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Compartment-specific aggregases direct distinct nuclear and cytoplasmic aggregate deposition

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 August 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript - although there is some disagreement in their evaluation of the broader significance of the findings. The referees also raise a number of significant technical and experimental concerns that will have to be addressed before they can support publication of a revised manuscript here.

In particular, you will need to provide further evidence to support the nuclear localisation of the INQ and to discuss the discrepancy with earlier reports on cytoplasmic localisation. If you have some data at hand concerning the relevance for the mammalian system, this would obviously strengthen the manuscript further but this point will not be an absolute requirement from our side. The referees also request mechanistic insight on the role for Btn2 and for ubiquitination of misfolded proteins. Regarding the comments on terminology used, I will leave it up to you to decide whether the terms 'aggregase' and 'INQ' stay in a revised manuscript. Furthermore, I would recommend that you leave the data on INQ formation following DNA damage in the manuscript (even if further functional follow up on that aspect of the study may fall outside the scope of the current work).

I understand that addressing all concerns raised will require an extensive effort on your side and I would understand it if you would rather wish to seek rapid publication in a less demanding venue. However, if you would be willing to undertake the effort to revise the manuscript along the lines

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laid out by the referees, we would be happy to consider a revised version of your manuscript. In light of the need for such substantial revision we can also offer to extend the deadline for this revised version to 6 months.

Given the referees' overall positive recommendations, I would invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication and please feel free to contact me with any questions. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Miller et al. present several lines of confusing evidence that compartment-specific 'aggregases' direct the deposition of misfolded proteins to specific compartments within the nucleus and cytoplasm in yeast. It is suggested that Hsp42 performs this function in the cytoplasm and Btn2 in the nucleus. Evidence is presented that the JUNQ compartment previously defined by Kaganovich and Frydman, is actually inside the nucleus, and should be termed the INQ. In my view, although these studies might be interesting to a specialized audience they are not of the broad significance that is usually associated with EMBO J. Moreover, I have concerns about several aspects of this confusing paper:

1. In previous studies, the Frydman and Kaganovich labs have provided multiple lines of evidence that the JUNQ is a conserved compartment that is also found in mammalian cells. By contrast, in the present study no evidence is provided that the INQ is also a relevant compartment in mammalian cells. Hence, the broader significance of this work is extremely unclear. Is the INQ just a phenomenon restricted to yeast? Should we abandon the the term JUNQ for mammalian cells too?

2. No convincing explanation or evidence is given to explain why the JUNQ was mistakenly assigned to the cytoplasm in previous work. There is a general failure to explain why in several careful studies (e.g. Spokoini et al., 2012) involving 3D reconsructions, the JUNQ is clearly visible outside the nucleus as originally described. In these studies, the nucleus is labeled by NLS-TFP and not simply DAPI stained. Thus, the present paper is extremely confusing and it is not clear that the JUNQ was truly misassigned to the cytoplasm. More effort is need to reconcile the present findings with previous studies.

3. It is suggested that Hsp42 and Btn2 are 'aggregases'. The suffix '-ase' suggests that they possess a catalytic activity in aggregate formation. However, Hsp42 and Btn2 also form part of the final aggregates and are thus consumed by the reaction. Hence, the term 'aggregase' is extremely misleading, confusing, and unhelpful as the proteins are not acting in a catalytic manner similar to enzyme. This term should be completely avoided.

4. The similarity between Btn2 and sHsps (Fig. 7A) is extremely marginal and not convincing at all. If the 100-215 region of Btn2 is similar truly similar to α-crystallin domain then it should display

activity in isolation. However, this is not tested. Is this region even required for Btn2 activity? Do point mutations in this region disrupt activity? Btn2 is also related in sequence to human Hook proteins, involved in aggresome formation and other transport activities. However, this similarity is completely ignored.

5. The INQ compartment appears to be stabilized by guanidinium hydrochloride, indicating that it is dissolved by the activity of Hsp104 (Fig. E5). However, INQ-specific Btn2 is subsequently shown to prevent aggregation dissolution by Hsp104 (Fig. 7F). This disconnect between the in vitro and in vivo data is disconcerting and adds to the general confusion created by this paper.

Referee #2:

In the manuscript "Compartment-specific aggregases direct distinct nuclear and cytoplasmic aggregate deposition" the Miller, Ho, Winkler, Khokhrina and colleagues provide evidence that the deposit for aggregated proteins that was previously believed to be juxtanuclear (JUNQ) (Kaganovich et al. 2008) is actually intranuclear. Therefore, they renamed this deposit "INQ" standing for intranuclear quality control compartment. Furthermore, they claim that sorting of aggregated proteins into INQ requires Sis1 and Nup42 mediated nuclear import of misfolded proteins followed by Btn2 mediated aggregation. Overall, this is an interesting manuscript with a large body of data, which on the flip side makes the manuscript a bit fragmented at places. Most of the experiments seem to be carefully done and the manuscript is well written. While most of the experiments are conducted in extreme conditions (i.e. by over-expressing exogenous proteins prone to misfold with simultaneous proteosomal inhibition and/or heat stress), the authors also provide some interesting hints that the INQ-pathway may be engaged during DNA replication stress.

The main argument that JUNQ-like structure resides inside the nucleus is well supported by both light and electron microscopic data. JUNQ was previously described as perinuclear solely based on co-localization with NLS and DAPI staining, which makes a distinction between intranuclear and perinuclear very difficult. Here, the authors used markers for nuclear pores as well as electron microscopy, allowing a more defined localization determination. Additionally, the authors used very similar conditions to study the localization of JUNQ in order to exclude experimental differences, although the possibility of coexisting nuclear and cytoplasmic JUNQ-like structures needs to be clarified. Data regarding the role of ubiquitination and nuclear import in this process is less convincing and would, in my opinion, require some additional experiments to back up the conclusions drawn (see below). The observation that Btn2 and Sis1 targets misfolded proteins to JUNQ was previously reported (Malinovska et al. 2012), however, novel and interesting in vitro evidence is provided demonstrating that Btn2 displays small heat shock protein like characteristics. Furthermore, the author's make an interesting link between DNA replication stress and the formation of INQ foci. Overall, this is an interesting manuscript on an important topic. However, some of the conclusions are not well justified in the current manuscript and need to be addressed before publication.

Specific points:

1. The authors found that Sis1-depleted cells contain more cytoplasmic deposits leading to the conclusion that Sis1 is required for the import of misfolded proteins into the nucleus. However, the images in Fig. 4 show an increased size of the nuclear deposit (INQ). Similarly the authors concluded from increased number of cytoplasmic VHL foci that Nup42 is required for nuclear import of misfolded VHL. Here, it is not clear whether deletion of these genes is indeed impairing the nuclear import or whether these cells are for example more stressed with a consequent increase in protein aggregation. Furthermore, it is also worrisome that all these experiments were performed in cells lacking Hsp42 without knowing how deletion of Hsp42 affects the flux for misfolded proteins between the nucleus and the cytoplasm. Additionally, since VHL is found in the cytoplasm and the nucleus prior to the shift to 37{degree sign}C, it is not clear whether the native or misfolded proteins are imported into the nucleus (i.e. is the INQ forming form pre-existing nuclear VHL or is there an active import of misfolded VHL into the nucleus that constitutes the INQ).
To solidify their conclusions and answer these questions of nuclear import of VHL, I strongly advice the authors to measure the flux rates of VHL import in the nucleus (after shut off of expression). This can be done for example by a FRAP experiment (bleaching the nuclear VHL

signal and measuring the recovery) or alternatively by using VHL tagged with a photoconvertible label. By conducting the experiment in Nup42 delete, Sis1 delete and Hsp42 delete, this experiment will allow a more careful assessment of the role of Nup42 and Sis1 in the nuclear import and to quantify the effect of deleting Hsp42 to the nuclear import of VHL.

2. It is shown that in wild type cells (at 370C with proteosomal inhibition) VHL forms one peripheral focus and one nuclear focus. From this the authors conclude that the nuclear puncta represents the JUNQ and the peripheral is the IPOD. It needs to be excluded that these two foci do not represent cytoplasmic JUNQ and a nuclear JUNQ (INQ). This should be tested by co-expressing the IPOD specific RNQ1 (Kaganovich et al. 2008) and VHL in these conditions. If the authors are correct there should be one nuclear focus (without RNQ1) and one cytoplasmic focus (with RNQ and VHL). However, if the cytoplasmic RNQ1 and VHL form distinct foci, it suggest that there are two JUNQ like structures, one cytoplasmic and one nuclear.

3. In the fractionation experiment described in Fig. 6 where the insoluble fraction is determined, an important control is missing. It is advisable to repeat the experiment by adding a soluble control protein (e.g. Pgk1 or free GFP).

3. The claim that ubiquitination is not required for INQ sorting is misleading. The phenotype in the non-ubiquitinated state (San1/Ubr1 delete) was there were more nuclear foci as compared to wild type, indicating that although nuclear aggregates still can form, they became fragmented, which might at least partially reflect a sorting defect. Therefore, in my opinion it would be safer to say that ubiquitination is not required for the formation of nuclear puncta (including INQ), but might promote their fusion. Text regarding these conclusions should be reformulated.

