript "Mitochondrial fission links ECM mechanotransduction to metabolic redox homeostasis and metastatic chemotherapy resistance" (NCB-D44591A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

As you will see from their comments (attached below) the referees find this work of interest, but Reviewer #1 has raised some important points that should be addressed in the revised manuscript. In particular, we would require sufficient toning down of any claims on antioxidant potential upon soft culture and on mitochondrial ROS on Nrf2, as well as clearly specifying the agents to which sensitivity may be restored (please see comments from Reviewer #1).

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

Sincerely,
Daryl

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Reviewer #1 (Remarks to the Author):

In their revised manuscript, the authors provide data with additional cell lines and excluded the role of Nrf1 in the process. They also provided robust measurements of tissue stiffness using Atomic Force Microscopy. Overall, the manuscript is improved. However, there remains some concerns pertaining to the general conclusion summarized in ExFig8. The authors should adjust their conclusion to better reflect their data:

On antioxidant potential: The model proposes that induction of Nrf2 creates more antioxidant potential for cells grown in soft matrix. Indeed, chronic activation of Nrf2 is expected to increase endogenous antioxidants and to decrease ROS but there's no data to demonstrate either of the above:
• Even though there is a 10 fold increase in intracellular cystine after 24 of soft culture (Fig1), there is no evidence that total GSH levels were increased. This would suggest that the cysteine may be routed to pyruvate, methionine or taurine synthesis.
• If cystine is routed to GSH synthesis one would expect an increase in reducing equivalents to buffer ROS. However, throughout the duration of soft matrix culture, ROS levels (both cyto (Fig1j) and mito (ExFig1c) ROS) stay elevated and decrease only when the matrix stiffens on day 14 (Fig7), at which point the Nrf2 program is not active anymore (Fig7g).
• If steady state ROS levels of soft cultured cells are elevated, they should be more sensitive, not less, to oxidants eg Cumene hydroperoxide (Fig2).
For the above reasons, the authors should refrain from drawing conclusions on the accumulation of antioxidant potential upon soft culture because there is no consensus from their data in this regard.

On chemoresistance: Nrf2 activates a xenobiotic stress response program in addition to an antioxidant program (https://cancerres.aacrjournals.org/content/74/24/7430). Therefore, the observation that Nrf2 is effective to mitigate response to cisplatin but not to doxorubicin is rather unexpected (ExFig6e,f). Nrf2 is known to mediate doxorubicin resistance. In fact, a well-established mechanism of doxorubicin cardiotoxicity is actually ROS induction. Therefore, the conclusion that inhibition of DRP1 and NRF2 can restore chemotherapy sensitivity in general is not supported by data presented. The data supports restoring sensitivity to select agents, and those agents should be specified in the abstract/main text.

The conclusion that Mitochondrial ROS is proximal to Nrf2 is not supported by the data. Mitochondrial, cytoplasmic and ER ROS are all increased upon soft matrix growth. While siDRP1 mitigates this increase, the measurements were made only for mitochondrial ROS.

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Reviewer #2 (Remarks to the Author):

The authors have thoroughly addressed all of my concerns. I think this is a very comprehensive and well done manuscript.

Reviewer #3 (Remarks to the Author):

Romani and coworkers show in their revised manuscript several new experiments. Firstly, the mechanism of matrix softness-induced mitochondrial fission is further analyzed. The authors show a dependency on Spire1c rather than on Inf2, which strengthens the observation that mitochondrial fission is a direct event caused by the co-localized peri-mitochondrial F-actin and not ER-ROS. In addition, with MDIVI-1 and Driptor1a the authors demonstrate more convincingly that mtROS is downstream of mitochondrial fission. This finding adds at least some novelty with respect to previous publications, which report similar findings as Romani et al.
The revised manuscript is also better structured and easier to read. The model for the ex vivo experiments on stiffened lung ECM is convincing, confirms previous experiments and with the Casp3 measurements points to a cell-autonomous effect in their experimental model system.

