Subcellular localization of the J-protein Sis1 regulates the heat shock response

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Re: JCB manuscript #202005165

Dr. David Pincus
University of Chicago
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Dear Dr. Pincus,

Thank you for submitting your manuscript entitled "Subcellular localization of the J-protein Sis1 regulates the heat shock response." Please accept our apologies for the delay in the processing of your manuscript.

The manuscript was assessed by expert reviewers, whose comments are appended to this letter. Overall, the reviewers were enthusiastic about the study and we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The main concern shared by reviewers is regarding the direct vs. indirect effects of Sis1 on Hsf1 activity. You will see that reviewer #1 asks for discussion of an alternative model of proteostasis collapse by Sis1 relocalization indirectly activating HSR by increasing Hsp70 clients while reviewer #2 requests that you show a physical link between Sis1 and Hsf1 either using a Sis1 mutant lacking a functional J-domain or by direct binding of purified proteins. We feel these requests are reasonable and every effort should be made to address them with new data. Reviewer #1 also raises questions regarding the strength of data for the proposed function of Sis1 in proteasomal degradation on nucleolus surface and the connections to ER and RCQ. Please address these concerns by either adding more definitive data or tone down conclusions and revise discussion accordingly.

Please be sure to also include a point-by-point rebuttal for all the items raised by the reviewers.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Judith Frydman, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Feder et al. study the interplay between protein quality control components, the heat shock response (HSR), and protein localization. Their primary conclusions are that (1) during heat shock, the HSR is activated by Sis1 re-localization away from Hsf1, causing loss of Hsp70 binding and derepression of Hsf1 (2) that Sis1 relocalization under stress is accompanied by relocalization of multiple quality control and protein synthesis proteins (3) both 1 and 2 require active translation. These major conclusions are strongly supported, while some more minor conclusions are not (see comments below). This work includes significant observations and conclusions and will be of interest to the protein quality control field and more generally to biologists studying stress responses and protein localization and compartmentalization. However, before publication the following issues must be addressed:

Figure 2E: Please explain why the initial increase in Hsf1 activity is the same in WT and Sis1OE cells. The model nicely recapitulates this, but the behavior should be explained in conceptual terms so that all readers can benefit from the insights of your model. Furthermore, it may be useful to include the behavior of an alternative model that proteostasis collapse by Sis1 relocalization indirectly activates the HSR by increasing Hsp70 clients. Does this alternative model fit the data equally well or worse than the Sis1/Hsp70/Hsf1 direct interaction model you propose? The comparison of alternate models would be more helpful for understanding observations than fitting just a single model.

Figure 4D: When was cycloheximide added? How does this explain the lack of nucleolar relocalization for Sis1? The authors state "This suggests that ongoing translation is required to trigger Sis1 re-localization

during heat shock, and is consistent with a role for nascent ribosomal proteins in Sis1 localization to the nucleolus." Nascent ribosomal proteins (those being actively translated) are in the cytosol (where the translating ribosomes are) so I'm not sure how the authors are connecting cytosolic nascent chains to nucleolar localization of Sis1. I can imagine some models, like fewer orphan ribosomal proteins in the nucleolus because translation has stopped while ribosome biogenesis continues, depleting the nucleoli of orphan ribosomal proteins. But this sort of speculation needs to be explicitly stated clearly (with timing info) and in the discussion section rather than the results section unless the reasoning serves as the basis for later experiments. Right now the discussion says "Sis1 spatial re-localization during heat shock suggests nascent proteins are also drivers (Figure 3D, E)." Again, what's the connection between nascent proteins and nucleoli? There seems to be a logical leap here that is not clear. Cycloheximide will block all nascent proteins in the cell, not just those going on to become ribosomes.

Figure 5E: The authors state that "The imaging data suggest that Sis1 forms a highly connected network with the proteasome and RQC on the surface of the ER." Is this really happening? To me it looks like Sis1 and Rtn1 may be adjacent in some cases, but Sis1 is not "at the surface of the ER". If Sis1 was at the surface, wouldn't it co-localize with Rtn1 more completely?