4. Based on the in vitro data and the effect of transiently expressing Btn2, the authors claim that after forming its duty as an "aggregase", the presence of Btn2 inhibits refolding of INQ substrates. If the authors wish to solidify this conclusion, the promoter of Btn2 could be exchanged by a constitutive active promoter (e.g. GDP) in order to test if INQ is now stabilized upon heat shock removal. It would be nice if the authors could speculate: does the transient expression peak of Btn2 possibly reflect the high turnover rate of INQ substrates?

5. The phenotype described in Fig. E5 should be quantified from several videos to justify the conclusions drawn.

6. Fig. 6A: a picture of a cell in a comparable cell cycle stage (G1) should be added.

7. The term "% cells with INQ" is not easy to understand. Perhaps there is a way to make the graphs more reader friendly.

8. Use either everywhere CPY or Prc1 as nomenclature (Fig. E8).

9. In the model of Fig. 9 it seems that Hsp42 and Hsp104 are switched in the cytoplasm. Hsp42 should be the aggregase and Hsp104 the disaggregase?

10. The finding that DNA replication stress induced by MMS treatment leads to INQ formation is very remarkable and would be interesting to follow up. It would be nice if the authors could speculate in the discussion why Hos2 aggregates into one nuclear focus (INQ) upon replication stress.

Referee #3:

The manuscript by Miller and colleagues "Compartment-specific aggregases direct distinct nuclear and cytoplasmic aggregate deposition" describes the characterization of the previously defined juxtanuclear quality control inclusion or 'JUNQ' as intranuclear rather than cytosolic or juxtanuclear.

In previous publications by other authors, notably the Frydman and Kaganovich labs, misfolded proteins in the cytosol have been shown to partition between two compartments: the JUNQ and IPOD. Generally, it has been assumed that both the JUNQ and IPOD are cytosolic quality control

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compartments due to the presumed cytosolic nature of the substrates examined. It is clear the IPOD is cytosolic. The JUNQ appears juxtanuclear cytosolic, but the markers that were used to define the JUNQ as juxtanuclear in past papers were chromatin, chromatin-associated histones, or a fluorescent protein fused to an NLS. Nuclear membrane markers had not been used, so it is conceivable that the JUNQ could be intranuclear rather than juxtanuclear.

In the studies here by Miller et al, the authors use a nuclear pore protein to delineate the nuclear membrane. With this measure, the authors demonstrate that the JUNQ is constrained by the nuclear membrane. The authors also use EM to demonstrate localization constrained by the nuclear membrane. This is the prinicipal significance of the study. The authors then go on to define chaperones that separate the intranuclear inclusions from the cytosolic inclusions. Notably demonstrating that Btn2 is crucial for sequestering proteins from the general nucleoplasm into the nuclear inclusion.

Prior to publication, there are a number of experimental and editorial concerns that need to be addressed.

Major concerns:

1. The authors adopt the term 'INQ' in what they believe is a better way to reflect the intranuclear localization of the 'JUNQ'. I think it is better to simply remain with JUNQ as the nomenclature because too many previous publications have already used this term. Changing the nomeclature would just increase confusion in the literature, akin to how gene names are often changed leading to multiple and often confusing names in the literature. Same for "CytoQ".

2. To be entirely convincing that the JUNQ is intranuclear, I think the authors need to do a 3D reconstruction of cells with a nuclear pore marker, DAPI-stained DNA and the JUNQ. The single plane images are suggestive, but the onus is on the authors to fully demonstrate intranuclear localization if they wish to counter what has been previously published.

3. For the microscopy in Figure 1A and B, we are only given a single cell to draw conclusions. I think more cell images should be included in the supplemental materials so that the readers can see the different cell-to-cell variations.

4. On page 9, "To provide further evidence for nuclear localization of INQ at higher resolution we performed immunoelectron microscopy of wt cells expressing GFP- Luciferase-DM-NLS and hsp42Δ cells expressing GFP-VHL (Figure 2A/B)". I think Figure E4 should go into Figure 2. The controls are important.

5. On page 11, "However, by ubiquitin immunostaining, we did not observe specific co-localization of ubiquitin and INQ (Figure 3A, Figure E7A)." The lack of ubiquitin immunostaining doesn't necessarily support that ubiquitin is not required for INQ formation. It could be the ubiquitylation is dynamic: required for formation of the INQ but removed once proteins are sequestered into the INQ. Also, while tGnd1-GFP forms nuclear inclusions in ubr1Δsan1Δ cells, this could simply be that the protein is rapidly degraded in wt cells but is stablized in ubr1Δsan1Δ cells and accumulates to much higher levels. Furthermore, loss of ubiquitylation through deletion of the QC degradation E3s doesn't exclude ubiquitylation by an E3 that is not involved in QC degradation. I think the authors need to be more cautious here. Especially when they overstate in the Discussion that "Our results revoke the current view of a ubiquitin-based sorting mechanism that targets ubiquitinated proteins to JUNQ (now INQ) and non-ubiquitinated proteins to IPOD (Kaganovich et al, 2008)." Unless they present a whole lot more data showing they can get ubiquitin colocalization with known structures and exhaust by all other methods that ubiquitin is not required for JUNQ formation, this section should be removed from the Results and Discussion because it is too inconclusive.

6. In Figure 3D, why did the authors switch to using histone H2B as the nuclear marker rather than the nuclear pore proteins? If they wish to demonstrate that tGnd1-GFP is intranuclear, they have to be consistent in the nuclear marker they use. Especially when the precise spatial localization is the foundational idea and claim of this manuscript. Using H2B is the same trap previous authors fell into.

7. Similarly, the authors need to use the nuclear pore marker for the Sis1 experiments in Figure 4B.

8. From Figure 4B, the authors conclude "These findings suggest that while Sis1 clearly plays an important role in nuclear import of misfolded proteins for INQ formation, other, so far unknown factors, are additionally involved." I'd suggest the authors be cautious here. The Sis tet deletion system is good, but it could be that remaining Sis1 protein levels function in the residual nuclear import, not another pathway. Furthermore, there are alternative interpretations of the data that would not support a role for Sis1 in nuclear import. For example, what if Sis1 is playing a role in maintaining solubility in the cytosol prior to nuclear import? In the absence of Sis1, the misfolded GFP-VHL proteins form inclusions in the cytosol and thus would be unable to transit the nuclear pore in the included state. This seems a more likely interpretation because >50% of the GFP-VHL is still within the nuclear inclusions even after depletion of Sis1.

9. More cell images are needed for Figure 5A and B. It would also be helpful to have the separate channel images in addition to the merged images.

10. Similar to my earlier points, a nuclear pore marker should be used in Figure 6A, B, D and E.

11. I think the DNA damage (MMS) experiments could be excluded from the manuscript. I understand why the authors wish to include them for physiological relevance. But, they seem rather preliminary compared to their other studies. And, at least in my opinion, they are distracting at the end the results section. For example, is Hos2 actually misfolded during MMS exposure? Does VHL become a better QC degradation substrate during MMS exposure? There are a ton of questions left unanswered after this section. And the section seems very incomplete. I'd rather the authors firm up all previous findings and make this a definitive intranuclear paper with strong chaperone data support.

12. The authors' use the term aggregate in the title and throughout the paper when they are discussing cellular inclusions. Aggregate has a very specific biochemical definition, which has not been demonstrated for the cellular inclusions. Have they demonstrated that the proteins in the nuclear and cytosolic inclusions are actually aggregated in the cell? Or, or they just concentrated in a specific cellular location? Separation into soluble and insoluble fractions does not necessarily indicate aggregation. They would need to assess the oligomeric state of the proteins in cells to be able to use the term aggregate for inclusion. The authors use the term appropriately in Figure 7 where they are biochemically looking at actual aggregates.

13. More cell images are needed for Figure E6A-C to solidify the claim that "INQ appears as a single focus of fluorescence adjacent to nucleolus and chromatin, opposite the spindle pole body". And, I think 3D reconstructions would be essential here.

Minor concerns:

1. The authors should be better in terms of scholarship. For example, if the authors cite Escusa-Toret et al 2013 (Nature Cell Biology 15: 1231-1243) for Q-bodies, they should also cite Spoikoni et al 2012 (Cell Reports 2: 738-747) for stress foci. The latter paper was published first and described the same phenomenon. Additionally, there are recent papers looking at inclusions of misfolded proteins in the yeast nucleus that are bounded by a nuclear membrane marker, most notably San1 substrates (MBoC 22: 2384-2395, JBC 288: 6130-6139, JCS 127: 1980-1991). These studies should be considered especially since the authors include the San1 substrates tGnd1-GFP and ΔssCPY*. It is not clear that the all nuclear inclusions formed by San1 substrates are the JUNQ, but it is probably likely and they should include something about this in the Discussion.

2. The manuscript would read much better if the authors standardized their language and presented their observations as cytosolic versus nuclear inclusions. Just a few examples:

On page 9, "In order to determine the relationship between formation of peripheral (cytosolic) and INQ aggregates we performed time-lapse microscopy." It would read better to the layperson as "In order to determine the relationship between formation of cytosolic and nuclear inclusions we performed time-lapse microscopy."