2nd November 2021

Dear Dr. Dupont,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Mitochondrial fission links ECM mechanotransduction to metabolic redox homeostasis and metastatic chemotherapy resistance" (NCB-D44591A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within one week). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Mitochondrial fission links ECM mechanotransduction to metabolic redox homeostasis and metastatic chemotherapy resistance". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

Nyx Hills
Staff
Nature Cell Biology

On behalf of

Daryl J.V. David, PhD

Senior Editor, Nature Cell Biology
Consulting Editor, Nature Communications
Nature Portfolio

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Reviewer #1:
Remarks to the Author:
In their revised manuscript, the authors provide data with additional cell lines and excluded the role of Nrf1 in the process. They also provided robust measurements of tissue stiffness using Atomic Force

Microscopy. Overall, the manuscript is improved. However, there remains some concerns pertaining to the general conclusion summarized in ExFig8. The authors should adjust their conclusion to better reflect their data:

On antioxidant potential: The model proposes that induction of Nrf2 creates more antioxidant potential for cells grown in soft matrix. Indeed, chronic activation of Nrf2 is expected to increase endogenous antioxidants and to decrease ROS but there's no data to demonstrate either of the above:
• Even though there is a 10 fold increase in intracellular cystine after 24 of soft culture (Fig1), there is no evidence that total GSH levels were increased. This would suggest that the cysteine may be routed to pyruvate, methionine or taurine synthesis.
• If cystine is routed to GSH synthesis one would expect an increase in reducing equivalents to buffer ROS. However, throughout the duration of soft matrix culture, ROS levels (both cyto (Fig1j) and mito (ExFig1c) ROS) stay elevated and decrease only when the matrix stiffens on day 14 (Fig7), at which point the Nrf2 program is not active anymore (Fig7g).
• If steady state ROS levels of soft cultured cells are elevated, they should be more sensitive, not less, to oxidants eg Cumene hydroperoxide (Fig2).
For the above reasons, the authors should refrain from drawing conclusions on the accumulation of antioxidant potential upon soft culture because there is no consensus from their data in this regard.

On chemoresistance: Nrf2 activates a xenobiotic stress response program in addition to an antioxidant program (https://cancerres.aacrjournals.org/content/74/24/7430). Therefore, the observation that Nrf2 is effective to mitigate response to cisplatin but not to doxorubicin is rather unexpected (ExFig6e,f). Nrf2 is known to mediate doxorubicin resistance. In fact, a well-established mechanism of doxorubicin cardiotoxicity is actually ROS induction. Therefore, the conclusion that inhibition of DRP1 and NRF2 can restore chemotherapy sensitivity in general is not supported by data presented. The data supports restoring sensitivity to select agents, and those agents should be specified in the abstract/main text.

The conclusion that Mitochondrial ROS is proximal to Nrf2 is not supported by the data. Mitochondrial, cytoplasmic and ER ROS are all increased upon soft matrix growth. While siDRP1 mitigates this increase, the measurements were made only for mitochondrial ROS.

Authors did not address concern#3 raised previously: Measurement of steady state NAPDH is not an indicator of PPP activity. This is particularly important given that soft matrix growth induces lipid synthesis, which consumes NADPH, as described in the author's 2019 publication. Thus, a change in PPP activity cannot be excluded as contributing mechanism.

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The authors have thoroughly addressed all of my concerns. I think this is a very comprehensive and

well done manuscript.

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The revised manuscript is also better structured and easier to read. The model for the ex vivo experiments on stiffened lung ECM is convincing, confirms previous experiments and with the Casp3 measurements points to a cell-autonomous effect in their experimental model system.

Author Rebuttal, first revision:

Reviewer #1 (Remarks to the Author):

In their revised manuscript, the authors provide data with additional cell lines and excluded the role of Nrf1 in the process. They also provided robust measurements of tissue stiffness using Atomic Force Microscopy. Overall, the manuscript is improved.

(Answer to the Reviewer) We thank the Reviewer for appreciating our efforts to provide an improved manuscript.

