SNAP29 mediates the assembly of histidine-induced CTP synthase filaments in proximity to the cytokeratin network

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MS TITLE: SNAP29 mediates the assembly of histidine-induced CTP synthase filaments along cytokeratin network

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers consider your observations of interest but at the same time raise a number of substantial criticisms that prevent me from accepting the paper at this stage. Most importantly, they find that the data to support a role of keratin in the assembly and activity of CTPS needs further investigations. Specifically, the reviewers request to stain the keratin network following knockdown of keratin 8 and 18, and to determine the activity of CTPS in keratin-deficient cells (Drosphila or keratinocytes derived mice lacking all type II keratins). Other points of concerns are the lack of quantitation, the selectivity of the drugs used to manipulate the three cytoskeletal systems (Fig. 2e), methodological deficits, etc, etc. Furthermore, the reviewers noted that several of the conclusions have been overstated.

It will be clear that these concerns and others raised by the reviewers are sufficiently significant for me not to be able to accept your manuscript in its current form. However, if you think that you

can provide additional experimental support for your conclusions and can adequately address the different points raised by the three reviewers, I will be pleased to receive a revised manuscript, which will then be sent to the original reviewers for re-review.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this interesting manuscript the authors demonstrate a previously unappreciated role for the cytokeratin network and the SNARE binding protein SNAP29 in the assembly of CTP synthase into cytoplasmic filaments.
The strongest aspect of the study is the multiple approaches to confirm SNAP29-CTPS-cytokeratin interactions including proximity biotinylation, PLA, and fluorescence co-localization studies. The greatest weakness is the lack of a clear model how these proteins might interact, given that SNAP29 is associated with vesicle trafficking but that CTP synthase filaments are not membrane associated. A second weakness is that conclusions are occasionally overstated. Nevertheless, I believe these results are worth publishing to allow the field to further investigate the role of the intermediate filament network in facilitating CTP synthase assembly. The quality of the data and statistical analysis is generally high and I would recommend publication after the authors address the following points.

Comments for the author

One important point not addressed by the authors is the potential role of SNAP29 in regulating CTPS methylation, a modification, first described by these same authors, that regulates CTPS assembly. Conversely, does methylation of CTPS affect SNAP29/CTPS binding? Determining whether SNAP29 regulates CTPS methylation on arginine 449 might provide the key mechanistic link between SNAP29 and CTPS assembly. The authors have previously used mass spectrometry to monitor methylation of this site.
Addressing this simple hypothesis would substantially increase the impact of this study.

Statements in the abstract such as “Inhibiting the SNARE recycling by N-ethylmaleimide (NEM) treatment dissembled [sic] the CTPS filaments…” imply that NEM effects on CTPS are mediated by SNARE recycling but the authors have not demonstrated this link. They have only shown that NEM leads to disassembly of the CTPS filaments. Likewise “This study links the cytokeratin network to the regulation of metabolism by compartmentation through SNAP29 involved SNARE recycling on the cytokeratin network during glutamine deprivation.” implies that the authors have demonstrated a role for SNARE recycling in CTPS compartmentalization, although they have only shown it is NEM-sensitive. NEM is a not specific inhibitor of the NSF. The authors should avoid such overstatements. Similarly, the authors cannot conclude that NEM “traps” SNAP29 on cytokeratins and “reduces availability” of SNAP29 to CTPS. Also “Collectively, the formation of CTPS filament mediated by SNAP29 is required to control the enzymatic activity of CTPS for adapting to Gln depletion stress.“ Again, the authors have not demonstrated that regulation of CTPS assembly is the mechanism by which SNAP29 reduces CTPS activity. This is just one potential mechanism and should not be overstated.
The description of how the proteomic data was evaluated was confusing. For example, 4 conditions are discussed in the text (EBSS alone, EBSS+His, EBSS+His+Gln, and EBSS+His without CTPS-APEX2) but Figure S2F presents a Venn diagram of only 3 groups and it is unclear from the labeling which they correspond to. Also, it is unclear why they are interested in the 26 “common candidates” in the INTERSECTION of the 3 groups since some conditions promote filaments and others disassemble them.

Also, what is the purpose of Fig 2A since Fig 1 already led the authors to SNAP29?

The SNAP29 mutant Drosophila experiment (Fig 2D) does not add significantly to the study. The actin staining of the mutant salivary gland tissues shows severely altered morphology. Snap29 has been shown to be involved in autophagy and membrane trafficking in Drosophila and so it is not clear if the lack of CTPS filaments is due to loss of SNAP29-CTPS interactions or indirectly through these other mechanisms.
Consequently, I would suggest removing them. Likewise, the non-quantitiative electron microscopy images in Fig 2E are much less compelling than the APEX2 biotinylation and PLA data. If included, the EM data should be more rigorous (e.g. quantitative analysis of labeling frequency).
Is the localization of IMPDH to cytokeratin CTPS dependent? If so, this would provide a mechanism at least for IMPDH recruitment to the cytokeratin network.

Why is KRT8 specifically involved? It could be informative to investigate if KRT8 knockdown disrupts the cytokeratin network more broadly than knockdown of other isoforms.

Finally, careful editing throughout the manuscript will reduce grammatical errors and improve the readability.

Minor points:
The legend to Fig 2A is very vague. “Proteomic profiling” is an inadequate description of what is shown.
The data in Figs 5B and 5G do not convincingly show colocalization. Perhaps a comparative experiment under EBSS versus EBSS+His would show a more convincing difference. Also, in Fig 5A, the figure does not seem to show association between CTPS and cytokeratin in knockdown cells as described in the text.

There is no control for total protein levels in Fig 5F. Does total SNAP29 increase on NEM treatment?

Fig 1 title should refer to “filament-associated” not “filament-related” proteins

The title of Fig 4 should also include the positive finding that cytokeratin disassembly disrupts CTPS assembly and not just focus on the negative data.

Fig S2 is labeled “identification of candidate genes…” but this is a proteomic study, not a genetic one.

Fig 3F should include quantification of KRT18 knockdown cells as well as KRT8.

Reviewer 2

Advance summary and potential significance to field

Cytidine 5’-triphosphate synthase (CTPS) forms filament-like structures under glutamine deprivation which render CTPS inactive. Here, the authors investigate how filament formation might be regulated. Based on high resolution colocalization, protein interaction, gene knockdown and mass spectrometry studies they identify the snare protein SNAP29 and keratins K8 and K18 as candidates involved in CTPS filament formation. They suggest a model in which under conditions of nutrient stress, CTPS filament formation proceeds along keratin filaments, somehow mediated by SNAP29.
This is an interesting concept which seems to fit the hypothesis of E. Lazarides wh coined IF as mechanical integrators of cellular space (1980). It predicts that cells that lack keratins CTPS activity is constitutively elevated. This could be easily tested, either in non-epithelial cells, in keratin-deficient cells (Kröger et al., 2013 JCB) or in Drosophila, which has no cytoplasmic IF proteins at all. The latter raises the question how general the keratin-dependent regulation of CTPS filament formation and activity might be.
I have a number of concerns with the data that require experimental work. These are detailed below.

Comments for the author

1. It is claimed that CTPS form along the keratin network. Still images (eg. SF 4F) and the videos provided clearly show that CTPS filaments co-align only with a small fraction of the keratin network. Why is this so, given the abundance of CTP synthase? Conversely, why are other CTPS filaments not recruited to the keratin network? At the very least, images of endogenous proteins showing partial colocalization need to quantified in a statistically meaningful manner.
2. In the first set of experiments, SNAP 29, among other proteins, is identified as a candidate that promotes CTPS filament formation. In contrast to prediction, the activity of CTPS was only moderately affected by His addition. The xplanation given is not very convincing. It would be more convincing to complement the knockdown with a SNAP29 overexpression study.
3. The hypothesis that CTPS filaments somehow interact with keratin filaments largely resides on the knockdown of keratin K8 which strongly diminishes CTPS filaments. It is very obvious (Fig 3C) that the K8 KD is very inefficient, unlike that of K18. In this setting, K8 KD seems to strongly diminish those filaments whereas that of K18 reportedly does not. It remains unknown whether K8 or K18 knockdown at all affect presence of keratins. If that were not the case, the entire argument would collapse.
To resolve this, the following aspects have to be addressed: a) clearly state which cells (HeLa, HEK or Hep) have been used, b) identify the endogenous keratins by western blotting with isotype-specific antibodies, c) stain the endogenous keratin network following KD of given endogenous keratin isotype.
4. In their proteomic experiments, the authors identify a range of keratins highly specific for terminally differentiated keratinocytes, such as K1, K9 and K10. To proof that these (and additional ones) are really present in the cells used, monospecific antibodies and/or Q-RT-PCR needs to be done. As the authors themselves point out, keratins, owing to their abundance, are likely contaminants.
5. How specific does 1,6-Hexanediol act on keratins?
6. The role of SNAP 29 in the ensemble of CTPS and keratins remains unclear. It will be necessary to understand whether SNAP29 can directly interact with keratins and with CTPS. In view of the known stickiness of keratins, I suggest to use keratin domains (head, rod tail) which are soluble and far less sticky than polymerization-competent intact proteins to map the interaction with SNAP29. In addition, it needs to be clarified whether SNAP29 directly interacts with CTP synthase.
Other issues:
8. The authors state (p6) "Previously, we found that CTPS filaments are important for endoreplication of salivary glands". How do SGs replicate?
9. (p7) The authors state "SNAP29 mutation in humans leads to CEDNIK (cerebral dysgenesis, neuropathy, ichthyosis and keratoderma) syndrome, which is related to the defective transportation of components in keratinocytes during epidermal differentiation, suggesting a possible involvement of intermediate filaments". However, it is not clear why keratins supposedly are involved in transport processes. At present, no motors are known, and they possess an apolar organization.
10. (p7) It is not clear what has been done here and what is shown "Imaging at different time point, CTPS displayed a spatiotemporal relocation along the cytokeratin (Fig. 3B)."
11. (p8) The statement "KRT8 and 18 are the most commonly found members of the keratin family in epithelial tissues, and they form heterodimers on the cytokeratin network" is not correct. K8 and K18 are most common in simple epithelia; they form a network via the assembly of heterodimers into ULF and into filaments.
12. (p9) The sentence "CTPS filament formation, SNAP29 interacts with CTPS along the cytokeratin network by superresolution imaging" is not correct. Might read: SRI suggests that during CTPS filament formation, ...
13. Please clarify the nature of the mutant "because a tetrameric mutant of CTPS (G148A)". Does the mutation stop assembly at the tetramer state?
14. What is "This dynamic availability of SNAP29"?
15. The phrase "Cytokeratins are the most diverse, which belong to the largest family among all types of intermediate filaments" is not correct. Possibly: Cytokeratins, which represent the largest family among all types of intermediate filaments, are the most diversified IF members.
16. The entire manuscript needs critical input by a native speaker. Also, in several places singular and plural are not correctly used.