Figures 3 and 5: The authors' data on the RQC is weak. Ltn1 and Cdc48 both have non-RQC roles (e.g. https://pubmed.ncbi.nlm.nih.gov/24616224/), so finding that Sis1 binds them both but not other RQC members Rqc1 and Rqc2 doesn't strongly implicate the RQC unless there is more data to link Sis1 to RQC. Partial co-localization of Ltn1-scarlet with Sis1 under stress also doesn't mean much because the author's haven't demonstrated that Ltn1-scarlet is functional. The only C terminal tag of Ltn1 that covers that this reviewer knows about is a C terminal HA tag, and C terminal FLAG tagging of Ltn1 results in loss of binding to all other RQC components (see https://pubmed.ncbi.nlm.nih.gov/20835226/ for evidence of both of these things). Unless that authors show that the Ltn1-scarlet tag covers, either by showing that Ltn1-scarlet still associates with other RQC members or that Ltn1-dependent RQC substrates are properly degraded in an Ltn1-scarlet background, then I would recommend deemphasizing RQC as a topic in this paper, probably removing the Ltn1-scarlet experiments entirely. The association of Sis1 with Ltn1 and Cdc48 is still interesting and should be reported but requires more followup to understand.

Page 21: "This implies that Sis1 mediates proteasomal degradation of chaperone clients on the surface of the nucleolus". This statement is too speculative for the results section and the idea should be moved to the discussion.

Figure 5F: This is a striking result. The authors say: "The short period of treatment (15 minutes) makes it unlikely that Sis1 anchor away pre- adapted cells to heat shock and thus precludes the stress. Rather, Sis1 appears to be necessary to recruit the proteasome. This implies that Sis1 mediates proteasomal degradation of chaperone clients on the surface of the nucleolus." I find each of these interpretations lacking or unclear as follows:

> The short period of treatment (15 minutes) makes it unlikely that Sis1 anchor away pre- adapted cells to heat shock and thus precluded the stress.

How long was the anchor away pretreatment? According to the legend, 15 minutes is the length of the heat shock. If anchor away pretreatment was 15 min and heat shock was also 15 min, then the total amount of time (30 min) is absolutely long enough for cells to pre-adapt to heat shock by the time the image was taken and explain the results. Even less time (15 minutes) would be enough for preadaptation to make a difference, as induction of heat shock messages is extremely rapid (some genes reach maximal transcription after heat shock within 5 minutes https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0017272, and translation of these messages can complete in minutes)

> Rather, Sis1 appears to be necessary to recruit the proteasome.

I think the authors mean Sis1 shuttling between the nucleus and cytosol is necessary for recruiting the proteasome to the cytosol (is this right?). If so, this would appear to be a role for Sis1 that is independent

of its role in activating the heat shock response, in which, according to the authors, Sis1 is stuck in the cytosol and doesn't perform its nuclear function. The authors' statement is both unclear and too speculative to appear in the results.

> This implies that Sis1 mediates proteasomal degradation of chaperone clients on the surface of the nucleolus.

The authors' have not shown that any proteasome client is dependent on Sis1. This is too speculative for the results (and should be very carefully articulated as speculation in the discussion).

The authors' findings significantly advance understanding of how cells respond to heat stress, and appears to be distinct from other chaperone-based (Hsp70/Hsp90/TRiC client overload) mechanisms of activating the HSR. However, it is unclear how often this mechanism is employed by cells. Aside from artificially inducing Sis1 relocalization, the only stress in which the authors show Sis1 relocalization is heat shock, a stress that is known to cause widespread changes in PQC machinery (including client overload of the previously mentioned chaperones). Thus, understanding the overall relevance of Sis1 relocalization in HSR activation would require many more experiments, including showing the degree to which other stressors activate the HSR via Sis1 relocalization. In fact, other studies have proposed diversity in the mechanisms by which different stressors activate the HSR. (e.g. https://pubmed.ncbi.nlm.nih.gov/21085490/, https://pubmed.ncbi.nlm.nih.gov/14612437/,https://rupress.org/jcb/article/217/11/3809/120659/Quantification-of-Hsp90-availability-reveals, https://www.biorxiv.org/content/10.1101/2020.03.29.014845v1). It would be useful to make this distinction clear in the discussion.