Also, "While apparent encounters of cytosolic aggregates and nuclear INQ foci were occasionally observed" would read better as "While apparent encounters of cytosolic and nuclear inclusions were occasionally observed"

Further, "The observation that INQ and peripheral aggregates form independently from one another" could be "The observation that nuclear and cytosolic inclusions form independently from one another"

3. In the Discussion: "Several observations indicate that substrate shuttling between cytosol and nucleus is actively biased toward transport into the nucleus." I don't think the observations indicate that there is shuttling between cytosol and nucleus, only that some cytosolic misfolded proteins are transported to the nucleus. Shuttling implies that some proteins move from the nucleus to the cytosol as well as from the cytosol to the nucleus.

5. In the Discussion: "This study establishes key features of cellular and molecular organisation of protein aggregation in yeast. This redraws the conceptual framework for protein quality control in eukaryotic cells." I think this is an overstatement. The previous JUNQ and IPOD studies developed the important conceptual framework that there is a bipartite system for sequestration of misfolded proteins. The work here redefines the location of the JUNQ, which does have new implications especially in terms of how misfolded cytosolic proteins are trafficked to the nucleus. In general, I thought the Discussion could use a lot of editing to remove overstatements and make it more concise.

1st Revision - authors' response 08 December 2014

Reply to referees' comments for manuscript EMBOJ-2014-89524 (Miller et al.).
Reviewers' comments are shown in italics.

We thank the reviewers for constructive comments and have carefully attended to all concerns and suggestions. We provide an extensive set of new experiments and analyses. Main additions to the revised manuscript include:

1) 3D reconstruction of cells based on fluorescence microscopy and expanded EM analysis of yeast thin sections, including the use of new misfolded reporter proteins. These experiments consolidate evidence indicating INQ/JUNQ resides inside the nucleus.
2) Demonstration of dynamic shuttling of misfolded reporter proteins between the cytosol and the nucleus using FLIP analyses.
3) Direct evidence for preferential nuclear import of misfolded proteins.
4) Demonstration that degradation kinetics of misfolded reporters are independent of substrate sequestration to a particular site. We show misfolded protein degradation in yeast wt and mutant cells forming either only CytoQ or INQ/JUNQ, or no inclusions at all is not different. A role for INQ/JUNQ in sorting sequestered proteins to proteasomal degradation is therefore unlikely. These further data support our initial findings indicating ubiquitination is not required for INQ/JUNQ sorting.
5) Further demonstration that aggregase effects are compartment-specific. Pronounced cytosolic VHL aggregation in *sti1Δ* cells is shifted to exclusive nuclear aggregation in *sti1Δ hsp42Δ* cells, demonstrating that VHL sequestration can be switched from the cytosol to the nucleus dependent on the availability of the cytosolic aggregase Hsp42, which we show is absent in the nucleus. These findings (i) disprove the original definition of Sti1 as an INQ/JUNQ sorting factor and (ii) suggest dynamic shuttling of VHL between compartments occurs.

Referee #1:

1. In previous studies, the Frydman and Kaganovich labs have provided multiple lines of evidence that the JUNQ is a conserved compartment that is also found in mammalian cells. By contrast, in the present study no evidence is provided that the INQ is also a relevant compartment in mammalian cells. Hence, the broader significance of this work is extremely unclear. Is the INQ just a phenomenon restricted to yeast? Should we abandon the term JUNQ for mammalian cells too?

A new outcome of our study is that the localizations of protein inclusions in yeast and mammalian cells differ. We have determined the localization of stress-induced GFP-VHL inclusions in mammalian cells and find these deposits locate outside the nucleus, beside the MTOC (microtubule organizing center) (new Figure E16). VHL inclusions localize next to Vimentin clusters, qualifying them as aggresomes (Kopito, 2000). Similar findings in mammalian cells are independently reported by the Kaganovich lab (Ogrodnik et al, 2014; Weisberg et al, 2012). In yeast VHL inclusions localize to either cytosolic sites or reside inside the nucleus opposite to the spindle pole body, the yeast MTOC equivalent. Our work exposes the existence of different sorting mechanisms operating in yeast and mammalian cells and explains these different localizations. In yeast, sequestration requires the compartment-specific aggregases, Hsp42 and Btn2, to target misfolded proteins to cytosolic or nuclear sites, respectively. Mammalian cells lack homologs of Hsp42 and Btn2 and instead use dynein-mediated protein sequestration transport of misfolded cargo along microtubule tracks to the juxtanuclear MTOC (Johnston et al, 2002). In contrast, INQ/JUNQ formation in yeast is independent from microtubules (Specht et al, 2011). Different mechanisms therefore actively sequester misfolded proteins in mammalian and yeast cells and target them to different cellular sites.

A simple, unifying mechanistic concept has been prominently published by Frydman and Kaganovich as applicable to eukaryotes from yeast through to mammalian cells. We demonstrate here that this is not the case since the INQ (former JUNQ) is a nuclear compartment specific to yeast.

2. No convincing explanation or evidence is given to explain why the JUNQ was mistakenly assigned to the cytoplasm in previous work. There is a general failure to explain why in several careful studies (e.g. Spokoini et al., 2012) involving 3D reconsructions, the JUNQ is clearly visible outside the nucleus as originally described. In these studies, the nucleus is labeled by NLS-TFP and not simply DAPI stained. Thus, the present paper is extremely confusing and it is not clear that the JUNQ was truly misassigned to the cytoplasm. More effort is need to reconcile the present findings with previous studies.

(Spokoini et al, 2012) state that the NLS-TFP labeling results indicate clearly cytosolic localization of JUNQ. However, re-examination shows the JUNQ marker GFP-VHL (in green) always at least partially overlaps (yellow) with the NLS-TFP (red) signal and in the same plane of focus, sometimes appears entirely intranuclear. See Fig. 1G of that study, and particularly, the 48 min and 240 min time points.

3D reconstructions led to the suggestion that JUNQ is located in a pocket in the outer-nuclear membrane (Spokoini et al., 2012, Fig. 3D). However, this may equally be interpreted as indicating JUNQ is intranuclear and is expulsing NLS-TFP, creating a hole within the otherwise homogenous nuclear NLS-TFP signal.

These findings are consistent with an intranuclear localization of JUNQ but have been interpreted in a unidirectional manner. Only the use of a nuclear envelope marker, as in our study (but not in others), allows unambiguous differentiation of cytosolic and nuclear localization.

Our immuno-EM analysis of yeast thin sections provides much higher resolution compared to previous reports relying on fluorescence microscopy. Specifically we detect nuclear aggregates of GFP-VHL and also endogenous aggregates stained by Hsp104-GFP in *hsp42Δ* cells (which form JUNQ/INQ exclusively) and confirm this identity by gold-labeling. The aggregates are clearly inside the continuous nuclear membrane. We have improved the labeling for Figure 2, delineating the position of the nuclear membrane. Invagination of the nuclear membrane as suggested by Spokoini et al (2012) would generate multiple nuclear membranes. These are not observed. These data together exclude invagination as an explanation of the images in Spokoini et al 2012.

We now further provide five consecutive serial EM sections of *hsp42Δ* cells, demonstrating the existence of GFP-VHL aggregates inside a continuous nuclear membrane across a segment 0.35 μm thick (New Figure E5). We also provide new EM images of yeast cells harboring intranuclear aggregates staining for GFP-VHL and Hsp104-GFP in the supplementary section. These images confirm our previous conclusions that JUNQ/INQ is an intranuclear quality control compartment (Figure E4).

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Finally, we have added Fig. 1C to the revised manuscript, showing a 3D reconstruction of yeast *hsp42Δ* cells expressing the INQ/JUNQ marker mCherry-VHL and the nuclear membrane marker GFP-Nup49. The reconstruction reconfirms the intranuclear localization of GFP-VHL aggregates.

3. It is suggested that Hsp42 and Btn2 are 'aggregases'. The suffix '-ase' suggests that they possess a catalytic activity in aggregate formation. However, Hsp42 and Btn2 also form part of the final aggregates and are thus consumed by the reaction. Hence, the term 'aggregase' is extremely misleading, confusing, and unhelpful as the proteins are not acting in a catalytic manner similar to enzyme. This term should be completely avoided.

In the chaperone field the terms "foldase", "holdase" and "disaggregase" describe distinct chaperone activities. These terms are well established and used in multiple original papers and reviews (Hipp et al, 2014; Kampinga & Craig, 2010; Saibil, 2013; Shibata & Morimoto, 2014; Sontag et al, 2014). Chaperones do not act catalytically but stoichiometrically and the definition of chaperone activities by these terms was never related to an enzymatic activity or the requirement for ATP. Our term "aggregase" describes the ability of Hsp42 and Btn2 to promote the formation of protein aggregates under physiological stress conditions best. This nomenclature conforms to established tradition in the field.

