nature research

Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Architecture and dynamics of a desmosome-endoplasmic reticulum complex
Corresponding author name(s): Dr Andrew Kowalczyk

Editorial Note:

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Dr Kowalczyk,

Thank you for submitting your manuscript, "Architecture and dynamics of a novel desmosome-endoplasmic reticulum organelle", to Nature Cell Biology, and thank you for your patience with the peer review process. The manuscript has now been seen by 4 referees, who are experts in intermediate filaments (Referee #1); cell-cell adhesion (Referee #2); ER (referee #3); FIB-SEM (Referee #4). As you will see from their comments (attached below), they found this work of potential interest, but have raised substantial concerns that in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

As per our standard editorial process, we have now discussed the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority. To guide the scope of the revisions, I have listed these points below. Our typical revision period is six months; 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 or anticipate any delays or issues addressing the reviews.

I should stress that the referees' concerns point to a premature dataset and concerns regarding the imaging data, systems used, and the functional relevance would need to be addressed with experiments and data. Reconsideration of the study for this journal and re-engagement of referees will depend on the strength of these revisions. In particular, in our view, strong revision efforts should be

dedicated to the following avenues:

A- quantitative and expanded analyses of the imaging data, resolving questions about the analyses of images across modalities and their interpretation, and addressing questions about the stability over time of the arrangement of the ER described:

Rev#1 "Results: By Fib-SEM ER tubules are found to be in close proximity to 16/32 desmosomes and in actual contact with 4/32 desmosomal plaques. While these statistics are unprecedented and impressive, what about those desmosomes for which an association with the ER is not seen? All if not most mature desmosomes are attached to a sizable amount of keratin IFs at their inner plaque, in epithelial cells, so why are the ER profiles "missing"? Presumably live imaging studies provide an explanation for this occurrence – the issue does come up later in the manuscript.

Results Fig. 2: The Fib-SEM data conveys what comes across as single keratin IFs traversing through ER profiles and (presumably) making contact at the inner rings of desmosomes. Comments? Accordingly it must be nearly impossible to identify such keratin filaments in TEM images, as is done in Fig. 2 E-F and G-L. Some of these assignments (through coloring) can be challenged, notably in frames E F, K and L. Comments? Note: This reviewer is struggling a bit to understand how the TEMs were generated for frames G-L. Perhaps a little schematic, as is often done when reporting of similar data, would help?

Results: The authors should show examples (and quantitation) of proximal contacts between ER and keratin filaments away from desmosomes – "data not shown" is not satisfying here given the importance of this finding."
"Results: The statistics regarding physical association/proximity between ER profiles and desosomal plaques are significant better when computed from live imaging recordings compared to Fib-SEB/EM analyses. Why is this?"
"ER and keratin IFs are both prominent elements in keratinocytes. To what extent does abundance account for the fact that they are physically proximal in many but not all instances? Related to this, to what extent are other prominent cytoskeletal elements in epithelial cells, such as F-actin, microtubules, and even adherens junctions, participating in the ER organization in epithelial cells?"

Rev#2 points #1-2-3

Rev#3 "The authors should consider adding quantitative information on how often ER tubules run parallel to keratin filaments and a supplement image showing Keratin contacts with planar ER they mention.

About half of the desmosomes in FIB-SEM are found in close (30nm) proximity. In describing Fig. 3 diffraction-limited imaging, the authors note that virtually all desmosomes were associated with ER. The EM and fluoresce microscopy observations should be reconciled: e.g. is this explainable by the limited resolution of spinning disk and thus a fraction of the two structures thought seem close are not interacting, and would that explain the fraction of unstably interacting/immobilised ER tubules? How FIB-SEM and fluorescence microscopy findings add up should be addressed.

In Fig. 4, the authors show ER tubule extension towards the reinitiated cell contact sites through light microscopy images showing the contacting components, but the cell contour is not visible. The same experiment, including phase contrast, plasma membrane or cytoplasm channel (e.g. as in Fig. 5), would be helpful to understand whether there is a cell projection forming and ER follows everywhere, or the tubule traverses ER-devoid cytoplasm reaching out to desmosomes (Is the tubule extension towards desmosome is different from general tubule extension with extending cell projection?). It may also provide a clearer picture of the sequence of events."

"How stable is the interaction beyond 2 minutes?"

Rev#4 "Fig1: on the interpretation of symmetrical organization: would there be a mean to quantify this from the segmentation? One would first need to determine the axis of symmetry to consider, e.g. one made by the junction, or an axis running perpendicular to the intermediate filaments. Then, mirror the ER tubules from one cell into the other, and measure the overlap between the projected ER and the actual ER of that cell.
The description of the mirrored organization seems subjective here, and biased towards the junction. For example, in figure 1A and in the region between the white and the blue box, one could also see a symmetrical organization of ER tubules, two branches from one cell almost in continuity with two branches from the other cell, yet, no desmosomes are visible there. What does the FIBSEM data reveal? Could that be the early stages of the formation of a new desmosome?

Video 1: the narrative in the result section does not really match the video caption, that focuses on the parallel arrangement of the IF bundle and ER, whilst the text describes the formation of ER tubule branches around the desmosome. Would be nice to coordinate this. Moreover, the ER/IF bundling here occurs in a cell protrusion that is forming the contact with the neighboring cell. One could thus argue that the "bundling" is a consequence of the space limitation created by the protrusion.
Figure 2 G-I shows a similar parallel association between ER tubules and intermediate filaments. Can the authors also show a movie of that region in order to reveal the plasma membrane and the cell organization at that spot? Is this another cell protrusion?"
"In panel B, the EM data shows a density at the contact points between an ER tubule and the bundle of intermediate filament. Is that making a ring? Any hypothesis on what it could be?"

B- Address all the revs' questions about the cell models used and requests for controls:

Rev#1 "Results: Do A431 keratinocytes stably co-expressing desmoplakin-EGFP and mApple-VAPB show normal kinetics and assembly/disassembly of cell-cell junctions in response to, for instance, manipulating extracellular calcium? How about adhesive strength?"

Rev#2 point #4

Rev#4 "They use cell lines expressing desmoplakin-EGFP. Is this over or endogenous expression?

"FigS2: typo in the legend: cryo-SIM (not cro-). Panel D shows intermediate filaments segmented in cyan. Not described in the legend. What is this cell line? Mitochondria labelling was not announced in the result section. FIB-SEM here is not done on the same cells as those described in figure S1. Why? Could the use of mitotraker alter the cell morphology?"
"Are the cells utilized for the live cell imaging the same as those imaged by FIB-SEM, importantly, comment the expression level of FP tagged proteins. Could this influence the desmosome formation? The ER/desmosome association?"

C- address the reviewers' questions for further analyses of the association of the ER with keratin filaments:

Rev#1 "Results / analysis of ER-keratin IF contacts using live imaging (Fig. 5). One would expect that, away from desmosomes, this association is NOT altered by the absence of Dsg2. Is this the case? This specific dimension of the analysis is not shared with the readers.

Results / keratin mutant / Fig. 6. The claim that keratin associates with ER whether in a properly polymerized or in an aggregated form rests on very partial data. This claim needs more rigorous substantiation."
"The authors should include data to substantiate the existence of the desmosome-keratin-ER physical triad in epithelial tissues in situ. For instance, well-curated TEM data from epidermis would likely fill that gap."

Rev#2 point #5

Rev#4 "On the concomitance of ER tubules and keratine bundle formation, quantitative analyses are missing to assess if this is a favored, induced behavior or not. Statements like "Following addition of calcium, some peripheral ER tubules extend and retract without any keratin filaments …" would benefit further quantification. Important to really support such statement: "Collectively, these experiments indicate that desmoplakin puncta formation and keratin filament assembly are initiated at sites of ER tubule extensions at nascent epithelial cell-cell contacts"."

D- examine the potential functional relevance of the observations:

Rev#2 point #6 and general comments (third paragraph of their review)

Rev#3 "The function of the ER in cell adhesion remains unclear. Establishing whether ER is necessary for desmosome formation/function and the ability to form cell junctions would advance toward this understanding. The authors should consider monitoring these upon, e.g. pulling the ER away from the periphery using the available RUSH-like tools, anchoring ER membrane to motor proteins, which were documented to be working for ER retrieval from the periphery."