However, there remains some concerns pertaining to the general conclusion summarized in ExFig8. The authors should adjust their conclusion to better reflect their data:

On antioxidant potential: The model proposes that induction of Nrf2 creates more antioxidant potential for cells grown in soft matrix. Indeed, chronic activation of Nrf2 is expected to increase endogenous antioxidants and to decrease ROS but there's no data to demonstrate either of the above:

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For the above reasons, the authors should refrain from drawing conclusions on the accumulation of antioxidant potential upon soft culture because there is no consensus from their data in this regard.

We thank the reviewer for pointing out these issues. It is surprising these were not brought about during the first revision, because the data the Reviewer discusses were already part of the original submission.

We respectfully disagree on the fact that our data do not support the notion of increased ability of cells to resist oxidative stress on a soft ECM. We define increased "antioxidant ability" or "antioxidant potential" as the increased NRF2-dependent ability of cells to functionally withstand oxidative stress by five treatments triggering different ROS species (cumene hydroperoxide, cisplatin, As2O3, Erastin, RSL3). Moreover, we also show by multiple assays that NRF2 is required to limit endogenous ROS production, and to limit oxidation of the reduced GSH pool (this is now in Fig. 3i,j). So, the NRF2 feedback system that we describe does actively reduce ROS and maintain the reduced pool of the GSH antioxidant - which can be thus considered "antioxidant potential" in our view.

As for the fact that cells have increased resistance even if they do not display increased GSH total levels, this is not a problem. We never stated that cysteine and glutathione synthesis is the ONLY factor that explains the ability of cells on a soft ECM to better resist oxidative stress. We used cystine uptake and glutathione redox potential as metabolic read-outs to study and demonstrate the relevance of upstream regulations, but we also think these are regulated by NRF2 as part of its broader antioxidant program. It is possible that in our cell lines NRF2 activity does not trigger enhanced steady-state GSH levels, but just a faster cycling (i.e. flow) of cysteine through the glutathione synthesis pathway, which will anyway result in heightened antioxidant defenses. It is also possible that direct disulfide exchange with exogenous cystine regenerates endogenous oxidized thiols, or that other NRF2-regulated enzymatic reactions contribute to set this increased antioxidant potential in parallel to glutathione (e.g. thioredoxins, HMOX-1, NQO1-mediated ubiquinone synthesis, increased glutamyl-amino acids). We carefully checked our claims to avoid any overinterpretation. Moreover, in response to the specific request of the Reviewer, we now reworded the abstract to better point out that the metabolic rewiring we observe is part of the NRF2 antioxidant program, and that NRF2 is responsible for increased resistance, and we included an explicit statement at the end of the paragraph "*A soft ECM empowers increased resistance to oxidative stress*":

...These results indicate that cells on a soft ECM have increased capacity to resist oxidative stress. This likely results from the global activation of the NRF2 antioxidant program, which includes but is not limited to cystine uptake and glutathione regeneration.

Finally, the Reviewer disapproves the experimental observation that elevation of steady state levels of ROS, and in particular of mitochondrial ROS, is associated with resistance to oxidants, and not to more

sensitivity. We also had the same Reviewer's expectation early on, because it was a simpler model, but we had to change our mind to make sense of the actual results that indicated a different phenomenon. Please note that a vast body of literature shows that an increase in ROS can cause increased resistance to oxidative stress, and that this happens in several cells and tissues, which is called "hormesis" or "mito-hormesis". We provided references in support of this concept, and we think this is the most conservative and fair interpretation of the experimental data.

On chemoresistance: Nrf2 activates a xenobiotic stress response program in addition to an antioxidant program (https://cancerres.aacrjournals.org/content/74/24/7430). Therefore, the observation that Nrf2 is effective to mitigate response to cisplatin but not to doxorubicin is rather unexpected (ExFig6e,f). Nrf2 is known to mediate doxorubicin resistance. In fact, a well-established mechanism of doxorubicin cardiotoxicity is actually ROS induction. Therefore, the conclusion that inhibition of DRP1 and NRF2 can restore chemotherapy sensitivity in general is not supported by data presented. The data supports restoring sensitivity to select agents, and those agents should be specified in the abstract/main text.