Minor comments:

Coloration of Fig 1C is confusing and inconsistent with the rest of the paper

Figure legends should say which fluorophores are being compared with MOC (I believe its YFP-mScarlet, but it's not explicitly stated)

Typo: Reference to "Fig 6 F,G" should be a reference to Fig 5F,G (p.20-21)

Reviewer #2 (Comments to the Authors (Required)):

This study provides two conceptually important contributions to the understanding of how Hsf1 is controlled by the Hsp70 chaperone system. First, the work puts the spotlight on the nuclear J-domain protein (JDP) Sis1 and presents data supporting the notion that Sis1 is a stress-titratable factor that recruits the negative regulator Hsp70 to Hsf1 in the nucleoplasm. Second, the study documents that heat shock triggers the redistribution of Sis1 from the nucleoplasm to the surfaces of the nucleolus and the ER suggesting that its activity is spatially controlled.

This study has the potential to become a key reference for Hsf1 regulation by chaperone titration. Moreover, in a timely manner the study contributes to the understanding of spatial quality control and its link to stress-induced transcription.

Major concerns

1. A central claim of this study is that Sis1 recruits Hsp70 to Hsf1. In support, depletion of Sis1 from the nucleus using anchor away results in activation of Hsf1 (Fig 1C-F) and also decreases the interaction with Ssa1/2 (Fig 2B). Yet, an alternative interpretation of these findings is that Sis1 depletion triggers instant and general protein misfolding in the nucleus via Hsp70 dysregulation. According to this reasoning, the misfolded proteins titrate available Hsp70 and thus Hsf1 becomes indirectly liberated from its repressing chaperone. This scenario based on indirect effects can unfortunately readily be extended to also explain

the Hsf1-repressive effect mediated by NLS-Sis1 (Fig 2C-E).

The key to distinguish a scenario in which Sis1 directly recruits Hsp70 to Hsf1 from the indirect alternative appears to be to establish a physical link between Sis1 and Hsf1. Since the described coIP approaches have failed to detect any interaction between Sis1 and Hsf1, perhaps more sensitive setups are required? For example, using a Sis1 mutant lacking a functional J-domain (deletion or HPD mutation) that cannot directly transfer its substrates to Hsp70. An alternative approach may involve assessing the interaction between purified Sis1 and Hsf1. In this regard it is worth noting that Masser et al 2019 eLife and Kmiecik, Le Breton & Mayer 2020 EMBO J include Sis1 and the related human JDP DnaJB1 when reconstituting the Hsf1-Hsp70 interaction in vitro.

2. Upon heat shock, Sis1-YFP rapidly relocalizes to form a ring around the nucleolus and a continuous ER network together with other protein quality controls components. Yet it is unclear if this relocalization is a required part of the chaperone titration mechanism that activates Hsf1 or a downstream event involving association of the Hsp70 system with aggregated/phase-separated misfolded proteins. In quantitative analysis, is the relocalization of Sis1 from the nucleoplasm immediate (like Hsf1-dependent transcription) or does it require somewhat longer time and thus represents downstream events? On a similar note, does Sis1 form similar structures when more specific Hsf1 activating regimes than heat shock are applied? An informative approach may involve activating Hsf1 using azetidine 2-carboxylic acid.

Minor concerns

3. Fig 1A: The results in this panel do not add much to the literature or to this study. It is well established that inactivation of SSA1, SSA2, HSC82 or YDJ1 result in Hsf1 activation. For readability, I suggest removing the data from the manuscript or alternatively keep it as supplementary data for the purpose of validation of the reporter and strain.

4. Fig 2B: The decreased interaction between Hsf1 and Ssa1/2 upon Sis1 depletion is a key observation in this study. It would be nice to see the ratios for each of the three replicates in the figure.

5. Fig 1 C-D/Fig S1B: As the authors rightly point out, the Ssa1/2-AA-GFP are impaired proteins and the anchor away setup is therefore unmeaningful for these constructs. Thus, the data probably should be removed from the manuscript not to confuse readers.