E- Rev#1, Rev#2, and Rev#4 provided thoughtful suggestions for the discussion and description of the results (e.g., 'Mirror-like" instead of more direct language) that should be considered.

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-portfolio/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 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. Thank you for considering NCB for your work.

Best wishes,

Melina

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Review of Kowalczyk manuscript for NCB

In this manuscript the authors describe a connection and physical interdependency between desmosomes, keratin filaments, and the ER. This is a completely novel finding with major implications for cell biology. The key conclusions are substantiated by robust and clear data. This reviewer supports publication and recommends that the manuscript be strengthened by consideration of the following comments.

Main comments

Main text / 2nd par and Results: Is the arrangement of ER and IFs similar ("mirror image like") or truly symmetrical (indeed) in opposing cells attached to one another via desmosomes? "Symmetrical" has a strong and specific meaning and likely does not (always) apply here. The authors should be careful about this, for instance, the use of "mirror-LIKE arrangement" is in my humble view acceptable.

Results: By Fib-SEM ER tubules are found to be in close proximity to 16/32 desmosomes and in actual contact with 4/32 desmosomal plaques. While these statistics are unprecedented and impressive, what about those desmosomes for which an association with the ER is not seen? All if not most mature desmosomes are attached to a sizable amount of keratin IFs at their inner plaque, in epithelial cells, so why are the ER profiles "missing"? Presumably live imaging studies provide an explanation for this occurrence – the issue does come up later in the manuscript.

Results Fig. 2: The Fib-SEM data conveys what comes across as single keratin IFs traversing through ER profiles and (presumably) making contact at the inner rings of desmosomes. Comments? Accordingly it must be nearly impossible to identify such keratin filaments in TEM images, as is done in Fig. 2 E-F and G-L. Some of these assignments (through coloring) can be challenged, notably in frames E F, K and L. Comments? Note: This reviewer is struggling a bit to understand how the TEMs were generated for frames G-L. Perhaps a little schematic, as is often done when reporting of similar data, would help?

Results: The authors should show examples (and quantitation) of proximal contacts between ER and keratin filaments away from desmosomes – "data not shown" is not satisfying here given the importance of this finding.

Results: Do A431 keratinocytes stably co-expressing desmoplakin-EGFP and mApple-VAPB show normal kinetics and assembly/disassembly of cell-cell junctions in response to, for instance, manipulating extracellular calcium? How about adhesive strength?

Results: The statistics regarding physical association/proximity between ER profiles and desosomal plaques are significant better when computed from live imaging recordings compared to Fib-SEB/EM analyses. Why is this?

Results / Dsg2 null A431 cells (Fig. 5): The statement "Similarly, peripheral ER tubules were organized parallel to cell-cell contacts rather than orthogonally to cell-cell borders" is confusing, given that the reader is previously told that Dsg2 null A431 cells do not form cell-cell contacts.

Results / analysis of ER-keratin IF contacts using live imaging (Fig. 5). One would expect that, away from desmosomes, this association is NOT altered by the absence of Dsg2. Is this the case? This specific dimension of the analysis is not shared with the readers.

Results / keratin mutant / Fig. 6. The claim that keratin associates with ER whether in a properly polymerized or in an aggregated form rests on very partial data. This claim needs more rigorous substantiation.

Broad issues: ER and keratin IFs are both prominent elements in keratinocytes. To what extent does abundance account for the fact that they are physically proximal in many but not all instances? Related to this, to what extent are other prominent cytoskeletal elements in epithelial cells, such as F-actin, microtubules, and even adherens junctions, participating in the ER organization in epithelial cells?

Relevance issue: The authors should include data to substantiate the existence of the desmosome-keratin-ER physical triad in epithelial tissues in situ. For instance, well-curated TEM data from epidermis would likely fill that gap.

Terry Lechler's laboratory (Duke) has recently published a thorough analysis of the desmosomal interactome (Mol Biol Cell 2020). One would predict, based on the current offering, that they have come across ER membrane proteins in their analysis (see Discussion in the current study). This should be addressed by the authors.

Request for speculation: What is the cell biological/physiological significance of attachment/association with desmosomes/IFs for ER function?

Other comments

Main text / first par: The nuclear envelope is also a significant site of physical/functional interactions for the ER (and a significant location for keratin IFs as well). Why is it not mentioned anywhere in this study?

Results: The authors should clearly define what they mean when using the terms "proximal" vs. "close contact" vs. "contact", in terms of physical distances (or lack thereof).

Reference 21 should be the original description of the R125C, EBS-causing allele in KRT14 – Coulombe et al Cell 1991.

Reviewer #2:
Remarks to the Author:
Summary of the key results: This study uses high resolution volume electron microscopy to discover previously unknown nanometer-level proximity between ER-tubules, keratin filaments and desmosomes. Live imaging analysis suggests this nanometer proximity constitutes bona fide physical contact, changing stability/mobility characteristics of ER tubules proximal to desmosomes versus non-associated ER-tubules.

Assessment: The conceptual implications forwarded in this study are original: Desmosomes and their associated keratin-network may instruct peripheral distribution of the ER network; alternatively, ER-tubule-desmosome interactions may drive desmosome assembly and organization (perhaps more directly that previously surmised). These ideas have potential to expand roles of the desmosomes-intermediate filament network to the structure and function of a major cellular organelle. The conceptual advance also has potential to be broadly significant, where cell-cell adhesion alteration may prove yet another upstream stress signal that the ER registers to restore homeostasis. Alternatively, peripheral ER-plasma membrane contacts may serve to organize other junction systems (not just the desmosome). No doubt, the authors are considering all of these ideas as they move this study forward.

While I appreciate that all good studies raise more questions than they answer, I think the authors need to address at least one aspect of functional relevance to justify elevating desmosomes and associated keratins to "organelle-status" with the ER. Most experiments presented here perturb desmosome/keratin function with consequences for ER morphology, but not function. Can the authors establish a consequence of losing the peripheral distribution of the ER for the latter's function? Alternatively, is there a way to perturb an ER protein that might drive desmosome proximity? Is it possible that this Darier's disease link between the SERCA2 calcium pump and desmosome dysfunction may be due to direct contact via SERCA2 and the desmosome? Do skin cells with mutated (or lacking) SERCA2 fail to peripherally distribute the ER to desmosome contacts (or is the SERCA2 loss simply driving a general ER stress that dampens bulk-protein translation which non-specifically impacts desmosomes)? Lastly, any claim that the desmosome-keratin network and ER function as an "organelle" raises the question as to how "promiscuous versus choosy" the ER is with regards to this junction-filament system—if such connections are similarly seen for other junction-membrane complexes (as suggested in reference #22), this discovery (while potentially important) may simply change our thinking about membrane proximal versus distal (nuclear) ER compartmentalization and functon(rather than the ER specifically being shaped by the desmosome-keratin filament system). The manuscript is clearly written; the figures/image quality is stunning and notable features described are reasonably quantified with appropriate statistics. I take minor issue with language/interpretation of some experiments and suggest further clarifications as detailed below.

Major Comments:

Regarding specificity/characteristics of the ER-tubule-desmosome interaction that may inform function:

1. It is clear that ER-tubule-desmosome (Desmoplakin) coincidence/proximity occurs during desmosome biogenesis at the contact (Fig. 4). It is also appears that this interaction occurs in more mature desmosomes (Fig. 1), but less clear is whether the ER-tubule-desmosome persists through

steady state. I ask this question because many images (e.g., Fig. 5A) appear to show membrane/ER connections independent of keratin filaments. Addressing this question would help with narrowing functional consequences of this interaction.
2. Are the ER tubules associated with desmosomes "rough"? That is, do they contain docked ribosomes for co-translational translocation of membrane proteins? Or is it "smooth", indicative of lipid function proximal to the junction? Might this move towards addressing functional consequences of the interaction?

3. In Fig. 1A, I see many ER-tubules ending blindly (presumably at the plasma membrane)—indicating that not all ER-tubules end in a desmosome. I recognize that some of the structures will build a desmosome, since the ER-keratin filament arrives first (Fig. 4). But it seems that not all of these ER tubules need to participate in desmosomes. This gets to the question as to how choosy ER-tubules are at the plasma membrane—do the prefer desmosomes, or do they target many different membrane complexes? Especially since on p5, line 5 it seems only half of the desmosomes are in close proximity to an ER tubule. Please clarify.