Reviewer 3

Advance summary and potential significance to field

This submission provides data at an important interface within cell biology, namely (macro) autophagy, stress responses and the involvement of the cytoskeleton. It reports an interaction between the SNARE family member, SNAP29, and the keratin network in HEp-2, HEK 293 and HeLa cells, which the authors propose is required for the formation of Cytidine 5'-Triphosphate Synthase (CTPS) filaments as part of the metabolic adaption to nutrient stress. In this case it is glutamine, an amino acid that in cancer cells helps maintain their reducing power as well as being essential for TCA cycle anaplerosis. CTP is an essential product from TCA cycle intermediates and is a coenzyme in pathways that produce for instance glycerophospholipids (phosphotidyl choline) and needed for N-linked protein glycosylation. The production of CTP from UTP by the enzyme CTPS is common to all life. CTPS forms filamentous structures of unknown function in both pro- and eu-karyotes. It has been reported that the polymerization inhibits CTPS activity and hence discovering how the filaments are formed and where in the cell would be an important discovery. The current submission claims to evidence the role of Intermediate Filaments in the assembly, a process that depends upon the association of SNAP29, a SNARE protein that is tightly associated with (macro)autophagy.
At first glance there are two significant issues. Firstly bacterial orthologues to intermediate filaments are usually membrane bound. Drosophila have nuclear lamina, but thus far no cytoplasmic intermediate filaments.
Secondly only a subset of the keratin filaments seem to provide the assembly platform for CTPS filament assembly. I think that the authors might argue that the complexity of a multicellular organism without an exoskeleton requires increased regulatory control and the association with a structure (intermediate filament) actively implicated in stress responses is therefore a logical extension of that control. SNAP29 and CTPS are biomarkers that evidence functional heterogeneity within the keratin network, a feature proven for all elements of the cytoskeleton. Therefore neither an evolutionary nor spatial selectivity are arguments to prevent consideration of this submission.

The report presents data to show that SNAP29 and CTPS can be co-immunoprecipitated and indeed can be colocalised by both high resolution fluorescence microscopy and immunogold electron microscopy methodologies.

There is some confusion about what is immunoprecipitation and what is co-precipitation. The Mass Spec approach seems to rely on an avidin-biotin co-precipitation approach, but uses fractions solubilized in 1% (w/v) SDS. As the SDS is diluted before the complex is isolated, there is the possibility that ad hoc functionally unimportant complexes form. It is difficult to advise how to control for this possibility as per se removing SNAP29/knockdown approach will diminish the signal for both functionally important and unimportant complexes. I do note though that for the immunogold labelling experiments presented in Figure 3, antibody controls and quantification were also missing and there appeared to be significant labelling of structures that were not filamentous in nature.

The other issue concerns the fractionation of the cells prior to immunoprecipitation using the keratin antibodies. The KRT lysis buffer will most likely retain the integrity of the keratin filaments, but solubilize the soluble fraction and those proteins associated with membranes. Therefore these are significant practical issues with the presented work and whilst the keratin knockdown approach mitigates partly the concern, the importance or otherwise of keratin 8 seems to have been somewhat overlooked within the submission. Indeed the knockdown of KRT8 (Fig. 3E)seemed to induce the formation of a single-perinuclear aggregate of CTPS.
After all there are several JCS papers and others too that link keratins to hepatic stress responses, to mitochondrial shape and function and to metabolic regulation which might pertinent to the effects seen on HEp-2 cells, the primary cell line investigated in these studies.

Drugs were used to manipulate the three elements of the cytoskeleton. The use of 1,6 hexanediol as a specific antagonist of intermediate filament networks is somewhat adventurous given the literature and the previous work of Heather Durham. Equally nocodazole and cytochalasin are not necessarily precise in their ability to selectively alter just one of the three cytoskeletal filament

systems. Rather given the role that SNAP29 plays in primary cilia formation, it might have been more interesting to investigate the correlation between pericentrin, SNAP29 and CPTS particularly in the KRT8 knockdown.

In summary therefore, the data presented are certainly provocative, but at this stage not convincing. This opinion should not be conflated with a conceptual conflict, rather this is a very exciting potential insight into the role of intermediate filaments. Several (to me) obvious experiments seem to be missing eg sequential extraction of the filamentous fraction from Hep-2 cells after incubating cultures in histidine and histidine/glutamine containing media; monitoring of (macro)autophagy, mitochondrial function and autophagosome formation or perhaps pursuing the ROI in SNAP29 responsible for binding to keratins.

Comments for the author

As detailed above the main issues for me are:
There is some confusion about what is immunoprecipitation and what is co-precipitation. The Mass Spec approach seems to rely on an avidin-biotin co-precipitation approach, but uses fractions solubilized in 1% (w/v) SDS. As the SDS is diluted before the complex is isolated, there is the possibility that ad hoc functionally unimportant complexes form. It is difficult to advise how to control for this possibility as per se removing SNAP29/knockdown approach will diminish the signal for both functionally important and unimportant complexes. I do note though that for the immunogold labelling experiments presented in Figure 3, antibody controls and quantification were missing and there appeared to be significant labelling of structures that were not filamentous in nature.

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Examples of syntactical issues, of which there were many throughout the manuscript:

Given that filament formation of CTPS is dynamic, posttranslational modifications, such as ubiquitination and methylation, are required for CTPS filament formation in human cancer cells (Pai et al., 2016). Commas misplaced?
Conventional method like co-immunoprecipitation assay have been challenging in identifying proteins interacting with CTPS filament - words missing?
western blotting - Proper noun needs capitalizing Exogenous CTPS1 (~100KDa) kDa?

.....a SNARE binding protein, known to involve in multiple protein trafficking processes - be involved in?
CTPS filaments assembles along Cytokeratin Network - assemble on the.....?

The second movie didn't play.

First revision

Author response to reviewers' comments

Point-by-point response to the Reviewers' comments

We appreciate the Reviewers' comments on our work, and we have addressed their questions by performing new experiments and have rewritten our manuscript to clarify some conclusions. Our responses are in red, and the quoted text from the manuscript are in green. The proteomic data sets are deposited to ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier **PXD010921**/ **PXD015507**:

Reviewer 1 Advance Summary and Potential Significance to Field:

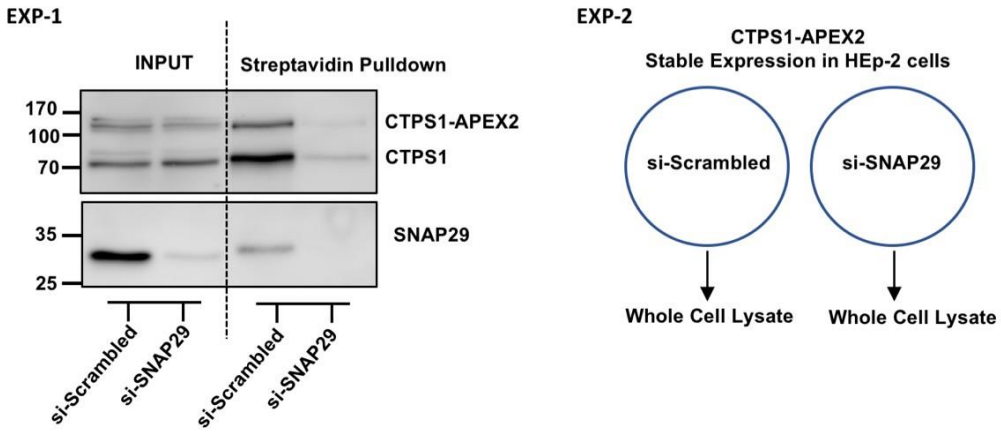
In this interesting manuscript the authors demonstrate a previously unappreciated role for the cytokeratin network and the SNARE binding protein SNAP29 in the assembly of CTP synthase into cytoplasmic filaments. The strongest aspect of the study is the multiple approaches to confirm SNAP29-CTPS-cytokeratin interactions including proximity biotinylation, PLA, and fluorescence co-localization studies. The greatest weakness is the lack of a clear model how these proteins might interact, given that SNAP29 is associated with vesicle trafficking but that CTP synthase filaments are not membrane associated. A second weakness is that conclusions are occasionally overstated. Nevertheless, I believe these results are worth publishing to allow the field to further investigate the role of the intermediate filament network in facilitating CTP synthase assembly. The quality of the data and statistical analysis is generally high and I would recommend publication after the authors address the following points.