4. The similarity between Btn2 and sHsps (Fig. 7A) is extremely marginal and not convincing at all. If the 100-215 region of Btn2 is similar truly similar to α-crystallin domain then it should display activity in isolation. However, this is not tested. Is this region even required for Btn2 activity? Do point mutations in this region disrupt activity? Btn2 is also related in sequence to human Hook proteins, involved in aggresome formation and other transport activities. However, this similarity is completely ignored.

Contrary to the reviewer's comment, the isolated α-crystallin of sHsp is not active. The N- and C-terminal extensions flanking the α-crystallin domains are crucial for sHsp oligomerization and substrate interaction, and therefore, chaperone activity. We agree with the reviewer that a more detailed mechanistic understanding of Btn2 function and the role of individual domains is desirable. We also agree, that the similarity between Btn2 and sHsps provided by the structure prediction algorithm is low and we would not have taken it into account without knowing the functional similarities of both proteins. Given these concerns and the extensive additional new data added to the revised manuscript we have therefore removed the initial biochemical characterization of Btn2 from the manuscript.

Amino acid sequence homology between Btn2 and Hook proteins (Hook2) is restricted to a C-terminal segment of Btn2 (aa 206-325) and a multitude of yeast proteins show much higher homology to human Hook2 (see result of Blast search below). The restricted sequence homology might indicate some functional homologies between Btn2 and Hook2. To alleviate the concern of the reviewer we have clarified the discussion as follows (p. 25) in the revised version:
"Mammalian cells lack a direct homolog of Btn2, but human Hook2 displays limited sequence similarity to a C-terminal region of Btn2 (Figure E17). Hook2 overproduction promotes aggresome formation by CFTR (Szebenyi et al, 2007), suggesting that the related Btn2 segment is involved in promoting protein aggregation".

S. cerevisiae WU-Blast2 search results using human Hook2 as query

S. cerevisiae BY4741 ORFs showing sequence homology to human Hook2	Score (bits)	E value
YDL058W_BY4741 USO1	46.9	7.9e-05
YFR031C_BY4741 SMC2	38.9	0.00016
YDR356W_BY4741 SPC110	40.3	0.00062
YLR309C_BY4741 IMH1...	34.3	0.24
YHR023W_BY4741 MYO1	41.3	0.0027
YNL091W_BY4741 NST1	41.0	0.0035
YKR095W_BY4741 MLP1	38.9	0.0043
YJR005C-A_BY4741 ORF Uncharacterized	30.4	0.0052
YGR169C-A_BY4741 ORF Uncharacterized	30.4	0.0052

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YGL086W_BY4741 MAD1	38.5	0.011
YLR383W_BY4741 SMC6	33.6	0.013
YNL079C_BY4741 TPM1	35.7	0.014
YLR206W_BY4741 ENT2	36.0	0.063
YMR124W_BY4741 ORF Uncharacterized	31.1	0.015
YFL008W_BY4741 SMC1	38.9	0.015
YOR195W_BY4741 SLK19	38.1	0.016
YOR216C_BY4741 RUD3	35.7	0.019
YKL054C_BY4741 DEF1	33.2	0.021
YIL138C_BY4741 TPM2	33.9	0.022
YIL149C_BY4741 MLP2	36.4	0.16
YLR086W_BY4741 SMC4	36.4	0.031
YJL074C_BY4741 SMC3	30.8	0.16
YKL082C_BY4741 RRP14	35.0	0.066
YER008C_BY4741 SEC3	36.7	0.073
YDR259C_BY4741 YAP6	30.4	0.075
YOR198C_BY4741 BFR1	34.3	0.12
YPL074W_BY4741 YTA6	35.0	0.13
YNL250W_BY4741 RAD50	34.3	0.13
YAL035W_BY4741 FUN12	34.6	0.21
YBR079C_BY4741 RPG1	30.8	0.22
YLR254C_BY4741 NDL1	31.1	0.25
YDR482C_BY4741 CWC21	29.3	0.37
YDL099W_BY4741 BUG1	30.4	0.43
YNL005C_BY4741 MRP7	29.7	0.43
YNR054C_BY4741 ESF2	26.9	0.49
YDR507C_BY4741 GIN4	30.8	1
YER036C_BY4741 ARB1	32.2	0.54
YEL043W_BY4741 ORF Verified	29.0	0.54
YHR135C_BY4741 YCK1	31.8	0.57
YIL127C_BY4741 RRT14	29.7	0.62
YCL029C_BY4741 BIK1	31.1	0.66
YPL255W_BY4741 BBP1	26.5	0.67
YPR178W_BY4741 PRP4	31.1	0.69
YKL179C_BY4741 COY1	28.6	0.69
YMR227C_BY4741 TAF7	31.5	0.71
YPR018W_BY4741 RLF2	31.5	0.72
YPL032C_BY4741 SVL3	29.3	0.73
YDL239C_BY4741 ADY3	33.2	0.77
YGR142W_BY4741 BTN2	31.8	0.81
YDL005C_BY4741 MED2	30.4	0.83

Sequence homology between *S. cerevisiae* Btn2 and human Hook2

Hook2: 538 LLKRKLEEHLQKLHEADLELQRKREYIE-ELEPPTDSSTARRIEELQHNLQKKDADLRAM 596
LLK L +H +++ E + + K IE +++ D E L+ +K +
Btn2: 206 LLKIGLVKHEEEISEGGIN-EPKMPIIESKIDESHDD--VNMSESLKEEEAEKAKEPLTK 262

Hook2: 597 EERYRRYVDKARMVMQTMEPKQRPAAGAPPELHSLRTQLRERDVRIR-HLEMDFEKSRSQ 655
E++ ++++++ R++ + ++ A E Q +E++ R++ E K +++
Btn2: 263 EDQIKKWIEEERLMQEESRKSEQEKAAKEDE----ERQKKEKEARLKARKESLINKQKTK 318

Hook2: 656 REQEEKL 662
R Q++KL

Btn2: 319 RSQQKKL 325

5. The INQ compartment appears to be stabilized by guanidinium hydrochloride, indicating that it is dissolved by the activity of Hsp104 (Fig. E5). However, INQ-specific Btn2 is subsequently shown to prevent aggregation dissolution by Hsp104 (Fig. 7F). This disconnect between the in vitro and in vivo data is disconcerting and adds to the general confusion created by this paper.

Btn2 is removed from INQ by proteasomal degradation (Malinovska et al, 2012), (also, this work). INQ dissolution correlates with Btn2 degradation (New Figure E14A/B). Btn2 *in vitro* inhibits protein disaggregation, predicting that *in vivo* Btn2 stabilization by addition of MG132 should stabilize INQ, which is indeed the case. These *in vivo* findings are entirely consistent with the previously *in vitro* data presented. There is no discrepancy between *in vitro* and *in vivo* data sets.

Referee #2:

While most of the experiments are conducted in extreme conditions (i.e. by over-expressing exogenous proteins prone to misfold with simultaneous proteosomal inhibition and/or heat stress) ...

We share the concern about relying on overproduced model proteins only. For this reason, we have included Hsp104-GFP to monitor endogenous aggregated yeast proteins and confirm key findings with endogenous protein (Fig. 2C/D, Fig. E2, Fig. E3B). Our experimental conditions of mild heat stress (38°C) without MG132 addition, which we consider physiological but not extreme stress, yields similar findings to heat stress (37°C) plus MG132 addition (Fig. 1C, Fig. 2C/D, Fig. E5).

The possibility of coexisting nuclear and cytoplasmic JUNQ-like structures needs to be clarified.

We have made a concerted effort to reconcile our findings with those of Frydman and Kaganovich. Careful re-examination and investigation however, simply provide no evidence for coexistence of nuclear and cytosolic JUNQ-like structures. Apart from different localizations, the clearly disparate nature of INQ/JUNQ and cytosolic aggregates is based on two further criteria: (i) in *hsp42Δ* cells ONLY ONE (and it is nuclear) INQ/JUNQ forms and (ii) Hsp42 co-localizes with all stress-induced cytosolic aggregates but not with INQ/JUNQ (Specht et al, 2011)(this work).

Specific points:

1-i. The authors found that Sis1-depleted cells contain more cytoplasmic deposits leading to the conclusion that Sis1 is required for the import of misfolded proteins into the nucleus. However, the images in Fig. 4 show an increased size of the nuclear deposit (INQ). Similarly the authors concluded from increased number of cytoplasmic VHL foci that Nup42 is required for nuclear import of misfolded VHL. Here, it is not clear whether deletion of these genes is indeed impairing the nuclear import or whether these cells are for example more stressed with a consequent increase in protein aggregation. Furthermore, it is also worrisome that all these experiments were performed in cells lacking Hsp42 without knowing how deletion of Hsp42 affects the flux for misfolded proteins between the nucleus and the cytoplasm.

As noted by the reviewer, the image in the original manuscript shows an increased size of INQ (in addition to cytosolic aggregates not observed under control conditions). The size of INQ formed upon Sis1 depletion however is variable. We therefore provide images of stress-induced GFP-VHL foci formed in absence of Sis1 in several cells, documenting this heterogeneity (new/modified Fig. 5A). The new images clarify that the variable sizes of INQ formed upon Sis1 depletion are overall, similar to control cells expressing Sis1.