4. Regarding the concept of a desmosome-keratin-ER "organelle"--Are the main features of this interaction seen in primary keratinocytes initiating cell-cell contact? I am insufficiently familiar with the A431 cells to know if the classical cadherin-catenin system is sufficiently robust to know whether this newly defined organelle is strange feature of this skin cancer cell line. Would you expect the density of ER-tubules at the periphery to be denser in keratinocytes than cells with less prominent desmosomes?

5. The pathogenic keratin mutant impacting ER structure is intriguing, but the authors noted that the ER at the periphery is tubular, whereas it is sheet-like more distal from the plasma membrane. This raises the question whether the effect of the keratin mutant reflects a new ER phenotype- or have these cells simply lost the mechanism to disperse the ER to the periphery (where the ER converts to its more nuclear proximal morphology)? Does the pathogenic keratin mutant disrupt the ER as much as loss of cell-cell contact? Or is there something visually different?

6. Muddled messaging: It is challenging to address functionality of these intriguing desmosome/ER contacts when the ER is already essential to membrane biogenesis (independently of direct contact with the desmosome, the ER is required to synthesize desmocollins/desmogleins, likely coordinate their co-translational assembly with plakoglobin/plakophilins/desmoplakin). Also challenging is considering how perturbation of ER/desmosome interactions would register an ER stress signal, when the vast majority of the ER may be unperturbed. Still, I encourage the authors to think through these options, and seek evidence for a functional consequence of a desmosome-keratin-ER network that rises beyond the important morphological analyses carried out thus far.

Minor Issues:

7. At ~lines 10/20, the authors speak of the "symmetrical configuration" and "mirror image" of ER relationship to the desmosome. I find this language a bit strong… since there is such variability in the ER structure proximal to the membrane. Maybe "quasi-symmetrical" is better?

8. It generally seems that the keratin intermediate filaments "pierce" or thread the ER tubule system, rather than guide or pattern it (Figures 1&2), especially since a full cell enface view is not shown and the co-alignment appears to change with junctional maturation.

9. Figure 4 seems to suggest that nascent ER-membrane contact occurs just before desmoplakin accumulates/coalesces, implying a critical role in desmosome biogenesis, but DP dots are everywhere (e.g., membrane distal), so ER "contact" appears not required for coalescence of DP. Please clarify,

given previous papers from the Green Lab showing DP coalescence further behind the membrane. Asked another way, is DP coalescence dependent on the ER network generally, rather than specifically at the contact?

10. Figure 4F-J appears mislabeled (DP instead of Krt14).

Reviewer #3:
Remarks to the Author:
In this manuscript, entitled Architecture and dynamics of a novel desmosome-endoplasmic reticulum organelle, the authors describe the hitherto unknown phenomenon of desmosome-endoplasmic reticulum close interaction. This is a significant addition toward a complete picture of the functional dynamic cell interior. They provide an exceptional quality electron microscopy imagery visualising this in a cleverly selected cell model with identifiable and manipulatable desmosomes. The authors demonstrate a close association of desmosomes and keratin intermediate filaments and provide characterisation info quantifying these interactions in space. They correlate their observations from fixed cells' FIB-SEM with dynamic imagery of fluorescence microscopy, adding a time dimension to their characterisation of the interaction of the ER-desmosome-keratin filament. This revealed critical details such as the stability of the interactions, the attraction of ER extending ER tubules towards newly forming desmosome-associated cell contacts, and ER tubules' immobilising effect upon desmosome ER docking. The study seems to leave the aspects of how the phenomenon is enabled or its functional purpose.

The study presentation, use of data analysis, rendering and statistics is overall excellent, and it represents a significant advance in cell biology worthy of high visibility publication (postrevision).

Comments:
The authors should consider modifying the title since the organelle interactions they describe may not constitute a conventional organelle

The authors should consider adding quantitative information on how often ER tubules run parallel to keratin filaments and a supplement image showing Keratin contacts with planar ER they mention.

About half of the desmosomes in FIB-SEM are found in close (30nm) proximity. In describing Fig. 3 diffraction-limited imaging, the authors note that virtually all desmosomes were associated with ER. The EM and fluoresce microscopy observations should be reconciled: e.g. is this explainable by the limited resolution of spinning disk and thus a fraction of the two structures thought seem close are not interacting, and would that explain the fraction of unstably interacting/immobilised ER tubules? How FIB-SEM and fluorescence microscopy findings add up should be addressed.

In Fig. 4, the authors show ER tubule extension towards the reinitiated cell contact sites through light microscopy images showing the contacting components, but the cell contour is not visible. The same experiment, including phase contrast, plasma membrane or cytoplasm channel (e.g. as in Fig. 5), would be helpful to understand whether there is a cell projection forming and ER follows everywhere, or the tubule traverses ER-devoid cytoplasm reaching out to desmosomes (Is the tubule extension towards desmosome is different from general tubule extension with extending cell projection?). It may also provide a clearer picture of the sequence of events.

How stable is the interaction beyond 2 minutes?

The authors should expand the discussion of the observations' meaning, e.g. Is the fraction of not ER-

associated desmosomes represent their different functional states or is ER, not an absolute requirement?
The function of the ER in cell adhesion remains unclear. Establishing whether ER is necessary for desmosome formation/function and the ability to form cell junctions would advance toward this understanding. The authors should consider monitoring these upon, e.g. pulling the ER away from the periphery using the available RUSH-like tools, anchoring ER membrane to motor proteins, which were documented to be working for ER retrieval from the periphery.

The authors seem to suggest a stress-sensing-related function of the phenomenon they have discovered in their Discussion. However, the justification of why this aspect, in particular, is the focus of the brief Discussion of the phenomenon's potential function is unclear.

Reviewer #4:
Remarks to the Author:
Authors use volume EM and live cell imaging to study at high subcellular resolution and the dynamics of the interactions between ER and desmosomes. They establish that desmosomes and intermediate filaments bundles are able to regulate the dynamics and the topology of the ER network.

Data quality is very high, especially FIB-SEM, which impressively reveals fine structures such as IF bundles. Such resolution is rarely achieved with this technique. The perturbation of the desmosomes and/or keratin filaments confirm the functional link between ER and cytoskeleton which seem involved also in pathological states, as suggested by results obtained on cellular models of skin epithelia disorders.

The paper is very well written, results supported by clear and concise figures, methods described in great and intelligible details.
The authors nevertheless might stress to much the hypothesized symmetric arrangement of ER of paired cells across desmosomes, which might not be strongly supported by data (see below). ER is indeed present on both sides, but a mirrored organization is discussable. They may want to tune down by avoiding too much repetition.

Some minor details

They use cell lines expressing desmoplakin-EGFP. Is this over or endogenous expression?

FigS1 ABC: ER is not symmetric ally organized. Same in TEM, the fact that ER is visible in both partner cells of the junction doesn't indicate a symmetrical organization.

FigS2: typo in the legend: cryo-SIM (not cro-). Panel D shows intermediate filaments segmented in cyan. Not described in the legend. What is this cell line? Mitochondria labelling was not announced in the result section. FIB-SEM here is not done on the same cells as those described in figure S1. Why? Could the use of mitotraker alter the cell morphology?
In panel B, the EM data shows a density at the contact points between an ER tubule and the bundle of intermediate filament. Is that making a ring? Any hypothesis on what it could be?

Fig1: on the interpretation of symmetrical organization: would there be a mean to quantify this from the segmentation? One would first need to determine the axis of symmetry to consider, e.g. one made by the junction, or an axis running perpendicular to the intermediate filaments. Then, mirror the ER tubules from one cell into the other, and measure the overlap between the projected ER and the actual ER of that cell.

The description of the mirrored organization seems subjective here, and biased towards the junction. For example, in figure 1A and in the region between the white and the blue box, one could also see a symmetrical organization of ER tubules, two branches from one cell almost in continuity with two branches from the other cell, yet, no desmosomes are visible there. What does the FIBSEM data reveal? Could that be the early stages of the formation of a new desmosome?