Reviewer 1 Comments for the Author:

One important point not addressed by the authors is the potential role of SNAP29 in regulating CTPS methylation, a modification, first described by these same authors, that regulates CTPS assembly. Conversely, does methylation of CTPS affect SNAP29/CTPS binding? Determining whether SNAP29 regulates CTPS methylation on arginine 449 might provide the key mechanistic link between SNAP29 and CTPS assembly. The authors have previously used mass spectrometry to monitor methylation of this site. Addressing this simple hypothesis would substantially increase the impact of this study.

Response: We agree that if SNAP29 regulates CTPS methylation, this would shed light on the mechanism of SNAP29-mediated regulation of CTPS filament formation. Therefore, we have used mass spectrometry to identify if methylation of CTPS on arginine 449 is regulated by SNAP29. We performed streptavidin pull-down from HEp-2 cells expressing CTPS1-APEX2 in wild-type cells or on a SNAP29 knockdown background and used targeted MS strategies such as AIMS (accurate inclusion mass screening) and PRM (Parallel Reaction Monitoring) followed by a two-dimensional LC system to detect CTPS1 methylation. To detect all peptides, regardless of the pull-down efficiency, we also used CTPS1-APEX2 overexpression cell lysates for the above analyses. Unfortunately, we could not detect arginine 449 methylation of CTPS1 under both conditions, indicating that methylation at arginine 449 of CTPS is not abundant. It is not clear whether this type of methylation of CTPS is required for filament formation, since no differences of this methylation was observed between EBSS and EBSS+His conditions in our previous experiment (Lin et al., 2018). Even though the mutation of 449 arginine residue affected filament formation, it is still open question whether methylation of CTPS at 449 arginine is required for filament formation. Furthermore, it is still

unclear at which step of CTPS filament formation involves methylation, and methylation of what proteins are required for filament formation. However, these are all very interesting future directions. The western blotting of streptavidin pull-down and scheme of over-expression of CTPS1-APEX2 are shown below.



Statements in the abstract such as “Inhibiting the SNARE recycling by N-ethylmaleimide (NEM) treatment dissembled [sic] the CTPS filaments…” imply that NEM effects on CTPS are mediated by SNARE recycling but the authors have not demonstrated this link. They have only shown that NEM leads to disassembly of the CTPS filaments. Likewise, “This study links the cytokeratin network to the regulation of metabolism by compartmentation through SNAP29 involved SNARE recycling on the cytokeratin network during glutamine deprivation.” implies that the authors have demonstrated a role for SNARE recycling in CTPS compartmentalization, although they have only shown it is NEM-sensitive. NEM is a not specific inhibitor of the NSF. The authors should avoid such overstatements. Similarly, the authors cannot conclude that NEM “traps” SNAP29 on cytokeratins and “reduces availability” of SNAP29 to CTPS. Also “Collectively, the formation of CTPS filament mediated by SNAP29 is required to control the enzymatic activity of CTPS for adapting to Gln depletion stress. “Again, the authors have not demonstrated that regulation of CTPS assembly is the mechanism by which SNAP29 reduces CTPS activity. This is just one potential mechanism and should not be overstated.

Response: We agree that the model of SNAP29 in CTPS filament formation is not completely clear, and NEM may have other effects than the inhibition of NSF; therefore, we modified the abstract to remove the sentences mentioned above.

The description of how the proteomic data was evaluated was confusing. For example, 4 conditions are discussed in the text (EBSS alone, EBSS+His, EBSS+His+Gln, and EBSS+His without CTPS-APEX2) but Figure S2F presents a Venn diagram of only 3 groups and it is unclear from the labeling which they correspond to. Also, it is unclear why they are interested in the 26 “common candidates” in the INTERSECTION of the 3 groups since some conditions promote filaments and others disassemble them.

Response: In the present study, our goal is to identify candidate proteins interacting with CTPS during filament formation. We used a quantitative proteomic approach, iTRAQ labeling, to identify the potential filament-interacting candidate proteins in the EBSS+His condition, and we divided EBSS+His (group 2) from the rest of the groups (EBSS (group 1), EBSS+His+Gln (group 3), and APEX alone (group 4)) to obtain a fold change ratio as represented in Fig. S2E. So, to identify the common proteins (i.e., 26 proteins represented in Fig. S2F) that showed a higher fold change ratio in all three combinations, group 2/group 1, group 2/group 3 and group 2/group 4 were further used for sh-RNA screening. To make our readers understand, we have now described the comparison of groups in the results and have modified the figure legend of Fig. S2E and the figure of Fig. S2F to include the meanings of HIS/EBSS, HIS/APEX and HIS/GLN.

Also, what is the purpose of Fig 2A since Fig 1 already led the authors to SNAP29?

Response: In order to confirm that SNAP29 is a candidate with a significant P value (<0.05) in the proteomic study, we performed 3 repeats to draw Fig. 2A.

The SNAP29 mutant Drosophila experiment (Fig 2D) does not add significantly to the study. The actin staining of the mutant salivary gland tissues shows severely altered morphology. Snap29 has been shown to be involved in autophagy and membrane trafficking in Drosophila and so it is not clear if the lack of CTPS filaments is due to loss of SNAP29-CTPS interactions or indirectly through these other mechanisms. Consequently, I would suggest removing them.

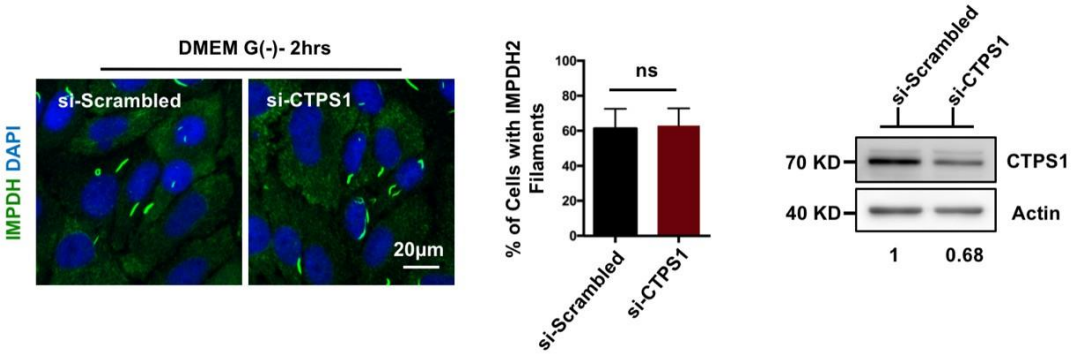
Response: We agree with the reviewer's suggestion, and we now have removed this data from the manuscript.

Likewise, the non-quantitiative electron microscopy images in Fig 2E are much less compelling than the APEX2 biotinylation and PLA data. If included, the EM data should be more rigorous (e.g. quantitative analysis of labeling frequency).

Response: We have now quantified the EM data. For Fig. 2E, the quantified data is provided in supplementary Fig. S3H. (*Manuscript reference line no: 183*)

Is the localization of IMPDH to cytokeratin CTPS dependent? If so, this would provide a mechanism at least for IMPDH recruitment to the cytokeratin network.

Response: We have now knocked down CTPS1 in HEp-2 cells; however, IMPDH2 filament formation remains unaffected in response to glutamine starvation.



Why is KRT8 specifically involved? It could be informative to investigate if KRT8 knockdown disrupts the cytokeratin network more broadly than knockdown of other isoforms.

Response: To answer the Reviewer's question, we performed immunostaining using a pan cytokeratin antibody and found that knockdown of KRT8 significantly reduced the cytokeratin network in HEp-2 cells. On the contrary, knockdown of KRT18 did not reduce the fluorescence intensity of the cytokeratin network. We have now provided this new data in supplementary Figure S5A-D. (*Manuscript reference line no: 214*)

Finally, careful editing throughout the manuscript will reduce grammatical errors and improve the readability.