6. Table S1: Since many strains are used in the manuscript, it would be helpful to link the strains to the data in the figure, for example by including the relevant strain names in the figure legends or by including information about the relevant figure panels in the strain list. The list also includes strains that do not appear to be part of the manuscript, for example strains expressing NES-Sis1.

7. Discussion, end of 2nd paragraph: For clarity I suggest that the two sentences are rephrased, "Second, the requirement for Sis1 [...] Hsp70-ADP near Hsf1 would be expected to drop, thereby repressing Hsf1". Hsp70-ADP is the regulatory species that binds Hsf1 but it is unlikely to engage Hsf1 (it probably has another substrate occupying its substrate binding site). Hsp70-ATP is the regulatory species that Sis1 can bind and potentially recruit to Hsf1.

Editorial comments

The main concern shared by reviewers is regarding the direct vs. indirect effects of Sis1 on Hsf1 activity. You will see that reviewer #1 asks for discussion of an alternative model of proteostasis collapse by Sis1 relocalization indirectly activating HSR by increasing Hsp70 clients while reviewer #2 requests that you show a physical link between Sis1 and Hsf1 either using a Sis1 mutant lacking a functional J-domain or by direct binding of purified proteins. We feel these requests are reasonable and every effort should be made to address them with new data.

To address whether the effects of Sis1 localization on Hsf1 activity are the direct result of de-repression in the absence of stress or the indirect result of proteostasis collapse, we used a “total-supernatant-pellet” differential centrifugation assay to monitor protein aggregation. We found that while heat shock at 39ºC triggers recruitment of Hsp70 the pellet fraction, Hsp70 remains in the supernatant after Sis1 is anchored away (new Figure S2C, D). Thus, depletion of Sis1 from the nucleus does not lead to bulk removal of Hsp70 from the soluble fraction, suggesting both that proteostasis remains largely intact and that there is no obvious reduction in the amount of Hsp70 available to repress Hsf1. These results, along with the imaging and RNA-seq data we included in the initial submission (showing that when Sis1 is anchored away, Hsp104-mKate doesn’t form foci and only Hsf1 target genes – not general stress genes – are induced), suggest that the increase in Hsf1 activity cannot be explained by proteostasis collapse.

To detect a transient interaction between Sis1 and Hsf1 and thereby establish the missing physical link, we took advantage of a mutant of Sis1 in which the HPD motif was mutated to alanine (HPD>AAA), as suggested. We performed anti-flag IPs of Hsf1 in cells expressing wild type Sis1 or HPD>AAA. While we were again unable to detect wild type Sis1 in the IP, we were

indeed able to co-IP HPD>AAA with Hsf1. This suggests that Sis1 and Hsf1 interact directly, and that Sis1 recognizes Hsf1 as a canonical client for delivery to Hsp70.

Reviewer #1 also raises questions regarding the strength of data for the proposed function of Sis1 in proteasomal degradation on nucleolus surface and the connections to ER and RCQ. Please address these concerns by either adding more definitive data or tone down conclusions and revise discussion accordingly.

We have toned down our conclusions and discussion of the putative role of Sis1 in degradation on the nucleolar and ER surfaces and the connection to RQC.

Reviewer #1

Figure 2E: Please explain why the initial increase in Hsf1 activity is the same in WT and Sis1OE cells. The model nicely recapitulates this, but the behavior should be explained in conceptual terms so that all readers can benefit from the insights of your model.

In the model, the reason that the initial increase in Hsf1 activity during heat shock is the same in WT and NLS-Sis1 is that Hsp70 becomes limiting immediately after heat shock – not Sis1. The model simulates heat shock as an instantaneous increase in unfolded proteins (UPs). Upon heat shock, the UPs initially greatly outnumber Hsp70, so no matter how much Sis1 is present, all the Hsp70 is bound by UPs. Once Hsf1 induces more Hsp70, the extra Sis1 serves to increase the affinity of Hsp70 for Hsf1 enabling rapid deactivation. This explanation has been added to the text.