Video 1: the narrative in the result section does not really match the video caption, that focuses on the parallel arrangement of the IF bundle and ER, whilst the text describes the formation of ER tubule branches around the desmosome. Would be nice to coordinate this. Moreover, the ER/IF bundling here occurs in a cell protrusion that is forming the contact with the neighboring cell. One could thus argue that the "bundling" is a consequence of the space limitation created by the protrusion.
Figure 2 G-I shows a similar parallel association between ER tubules and intermediate filaments. Can the authors also show a movie of that region in order to reveal the plasma membrane and the cell organization at that spot? Is this another cell protrusion?

The demonstration of IF bundles traversing ER sheets is impressive (fig 2A-C and video3).

Are the cells utilized for the live cell imaging the same as those imaged by FIB-SEM, importantly, comment the expression level of FP tagged proteins. Could this influence the desmosome formation? The ER/desmosome association?

On the concomitance of ER tubules and keratine bundle formation, quantitative analyses are missing to assess if this is a favored, induced behavior or not. Statements like "Following addition of calcium, some peripheral ER tubules extend and retract without any keratin filaments …" would benefit further quantification. Important to really support such statement: "Collectively, these experiments indicate that desmoplakin puncta formation and keratin filament assembly are initiated at sites of ER tubule extensions at nascent epithelial cell-cell contacts".

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]."

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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.

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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.

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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

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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.

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Author Rebuttal to Initial comments

Response to Reviewers' Comments

Reviewer #1:

In this manuscript the authors describe a connection and physical interdependency between desmosomes, keratin filaments, and the ER. This is a completely novel finding with major implications for cell biology. The key conclusions are substantiated by robust and clear data. This reviewer supports publication and recommends that the manuscript be strengthened by consideration of the following comments.

Authors' response: We thank the reviewer for the encouraging assessment of the findings.

Main comments

Main text / 2nd par and Results: Is the arrangement of ER and IFs similar ("mirror image like") or truly symmetrical (indeed) in opposing cells attached to one another via desmosomes? "Symmetrical" has a strong and specific meaning and likely does not (always) apply here. The authors should be careful about this, for instance, the use of "mirror-LIKE arrangement" is in my humble view acceptable.

Authors' Response: We attempted to convey in simple terms that ER tubules are seen on either side of a desmosomal cell-cell contact, much like keratin filaments. We have changed the terminology to "mirror image-like arrangements" or "paired ER structures" rather than mirror image. In addition, we include new data that quantitatively assess the presence of ER on each cytoplasmic face of the desmosome to

quantify the mirror image-like organization of the desmosome-ER complex (Extended Data Fig. 4 and Supplementary Figure 4).

Results: By Fib-SEM ER tubules are found to be in close proximity to 16/32 desmosomes and in actual contact with 4/32 desmosomal plaques. While these statistics are unprecedented and impressive, what about those desmosomes for which an association with the ER is not seen? All if not most mature desmosomes are attached to a sizable amount of keratin IFs at their inner plaque, in epithelial cells, so why are the ER profiles "missing"? Presumably live imaging studies provide an explanation for this occurrence – the issue does come up later in the manuscript.

Authors' Response: ER tubules are highly dynamic, and it is possible that some ER tubules were retracted from desmosomes at the moment of sample fixation for subsequent FIB-SEM. In addition, as discussed below, when we relax the criteria for association from 30nm to 250nm, we observe that ~94% of desmosomes (31/33) are in proximity to ER. These data are more easily compared to optical imaging indicating that ER tubules are associated with virtually all desmosomes and are stabilized by keratin filament bundles attached to desmosomes.

Results Fig. 2: The Fib-SEM data conveys what comes across as single keratin IFs traversing through ER profiles and (presumably) making contact at the inner rings of desmosomes. Comments? Accordingly it must be nearly impossible to identify such keratin filaments in TEM images, as is done in Fig. 2 E-F and G-L. Some of these assignments (through coloring) can be challenged, notably in frames E F, K and L. Comments? Note: This reviewer is struggling a bit to understand how the TEMs were generated for frames G-L. Perhaps a little schematic, as is often done when reporting of similar data, would help?

Authors' Response: We apologize for the lack of clarity here. New images in Fig. 2 and Extended Data Fig. 3 show orthoslices of FIB-SEM data, which enables identification of keratin filaments as electron-dense structures. No TEM imaging is done in this figure. We have also included raw FIB-SEM data in Fig. 2 that shows identifiable keratin filaments. Also included is a better view that makes this point, along with a new movie (Supplementary Videos 2 and 3). We avoid segmenting the inner dense plaque (IDP), particularly in the 4x4x4nm^3 FIB-SEM dataset, as these regions appear "hazy". Thus, we are not confident in truly delineating the IDP. However, we do notice these keratin filaments terminating at this hazy IDP.

Results: The authors should show examples (and quantitation) of proximal contacts between ER and keratin filaments away from desmosomes – "data not shown" is not satisfying here given the importance of this finding.

We present new images, segmentations, and quantification of contacts between ER-keratin vs ER-microtubules at regions proximal (within 250nm) and distal (>1μm) to desmosomes (Fig. 2K, Extended Data Fig. 4, Extended Data Fig. 5, and Supplementary Figure 4). Additional examples of ER-keratin contacts away from desmosomes are visible in Supplementary Figure 3.

Results: Do A431 keratinocytes stably co-expressing desmoplakin-EGFP and mApple-VAPB show normal kinetics and assembly/disassembly of cell-cell junctions in response to, for instance, manipulating extracellular calcium? How about adhesive strength?

Authors' Response: We have not seen qualitative differences in desmosome formation kinetics. Desmosome kinetics are on par with previous reports using A431 cell lines (Godsel et al., 2005). In addition, we have now used lumenal ER markers and see a similar ER-keratin association as obtained with cells expressing VAPB [Redacted].. We are currently manipulating the expression of several ER proteins to determine which molecules mediate ER-desmosome associations and how perturbation of these proteins affects cell adhesion. We believe that these experiments are beyond the scope of this manuscript.

[Redacted]

Results: The statistics regarding physical association/proximity between ER profiles and desosomal plaques are significant better when computed from live imaging recordings compared to Fib-SEB/EM analyses. Why is this?

Authors' Response: These differences are due to the resolution differences of the approaches. In the case of our optical imaging system, x/y resolution is in the range of ~250nm whereas the FIB-SEM approaches 4nm isotropic resolution. Thus, desmosomes and ER that exhibit pixel overlap by optical imaging (i.e., in "contact") might not be scored within 30 nm when assessed by FIB-SEM. In the original manuscript, we reported that 50% of desmosomes (16/32) in the FIB-SEM datasets are within 30nm of an ER tubule, but have corrected this number to 42% after improved denoising of FIB-SEM data using an updated version of Noise2Void (N2V2). We now include quantification that demonstrates 94% of desmosomes (n=31/ 33) are within 250nm of an ER tubule when assessed by FIB-SEM (Results, page 4). This new analysis reconciles the differences between the imaging modalities.

Results / Dsg2 null A431 cells (Fig. 5): The statement "Similarly, peripheral ER tubules were organized parallel to cell-cell contacts rather than orthogonally to cell-cell borders" is confusing, given that the reader is previously told that Dsg2 null A431 cells do not form cell-cell contacts.

Authors' Response: A431 cells lacking desmosomes continue to form cell-cell interactions mediated by adherens junctions. We apologize for the inconsistency in terminology. In this context, cell-cell contact and cell-cell border mean the same thing, i.e., the contact between the plasma membranes of two or more cells. We have tried to clarify this point and the terminology in the revised manuscript.

Results / analysis of ER-keratin IF contacts using live imaging (Fig. 5). One would expect that, away from desmosomes, this association is NOT altered by the absence of Dsg2. Is this the case? This specific dimension of the analysis is not shared with the readers.

Authors' response: Two possibilities might explain our observation that ER dynamics change at regions of the cell distal to cell-cell contacts when desmosomes are absent. First, it is possible that keratin filaments become more dynamic in all regions of the cell when desmosomes are absent. ER dynamics would increase as keratin dynamics increase. Alternatively, or in addition, it is possible that global

cellular ER dynamics increase when desmosomes are disrupted due to some change in ER-keratin associations globally throughout the cell, or through some other unknown mechanism.

Results / keratin mutant / Fig. 6. The claim that keratin associates with ER whether in a properly polymerized or in an aggregated form rests on very partial data. This claim needs more rigorous substantiation.