Response: We thank the Reviewer for the suggestions, and this manuscript has now been edited by a professional editing office.

Minor points:

The legend to Fig 2A is very vague. "Proteomic profiling" is an inadequate description of what is shown.

Response: We have now changed the subtitle of the figure legend of 2A to "Shown is a volcano plot comparing the log2 fold changes (x-axis) versus the -log10 p values (y-axis) for each protein identified in the 6-plex tandem-mass-tag (TMT) labeled proteomic profiling of HEp-2 cells expressing FLAG-CTPS1-APEX2 cultured in EBSS and EBSS+His at 6 hrs." (*Manuscript reference line no: 884*)

The data in Figs 5B and 5G do not convincingly show colocalization. Perhaps a comparative experiment under EBSS versus EBSS+His would show a more convincing difference. Also, in Fig 5A, the figure does not seem to show association between CTPS and cytokeratin in knockdown cells as described in the text.

Response: The images of Fig. 5B were from the proximity labeling experiment of CTPS and FLAG-SNAP29, and the positive results (green signal) indicated that CTPS and SNAP29 were located proximally to each other. The green signals were very strong, and image in Fig. 5B was only one single section (the red signal (mCherry-KRT8) at some places was not very strong), so we do not expect to see that all green signal turns into yellow signal. However, we described this as "The PLA signal of CTPS and FLAG-SNAP29 located proximally to the cytokeratin network." Similarly, in Fig. 5G, the yellow signals of the overlap of SNAP29 and KRT8 can be visualized by enlarging the images. However, we agree that some SNAP29 complexes were not closely associated with KRT8. Giving that SNAP29 has multiple roles in cells, this result is expected. We agreed that the "knockdown control" cell description was confusing, so we have now removed the word "knockdown" and kept "whereas in control cells", which showed the association (*Manuscript reference line no: 252*). Our results indicated that EBSS stress recruited SNAP29 to locate proximally with cytokeratin (Fig. S6F), and histidine- mediated posttranslational modifications may facilitate the CTPS filament assembly. Therefore, the images of CTPS/SNAP29 related to cytokeratin were similar between EBSS and EBSS+ His.

There is no control for total protein levels in Fig 5F. Does total SNAP29 increase on NEM treatment?

Response: Due to space constraints, we initially did not show the total protein levels. However, now we have provided the total protein control for SNAP29 in Fig. 5F. NEM treatment doesn't increases the total protein for SNAP29 in Fig. 5I.

Fig 1 title should refer to "filament-associated" not "filament-related" proteins

Response: We have now updated the title for Fig. 1 as "filament-associated" instead of "filament related". (*Manuscript reference line no: 864*)

The title of Fig 4 should also include the positive finding that cytokeratin disassembly disrupts CTPS assembly and not just focus on the negative data.

Response: We thank the Reviewer for the reminder, and we now have updated the title for Fig. 4 to "CTPS filament formation is affected by cytokeratin disassembly". (*Manuscript reference line no: 937*)

Fig S2 is labeled "identification of candidate genes…" but this is a proteomic study, not a genetic one.

Response: We have now changed the title to "Discovery of candidate genes …".

Fig 3F should include quantification of KRT18 knockdown cells as well as KRT8.

Response: Now we have included the quantified data for CTPS filament formation under KRT18 knockdown in Fig. 3D (previous figure Fig. 3F) together with the KRT8 knockdown data. (*Manuscript reference line no: 213*)

Reviewer 2 Advance Summary and Potential Significance to Field:

Cytidine 5'-triphosphate synthase (CTPS) forms filament-like structures under glutamine deprivation which render CTPS inactive. Here, the authors investigate how filament formation might be regulated. Based on high resolution colocalization, protein interaction, gene knockdown and mass spectrometry studies they identify the snare protein SNAP29 and keratins K8 and K18 as candidates involved in CTPS filament formation. They suggest a model in which under conditions of nutrient stress, CTPS filament formation proceeds along keratin filaments, somehow mediated by SNAP29.This is an interesting concept which seems to fit the hypothesis of E. Lazarides wh coined IF as mechanical integrators of cellular space (1980). It predicts that cells that lack keratins CTPS activity is constitutively elevated. This could be easily tested, either in non-epithelial cells, in keratin-deficient cells (Kröger et al., 2013 JCB) or in Drosophila, which has no cytoplasmic IF proteins at all. The latter raises the question how general the keratin-dependent regulation of CTPS filament formation and activity might be. I have a number of concerns with the data that require experimental work. These are detailed below.

Response: This study demonstrated that keratin network plays a role in regulating CTPS filament formation. Interestingly, CTPS filaments were observed in *Drosophila* tissues where recognizable cytoplasmic intermediate filaments (IF) were absent. It has been suggested that other types of protein may perform crucial functions of IF (Herrmann and Strelkov, 2011). Indeed, an atypical Tropomyosin was identified with intermediate filament-like properties in *Drosophila* (Cho et al., 2016). One possibility is that non-canonical keratin proteins may involve in CTPS filament formation in cells where keratins are not present.

Reviewer 2 Comments for the Author:

1. It is claimed that CTPS form along the keratin network. Still images (eg. SF 4F) and the videos provided clearly show that CTPS filaments co-align only with a small fraction of the keratin network. Why is this so, given the abundance of CTP synthase? Conversely, why are other CTPS filaments not recruited to the keratin network? At the very least, images of endogenous proteins showing partial colocalization need to quantified in a statistically meaningful manner.

Response: In Fig. S4F, we presented a single section image using LSM confocal 780 to obtain a clear image of the cytokeratin network. The fluorescence intensities of CTPS (green fluorescence) were enriched at particular locations along the network; however, we do not know how CTPS selected the locations for filament assembly. Since the cytokeratin network is not homogenous, we did not expect to see merged yellow signals in all filaments. In Fig. 3F, the merged image of several sections showed that all of the CTPS filaments co-aligned with cytokeratin network when using mCherry-tagged KRT18 in HEp-2cells. Furthermore, we have now statistically quantified the percentages of CTPS associated with DAB stained KRT18 filaments (Fig. 5C) using the EM images and have provided this new set of data in supplementary Fig. S6E. (*Manuscript reference line no: 259*)

2. In the first set of experiments, SNAP29, among other proteins, is identified as a candidate that promotes CTPS filament formation. In contrast to prediction, the activity of CTPS was only moderately affected by His addition. The explanation given is not very convincing. It would be more convincing to complement the knockdown with a SNAP29 overexpression study.

Response: We thank the Reviewer for this suggestion. Now, we have provided this new set of data in supplementary Figs. S3E and S3F. We found that the effect of SNAP29 RNAi, which relaxed the suppression of CTPS enzymatic activity, was reversed by overexpression of a RNAi-resistant SNAP29 construct. (*Manuscript reference line no: 172*)

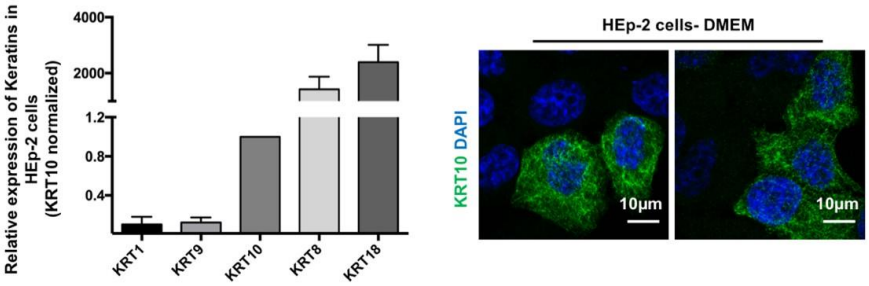
3. The hypothesis that CTPS filaments somehow interact with keratin filaments largely resides on the knockdown of keratin K8 which strongly diminishes CTPS filaments. It is very obvious (Fig 3C) that the K8 KD is very inefficient, unlike that of K18. In this setting, K8 KD seems to strongly diminish those filaments whereas that of K18 reportedly does not. It remains unknown whether K8 or K18 knockdown at all affect presence of keratins. If that were not the case, the entire argument would collapse. To resolve this, the following aspects have to be addressed:
a) clearly state which cells (HeLa, HEK or Hep) have been used, b) identify the endogenous keratins by western blotting with isotype-specific antibodies, c) stain the endogenous keratin network

following KD of given endogenous keratin isotype.

Response: To answer the Reviewer's question, a) we have included the cell name HEp2 in the figure S5A-C legend; b) we have identified the endogenous keratins by western blotting with isotype-specific antibodies in Fig. S5 A and B; c) we have performed immunostaining using a pan cytokeratin antibody and found that partial knockdown of KRT8 significantly reduced the cytokeratin network in HEp-2 cells. On the contrary, knockdown of KRT18 did not reduce the fluorescence intensity of the cytokeratin network. These results are consistent with the hypothesis that the function of KRT18 may be compensated by other isotypes of KRTs, such as KRT19. We have now provided this new data in supplementary Fig. S5A-D. (*Manuscript reference line no: 213*)

4. In their proteomic experiments, the authors identify a range of keratins highly specific for terminally differentiated keratinocytes, such as K1, K9 and K10. To proof that these (and additional ones) are really present in the cells used, monospecific antibodies and/or Q-RT- PCR needs to be done. As the authors themselves point out, keratins, owing to their abundance, are likely contaminants.