It is very possible that doxorubicin induces oxidative stress in the heart. However, we took care to experimentally check whether doxorubicin toxicity was functionally dependent on ROS, which was not the case in our systems (ED Fig. 7e). Then, of course we are not claiming that inhibition of DRP1 and NRF2 can restore chemotherapy sensitivity in general. We used "ROS-dependent chemotherapy" in the text, and now introduced "cisplatin" in the abstract to avoid misinterpretations.

The conclusion that Mitochondrial ROS is proximal to Nrf2 is not supported by the data. Mitochondrial, cytoplasmic and ER ROS are all increased upon soft matrix growth. While siDRP1 mitigates this increase, the measurements were made only for mitochondrial ROS.

There are two points here:

1) mtROS is not proximal to NRF2.

ROS activate NRF2 through KEAP1. An extensive literature supports the view that ROS modify multiple KEAP1 cysteine residues in a highly combinatorial and context-dependent manner, but all agree that KEAP1 modification by ROS results in decreased ability of KEAP1 to bind and inhibit NRF2. In line, we provided evidence in the revision that actomyosin regulates the amount of endogenous KEAP1/NRF2 protein complexes (Fig. 3f), and that KEAP1 is present and active in our cells (ED Fig. 5c,d,e), which is a prerequisite for its inhibition on a soft ECM. This new data reinforces previous results on nuclear accumulation of NRF2, on activation of NRF2 target genes, and on extensive functional NRF2 requirements for the downstream phenotypes (i.e. cystine uptake, glutathione redox potential, survival). The same phenotypes are also regulated by mtROS, and by upstream players that regulate mtROS production (DRP1, Spire1C, MIEF1/2). So, we believe our data are fully coherent with the proposed model where mtROS is causative for regulating NRF2 activity.

2) We only provide evidence that DRP1 regulates mtROS, and not other types of ROS.

We measured mitochondrial (MitoSOX) and cytoplasmic (DCFDA) ROS upon DRP1 knockdown and inhibition, and they are equally rescued. We did not include the DCFDA data as we thought this was redundant. Anyway, MitoTEMPO and DRP1 inhibition have coherent effects on NRF2 target genes, cystine uptake, and mtROS production, indicating the DRP1/mtROS axis is sufficient to explain the phenotypes we aimed at understanding. TUDCA (i.e. ER ROS) was not able to rescue mtROS, and we thus came to the conclusion that ER ROS represent a secondary response. It might be well possible that ER ROS activate other types of responses, but this is outside of the scope of this manuscript.

Authors did not address concern#3 raised previously: Measurement of steady state NAPDH is not an indicator of PPP activity. This is particularly important given that soft matrix growth induces lipid synthesis, which consumes NADPH, as described in the author's 2019 publication. Thus, a change in PPP activity cannot be excluded as contributing mechanism.

The reviewer originally asked to measure the PPP pathway as an alternative mechanism to explain why cells on a soft ECM display enhanced ROS. The Reviewer explicitly pointed towards PPP-dependent renewal of NADPH as the relevant parameter, which we measured, and this doesn't change, based on the established iNap1 ratiometric sensor and compared to the positive controls. It is true that soft matrix induces lipid synthesis, and that this consumes NADPH. Since NADPH is not changed, we must conclude that cells have the ability to accommodate both lipid synthesis and glutathione regeneration by rewiring other metabolic pathways. How this happens might be an interesting point to address in future. Anyway, we do not exclude PPP activity as a contributing mechanism, in addition to modulation of mitochondrial dynamics and associated mtROS.

Interpretation of the revised data is further complicated by numerous mislabelling of figure panels for example:
Figure 7C is described in the text to be glutathione oxidation data. However, the actual figure dispalys cleaved caspase 3 quantification.
Extended 5B displays Mito-tracker data, but is described in the manuscript to be Nrf2 gene signature

This has been corrected, thanks for pointing this out.

Final Decision Letter:

Dear Dr Dupont,

I am pleased to inform you that your manuscript, "Mitochondrial fission links ECM mechanotransduction to metabolic redox homeostasis and metastatic chemotherapy resistance", has

now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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Please feel free to contact us if you have any questions.

With kind regards,

Daryl J.V. David, PhD

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