Authors' response: We have now included quantitative analyses demonstrating that ~75-79% of the keratin aggregates maintain contact with ER membranes over a 2-minute time course (Extended Data Fig. 10I).

Broad issues: ER and keratin IFs are both prominent elements in keratinocytes. To what extent does abundance account for the fact that they are physically proximal in many but not all instances? Related to this, to what extent are other prominent cytoskeletal elements in epithelial cells, such as F-actin, microtubules, and even adherens junctions, participating in the ER organization in epithelial cells?

Authors' Response: Microtubules have well-established roles in ER tubule elongation and transport, and actin has recently been shown to regulate ER recruitment to endosomes, facilitating endosomal fission in COS-7 cells (Striepen and Voeltz, 2022). We are not able to reliably identify actin in our FIB-SEM images. New data shown in Extended Data Fig. 5 indicate that ER makes more frequent and larger contacts with keratin filaments than with microtubules, particularly near the desmosome. The observation that ER-keratin contacts are larger than ER-microtubule contacts suggests that simple abundance of these structures does not explain the stability and geometry of their physical proximity.

With respect to adherens junctions, we believe it is likely that these structures also influence ER organization. Our preliminary experiments suggest that ER retracts from the cell periphery when AJs are disrupted. We respectfully suggest that the structural and function influence of AJs and actin on ER be left for subsequent analysis as this issue will take considerable time to carefully analyze.

Relevance issue: The authors should include data to substantiate the existence of the desmosome-keratin-ER physical triad in epithelial tissues in situ. For instance, well-curated TEM data from epidermis would likely fill that gap.

Authors' Response: New TEM data documenting ER-desmosome associations in several tissues is included in the revised manuscript, including rat skin and colon (Extended Data Fig. 2).

Terry Lechler's laboratory (Duke) has recently published a thorough analysis of the desmosomal interactome (Mol Biol Cell 2020). One would predict, based on the current offering, that they have come across ER membrane proteins in their analysis (see Discussion in the current study). This should be addressed by the authors.

Authors' Response: We thank the reviewer for raising this point. Indeed, the MBC 2020 proteomics work from the Lechler lab identified a number of ER proteins as desmoplakin binding partners, including the ER shaping protein reticulon-4 and the calcium pump SERCA2. This work is now cited and discussed.

Request for speculation: What is the cell biological/physiological significance of attachment/association with desmosomes/IFs for ER function?

Authors' Response: We include new data linking disruption of keratin filament organization and desmosomal adhesion to ER stress pathways (Fig. 7). Please see reply to R2 below for further details.

Other comments

Main text / first par: The nuclear envelope is also a significant site of physical/functional interactions for the ER (and a significant location for keratin IFs as well). Why is it not mentioned anywhere in this study?

Authors' Reply: We agree and now include mention of previous studies demonstrating a physical contact between keratin and the nuclear envelope (which is an extension of the ER network) (Jorgens et al., 2017), as well as the effect of KRT14 loss on the size and shape of the nucleus in epidermal keratinocytes (Lee et al., 2012) in the Discussion section.

Results: The authors should clearly define what they mean when using the terms "proximal" vs. "close contact" vs. "contact", in terms of physical distances (or lack thereof).

Authors' Response: We have tried to clarify these terms throughout the manuscript in the context of different imaging modalities. For FIB-SEM datasets, we have now included definitions for "proximal" (within 250nm) and "distal" (>1000nm) to a desmosome. We also clarify whether two structures/ organelle classes are in physical contact (assessed in FIB-SEM datasets) or within a certain distance from an organelle class. For optical imaging data, we have softened the term "DP-ER contact" to "DP-ER colocalization", to differentiate between the 4-8nm x-y resolution of the FIB-SEM data and the ~250nm x-y resolution of confocal microscopy data.

Reference 21 should be the original description of the R125C, EBS-causing allele in KRT14 – Coulombe et al Cell 1991.

Authors' Response: The primary and corresponding author are both well aware of this publication and use the paper routinely in research seminars and didactic lectures. We apologize for the omission and now include the citation.

Reviewer #2:

Summary of the key results: This study uses high resolution volume electron microscopy to discover previously unknown nanometer-level proximity between ER-tubules, keratin filaments

and desmosomes. Live imaging analysis suggests this nanometer proximity constitutes bona fide physical contact, changing stability/mobility characteristics of ER tubules proximal to desmosomes versus non-associated ER-tubules.

Assessment: The conceptual implications forwarded in this study are original: Desmosomes and their associated keratin-network may instruct peripheral distribution of the ER network; alternatively, ER-tubule-desmosome interactions may drive desmosome assembly and organization (perhaps more directly that previously surmised). These ideas have potential to expand roles of the desmosomes-intermediate filament network to the structure and function of a major cellular organelle. The conceptual advance also has potential to be broadly significant, where cell-cell adhesion alteration may prove yet another upstream stress signal that the ER registers to restore homeostasis. Alternatively, peripheral ER-plasma membrane contacts may serve to organize other junction systems (not just the desmosome). No doubt, the authors are considering all of these ideas as they move this study forward.

While I appreciate that all good studies raise more questions than they answer, I think the authors need to address at least one aspect of functional relevance to justify elevating desmosomes and associated keratins to “organelle-status” with the ER. Most experiments presented here perturb desmosome/keratin function with consequences for ER morphology, but not function. Can the authors establish a consequence of losing the peripheral distribution of the ER for the latter’s function? Alternatively, is there a way to perturb an ER protein that might drive desmosome proximity? Is it possible that this Darier’s disease link between the SERCA2 calcium pump and desmosome dysfunction may be due to direct contact via SERCA2 and the desmosome? Do skin cells with mutated (or lacking) SERCA2 fail to peripherally distribute the ER to desmosome contacts (or is the SERCA2 loss simply driving a general ER stress that dampens bulk-protein translation which non-specifically impacts desmosomes)?

Lastly, any claim that the desmosome-keratin network and ER function as an “organelle” raises the question as to how “promiscuous versus choosy” the ER is with regards to this junction-filament system—if such connections are similarly seen for other junction-membrane complexes (as suggested in reference #22), this discovery (while potentially important) may simply change our thinking about membrane proximal versus distal (nuclear) ER compartmentalization and functon(rather than the ER specifically being shaped by the desmosome-keratin filament system). The manuscript is clearly written; the figures/image quality is stunning and notable features described are reasonably quantified with appropriate statistics. I take minor issue with language/interpretation of some experiments and suggest further clarifications as detailed below.

Authors’ response: We thank the reviewer for the overall positive assessment of the data and presentation. Most experiments presented in the original submission perturb desmosome/keratin function with consequences for ER morphology, but not function. We speculated that ER association with desmosomes might function as a stress sensor. To test this possibility directly, we disrupted desmosomes using IgG isolated from patients with the skin disease pemphigus vulgaris. In this disease, IgG directed

against the desmosomal cadherin desmoglein-3 (Dsg3) are well known to cause desmosome disruption, keratin filament retraction and loss of adhesion. Keratinocytes treated with IgG that block Dsg3 adhesive function exhibited an ER stress response with the induction of XBP1s, HSPA5, and ATF3 (Fig. 7M). We also demonstrate that A431 cells expressing the K14^{R125C} aggregation mutant show an induction of the ER stress marker, ATF3 (Fig. 7L). These new data indicate that one function of the ER-desmosome complex is to couple cell adhesion to ER stress pathways.

Further, we provide new experiments with thapsigargin (TG), an inhibitor of the ER calcium pump SERAC2, which has been shown to inhibit desmosome formation in cultured cells (Hobbs et al., 2011). When we pre-treat cells with TG followed by a calcium switch, no desmoplakin puncta appear at ~51% of cell-cell contacts. However, ER tubules still extended toward the cell periphery (Extended Data Fig. 9). These data suggest a role for ER tubule regulation of local calcium in desmosome assembly.

As outlined in the discussion, we predict that the ER-desmosome association will function in other aspects of cell physiology including plasma membrane lipid regulation and calcium homeostasis. We respectfully suggest that revealing the entire range of functions of this complex will be the subject of future studies by our group and others.