Response: We now have performed RT-qPCR to show the mRNA expression of KRT10, 9, 1, 8 and 18 in HEp-2 cells which were used for proteomic analysis in our manuscript. RT- qPCR shows that KRT9 and 1 were expressed at very low levels, KRT10 was expressed at a moderate level and KRT8 and 18 were expressed at very high levels. We have further validated the expression of KRT10 by performing immunostaining, and the data is as follows.

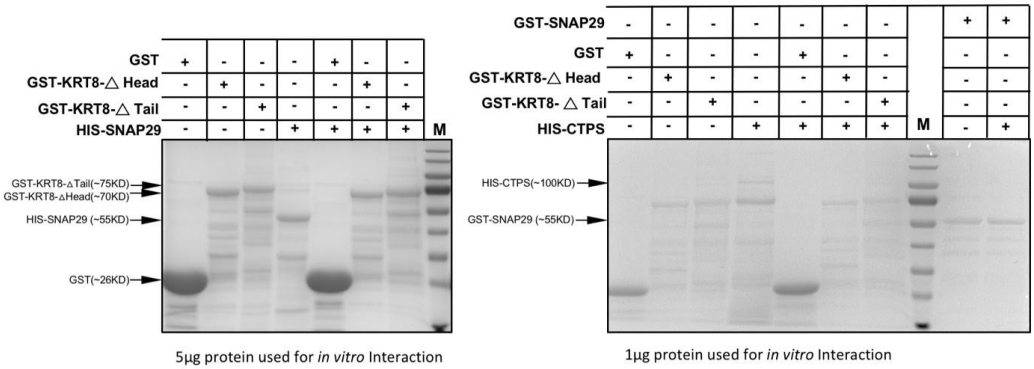


5. How specific does 1,6-Hexanediol act on keratins?

Response: Lin et al. (Toxic PR Poly-Dipeptides Encoded by the C9orf72 Repeat Expansion Target LC Domain Polymers, 2016, Cell 167, 789–802) found that cytoplasmic organization of keratin and vimentin intermediate filaments changed with 1,6-hexanediol treatment. So, we used 1,6-hexanediol to dissemble the cytokeratin network.

6. The role of SNAP29 in the ensemble of CTPS and keratins remains unclear. It will be necessary to understand whether SNAP29 can directly interact with keratins and with CTPS. In view of the known stickiness of keratins, I suggest to use keratin domains (head, rod tail) which are soluble and far less sticky than polymerization-competent intact proteins to map the interaction with SNAP29. In addition, it needs to be clarified whether SNAP29 directly interacts with CTP synthase.

Response: To answer the Reviewer's question, we now have performed *in vitro* interaction assay between HIS-tagged SNAP29 and GST fusion proteins containing truncated Head or Tail domain of KRT8. However, we didn't detect a direct interaction of SNAP29 with truncated KRT8. Similarly, HIS-tagged CTPS1 did not show a direct interaction with truncated KRT8. We do have a challenge in purifying HIS-tagged or GST fusion protein of CTPS1 in its native conformation. As most of the protein was in inclusion body so we used only 1ug protein to perform the *in vitro* interaction assay. Furthermore, GST SNAP29 fusion protein did not show a direct interaction with HIS-tagged CTPS1 either. Since the interaction only increases during stress conditions, there might be more levels of regulations involving post translational modifications and other protein complex.



5μg protein used for *in vitro* Interaction

1μg protein used for *in vitro* Interaction

Other issues:

8. The authors state (p6) "Previously, we found that CTPS filaments are important for endoreplication of salivary glands". How do SGs replicate?

Response: In *Drosophila*, there are several tissues that undergo endo-replication to amplify particular genes for special physiological functions, such as the production of secretory glycoproteins by salivary glands.

9. (p7) The authors state "SNAP29 mutation in humans leads to CEDNIK (cerebral dysgenesis, neuropathy, ichthyosis and keratoderma) syndrome, which is related to the defective transportation of components in keratinocytes during epidermal differentiation, suggesting a possible involvement of intermediate filaments". However, it is not clear why keratins supposedly are involved in transport processes. At present, no motors are known, and they possess an apolar organization.

Response: We agree that due to lack of polarity in keratin filaments and no known motor proteins associated with these networks, they are not yet considered to be involved in transportation processes. We have now removed "suggesting a possible involvement of intermediate filaments" from the sentence in the results section. (*Manuscript reference line no:190*)

10. (p7) It is not clear what has been done here and what is shown "Imaging at different time point, CTPS displayed a spatiotemporal relocation along the cytokeratin (Fig. 3B)."

Response: In Fig. 3B, we showed that CTPS enrichment along the cytokeratin network increased in a time-dependent fashion with histidine supplementation in EBSS medium. To make it clear for the readers, we have now re-written the sentence as: "Imaging at different time points, CTPS displayed a spatiotemporal association along the cytokeratin network (Fig. 3B)." (*Manuscript reference line no: 199)*

11. (p8) The statement "KRT8 and 18 are the most commonly found members of the keratin family in epithelial tissues, and they form heterodimers on the cytokeratin network" is not correct. K8 and K18 are most common in simple epithelia; they form a network via the assembly of heterodimers into ULF and into filaments.

Response: We have now rewritten the sentence according to the Reviewer's suggestion. "K8 and K18 are most common in simple epithelia; they form a network via the assembly of heterodimers into non-polar unit-length filaments (ULF) and into intermediate filaments (Snider and Omary, 2014)." (*Manuscript reference line no: 210*)

12. (p9) The sentence "CTPS filament formation, SNAP29 interacts with CTPS along the cytokeratin network by superresolution imaging" is not correct. Might read: SRI suggests that during CTPS filament formation, ...

Response: We have now rewritten the sentence as "Super-resolution imaging suggests that during CTPS filament formation, SNAP29 interacts with CTPS along the cytokeratin network." (*Manuscript reference line no: 275*)

13. Please clarify the nature of the mutant "because a tetrameric mutant of CTPS (G148A)". Does the mutation stop assembly at the tetramer state?

Response: We thank the Reviewer for the suggestion, and the G148A mutation was first used to understand the compositional complexity (monomers, dimers and tetramers) of yeast CTPS filaments (Noree et al., 2014). This mutation overlaps with the UTP binding site, which is critical for activity and tetrameric state (Goto et al., 2004). In our previous publication, we also showed that the CTP/UTP ratio is lower in HEK 293T cells overexpressing CTPS-G148A constructs (Lin et al., 2018). We have now changed the sentence as follows: "Moreover, this assembly along cytokeratin might also require proper conformation of the CTPS protein because the G148A CTPS1 mutant, which cannot form tetramer, could not assemble on the cytokeratin network (Fig. S6F)" (*Manuscript reference line no: 280*)

14. What is "This dynamic availability of SNAP29"?

Response: Our results suggests that SNAP29 interacts with the cytokeratin network in a time-dependent fashion in response to nutrient stress. We think that this interaction of SNAP29 with cytokeratin is dynamic in nature. To make it clear for readers, we have now removed the word dynamic from this sentence. (*Manuscript reference line no: 289)*

15. The phrase "Cytokeratins are the most diverse, which belong to the largest family among all types of intermediate filaments" is not correct. Possibly: Cytokeratins, which represent the largest family among all types of intermediate filaments, are the most diversified IF members.

Response: We have now rewritten the sentence according to the Reviewer's suggestion. (*Manuscript reference line no: 301*)

16. The entire manuscript needs critical input by a native speaker. Also, in several places singular and plural are not correctly used.

Response: We thank the Reviewer for the suggestions, and this manuscript has now been edited by a professional editing office.