Furthermore, it may be useful to include the behavior of an alternative model that proteostasis collapse by Sis1 relocalization indirectly activates the HSR by increasing Hsp70 clients. Does this

alternative model fit the data equally well or worse than the Sis1/Hsp70/Hsf1 direct interaction model you propose? The comparison of alternate models would be more helpful for understanding observations than fitting just a single model.

We elected not to pursue an alternative computational model based on proteostasis collapse for two reasons. First, the current model does not actually simulate any of the key aspects of proteostasis (protein synthesis, folding and degradation). Currently, UPs just appear at a set amount at time 0 that depends on the temperature. We are working on a new input function for the model that explicitly simulates proteostasis to allow us to explore a broader range of physiological inputs, but this will be included in a future study. Second, we have generated new data suggesting that proteostasis remains intact when we anchor away Sis1 (see the response to the editorial comments above), obviating the need to explore this scenario in silico.

Figure 4D: When was cycloheximide added? How does this explain the lack of nucleolar relocalization for Sis1? The authors state "This suggests that ongoing translation is required to trigger Sis1 re-localization during heat shock, and is consistent with a role for nascent ribosomal proteins in Sis1 localization to the nucleolus." Nascent ribosomal proteins (those being actively translated) are in the cytosol (where the translating ribosomes are) so I'm not sure how the authors are connecting cytosolic nascent chains to nucleolar localization of Sis1. I can imagine some models, like fewer orphan ribosomal proteins in the nucleolus because translation has stopped while ribosome biogenesis continues, depleting the nucleoli of orphan ribosomal proteins. But this sort of speculation needs to be explicitly stated clearly (with timing info) and in the discussion section rather than the results section unless the reasoning serves as the basis for later experiments. Right now the discussion says "Sis1 spatial re-localization during heat shock suggests nascent proteins are also drivers (Figure 3D, E)." Again, what's the connection between nascent proteins and nucleoli? There seems to be a logical leap here that is not clear.

Cycloheximide will block all nascent proteins in the cell, not just those going on to become ribosomes.

We added cycloheximide 5 minutes before heat shock. We misused the term “nascent” – which specifically signifies proteins still being translated on the ribosome – when we meant “newly synthesized” to refer to the un-incorporated ribosomal proteins we hypothesize to be the molecules that trigger Sis1 localization to the nucleolus. The reviewer’s proposed model involving fewer orphan ribosomal proteins is precisely what we had in mind. We have corrected the text.

Figure 5E: The authors state that "The imaging data suggest that Sis1 forms a highly connected network with the proteasome and RQC on the surface of the ER." Is this really happening? To me it looks like Sis1 and Rtn1 may be adjacent in some cases, but Sis1 is not "at the surface of the ER". If Sis1 was at the surface, wouldn't it co-localize with Rtn1 more completely?

The image analysis reveals that 13% of Sis1 signal overlaps with Rtn1 under nonstress conditions and 60% colocalizes upon heat shock. Aside from the peri-nucleolar Sis1, there is very little Sis1 signal that doesn’t co-localize with Rtn1. We have added a 2-color merge to the supplement to show Sis1 and Rtn1 to make this more evident (new Figure S6D).

Figures 3 and 5: The authors' data on the RQC is weak. Ltn1 and Cdc48 both have non-RQC roles (e.g. https://pubmed.ncbi.nlm.nih.gov/24616224/), so finding that Sis1 binds them both but not other RQC members Rqc1 and Rqc2 doesn't strongly implicate the RQC unless there is more data to link Sis1 to RQC. Partial co-localization of Ltn1-scarlet with Sis1 under stress also doesn't mean much because the author's haven't demonstrated that Ltn1-scarlet is functional. The only C terminal tag of Ltn1 that covers that this reviewer knows about is a C terminal HA tag, and C terminal FLAG tagging of Ltn1 results in loss of binding to all other RQC components

(see https://pubmed.ncbi.nlm.nih.gov/20835226/ for evidence of both of these things). Unless that authors show that the Ltn1-scarlet tag covers, either by showing that Ltn1-scarlet still associates with other RQC members or that Ltn1-dependent RQC substrates are properly degraded in an Ltn1-scarlet background, then I would recommend deemphasizing RQC as a topic in this paper, probably removing the Ltn1-scarlet experiments entirely. The association of Sis1 with Ltn1 and Cdc48 is still interesting and should be reported but requires more followup to understand.