Major Comments:

Regarding specificity/characteristics of the ER-tubule-desmosome interaction that may inform function:

1. It is clear that ER-tubule-desmosome (Desmoplakin) coincidence/proximity occurs during desmosome biogenesis at the contact (Fig. 4). It is also appears that this interaction occurs in more mature desmosomes (Fig. 1), but less clear is whether the ER-tubule-desmosome persists through steady state. I ask this question because many images (e.g., Fig. 5A) appear to show membrane/ER connections independent of keratin filaments. Addressing this question would help with narrowing functional consequences of this interaction.

Authors' Response: We agree that there are numerous ER-membrane interactions that occur independently of desmosomes and keratin filaments. Data in Fig. 3C-F show that ER present at steady state desmosomes is more stable than ER at non-desmosomal regions near the plasma membrane. In addition, the data in Fig. 6C-F (old Fig. 5) indicate that disrupting desmosomes increases the dynamic behavior of ER. Collectively, the data indicate that ER associates with desmosomes during initial formation and is then stabilized at these sites as desmosomes reach steady state.

2. Are the ER tubules associated with desmosomes "rough"? That is, do they contain docked ribosomes for co-translational translocation of membrane proteins? Or is it "smooth", indicative of lipid function proximal to the junction? Might this move towards addressing functional consequences of the interaction?

Authors' Response: Newly added segmentations for ribosomes in FIB-SEM datasets indicate that ER tubules near desmosomes do have ribosomes, but that most of the tubules that extend toward the desmosome plaque and plasma membrane are relatively smooth ER tubules (Supplementary Figure 2). It

has been reported that RNA processing machinery/ RISC complexes are present at adherens junctions (Kourtidis et al., 2017), but this has not yet been reported for desmosomes.

3. In Fig. 1A, I see many ER-tubules ending blindly (presumably at the plasma membrane)—indicating that not all ER-tubules end in a desmosome. I recognize that some of the structures will build a desmosome, since the ER-keratin filament arrives first (Fig. 4). But it seems that not all of these ER tubules need to participate in desmosomes. This gets to the question as to how choosy ER-tubules are at the plasma membrane—do the prefer desmosomes, or do they target many different membrane complexes? Especially since on p5, line 5 it seems only half of the desmosomes are in close proximity to an ER tubule. Please clarify.

Authors' Response: Our data indicate that some ER tubules extend out to desmosomes, and some extend to non-desmosomal regions of the plasma membrane. The revised manuscript includes new segmentations in the FIB-SEM datasets showing that some ER tubules near the plasma membrane are in close proximity to organelles such as endosomes and occasionally with microtubules (Fig. 2, Extended Data Fig. 4, Extended Data Fig. 5, and Supplementary Figure 4). We also agree that some of these ER tubules that are not visibly associated with desmosomes could represent ER tubules destined to participate in desmosome assembly. Thus, while ER associates with other structures near the plasma membrane, quantitative analysis indicates that the vast majority of desmosomes (31/33) analyzed by FIB-SEM are associated with or proximal to ER.

4. Regarding the concept of a desmosome-keratin-ER "organelle"--Are the main features of this interaction seen in primary keratinocytes initiating cell-cell contact? I am insufficiently familiar with the A431 cells to know if the classical cadherin-catenin system is sufficiently robust to know whether this newly defined organelle is strange feature of this skin cancer cell line. Would you expect the density of ER-tubules at the periphery to be denser in keratinocytes than cells with less prominent desmosomes?

Authors' Response: Yes, the main features of the ER-desmosome association occur in multiple epithelial cell types and tissues as observed by both light microscopy (Extended Data Fig. 1) and by transmission EM imaging of both cultured cells and tissues (Extended Data Fig. 2). Based on the observation that knocking out Dsg2 in A431 cells, we do predict that ER peripheral organization would be different in various epithelial cell types. However, ER association with desmosomes appears to be a feature of most or all desmosomes in different cell types.

5. The pathogenic keratin mutant impacting ER structure is intriguing, but the authors noted that the ER at the periphery is tubular, whereas it is sheet-like more distal from the plasma membrane. This raises the question whether the effect of the keratin mutant reflects a new ER phenotype- or have these cells simply lost the mechanism to disperse the ER to the periphery (where the ER converts to its more nuclear proximal morphology)? Does the pathogenic keratin mutant disrupt the ER as much as loss of cell-cell contact? Or is there something visually different?

Authors' response: The data suggest that the disruption of desmosomes and expression of the K14 mutant cause two different effects. The Dsg2KO cell line exhibits a decrease in the density of peripheral ER tubules proximal to the plasma membrane. In contrast, expression of the K14 EBS mutant causes loss of tubules and more planar/sheet-like ER. Additional studies will be needed to understand the underlying basis for these changes in ER morphology.

6. Muddled messaging: It is challenging to address functionality of these intriguing desmosome/ER contacts when the ER is already essential to membrane biogenesis (independently of direct contact with the desmosome, the ER is required to synthesize desmocollins/desmogleins, likely coordinate their co-translational assembly with plakoglobin/plakophilins/desmoplakin). Also challenging is considering how perturbation of ER/desmosome interactions would register an ER stress signal, when the vast majority of the ER may be unperturbed. Still, I encourage the authors to think through these options, and seek evidence for a functional consequence of a desmosome-keratin-ER network that rises beyond the important morphological analyses carried out thus far.

Authors' Response: We thank the reviewer for these suggestions. As discussed above, new data are shown in Fig. 7 linking desmosome and keratin perturbations with ER stress signaling.

Minor Issues:

7. At ~lines 10/20, the authors speak of the "symmetrical configuration" and "mirror image" of ER relationship to the desmosome. I find this language a bit strong… since there is such variability in the ER structure proximal to the membrane. Maybe "quasi-symmetrical" is better?

Authors' Response: Please see response above to R1. We have changed the terminology to "mirror image-like arrangements" or "paired ER structures" rather than mirror image. In addition, we include new data that quantitatively assess the presence of ER on each cytoplasmic face of the desmosome to quantify the mirror image-like organization of the desmosome-ER complex (Extended Data Fig. 4 and Supplementary Figure 4).

8. It generally seems that the keratin intermediate filaments "pierce" or thread the ER tubule system, rather than guide or pattern it (Figures 1&2), especially since a full cell enface view is not shown and the co-alignment appears to change with junctional maturation.

Authors' Response: At present, we cannot discern whether keratin filaments pierce ER tubules, but new quantification in Extended Data Fig. 4 and Supplementary Figure 4 suggest that ER and keratin share a paired "symmetry" compared to microtubules or endosomes, particularly at more mature desmosomes, i.e., presence of radial keratin filament bundles on either cytoplasmic face of the desmosome.

9. Figure 4 seems to suggest that nascent ER-membrane contact occurs just before desmoplakin accumulates/coalesces, implying a critical role in desmosome biogenesis, but DP dots are everywhere (e.g., membrane distal), so ER "contact" appears not required for coalescence of DP. Please clarify, given previous papers from the Green Lab showing DP coalescence further behind the membrane. Asked another way, is DP coalescence dependent on the ER network generally, rather than specifically at the contact?

Authors' response: We thank the reviewer for this comment. We are likewise intrigued by the timing of the DP coalescence at sites of ER tubule extension. There is a growing body of evidence, especially for tight junctions, that phase condensation drives junction organization. We suspect that ER might regulate this kind of process for DP at nascent junctions. Interestingly, live cell imaging indicates that DP dots in the cytoplasm are in continual contact with ER tubules (not shown). We are very keen to address the role of ER in regulating DP coalescence at nascent cell contacts and within the cell interior as we move forward with this project.

10. Figure 4F-J appears mislabeled (DP instead of Krt14).

Authors' response: The error has been corrected.

Reviewer #3:

Remarks to the Author:

In this manuscript, entitled Architecture and dynamics of a novel desmosome-endoplasmic reticulum organelle, the authors describe the hitherto unknown phenomenon of desmosome-endoplasmic reticulum close interaction. This is a significant addition toward a complete picture of the functional dynamic cell interior. They provide an exceptional quality electron microscopy imagery visualising this in a cleverly selected cell model with identifiable and manipulatable desmosomes. The authors demonstrate a close association of desmosomes and keratin intermediate filaments and provide characterisation info quantifying these interactions in space. They correlate their observations from fixed cells' FIB-SEM with dynamic imagery of fluorescence microscopy, adding a time dimension to their characterisation of the interaction of the ER-desmosome-keratin filament. This revealed critical details such as the stability of the interactions, the attraction of ER extending ER tubules towards newly forming desmosome-associated cell contacts, and ER tubules' immobilising effect upon desmosome ER docking. The study seems to leave the aspects of how the phenomenon is enabled or its functional purpose.