Reviewer 3 Advance Summary and Potential Significance to Field:

This submission provides data at an important interface within cell biology, namely (macro) autophagy, stress responses and the involvement of the cytoskeleton. It reports an interaction between the SNARE family member, SNAP29, and the keratin network in HEp-2, HEK 293 and HeLa cells, which the authors propose is required for the formation of Cytidine 5'- Triphosphate Synthase (CTPS) filaments as part of the metabolic adaption to nutrient stress. In this case it is glutamine, an amino acid that in cancer cells helps maintain their reducing power as well as being essential for TCA cycle anaplerosis. CTP is an essential product from TCA cycle intermediates and is a coenzyme in pathways that produce for instance glycerophospholipids (phosphotidyl choline) and needed for N-linked protein glycosylation. The production of CTP from UTP by the enzyme CTPS is common to all life. CTPS forms filamentous structures of unknown function in both pro- and eu-karyotes. It has been reported that the polymerization inhibits CTPS activity and hence discovering how the filaments are formed and where in the cell would be an important discovery. The current submission claims to evidence the role of Intermediate Filaments in the assembly, a process that depends upon the association of SNAP29, a SNARE protein that is tightly associated with (macro)autophagy. At first glance there are two significant issues. Firstly bacterial orthologues to intermediate filaments are usually membrane bound. Drosophila have nuclear lamina, but thus far no cytoplasmic intermediate filaments. Secondly only a subset of the keratin filaments seem to provide the assembly platform for CTPS filament assembly. I think that the authors might argue that the complexity of a multicellular organism without an exoskeleton requires increased regulatory control and the association with a structure (intermediate filament) actively implicated in stress responses is therefore a logical extension of that control. SNAP29 and CTPS are biomarkers that evidence functional heterogeneity within the keratin network, a feature proven for all elements of the

cytoskeleton. Therefore neither an evolutionary nor spatial selectivity are arguments to prevent consideration of this submission. The report presents data to show that SNAP29 and CTPS can be co-immunoprecipitated and indeed can be colocalised by both high resolution fluorescence microscopy and immunogold electron microscopy methodologies.

There is some confusion about what is immunoprecipitation and what is co-precipitation. The Mass Spec approach seems to rely on an avidin-biotin co-precipitation approach, but uses fractions solubilized in 1% (w/v) SDS. As the SDS is diluted before the complex is isolated, there is the possibility that ad hoc, functionally unimportant complexes form. It is difficult to advise how to control for this possibility as per se, removing SNAP29/knockdown approach will diminish the signal for both functionally important and unimportant complexes. I do note though that for the immunogold labelling experiments presented in Figure 3, antibody controls and quantification were also missing and there appeared to be significant labelling of structures that were not filamentous in nature.

The other issue concerns the fractionation of the cells prior to immunoprecipitation using the keratin antibodies. The KRT lysis buffer will most likely retain the integrity of the keratin filaments, but solubilize the soluble fraction and those proteins associated with membranes. Therefore these are significant practical issues with the presented work and whilst the keratin knockdown approach mitigates partly the concern, the importance or otherwise of keratin 8 seems to have been somewhat overlooked within the submission. Indeed the knockdown of KRT8 (Fig. 3E) seemed to induce the formation of a single-perinuclear aggregate of CTPS. After all there are several JCS papers and others too that link keratins to hepatic stress responses, to mitochondrial shape and function and to metabolic regulation which might pertinent to the effects seen on HEp-2 cells, the primary cell line investigated in these studies.

Drugs were used to manipulate the three elements of the cytoskeleton. The use of 1,6 hexanediol as a specific antagonist of intermediate filament networks is somewhat adventurous given the literature and the previous work of Heather Durham. Equally nocodazole and cytochalasin are not necessarily precise in their ability to selectively alter just one of the three cytoskeletal filament systems. Rather given the role that SNAP29 plays in primary cilia formation, it might have been more interesting to investigate the correlation between pericentrin, SNAP29 and CPTS particularly in the KRT8 knockdown.

In summary therefore, the data presented are certainly provocative, but at this stage not convincing. This opinion should not be conflated with a conceptual conflict, rather this is a very exciting potential insight into the role of intermediate filaments. Several (to me) obvious experiments seem to be missing eg sequential extraction of the filamentous fraction from Hep-2 cells after incubating cultures in histidine and histidine/glutamine containing media; monitoring of (macro)autophagy, mitochondrial function and autophagosome formation or perhaps pursuing the ROI in SNAP29 responsible for binding to keratins.

Reviewer 3 Comments for the Author:

As detailed above the main issues for me are: There is some confusion about what is immunoprecipitation and what is co-precipitation. The Mass Spec approach seems to rely on an avidin-biotin co-precipitation approach, but uses fractions solubilized in 1% (w/v) SDS. As the SDS is diluted before the complex is isolated, there is the possibility that ad hoc, functionally unimportant complexes form. It is difficult to advise how to control for this possibility as per se, removing SNAP29/knockdown approach will diminish the signal for both functionally important and unimportant complexes.

Response: We agree with the Reviewer that the mass spectrometry analysis is just a screen, which may identify CTPS-related or unrelated proteins. Since CTPS in filaments is very difficult to be isolate in the soluble fraction, we applied 1% SDS to dissolve filament components after APEX-mediated proximity labeling. So as not to reduce the pull-down efficiency of streptavidin to biotin, we diluted the buffer to be 0.1% SDS. SNAP29 knockdown could affect different complexes; however, we also used PLA and immunofluorescence staining to demonstrate the relationship between SNAP29, CTPS and cytokeratin.

I do note though that for the immunogold labelling experiments presented in Figure 3, antibody controls and quantification were missing and there appeared to be significant labelling of structures that were not filamentous in nature.

Response: For Fig. 3H, we have now quantified the percentage of keratin immunogold on filamentous structures as in Fig. S6B, and the antibody control is in Fig. S3Ga. (*Manuscript reference line no: 235*)

The other issue concerns the fractionation of the cells prior to immunoprecipitation using the keratin antibodies. The KRT lysis buffer will most likely retain the integrity of the keratin filaments, but solubilize the soluble fraction and those proteins associated with membranes. Therefore these are significant practical issues with the presented work and whilst the keratin knockdown approach mitigates partly the concern, the importance or otherwise of keratin 8 seems to have been somewhat overlooked within the submission. Indeed the knockdown of KRT8 (Fig. 3E) seemed to induce the formation of a single-perinuclear aggregate of CTPS. After all there are several JCS papers and others too that link keratins to hepatic stress responses, to mitochondrial shape and function and to metabolic regulation which might pertinent to the effects seen on HEp-2 cells, the primary cell line investigated in these studies.

Response: In the immunoprecipitation experiment of KRT8, we applied sonication prior to immunoprecipitation, and SNAP29 was detected. However, we did not detect SNAP29 association in KRT8 IP without sonication, indicating that the soluble fraction of KRT8 is not strongly associated with SNAP29. The single-perinuclear aggregation of CTPS was seen in some cells under glutamine deprivation conditions, and it is not specific under KRT8 knockdown conditions.

Drugs were used to manipulate the three elements of the cytoskeleton. The use of 1,6 hexanediol as a specific antagonist of intermediate filament networks is somewhat adventurous given the literature and the previous work of Heather Durham. Equally nocodazole and cytochalasin are not necessarily precise in their ability to selectively alter just one of the three cytoskeletal filament systems. Rather given the role that SNAP29 plays in primary cilia formation, it might have been more interesting to investigate the correlation between pericentrin, SNAP29 and CPTS particularly in the KRT8 knockdown.

Response: In a previous study, to rule out the possibility that CTPS filaments are primary cilia, they were co-stained with pericentrin antibodies, which recognize centrosome. Since centrosome was not located at the end of the rod-like CTPS filament structure, they were not considered to be primary cilia (Carcamo et al., 2011).

In summary therefore, the data presented are certainly provocative, but at this stage not convincing. Several obvious experiments seem to be missing eg sequential extraction of the filamentous fraction from Hep-2 cells after incubation in Histidine and Histidine/glutamine containing media; monitoring of (macro)autophagy, mitochondrial function and autophagosome formation or perhaps pursuing the ROI in SNAP29 responsible for binding to keratins.

Response: We acknowledge the Reviewer's concerns, and monitoring of autophagy, mitochondrial function and autophagosome formation are all interesting future directions.

Examples of syntactical issues, of which there were many throughout the manuscript: Given that filament formation of CTPS is dynamic, posttranslational modifications, such as ubiquitination and methylation, are required for CTPS filament formation in human cancer cells (Pai et al., 2016). Commas misplaced? Conventional method like co-immunoprecipitation assay have been challenging in identifying proteins interacting with CTPS filament - words missing? western blotting - Proper noun needs capitalizing Exogenous CTPS1 (~100KDa) kDa?.....a SNARE binding protein, known to involve in multiple protein trafficking processes - be involved in? CTPS filaments assembles along Cytokeratin Network - assemble on the.....?

Response: We thank the Reviewer for the suggestions, and this manuscript has now been edited by a professional editing office.

The second movie didn't play.

Response: It can be played now.

References:

Carcamo, W.C., M. Satoh, H. Kasahara, N. Terada, T. Hamazaki, J.Y. Chan, B. Yao, S. Tamayo, Covini, C.A. von Muhlen, and E.K. Chan. 2011. Induction of cytoplasmic rods and rings structures by inhibition of the CTP and GTP synthetic pathway in mammalian cells. *PloS one*. 6:e29690.

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Goto, M., R. Omi, N. Nakagawa, I. Miyahara, and K. Hirotsu. 2004. Crystal structures of CTP synthetase reveal ATP, UTP, and glutamine binding sites. *Structure*. 12:1413-1423.