We also investigated the possibility that Nup42 deletion provokes protein folding stress. Neither *nup42*Δ nor *nup42*Δ *hsp42*Δ cells form visible aggregates at 30°C (Fig. 4A/B) and do not exhibit increased levels of Hsp104 compared to wt cells (Fig. E10D), arguing against a generally stressed state of these cells.

We employed *hsp42Δ* cells because these form only the INQ compartment after being stressed,

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enabling identification of other cellular activities involved in INQ formation. Whether and how Hsp42 affects the flux of misfolded proteins between cytosol and nucleus is an interesting question. Hsp42 may directly affect nuclear transport of substrates by inhibiting nuclear import factors, thereby directing substrates to cytosolic deposition sites. There is, however, currently no data supporting such scenario. Alternatively, Hsp42 may indirectly affect nuclear import and flux of misfolded proteins, by sequestering misfolded proteins into cytosolic deposits before these can be transported into the nucleus. This role of Hsp42 is well established by work from us and others (Escusa-Toret et al, 2013; Malinovska et al, 2012; Specht et al, 2011). Furthermore, the absence of Hsp42 from the nucleus, shown in this study, also argues against a function of Hsp42 in controlling nuclear trafficking. In further support of such indirect role of Hsp42, we report new findings specifying the role of Hsp42 in defining aggregate position in yeast cells lacking the Hsp70/90 co-chaperone Sti1 (new Figure 5B). Sti1 was originally described as a crucial sorting factor for INQ/JUNQ, as *sti1Δ* cells exclusively form peripheral aggregates in the cytosol (Q-bodies) (Kaganovich et al, 2008). We confirm this phenotype but show in addition that INQ/JUNQ formation is restored in *sti1Δ hsp42Δ* mutants. This demonstrates that (i) Sti1 is not a INQ/JUNQ sorting factor and (ii) the site of protein aggregation is simply determined by the availability of compartment-specific aggregases (e.g. Hsp42), which indirectly interfere with dynamic nuclear shuttling of misfolded proteins by sequestering them into cytosolic or nuclear inclusions. These new findings have been added to the revised manuscript and are discussed as follows:

"The Hsp70/Hsp90 co-chaperone Sti1 has been implicated in JUNQ (INQ) targeting since mCherry-VHL exclusively forms peripheral cytosolic aggregates in *sti1Δ* cells (Kaganovich et al, 2008). We have compared deposition of misfolded GFP-VHL upon stress application in *sti1Δ* and *sti1Δ hsp42Δ* mutant cells. In agreement with earlier findings in *sti1Δ* cells GFP-VHL was most frequently deposited at CytoQs compared to wt, though INQ formation was not completely abolished (Figure 5B). In contrast, in *sti1Δ hsp42Δ* knockouts GFP-VHL was almost exclusively targeted to INQ, as in *hsp42Δ* cells (Figure 5B). These data exclude an essential function for Sti1 in INQ targeting, but underline a crucial role in cellular protein quality control. The change in localization observed for aggregated VHL from cytosol to nucleus upon deleting *hsp42* in *sti1Δ* cells (Figure 5B) indicates that Hsp42 only indirectly affects the import of misfolded proteins, by sequestering these in cytosolic deposits. We conclude that substrate deposition is switched to either CytoQ or INQ dependent on the status of the protein quality system and availability of Hsp42."

1-ii. *Additionally, since VHL is found in the cytoplasm and the nucleus prior to the shift to 37°C, it is not clear whether the native or misfolded proteins are imported into the nucleus (i.e. is the INQ forming form pre-existing nuclear VHL or is there an active import of misfolded VHL into the nucleus that constitutes the INQ).*

To solidify their conclusions and answer these questions of nuclear import of VHL, I strongly advice the authors to measure the flux rates of VHL import in the nucleus (after shut off of expression). This can be done for example by a FRAP experiment (bleaching the nuclear VHL signal and measuring the recovery) or alternatively by using VHL tagged with a photoconvertible label. By conducting the experiment in Nup42 delete, Sis1 delete and Hsp42 delete, this experiment will allow a more careful assessment of the role of Nup42 and Sis1 in the nuclear import and to quantify the effect of deleting Hsp42 to the nuclear import of VHL.

We have measured the dynamics of nuclear import and export of mCherry-VHL and tGnd1-GFP. The suggested approach using FRAP turned out to be difficult because of the low levels of mCherry-VHL due to constant degradation and the small size of the yeast nucleus. We could not bleach the whole nucleus without retaining substantial cytosolic fluorescence to obtain kinetic data of sufficient quality that would allow us to determine nuclear import rates. We instead performed FLIP experiments (new Fig. 4C, new Fig. E11), which are also suited to determine the nuclear shuttling behavior of misfolded protein reporters. FLIP analyses show that mCherry-VHL and tGnd1-GFP shuttle between cytosol and nucleus, explaining dual nuclear and cytosolic aggregation.

Since VHL and tGnd1 represent constitutively misfolded proteins, they do not allow to discriminate between import of folded and misfolded conformers. To address the concern of the reviewer we used hyperlabile Luciferase-DM-GFP (R188Q/R261Q) as reporter and expressed it at 30°C and 37°C in *hsp42Δ btn2Δ* cells. This allows conditional induction of Luciferase-DM-GFP misfolding at

37°C.

We employed *hsp42Δ btn2Δ* cells so that Luciferase aggregation, which would otherwise perturb the analysis, is prevented. This allows direct comparison of Luciferase-DM-GFP localization at permissive (folding) and non-permissive (misfolding) temperatures. Luciferase-DM-GFP remained diffuse at both temperatures and was clearly enriched in the nucleus at 37°C, indicating that heat-induced protein misfolding leads to increased nuclear import. These data were added to the revised manuscript as new Fig. 6D.

In strong support, *nup42Δ* cells defective in nuclear import of misfolded VHL show delayed INQ formation, which is in line with the localization of Nup42 at the cytosolic side of nuclear pores (Fig. 4A/B). The precise mechanism of nuclear import of misfolded proteins however, is not the main focus of this manuscript. A more thorough analysis of the mechanism of nuclear import of misfolded proteins remains beyond the scope of this already very extensive manuscript.
The observed change in localization of aggregated VHL from cytosol to nucleus upon deleting *hsp42* in *sti1Δ* cells (New Fig. 5B) indicates that Hsp42 is only indirectly affecting the import of misfolded proteins by sequestering them in cytosolic deposits.

2. It is shown that in wild type cells (at 370C with proteosomal inhibition) VHL forms one peripheral focus and one nuclear focus. From this the authors conclude that the nuclear puncta represents the JUNQ and the peripheral is the IPOD. It needs to be excluded that these two foci do not represent cytoplasmic JUNQ and a nuclear JUNQ (INQ). This should be tested by co-expressing the IPOD specific RNQ1 (Kaganovich et al. 2008) and VHL in these conditions. If the authors are correct there should be one nuclear focus (without RNQ1) and one cytoplasmic focus (with RNQ and VHL). However, if the cytoplasmic RNQ1 and VHL form distinct foci, it suggest that there are two JUNQ like structures, one cytoplasmic and one nuclear.

There is a misunderstanding in the reviewer´s comment (and in some of the published papers on this topic, e.g. (Oling et al, 2014)) as follows. The term IPOD describes aggregates in the cytosol, which are distinct from the peripheral, Hsp42-dependent aggregates, also termed Q-bodies (Escusa-Toret et al, 2013; Specht et al, 2011). The deposition of amyloidogenic substrates (e.g. Rnq1) is Hsp42-independent and, consistently, Hsp42 does not colocalize with the IPOD marker Rnq1 (Specht et al, 2011). Peripheral, cytosolic aggregates (Q-bodies), which rely on Hsp42 for formation and co-localize with Hsp42, are therefore distinct from the IPOD. Similarly, Frydman and coworkers did not observe colocalization of GFP-VHL with Rnq1-CHFP (Escusa-Toret et al, 2013), exactly addressing the reviewer`s suggestion. Together these data support the existence of three distinct deposits in yeast cells: IPOD, CytoQ and INQ. INQ and CytoQ are specific, distinct aggregation compartments, which do not represent cytoplasmic and nuclear JUNQ-like structures on the following bases: (i) demonstrably different dependencies on Hsp42 and Btn2; (ii) co-localization (CytoQ) and non-localization (INQ) with Hsp42. We have revised our discussion to clarify this issue and add IPOD as a third compartment specific for amyloidogenic substrates to Figure 9

3. In the fractionation experiment described in Fig. 6 where the insoluble fraction is determined, an important control is missing. It is advisable to repeat the experiment by adding a soluble control protein (e.g. Pgk1 or free GFP).

Both model substrates tested are completely soluble at 30°C so the experiment is already internally well controlled. We now further demonstrate by western blot analysis that an additional control protein (actin) remains soluble after heat stress (see revised Figure 6 and Figure E13).