The study presentation, use of data analysis, rendering and statistics is overall excellent, and it represents a significant advance in cell biology worthy of high visibility publication (postrevision).

Authors' Response: We thank the reviewer for the encouraging assessment of the primary findings in the manuscript.

Comments:

The authors should consider modifying the title since the organelle interactions they describe may not constitute a conventional organelle

Authors' response: The title and any other instances referring to this association as an "organelle" have been modified to refer to this structure as an ER-desmosome-keratin "complex".

The authors should consider adding quantitative information on how often ER tubules run parallel to keratin filaments and a supplement image showing Keratin contacts with planar ER they mention.

Authors' Response: We now include representative figure of planar ER and keratin in regions distal to desmosomes (Supplementary Figure 3). We have removed reference to the "parallel" orientation of ER and keratin arrangements to avoid overstating the frequency of that morphology.

About half of the desmosomes in FIB-SEM are found in close (30nm) proximity. In describing Fig. 3 diffraction-limited imaging, the authors note that virtually all desmosomes were associated with ER. The EM and fluoresce microscopy observations should be reconciled: e.g. is this explainable by the limited resolution of spinning disk and thus a fraction of the two structures thought seem close are not interacting, and would that explain the fraction of unstably interacting/immobilised ER tubules? How FIB-SEM and fluorescence microscopy findings add up should be addressed.

Authors' Response: Please see response to Rev#1 comment above regarding reconciling the FIB-SEM and optical data sets. Yes, we do believe that the resolution differences explain the discrepancy. New analysis shows that relaxing the criteria for "association" in the FIB-SEM data sets from 30nm to 250nm results in good alignment between the data sets. Please see Results, page 5.

In Fig. 4, the authors show ER tubule extension towards the reinitiated cell contact sites through light microscopy images showing the contacting components, but the cell contour is not visible. The same experiment, including phase contrast, plasma membrane or cytoplasm channel (e.g. as in Fig. 5), would be helpful to understand whether there is a cell projection forming and ER follows everywhere, or the tubule traverses ER-devoid cytoplasm reaching out to desmosomes (Is the tubule extension towards desmosome is different from general tubule extension with extending cell projection?). It may also provide a clearer picture of the sequence of events.

Authors' Response: New data are provided using fluorescently tagged WGA to highlight the plasma membrane as cell-cell contact is initiated (Supplementary Figure 5). The data show that cell membranes of adjacent cells contact each other prior to the extension of ER tubules to cell-cell contacts.

How stable is the interaction beyond 2 minutes?

Authors' Response: In Extended Data Fig. 8, we observe that ER tubules interact with fusing desmosomes and maintain contact with newly-fused desmosomes over a 30 min time course. One caveat to these longer time course experiments is that imaging is taken at 30 sec intervals to avoid phototoxicity and photobleaching, so we are very likely missing some ER-desmosome dissociation events. However, new data in the revised manuscript demonstrates that ER tubules remain at desmosomes after disruption of microtubules, even as the peripheral ER at cell-free edges retracts toward the nucleus (New Fig. 4, Extended Data Fig. 7). Together, these findings suggest a tight and stable association between ER and the keratin-desmosome complex.

The authors should expand the discussion of the observations' meaning, e.g. Is the fraction of not ER-associated desmosomes represent their different functional states or is ER, not an absolute requirement?

Authors' response: This is a challenging issue to address because we do not yet have methodology to assess function of individual desmosomes. We do agree that this is an important issue and will explore approaches to distinguish functional states of desmosomes that are and are not ER associated as part of future studies.

The function of the ER in cell adhesion remains unclear. Establishing whether ER is necessary for desmosome formation/function and the ability to form cell junctions would advance toward this understanding. The authors should consider monitoring these upon, e.g. pulling the ER away from the periphery using the available RUSH-like tools, anchoring ER membrane to motor proteins, which were documented to be working for ER retrieval from the periphery.

Authors' Response: In the revised manuscript, we demonstrate a link between desmosome and ER function as part of a cellular stress response, and we provide data suggesting a potential role for ER regulation of calcium in desmosome assembly (Please see response above to R2 and Fig. 7L, M and Extended Data Fig. 9. We thank the reviewer for the suggestion of the RUSH-like tools and we will employ this approach in further studies to understand how ER tubules influence desmosome assembly and disassembly.

The authors seem to suggest a stress-sensing-related function of the phenomenon they have discovered in their Discussion. However, the justification of why this aspect, in particular, is the focus of the brief Discussion of the phenomenon's potential function is unclear.

Authors' response: There are several hints in the desmosome and keratin literature suggesting that ER stress is associated with dysfunction of these adhesion and cytoskeletal elements. In the revised manuscript, we tested this possibility directly by disrupting keratin filaments or desmosomes and measuring ER stress responses. These new data are included in Fig. 7 L and M of the revised manuscript.

Reviewer #4:

Authors use volume EM and live cell imaging to study at high subcellular resolution and the dynamics of the interactions between ER and desmosomes. They establish that desmosomes

and intermediate filaments bundles are able to regulate the dynamics and the topology of the ER network.

Data quality is very high, especially FIB-SEM, which impressively reveals fine structures such as IF bundles. Such resolution is rarely achieved with this technique. The perturbation of the desmosomes and/or keratin filaments confirm the functional link between ER and cytoskeleton which seem involved also in pathological states, as suggested by results obtained on cellular models of skin epithelia disorders.

The paper is very well written, results supported by clear and concise figures, methods described in great and intelligible details.

The authors nevertheless might stress to much the hypothesized symmetric arrangement of ER of paired cells across desmosomes, which might not be strongly supported by data (see below). ER is indeed present on both sides, but a mirrored organization is discussable. They may want to tune down by avoiding too much repetition.

Authors' Response: We thank the reviewer for the encouraging assessment of the manuscript and data quality. Responses to specific concerns are outlined below.

Some minor details

They use cell lines expressing desmoplakin-EGFP. Is this over or endogenous expression?

Author's response: The A431 cell lines express exogenous DP-EGFP in addition to endogenous DP and have been used extensively in previous studies (Godsel et al., 2005; Broussard et al., 2017). Western blot data demonstrated that DP-EGFP was expressed at 1/7th the levels of endogenous DP (Godsel et al., 2005). Western blot data for both DP-EGFP and mApple-VAPB are now shown in (Supplementary Figure 6). Importantly, TEM of parental A431 cells and new transmission EM of several tissues verifies that the ER associates with desmosomes in cells lacking any exogenous protein expression and in epithelial tissues (Extended Data Fig. 2). [Redacted].

FigS1 ABC: ER is not symmetric ally organized. Same in TEM, the fact that ER is visible in both partner cells of the junction doesn't indicate a symmetrical organization.

Authors' response: We agree and have modified the terminology to "mirror image-like" arrangement or "paired ER structures". In addition, new quantitative data in Extended Data Fig. 4 and Supplementary Figure 4 suggest that ER and keratin share a paired "symmetry" compared to microtubules or endosomes.

FigS2: typo in the legend: cryo-SIM (not cro-). Panel D shows intermediate filaments segmented in cyan. Not described in the legend. What is this cell line? Mitochondria labelling was not announced in the result section.

Authors' Response: These omissions have been corrected. The cell line is A431 cells expressing DP-EGFP and mApple-VAPB where mitochondria are marked using MitoView 650. We now include more

detailed information in the Methods section describing how mitochondria are used as a landmark to align Cryo-SIM and FIB-SEM images.

FIB-SEM here is not done on the same cells as those described in figure S1. Why? Could the use of mitotraker alter the cell morphology?

Authors' Response: We performed FIB-SEM using A431 cells expressing DP-EGFP and mApple-VAPB so that we could use a CLEM approach. We conducted transmission EM on parental A431 cells to verify that ER was present at desmosomes in the parental line and that this association between ER and desmosomes was not caused by exogenous protein expression. This issue is further addressed with new data showing ER-desmosome associations endogenously in various tissues (Extended Data Fig. 2). We apologize for an error in methodological detail in the original submission. While MitoTracker was used to label mitochondria in one FIB-SEM dataset, this dataset was not included in this manuscript. We have updated the methods section to specify the correct mitochondrial dye (MitoView 650). In either case, we are not aware of global changes in cell morphology caused by the use of MitoTracker or MitoView 650.