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Lin, W.C., A. Chakraborty, S.C. Huang, P.Y. Wang, Y.J. Hsieh, K.Y. Chien, Y.H. Lee, C.C. Chang, Y. Tang, Y.T. Lin, C.S. Tung, J.D. Luo, T.W. Chen, T.Y. Lin, M.L. Cheng, Y.T. Chen, C.T. Yeh, J.L. Liu, L.Y. Sung, M.S. Shiao, J.S. Yu, Y.S. Chang, and L.M. Pai. 2018. Histidine-Dependent Protein Methylation Is Required for Compartmentalization of CTP Synthase. *Cell reports*. 24:2733-2745.e2737.

Noree, C., E. Monfort, A.K. Shiau, and J.E. Wilhelm. 2014. Common regulatory control of CTP synthase enzyme activity and filament formation. *Mol Biol Cell*. 25:2282-2290.

Snider, N.T., and M.B. Omary. 2014. Post-translational modifications of intermediate filament proteins: mechanisms and functions. *Nat Rev Mol Cell Biol*. 15:163-177.

Professional editing certificate:

Second decision letter

MS ID#: JOCES/2019/240200

MS TITLE: SNAP29 mediates the assembly of histidine-induced CTP synthase filaments along the cytokeratin network

AUTHORS: Archan Chakraborty, Wei-Cheng Lin, Yu-Tsun Lin, Kuang-Jing Huang, Pei-Yu Wang, Yi-Feng Chang, Hsiang-Iu Wang, Kung-Ting Ma, Chun-Yen Wang, Xuan-Rong Huang, Yen-Hsien Lee, Bi-Chang Chen, Ya-Ju Hsieh, Kun-Yi Chien, Tzu-Yang Lin, Ji-Long Liu, Li-Ying Sung, Jau-Song Yu, Yu-Sun Chang, and Li-Mei Pai
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

We have sent your revised manuscript to the original reviewers. As you will see, reviewer #1 made only a few comments and recommended publication. Reviewers #2 and #3 indicated that the revised manuscript has improved but that the important questions concerning the specificity and the role of SNAP29 in the interaction of CTPS with keratin filaments have remained unanswered. Therefore, the significance of your findings remains unclear.

I agree with the critical reviewers that further information on how CTPS interacts with keratin filaments is needed before we can consider your manuscript for publication. In revising your manuscript, I would be grateful if you would list how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box online. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors demonstrate a previously unappreciated role for the cytokeratin network and the SNARE binding protein SNAP29 in the assembly of CTP synthase into cytoplasmic filaments. The strongest aspect of the study is the multiple approaches to confirm SNAP29-CTPS-cytokeratin interactions including proximity biotinylation, PLA, and fluorescence co-localization studies. A weakness is the lack of a clear model how these proteins might interact, given that SNAP29 is associated with vesicle trafficking but that CTP synthase filaments are not membrane associated. Nevertheless, I believe the revised manuscript should be published to inspire further investigation of the role of the intermediate filament network in facilitating CTP synthase assembly. The quality of the data and statistical analysis is generally high.

Comments for the author

I am satisfied that the authors have addressed the issues raised in my prior review and I recommend the manuscript be published. I have 2 minor comments, however.

In the point-by-point response, the authors state that in response to the reviewer comment they found that IMPDH assembly on the cytokeratin network was independent of CTPS. The data was not included in the revised manuscript but was shown in the letter. Unfortunately, the knockdown of CTPS was not particularly strong (~30%) so the significance of this negative result is unclear and the authors are perhaps wise to leave it out. Nevertheless, this is an interesting question that deserves follow up. Are IMPDH filaments cytokeratin-dependent? Are they SNAP29-dependent? This is probably beyond the scope of the current manuscript but by including the preliminary IMPDH data, the authors unsatisfyingly raise, but leave unanswered, obvious questions.

The meanings of error bars and asterisks are not given for Supplementary Figure S3

Reviewer 2

Advance summary and potential significance to field

The authors have undertaken efforts to address extensive reviewers' concerns and thereby have improved the manuscript. I remain very concerned about the interaction between CTPS and components of the keratin cytoskeleton. All data shown are based on imaging methods; attempts to show direct interactions by pulldown of KRT8, CTPS and SNAP29 have so far not been successful. Thus, in line with the occurrence of CTPS filaments in phylae which lack intermediate filament proteins, it remains well possible that close proximity between a subset of IF and CTPS occurs, however without direct interaction. The ultimate challenge which is beyond the scope of this

manuscript, remains to examine whether CTPS activity functionally depends on the state/and or presence of keratins.
Comments:
Title: I suggest to replace "along" by "in proximity to"
Hexanediol data lines 307-310: Given the lack of proven specificity of Hexanediol the statement "suggesting that CTPS filament formation may 310 depend on the integrity of the cytokeratin network" should be toned down. The wording in the results section is more appropriate.

Comments for the author

I remain very concerned about the interaction between CTPS and components of the keratin cytoskeleton. All data shown are based on imaging methods; attempts to show direct interactions by pulldown of KRT8, CTPS and SNAP29 have so far not been successful. This is the more important in view of the fact that SNAP29 is a transmembrane protein whereas CTPS and keratins are not. Thus, in line with the occurrence of CTPS filaments in phylae which lack intermediate filament proteins, it remains well possible that close proximity between a subset of IF and CTPS occurs, however without direct interaction.
Despite my concern, I accept a decision of publishing the data, as other groups might be interested to examine the functional significance of keratin-CTPS-SNAP29 interactions.

Reviewer 3

Advance summary and potential significance to field

I would like to thank the authors for their very detailed responses to all three reviewers. The manuscript has improved significantly, but for me the most important issue that remains to be addressed is specificity. The new data and revised manuscript do not answer this question and therefore I have become more skeptical of the significance of these observations presented by

The main point is that CTPS only associates with a small sub-portion of the keratin filament network in Hep2 cells.
The definition, properties, function of this sub-portion of the keratin network is unknown. Therefore the specificity or otherwise can not be assessed.
The movie data (eg Movie2) compel the view that there's a significant soluble pool of CTPS. This pool as shown by the fluorescence signal suggests a range of sizes to the CTPS material. There is also a non-keratin filament associated CTPS filament, suggesting that the proposed keratin association is not entirely keratin mediated rather could also involve microtubules, actin, their associated proteins or perhaps be related to the bundling of keratin filaments.
In summary, I dont doubt the data presented and I would like to thank the authors for their professional approach to the comments made by the referees, but I am unconvinced of the proposed significance of the data. Key points of revision suggested by us as referees could not be completed (eg CTPS methylation; in vitro binding assays) The authors need to identify what is special about the cytoskeletal association of some of the CTPS pool and why SNAP29 is needed for this association. Whether keratins, keratin bundles, other intermediate filament or cytoskeletal elements are needed remains to be evidenced.

Comments for the author

Revision will be tough because of the limited and transient nature of the physical interaction. Is the CTPS1 filament formation due to entrapment in the keratin filament bundles or is it a specific, function-mediate association and assembly.

Second revision

Author response to reviewers' comments

Point-by-point response to the Reviewers' comments

We appreciate the Reviewers' comments on our work. Our responses are in red, and the quoted text from the manuscript are in green.

Reviewer 1 Advance Summary and Potential Significance to Field:

The authors demonstrate a previously unappreciated role for the cytokeratin network and the SNARE binding protein SNAP29 in the assembly of CTP synthase into cytoplasmic filaments. The strongest aspect of the study is the multiple approaches to confirm SNAP29-CTPS- cytokeratin interactions including proximity biotinylation, PLA, and fluorescence co- localization studies. A weakness is the lack of a clear model how these proteins might interact, given that SNAP29 is associated with vesicle trafficking but that CTP synthase filaments are not membrane associated. Nevertheless, I believe the revised manuscript should be published to inspire further investigation of the role of the intermediate filament network in facilitating CTP synthase assembly. The quality of the data and statistical analysis is generally high.

Reviewer 1 Comments for the Author:

I am satisfied that the authors have addressed the issues raised in my prior review and I recommend the manuscript be published. I have 2 minor comments, however.

In the point-by-point response, the authors state that in response to the reviewer comment they found that IMPDH assembly on the cytokeratin network was independent of CTPS. The data was not included in the revised manuscript but was shown in the letter. Unfortunately, the knockdown of CTPS was not particularly strong (~30%) so the significance of this negative result is unclear and the authors are perhaps wise to leave it out. Nevertheless, this is an interesting question that deserves follow up. Are IMPDH filaments cytokeratin-dependent? Are they SNAP29-dependent? This is probably beyond the scope of the current manuscript but by including the preliminary IMPDH data, the authors unsatisfyingly raise, but leave unanswered, obvious questions.

Response: We appreciate the Reviewer 1's support of our work to publication. We agree that whether IMPDH filament depends on CTPS is an important question, so we now have included this data in supplementary Fig. S4D and S4E. (*Manuscript text reference Line no: 203-205*)

The meanings of error bars and asterisks are not given for Supplementary Figure S3

Response: We thank Reviewer for the critical reading and we have now described the statistical analysis in the Figure legend of Fig. S3F.