4. The claim that ubiquitination is not required for INQ sorting is misleading. The phenotype in the non-ubiquitinated state (San1/Ubr1 delete) was there were more nuclear foci as compared to wild type, indicating that although nuclear aggregates still can form, they became fragmented, which might at least partially reflect a sorting defect. Therefore, in my opinion it would be safer to say that ubiquitination is not required for the formation of nuclear puncta (including INQ), but might promote their fusion. Text regarding these conclusions should be reformulated.

Our previous data representation in Fig. 3D was not sufficiently clear, leading to misinterpretation. Yeast wt cells expressing tGnd1-GFP do not exhibit fluorescent foci. Only <u>one</u> nuclear aggregate is formed in *ubr1Δ san1Δ* mutants; the number of foci given in Fig. 3D includes both cytosolic and

nuclear aggregates. 93% of cells harboring two tGnd1-GFP foci, one of them is nuclear. Multiple nuclear aggregates are not present in *ubr1Δ san1Δ* cells and ubiquitination of tGnd1-GFP is neither required for INQ formation nor for aggregate fusion. We also add new data showing that nuclear aggregation of tGnd1-GFP is no longer observed in *ubr1Δ san1Δ btn2*Δ triple mutant cells which lack the nuclear aggregase Btn2 (New Fig. 3D). This finding further validates INQ formation by non-ubiquitinated tGnd1-GFP and excludes ubiquitination as general sorting signal.

5. Based on the in vitro data and the effect of transiently expressing Btn2, the authors claim that after forming its duty as an "aggregase", the presence of Btn2 inhibits refolding of INQ substrates. If the authors wish to solidify this conclusion, the promoter of Btn2 could be exchanged by a constitutive active promoter (e.g. GDP) in order to test if INQ is now stabilized upon heat shock removal. It would be nice if the authors could speculate: does the transient expression peak of Btn2 possibly reflect the high turnover rate of INQ substrates?

Concerning INQ stability: Constitutive overproduction of Btn2 alone induces protein aggregation in non-stressed yeast cells (Malinovska et al, 2012), limiting the readout of the suggested experiment. In support of the reviewer´s suggestion and our hypothesis, we show that MG132 addition stabilizing Btn2 also stabilizes INQ (New Fig. E14A/B), indicating that continuous presence of the Btn2 aggregase prevents INQ disintegration. The transient accumulation of Btn2 therefore seems necessary to restrict INQ formation to periods of immediate stress, either to allow for efficient solubilization of sequestered substrates or to limit potentially linked signaling processes as discussed below. These clarifications have been added to the revised version of the manuscript – see discussion, p. 25.

Concerning INQ substrate turnover: We cannot link the turnover rates of unstable proteins to INQ sequestration. While mCherry-VHL and hyperlabile Luciferase-DM-GFP-NLS are sequestered at INQ, we now show that substrate degradation rates remain unchanged in *btn2Δ* cells in which substrate sequestration at INQ is abolished (new Fig. 6C). This finding is consistent with our previous results showing that ubiquitination is not an INQ/JUNQ sorting signal. Whether misfolded proteins are degraded by the proteasome or refolded by chaperones is therefore not determined by sequestration but decided after substrate solubilisation, and is determined only by intrinsic substrate features. These clarifications are now included in the revised manuscript discussion section, p. 24.

6. The phenotype described in Fig. E5 should be quantified from several videos to justify the conclusions drawn.

We have added the quantifications from > 25 cells to new Fig. E6 as requested.

7. Fig. 6A: a picture of a cell in a comparable cell cycle stage (G1) should be added.

We have added an appropriate picture to Fig. 6A.

8. The term "% cells with INQ" is not easy to understand. Perhaps there is a way to make the graphs more reader friendly.

"% cells with INQ" refers to cells harboring one or multiple aggregates and determines the fraction harboring an INQ compartment. This clarification has been added to the revised manuscript.

9. Use either everywhere CPY or Prc1 as nomenclature (Fig. E8).

We are now only using CPY.

10. In the model of Fig. 9 it seems that Hsp42 and Hsp104 are switched in the cytoplasm. Hsp42 should be the aggregase and Hsp104 the disaggregase?

Thank you. We have changed the model accordingly.

10. The finding that DNA replication stress induced by MMS treatment leads to INQ formation is very remarkable and would be interesting to follow up. It would be nice if the authors could speculate in the discussion why Hos2 aggregates into one nuclear focus (INQ) upon replication

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stress.

These aspects have been incorporated in the revised discussion: Nuclear, non-canonical DNA stress foci formed upon MMS treatment so far include the deacetylase Hos2, protein phosphatase Pph21, the Hsp40 co-chaperone Apj1 and Cmr1, a protein linked to DNA repair. The roles of these proteins in DNA damage response so far remain unclear (Tkach et al, 2012). Hos2 foci may represent sites of DNA damage-induced transcription (Tkach et al, 2012). Hos2 may also facilitate chromatin remodeling to enable binding of chromatin-associated factor Cmr1 to damaged DNA (Choi et al, 2012; Tkach et al, 2012).

We show formation of these DNA stress foci relies on Btn2, a highly unstable, stress-inducible protein. We speculate that Btn2 provides a scaffold allowing integration of different stress stimuli, facilitating orchestration and organization of downstream processes. These may include control of DNA damage checkpoints, with degradation of Btn2 limiting signaling to periods of immediate stress.

Referee #3:

Major concerns:

1. The authors adopt the term 'INQ' in what they believe is a better way to reflect the intranuclear localization of the 'JUNQ'. I think it is better to simply remain with JUNQ as the nomenclature because too many previous publications have already used this term. Changing the nomenclature would just increase confusion in the literature, akin to how gene names are often changed leading to multiple and often confusing names in the literature. Same for "CytoQ".

We understand the point but think it counterproductive to retain a term previously defined by and reflecting inaccurate data interpretation and conclusions. Specifically, the aggregates are inside the nucleus, not juxtanuclear. The term JUNQ cannot therefore remain. The use of different terms, often poorly defined, is currently confusing. For instance the term “Q-bodies” has been recently introduced (Escusa-Toret et al, 2013) to describe what we referred to earlier as "peripheral aggregates" (Specht et al, 2011), but the relationship of those aggregates to JUNQ has not been addressed in the original study and Q-bodies were later suggested to represent JUNQ precursors (Sontag et al, 2014), which we demonstrate here to be incorrect.

We strongly suggest an updated nomenclature for this research field. Our work demonstrates cellular localization is a main point of systemically relevant difference between stress-induced foci. This is appropriately reflected in the terms “INQ” and “CytoQ”. These terms clarify and simplify aggregate terminology, and are immediately informative with regard to specific localization. We therefore consider these updated terms worth maintaining.

2. To be entirely convincing that the JUNQ is intranuclear, I think the authors need to do a 3D reconstruction of cells with a nuclear pore marker, DAPI-stained DNA and the JUNQ. The single plane images are suggestive, but the onus is on the authors to fully demonstrate intranuclear localization if they wish to counter what has been previously published.

We now provide 3D reconstructions of wt and *hsp42Δ* cells expressing mCherry-VHL and GFP-Nup49 (new Fig. 1C). The 3D model confirms the intranuclear localization of INQ/JUNQ. We further provide serial section EM analysis of five consecutive thin sections of hsp42Δ cells, demonstrating GFP-VHL aggregates inside a continuous nuclear membrane across a segment of 0.35 μm thickness (New Figure E5). New EM images of various yeast cells further confirm our previous conclusions that INQ/JUNQ resides inside the nucleus. These are provided in the supplementary data section (Figure E4E).

3. For the microscopy in Figure 1A and B, we are only given a single cell to draw conclusions. I think more cell images should be included in the supplemental materials so that the readers can see the different cell-to-cell variations.

We now provide statistics for Figure 1A and B in the main text: “Co-localization between mCherry-

VHL and GFP-Luciferase-DM-NLS was observed in 92% of wt cells (n=104) and 99% of *hsp42Δ* cells (n=100)"; "97% of GFP-VHL foci formed in *hsp42Δ* cells were located inside the nucleus and 3% of foci formed at the nuclear envelope (n=100)."

4. On page 9, "To provide further evidence for nuclear localization of INQ at higher resolution we performed immunoelectron microscopy of wt cells expressing GFP- Luciferase-DM-NLS and hsp42Δ cells expressing GFP-VHL (Figure 2A/B)". I think Figure E4 should go into Figure 2. The controls are important.

Integrating those control images in Figure 2 will require a reduction of image sizes, which will affect data significance. We therefore prefer to keep these controls in the supplementary section.

5. On page 11, "However, by ubiquitin immunostaining, we did not observe specific co-localization of ubiquitin and INQ (Figure 3A, Figure E7A)." The lack of ubiquitin immunostaining doesn't necessarily support that ubiquitin is not required for INQ formation. It could be the ubiquitylation is dynamic: required for formation of the INQ but removed once proteins are sequestered into the INQ. Also, while tGnd1-GFP forms nuclear inclusions in ubr1Δsan1Δ cells, this could simply be that the protein is rapidly degraded in wt cells but is stabilized in ubr1Δsan1Δ cells and accumulates to much higher levels. Furthermore, loss of ubiquitylation through deletion of the QC degradation E3s doesn't exclude ubiquitylation by an E3 that is not involved in QC degradation. I think the authors need to be more cautious here. Especially when they overstate in the Discussion that "Our results revoke the current view of a ubiquitin-based sorting mechanism that targets ubiquitinated proteins to JUNQ (now INQ) and non-ubiquitinated proteins to IPOD (Kaganovich et al, 2008)." Unless they present a whole lot more data showing they can get ubiquitin colocalization with known structures and exhaust by all other methods that ubiquitin is not required for JUNQ formation, this section should be removed from the Results and Discussion because it is too inconclusive.