In panel B, the EM data shows a density at the contact points between an ER tubule and the bundle of intermediate filament. Is that making a ring? Any hypothesis on what it could be?

Authors' Response: We sometimes observe these densities at sites of ER-keratin filament contact that appear to be sites of ER constriction. These could represent clusters of proteins mediating interactions between the ER membrane and the keratin bundle, but we do not yet have a clear understanding of different substructures along the ER-keratin interface.

Fig1: on the interpretation of symmetrical organization: would there be a mean to quantify this from the segmentation? One would first need to determine the axis of symmetry to consider, e.g. one made by the junction, or an axis running perpendicular to the intermediate filaments. Then, mirror the ER tubules from one cell into the other, and measure the overlap between the projected ER and the actual ER of that cell.

Authors' response: As mentioned above, we agree and have modified the terminology to "mirror image-like" arrangement or "paired ER structures". In addition, new quantitative data in Extended Data Fig. 4 and Supplementary Figure 4 suggest that ER and keratin share a paired "symmetry" compared to microtubules or endosomes.

The description of the mirrored organization seems subjective here, and biased towards the junction. For example, in figure 1A and in the region between the white and the blue box, one could also see a symmetrical organization of ER tubules, two branches from one cell almost in continuity with two branches from the other cell, yet, no desmosomes are visible there. What does the FIBSEM data reveal? Could that be the early stages of the formation of a new desmosome?

Authors' Response: We agree that mirrored organizations can occur at regions where we cannot confirm the presence of a desmosome. This phenomenon could reflect a region of the cell where desmosome

formation is being initiated, as suggested, it could be a site where another adhesive cell-cell junction such as adherens junction is organizing ER, or due to some other unknown phenomenon.

Video 1: the narrative in the result section does not really match the video caption, that focuses on the parallel arrangement of the IF bundle and ER, whilst the text describes the formation of ER tubule branches around the desmosome. Would be nice to coordinate this. Moreover, the ER/IF bundling here occurs in a cell protrusion that is forming the contact with the neighboring cell. One could thus argue that the "bundling" is a consequence of the space limitation created by the protrusion.

Authors' Response: We apologize for the inconsistency between the caption and the video and have corrected this issue. The ER-keratin interactions occur throughout the cell and not just within protrusions, so we don't think that spatial constraints explain the association. New data are provided that quantify the size of ER-keratin associations from various cellular locations (Extended Data Fig. 5).

Figure 2 G-I shows a similar parallel association between ER tubules and intermediate filaments. Can the authors also show a movie of that region in order to reveal the plasma membrane and the cell organization at that spot? Is this another cell protrusion?

Authors' Response: It does appear that the ER tubule and keratin filaments are in an enclosed PM protrusion, possibly explaining the extended "parallel" association of these structures. However, we believe that this enclosed space does not necessarily explain the close association of ER and keratin filaments. We show new quantification of ER-Keratin contact number and size compared to ER-microtubule contact number and size (Fig. 2K and Extended Data Fig. 5). These new data suggest that ER preferentially interacts with keratin filaments at desmosome-proximal regions. We have also included new videos (Supplementary Videos 1 and 2) that instead show ER-keratin associations that are not in protrusions to avoid overstating the "parallel" nature of these interactions.

The demonstration of IF bundles traversing ER sheets is impressive (fig 2A-C and video3).

Authors' response: We appreciate the encouraging assessment of the imaging.

Are the cells utilized for the live cell imaging the same as those imaged by FIB-SEM, importantly, comment the expression level of FP tagged proteins. Could this influence the desmosome formation? The ER/desmosome association?

Authors' Response: Yes, A431 cells expressing mApple-VAPB and DP-EGFP were used for both live imaging and for FIB-SEM to allow for comparisons between the different imaging modalities and to permit a CLEM workflow on cyro-fixed cells. With regards to expression levels of tagged proteins, please see response above. New data are also included showing that ER associates with desmosomes in a variety of cell types and tissues (Extended Data Fig. 2). Based on this analysis, we conclude that expression of the exogenous proteins is not causing ER-desmosome associations nor causing large scale changes in cell adhesion.

On the concomitance of ER tubules and keratin bundle formation, quantitative analyses are missing to assess if this is a favored, induced behavior or not. Statements like “Following addition of calcium, some peripheral ER tubules extend and retract without any keratin filaments …” would benefit further quantification. Important to really support such statement: “Collectively, these experiments indicate that desmoplakin puncta formation and keratin filament assembly are initiated at sites of ER tubule extensions at nascent epithelial cell-cell contacts”.

Authors’ Response: We have clarified this statement. Further, new quantitative assessment of ER and keratin extensions during initiation of cell-cell contact indicates that ER extension precedes keratin filament appearance in 2/ 40 cells initiating contact, while ER and keratin filaments extend simultaneously in 38/40 cells where ER tubule extension was observed at nascent cell-cell contacts.

Decision Letter, first revision:

Our ref: NCB-A48981A

2nd March 2023

Dear Dr. Kowalczyk,

Thank you very much for submitting your revised manuscript "Architecture and dynamics of a novel desmosome-endoplasmic reticulum complex" (NCB-A48981A). 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 comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, could you please email us a copy of the file in an editable format (Microsoft Word or LaTex)? We cannot proceed with PDFs at this stage. Thank you in advance.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about 1-2 weeks. 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,

Melina

Melina Casadio, PhD

Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

Reviewer #1 (Remarks to the Author):

The authors have significantly strengthened their manuscript through substantive revisions to data and their interpretation. The data is (still) beautiful and clear, and the revised narrative is appropriately more reserved. This manuscript reports on a completely novel reality with significant and broad impact for cell biology. This reviewer supports publication, with great enthusiasm.

Reviewer #2 (Remarks to the Author):

This revision has thoughtfully addressed all of my questions/concerns. New evidence that ER-stress is increased in the absence of desmosome-contacts or keratin mutants is interesting even if I don't fully understand the greater meaning. Nice study that will open up new ways to think about desmosome biogenesis and functions in epithelial.

Reviewer #3 (Remarks to the Author):

The authors addressed the comments comprehensively, and I have no objections to advancing this manuscript towards publication.

Reviewer #4 (Remarks to the Author):

This is another version of the paper, result of a thorough rewriting upon comments made by 4 reviewers.

I must say that I am impressed by the depth and seriousness of the responses to all 4 reviews, which were challenging.
In my opinion the paper is now greatly improved and will most certainly represent a reference in the field for the years to come.

I am of course satisfied by the way my specific comments have been addressed and have nothing else to add at this stage.

Looking forward to seeing this work published, and also to see how the work will be used by the field for further researches.

Decision Letter, second revision:

Our ref: NCB-A48981A

10th March 2023

Dear Dr. Kowalczyk,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Architecture and dynamics of a novel desmosome-endoplasmic reticulum complex" (NCB-A48981A). 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 two weeks). 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 "Architecture and dynamics of a novel desmosome-endoplasmic reticulum complex". 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,

Kendra Donahue

Staff
Nature Cell Biology

On behalf of

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

Reviewer #1:
Remarks to the Author:
The authors have significantly strengthened their manuscript through substantive revisions to data and their interpretation. The data is (still) beautiful and clear, and the revised narrative is appropriately more reserved. This manuscript reports on a completely novel reality with significant and broad impact for cell biology. This reviewer supports publication, with great enthusiasm.

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I must say that I am impressed by the depth and seriousness of the responses to all 4 reviews, which were challenging.
In my opinion the paper is now greatly improved and will most certainly represent a reference in the field for the years to come.

I am of course satisfied by the way my specific comments have been addressed and have nothing else to add at this stage.

Looking forward to seeing this work published, and also to see how the work will be used by the field for further researches.

Final Decision Letter:

Dear Dr Kowalczyk,

I am pleased to inform you that your manuscript, "Architecture and dynamics of a desmosome-endoplasmic reticulum complex", has now been accepted for publication in Nature Cell Biology. Congratulations on your beautiful study!

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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Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

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