Reviewer 2 Advance Summary and Potential Significance to Field:

The authors have undertaken efforts to address extensive reviewers' concerns and thereby have improved the manuscript. I remain very concerned about the interaction between CTPS and components of the keratin cytoskeleton. All data shown are based on imaging methods; attempts to show direct interactions by pulldown of KRT8, CTPS and SNAP29 have so far not been successful. Thus, in line with the occurrence of CTPS filaments in phylae which lack intermediate filament proteins, it remains well possible that close proximity between a subset of IF and CTPS occurs, however without direct interaction. The ultimate challenge which is beyond the scope of this manuscript, remains to examine whether CTPS activity functionally depends on the state/and or presence of keratins.

Comments:
Title: I suggest to replace "along" by "in proximity to"
Hexanediol data lines 307-310: Given the lack of proven specificity of Hexanediol the statement

"suggesting that CTPS filament formation may 310 depend on the integrity of the cytokeratin network" should be toned down. The wording in the results section is more appropriate.

Response: We thank Reviewer 2' suggestions, and the title of the manuscript has been changed to be "SNAP29 mediates the assembly of histidine-induced CTP synthase filaments in proximity to the cytokeratin network". The line of 307-310 in discussion section have been modified according to reviewer's suggestion as "These results suggest a possibility that CTPS filament formation may relate to the cytokeratin network". (*Manuscript text reference Line no: 311-312*)

Reviewer 2 Comments for the Author:

I remain very concerned about the 1) interaction between CTPS and components of the keratin cytoskeleton. 2) All data shown are based on imaging methods; 3) attempts to show direct interactions by pulldown of KRT8, CTPS and SNAP29 have so far not been successful. 4) This is the more important in view of the fact that SNAP29 is a transmembrane protein whereas CTPS and keratins are not. Thus, in line with the occurrence of CTPS filaments in phylae which lack intermediate filament proteins, it remains well possible that close proximity between a subset of IF and CTPS occurs, however without direct interaction.

Despite my concern, I accept a decision of publishing the data, as other groups might be interested to examine the functional significance of keratin-CTPS-SNAP29 interactions.

Response: We appreciate Reviewer 2's support on our manuscript to be published to lead researchers to further investigate the interaction between keratin-CTPS-SNAP29.
1) We believe that the experimental tool we used, including APEX to show the association of CTPS filament with keratin is relatively specific. The length of CTPS filaments are around 5-10 um in human cancer cells, and are mainly maintained in a linear structure. Therefore, previously several studies had investigated the association with actin microfilament, and microtubule cytoskeleton, and learned that there is no co-localization or proximity between these structures. The vimentin, a member in intermediate filament family was investigated as well, and the result was negative (ref). Here, we identified keratin 8, and 18 as candidates for enhancing CTPS filament formation through SNAP29. SNAP29 has been reported to be involved in transportation inside keratinocytes, and several keratin isoforms were simultaneously identified in our results of proteomic analysis of CTPS-APEX2-mediated biotinylated proteins. We further confirm this proximity by reverse approach using keratin 18-APEX2 to pull down SNAP29 and CTPS.
2) In addition to the co-localization in immunostaining fluorescence images, the proximity ligation assay (PLA) which only occurs between two proteins within 40 nm, also demonstrated the substantial specificity of the assay. Importantly, the ultrastructure images from electronic microscopy showed that filamentous CTPS (DAB staining of Flag-CTPS- APEX2) locates on filamentous structures that are labeled by anti- keratin 8. Since we do not have a biochemical assay to show direct interactions, we agree that "proximity" as suggested by Reviwer#2 is a more precise word to describe the relationship between CTPS and keratin.
3) One of the possibilities that in vitro assay did not show direct interactions between CTPS and keratin or SNAP29, could be due to that one or more required proteins for the interaction of CTPS-SNAP29-keratin may be missing in the in vitro assay. Alternatively, the interactions between these molecules could be transient and, therefore, the complex cannot be pulled down in in vitro conditions. A third possibility is that post-translational modifications might be required for the interaction in vivo, which is not present in vitro. We also agree that a subset of IF and CTPS are at a close proximity without direct interaction as suggested by Reviewer #2.
4) We believe that this stress dependent association of keratin-CTPS-SNAP29 must have more levels of regulation, probably involving post translational modification and other protein complexes in the assembly process. Therefore, the complexity of association between CTPS filament and keratin network may not be revealed by just the study presented here. However, the novelty of this study may lead others to further investigate a previously unappreciated role of the cytokeratin network in the regulation of metabolic compartmentalization.

Reviewer 3 Advance Summary and Potential Significance to Field:

I would like to thank the authors for their very detailed responses to all three reviewers. The manuscript has improved significantly, but for me 1) the most important issue that remains to be addressed is specificity. The new data and revised manuscript do not answer this question and therefore I have become more skeptical of the significance of these observations presented by. 2) The main point is that CTPS only associates with a small sub-portion of the keratin filament network in Hep2 cells. The definition, properties, function of this sub-portion of the keratin network is unknown. Therefore the specificity or otherwise cannot be assessed.

Response: We believe that the association between CTPS filament and keratin is relatively specific, which have already been described in the responses to Reviewer#2. Therefore, we will not reiterate it. Please see responses in the above.

The movie data (eg Movie2) compel the view that there's a significant soluble pool of CTPS. This pool as shown by the fluorescence signal suggests a range of sizes to the CTPS material. There is also a non-keratin filament associated CTPS filament, suggesting that the proposed keratin association is not entirely keratin mediated rather could also involve microtubules, actin, their associated proteins or perhaps be related to the bundling of keratin filaments.

Response: The movie 2 was made from 3 sections of live image taken from confocal microscope (Andor Dragonfly) to show a close proximity between CTPS filament and the cytokeratin network. However, we agree that not all CTPS signals were overlapped with keratin signals since the 3 sections may not include entire keratin network. Moreover, we did not see that CTPS filaments associate with microtubule, actin or vimentin network, which is consistent with a previous study (Carcamo et al., 2011), suggesting a selectivity of the assay.

In summary, 1) I dont doubt the data presented and I would like to thank the authors for their professional approach to the comments made by the referees, but I am unconvinced of the proposed significance of the data. Key points of revision suggested by us as referees could not be completed (eg 2) CTPS methylation; 3) in vitro binding assays) The authors need to identify 4) what is special about the cytoskeletal association of some of the CTPS pool and why SNAP29 is needed for this association. 5) Whether keratins, keratin bundles, other intermediate filament or cytoskeletal elements are needed remains to be evidenced.

Response: 1) We thank Reviewer 3 for the recognition of our data and efforts in previous revision.
2) The question about CTPS methylation raised by Reviewer 1 was to help us further find a potential mechanism by which SNAP29 regulates the CTPS filament formation. However, the role of methylation in which step of the process of CTPS filament formation remains unclear. In addition, methylation could occur on other related proteins. As CTPS arginine 449 methylation was not detectable, which suggested that this modification is less abundant or it could be transient due to the present of demethylation events. Therefore, further studies on identification of methylated proteins in CTPS filament formation processes may shed lights on this mechanism.
3) The Reviewer 2 suggested to analyze the potential direct bindings between CTPS, keratin 8, and SNAP29 to further clarify the roles of these components. Unfortunately, in vitro assay did not show direct interactions between CTPS and keratin or SNAP29 for potential reasons that we have described in the above (response to Reviewer#2 the third response).
4) We believe that the role of SNAP29 is crucial for modulating the enzymatic activity of CTPS, which has been demonstrated by SNAP29 knockdown approach. The RNAi resistant SNAP29 rescued this modulation of CTPS activity in our revision, which improved the significance of the RNAi knockdown results. These results suggest the significance of the association between Keratin-CTPS-SNAP29.
5) The involvement of other cytoskeletal elements was discussed in the above (response to Reviewer# 2 the first response).
This is a pilot study in understanding the CTPS filament formation, we used the proximity approach to identify CTPS associated proteins, and verified their roles in filament formation. Indeed, there are many interesting questions for further studies, however, they are probably beyond the scope of this manuscript.

Reviewer 3 Comments for the Author:

Revision will be tough because of the limited and transient nature of the physical interaction. Is the CTPS1 filament formation due to entrapment in the keratin filament bundles or is it a specific, function-mediate association and assembly.

Response: We appreciate the Reviewer 3 for understanding the challenges of investigating this novel dynamic assembly of CTPS filament. Our data demonstrated that the association of CTPS filament with keratin is relatively specific as we discussed in the above.

Carcamo, W.C., M. Satoh, H. Kasahara, N. Terada, T. Hamazaki, J.Y. Chan, B. Yao, S. Tamayo, G. Covini, C.A. von Muhlen, and E.K. Chan. 2011. Induction of cytoplasmic rods and rings structures by inhibition of the CTP and GTP synthetic pathway in mammalian cells. *PloS one*. 6:e29690.

Third decision letter

MS ID#: JOCES/2019/240200

MS TITLE: SNAP29 mediates the assembly of histidine-induced CTP synthase filaments in proximity to the cytokeratin network

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ARTICLE TYPE: Research Article

I have read your revised manuscript and taken notice of your answers to the comments of the reviewers. After careful consideration, I have decided to accept your paper for publication in the Journal of Cell Science.