We have added new experiments, further supporting our interpretation. Taking all evidence together (see below) we strongly suggest we can maintain our claim that ubiquitination is not the essential sorting principle for aggregating proteins. We nonetheless have softened our statement concerning the role of ubiquitination for aggregate sorting in the discussion section as follows: "Our results challenge the current view that an ubiquitin-based sorting mechanism is essential for targeting ubiquitinated proteins to JUNQ (now INQ)."

First, in *ubr1Δ san1Δ* mutant cells tGnd1-GFP is no longer ubiquitinated and entirely stabilized (Fig. 3B/C), in agreement with the literature (Heck et al, 2010). There is no evidence that tGnd1-GFP is ubiquitinated by another E3. Stabilized, non-ubiquitinated tGnd1-GFP forms CytoQ and INQ deposits in *ubr1Δ san1Δ* cells, demonstrating that ubiquitination is not a prerequisite for protein aggregation and CytoQ or INQ targeting. These findings are in line with studies demonstrating nuclear aggregation of misfolded San1 substrates upon stabilization in *san1Δ* cells (Fredrickson et al, 2013; Fredrickson et al, 2011).

Second, accumulation of tGnd1-GFP in *ubr1Δ san1Δ* mutant cells does not lead to a general breakdown of the aggregate sorting system, with uncontrolled nuclear aggregation. We demonstrate that this sorting system is operative during heat shock, which causes a massive increase in the amount of multiple misfolded protein species. To ensure specificity of nuclear tGnd1-GFP aggregation we monitored tGnd1-GFP localization in *ubr1Δ san1Δ btn2Δ* cells lacking the nuclear aggregase. The amount of accumulated tGnd1-GFP in these cells was similar to *ubr1Δ san1Δ* controls. However, nuclear tGnd1-GFP foci were no longer observed in the triple mutant demonstrating that (i) tGnd1-GFP is deposited at the INQ in *ubr1Δ san1Δ* and (ii) tGnd1-GFP deposition is not caused by uncontrolled aggregation but is subject to Btn2-controlled sequestration. Similar results were obtained for ΔssCPY*-GFP. These new findings (new Fig. 3D and new Fig. E8D) further support our conclusion that ubiquitination is not required for INQ/JUNQ deposition.

Third, JUNQ was also originally suggested to target sequestered proteins primarily for degradation, consistent with the claim that JUNQ sorting requires substrate ubiquitination (Kaganovich et al, 2008). We now determined the stability of two additional substrates, mCherry-VHL and GFP-Luciferase-DM-NLS in yeast wt, *hsp42Δ*, *btn2Δ* and *hsp42Δ btn2Δ* mutant cells (new Fig. 6C). Kinetics of substrate degradation are almost identical for all tested strains, demonstrating that INQ

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formation (abrogated in *btn2Δ* cells and sole deposit in *hsp42Δ* cells) does not impact on protein degradation. These new findings provide further indication that the fate of misfolded proteins is not decided at the stage of sequestration but after substrate solubilisation. These clarification are added to the revised manuscript discussion section

6. In Figure 3D, why did the authors switch to using histone H2B as the nuclear marker rather than the nuclear pore proteins? If they wish to demonstrate that tGnd1-GFP is intranuclear, they have to be consistent in the nuclear marker they use. Especially when the precise spatial localization is the foundational idea and claim of this manuscript. Using H2B is the same trap previous authors fell into.

We agree with the reviewer that the nuclear envelope staining is important to provide convincing evidence for intranuclear localization of INQ. In the original paper we used the envelope marker Nsp1 (Fig. E8E), and now add another envelope marker Nic96-mCherry (new Fig. 3D, Fig. E8D), and reconfirm that tGnd1-GFP foci are intranuclear. Notably, quantification of INQ formation based on either Htb1-mCherry or Nic96-mCherry signals yields comparable results (Fig. 3D/ Fig. E8D) indicating that close vicinity of, for example, tGnd1-foci to DNA (stained by Htb1-mCherry) is a characteristic feature of INQ formation. In addition to results with nuclear envelope markers we show that tGnd1-GFP foci frequently do not co-localize with Hsp42, which is excluded from the nucleus (INQ) (Fig. E8E).) Furthermore, EM of cryo-sections of *ubr1Δ san1Δ* cells expressing tGnd1-GFP reveal amorphous, electron-dense structures representing tGnd1-GFP aggregates, within a continuous nuclear membrane. This confirms intranuclear INQ localization. These new data are presented in new Figure E9.

7. Similarly, the authors need to use the nuclear pore marker for the Sis1 experiments in Figure 4B.

According to suggestion we used Nsp1 antibodies as nuclear envelope marker. We demonstrate that intranuclear GFP-VHL or ΔssCPY*-GFP foci are still formed upon Sis1 depletion (Fig. E12C), and provide respective statistics for GFP-VHL based on > 65 cells.

8. From Figure 4B, the authors conclude "These findings suggest that while Sis1 clearly plays an important role in nuclear import of misfolded proteins for INQ formation, other, so far unknown factors, are additionally involved." I'd suggest the authors be cautious here. The Sis tet deletion system is good, but it could be that remaining Sis1 protein levels function in the residual nuclear import, not another pathway. Furthermore, there are alternative interpretations of the data that would not support a role for Sis1 in nuclear import. For example, what if Sis1 is playing a role in maintaining solubility in the cytosol prior to nuclear import? In the absence of Sis1, the misfolded GFP-VHL proteins form inclusions in the cytosol and thus would be unable to transit the nuclear pore in the included state. This seems a more likely interpretation because >50% of the GFP-VHL is still within the nuclear inclusions even after depletion of Sis1.

The depletion of Sis1 after 20 h doxycycline treatment is very strong and the protein is no longer detectable by western blot analysis. We therefore consider significant nuclear import of misfolded proteins by residual Sis1 unlikely. We emphasize that the experimental conditions used here were exactly as those of Park et al., Cell 2013, which depleted Sis1 to the same degree. This suggests that Sis1 is not strictly essential for nuclear import of misfolded substrate, unless extremely low levels of remaining Sis1 maintain nuclear import.

We agree with the reviewer that a cytosolic chaperone function of Sis1, keeping misfolded proteins in a soluble and transport-competent state, also explains the observed phenotype. The role of Sis1 is potentially complex and we therefore follow reviewer suggestion for caution in our statements. We have changed the discussion section accordingly: "Sis1 depletion may however also indirectly affect INQ formation by reducing the protective capacity of cytosolic protein quality control causing aggregation of GFP-VHL before it can be encountered by nuclear import factors" and we state that "even efficient Sis1 depletion does not completely block misfolded protein import into the nucleus, suggesting that additional factors may be involved in this process as well".

9. More cell images are needed for Figure 5A and B. It would also be helpful to have the separate channel images in addition to the merged images.

We provide more cell images (Figure E10) and show separate channel images as requested.

10. Similar to my earlier points, a nuclear pore marker should be used in Figure 6A, B, D and E.

We now confirm the nuclear localization of GFP-VHL and GFP-Luciferase-DM-NLS by staining the nuclear membrane with antibodies raised against the nucleoporin Nsp1 and provide statistic analyses (New Fig. 6A).

11. I think the DNA damage (MMS) experiments could be excluded from the manuscript. I understand why the authors wish to include them for physiological relevance. But, they seem rather preliminary compared to their other studies. And, at least in my opinion, they are distracting at the end the results section. For example, is Hos2 actually misfolded during MMS exposure? Does VHL become a better QC degradation substrate during MMS exposure? There are a ton of questions left unanswered after this section. And the section seems very incomplete. I'd rather the authors firm up all previous findings and make this a definitive intranuclear paper with strong chaperone data support.

We agree these DNA damage data are preliminary and raise a multiplicity of highly interesting questions. Nonetheless, our observation that protein and DNA specific stress conditions trigger the formation of an intranuclear inclusion that relies on unstable Btn2 is an intriguing and highly interesting finding. We prefer to present this in our manuscript since it is conceptually exciting in relation to the broader cellular context of protein quality control. We speculate that Btn2 provides a scaffold allowing integration of different stress stimuli, facilitating orchestration and organization of downstream processes. Such processes could include control of DNA damage checkpoints, where degradation of Btn2 could limit signaling to periods of immediate stress. These aspects have been added to the discussion in the revised manuscript.

12. The authors' use the term aggregate in the title and throughout the paper when they are discussing cellular inclusions. Aggregate has a very specific biochemical definition, which has not been demonstrated for the cellular inclusions. Have they demonstrated that the proteins in the nuclear and cytosolic inclusions are actually aggregated in the cell? Or, or they just concentrated in a specific cellular location? Separation into soluble and insoluble fractions does not necessarily indicate aggregation. They would need to assess the oligomeric state of the proteins in cells to be able to use the term aggregate for inclusion. The authors use the term appropriately in Figure 7 where they are biochemically looking at actual aggregates.

Fluorescent foci of misfolded model proteins observed and analyzed in this study co-localize with Hsp104, the central component of the disaggregation machinery in yeast cells. Accordingly, disintegration of the foci depend on Hsp104 activity (Fig. E6). Stress-induced structures detected in EM of yeast thin sections appear as electron dense and amorphous structures. These are key characteristics of protein aggregates and qualify the foci as protein aggregates.

13. More cell images are needed for Figure E6A-C to solidify the claim that "INQ appears as a single focus of fluorescence adjacent to nucleolus and chromatin, opposite the spindle pole body". And, I think 3D reconstructions would be essential here.

Additional images and a 3D reconstruction are presented (new Figure E7A-C), reconfirming the specific localization of INQ within the nucleus.

Minor concerns:

1. The authors should be better in terms of scholarship. For example, if the authors cite Escusa-Toret et al 2013 (Nature Cell Biology 15: 1231-1243) for Q-bodies, they should also cite Spoikoni et al 2012 (Cell Reports 2: 738-747) for stress foci. The latter paper was published first and described the same phenomenon. Additionally, there are recent papers looking at inclusions of misfolded proteins in the yeast nucleus that are bounded by a nuclear membrane marker, most notably San1 substrates (MBoC 22: 2384-2395, JBC 288: 6130-6139, JCS 127: 1980-1991). These studies should be considered especially since the authors include the San1 substrates tGnd1-GFP and ΔssCPY. It is not clear that the all nuclear inclusions formed by San1 substrates are the JUNQ, but it is probably likely and they should include something about this in the Discussion.*

We appreciate the reviewer comment and have added the appropriate literature. The nuclear aggregation of stabilized San1 substrates in *san1Δ* cells observed in these publications agrees with the findings presented here and underlines our finding that INQ formation does not rely on substrate ubiquitination.

2. The manuscript would read much better if the authors standardized their language and presented their observations as cytosolic versus nuclear inclusions. Just a few examples:

On page 9, "In order to determine the relationship between formation of peripheral (cytosolic) and INQ aggregates we performed time-lapse microscopy." It would read better to the layperson as "In order to determine the relationship between formation of cytosolic and nuclear inclusions we performed time-lapse microscopy."

Also, "While apparent encounters of cytosolic aggregates and nuclear INQ foci were occasionally observed" would read better as "While apparent encounters of cytosolic and nuclear inclusions were occasionally observed"

Further, "The observation that INQ and peripheral aggregates form independently from one another" could be "The observation that nuclear and cytosolic inclusions form independently from one another"

Following reviewer suggestion we have standardized language and now consistently refer to cytosolic aggregates as CytoQ. As discussed above we maintain the term “aggregate”, since foci co-localize with the disaggregase Hsp104 and require Hsp104 activity for disintegration.

3. In the Discussion: "Several observations indicate that substrate shuttling between cytosol and nucleus is actively biased toward transport into the nucleus." I don't think the observations indicate that there is shuttling between cytosol and nucleus, only that some cytosolic misfolded proteins are transported to the nucleus. Shuttling implies that some proteins move from the nucleus to the cytosol as well as from the cytosol to the nucleus.

We provide new data demonstrating by FLIP experiments that mCherry-VHL and tGnd1-GFP dynamically shuttle between cytosol and nucleus (New Fig.4C and new Fig. E11).

5. In the Discussion: "This study establishes key features of cellular and molecular organization of protein aggregation in yeast. This redraws the conceptual framework for protein quality control in eukaryotic cells." I think this is an overstatement. The previous JUNQ and IPOD studies developed the important conceptual framework that there is a bipartite system for sequestration of misfolded proteins. The work here redefines the location of the JUNQ, which does have new implications especially in terms of how misfolded cytosolic proteins are trafficked to the nucleus. In general, I thought the Discussion could use a lot of editing to remove overstatements and make it more concise.

We agree with the reviewer that the concept of distinct deposition sites in yeast cells for misfolded proteins was established earlier by the pioneering work from J. Frydman and coworkers (Kaganovich et al, 2008). We show here that the original model, however, suffers from oversimplifications and includes erroneous conclusions and predictions. Our work changes key features of the current model of protein aggregation. The nuclear localization of INQ has a profound impact on the mechanism of INQ formation as it must involve nuclear transport of misfolded proteins, a process that did not play any role in the previous model. Furthermore, we demonstrate that ubiquitination is not required for INQ formation and, accordingly, preventing substrates from being sequestered at the INQ does not affect degradation kinetics. These findings call the previous model of INQ/JUNQ representing a major site of proteasomal degradation into question. Finally, we identify Hsp42 and Btn2 as compartment-specific aggregases. We therefore strongly believe that the presented work will have a profound influence on researchers studying protein aggregation in eukaryotic cells.
We have revised the discussion taking these points into account and to meet the reviewer’s comments: “This study determines the molecular organisation of protein aggregation in yeast cells and the function of critical factors in controlling protein aggregation (Figure 9).”

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2nd Editorial Decision 19 December 2014

Thank you for submitting a revised version of your manuscript to The EMBO Journal. Your study has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all main criticisms have been sufficiently addressed and consequently recommend your study for publication here. However, before we can proceed to officially accept your manuscript, I have to ask you address the following minor points in a final revision of the manuscript:

-> Please comment on points 1 and 3 raised by referee #3.

Thank you again for the opportunity to consider your work for publication. I look forward to receiving your final revision.

REFEREE REPORTS

Referee #2:

I am fully satisfied by the way the authors have addressed the reviewers comments. This a very interesting piece of work.

Referee #3:

I thought the authors' responses to my criticisms were well considered and the new experiments elevate the paper. I would like to see the new version of the manuscript published.

Just a few minor points that I leave to the editor to decide. And I am okay if these are not addressed in the final version, but they would be good to see.

1. Thinking about nuclear shuttling, what are the sizes of mCherry-VHL, GFP-VHL, GFP-Ubc9ts, and tGnd1-GFP? If they are around the nuclear pore size passive diffusion limit (~45kDa), it would be worth pointing this out in the manuscript. Passive shuttling could certainly apply here for some substrates. Maybe a supplemental table that lists the kDa sizes of the substrates used in this study would be helpful?

The only reason I bring this up is that there are some general misconceptions about nuclear import/export of misfolded proteins. Given that the main point of this paper is on nuclear localization, it would be a great venue to at least point out passive versus direct import and how small proteins can shuttle back and forth.

2. I would still like the authors to be more considerate with the term 'aggregate' and 'aggregation'

when discussing in vivo results. 'Inclusion' or 'inclusion formation' are better terms for in vivo observations. Microscopy can only reveal included states, which could be protein aggregation or not. But, this is a semantic issue and not worth holding up an otherwise intriguing paper for publication.

3. The Lindquist lab had a paper from 20 years ago using EM to show that inclusions form in the nucleus after heat shock, and more so without Hsp104 (http://www.ncbi.nlm.nih.gov/pubmed/7984243). It would be good to acknowledge and cite this paper when discussing Hsp104 and heat shock.

2nd Revision - authors' response 08 January 2015

Reply to referees' comments for manuscript EMBOJ-2014-89524 (Miller et al.).

Reviewers' comments are shown in italics.

Referee #3:

1. Thinking about nuclear shuttling, what are the sizes of mCherry-VHL, GFP-VHL, GFP-Ubc9ts, and tGnd1-GFP? If they are around the nuclear pore size passive diffusion limit (~45kDa), it would be worth pointing this out in the manuscript. Passive shuttling could certainly apply here for some substrates. Maybe a supplemental table that lists the kDa sizes of the substrates used in this study would be helpful?

The only reason I bring this up is that there are some general misconceptions about nuclear import/export of misfolded proteins. Given that the main point of this paper is on nuclear localization, it would be a great venue to at least point out passive versus direct import and how small proteins can shuttle back and forth.

We added the new Table E1 summarizing the molecular weights of all used fluorescent reporters, which all exceed the size limit for passive nuclear trafficking. We refer to this point in the main text on page 14, lines 22-24:

"The sizes of the chosen fluorescent reporters are beyond the size limit (45 kDa) allowing for passive diffusion in and out the nucleus, indicating active import and export processes (Table E1)."

3. The Lindquist lab had a paper from 20 years ago using EM to show that inclusions form in the nucleus after heat shock, and more so without Hsp104 (http://www.ncbi.nlm.nih.gov/pubmed/7984243). It would be good to acknowledge and cite this paper when discussing Hsp104 and heat shock.

We now refer to the indicated reference on page 11, lines 3-5:
"Our findings are consistent with early reports, showing increased levels of nuclear aggregates upon heat shock in *hsp104Δ* mutant cells (Parsell et al, 1994)."

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