We appreciate the expertise of the reviewer and have removed the Ltn1 imaging data from the paper and de-emphasized the connection to RQC.

Page 21: "This implies that Sis1 mediates proteasomal degradation of chaperone clients on the surface of the nucleolus". This statement is too speculative for the results section and the idea should be moved to the discussion.

This speculative statement has been removed from the results.

Figure 5F: This is a striking result. The authors say: "The short period of treatment (15 minutes) makes it unlikely that Sis1 anchor away pre- adapted cells to heat shock and thus precludes the stress. Rather, Sis1 appears to be necessary to recruit the proteasome. This implies that Sis1 mediates proteasomal degradation of chaperone clients on the surface of the nucleolus." I find each of these interpretations lacking or unclear as follows:

We agree with the reviewer that our assumption that the cells could not have pre-adapted is too strongly stated and have added this alternative interpretation. We have also removed speculation that Sis1 participates in proteasomal degradation of chaperone clients.

> The short period of treatment (15 minutes) makes it unlikely that Sis1 anchor away pre-adapted cells to heat shock and thus precluded the stress.

How long was the anchor away pretreatment? According to the legend, 15 minutes is the length of the heat shock. If anchor away pretreatment was 15 min and heat shock was also 15 min, then the total amount of time (30 min) is absolutely long enough for cells to pre-adapt to heat shock by the time the image was taken and explain the results. Even less time (15 minutes) would be enough for preadaptation to make a difference, as induction of heat shock messages is extremely rapid (some genes reach maximal transcription after heat shock within 5 minutes https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0017272, and translation of these messages can complete in minutes)

The pretreatment was 15 minutes, so the reviewer is correct that the total amount of time before the images were captured was 30 minutes. The reason we think it is unlikely that the cells have pre-adapted is that it takes 15 minutes of rapamycin treatment before Sis1 is quantitatively depleted from the nucleus in >90% of cells. Unlike heat shock, which certainly induces near-instantaneous Hsf1 activation, anchoring away Sis1 would be expected to result in slower Hsf1 activation. Supporting this, our RNA-seq data shows that Hsf1 target gene transcript levels don't peak until 30 minutes of treatment with rapamycin, with only a modest induction of Hsf1 targets at 15 minutes (Fig. S3). However, it remains possible that cells have pre-adapted to some extent, and we have added this interpretation to the text.

> Rather, Sis1 appears to be necessary to recruit the proteasome.
I think the authors mean Sis1 shuttling between the nucleus and cytosol is necessary for recruiting the proteasome to the cytosol (is this right?). If so, this would appear to be a role for Sis1 that is independent of its role in activating the heat shock response, in which, according to the authors, Sis1 is stuck in the cytosol and doesn't perform its nuclear function. The authors' statement is both unclear and too speculative to appear in the results.

We meant that unanchored Sis1 is required for Rpn1 to re-localize in response to heat shock both within the nucleus and to the cytosolic foci, implying that free Sis1 is an upstream factor required for recruitment of the proteasome to these sites. Indeed, this would constitute a distinct function from the role of Sis1 in Hsf1 regulation. We have stated this along with the alternative interpretation of pre-adaptation.

> *This implies that Sis1 mediates proteasomal degradation of chaperone clients on the surface of the nucleolus.*
> *The authors' have not shown that any proteasome client is dependent on Sis1. This is too speculative for the results (and should be very carefully articulated as speculation in the discussion).*

We have removed this statement.

The authors' findings significantly advance understanding of how cells respond to heat stress, and appears to be distinct from other chaperone-based (Hsp70/Hsp90/TRiC client overload) mechanisms of activating the HSR. However, it is unclear how often this mechanism is employed by cells. Aside from artificially inducing Sis1 relocalization, the only stress in which the authors show Sis1 relocalization is heat shock, a stress that is known to cause widespread changes in PQC machinery (including client overload of the previously mentioned chaperones). Thus, understanding the overall relevance of Sis1 relocalization in HSR activation would require many more experiments, including showing the degree to which other stressors activate the HSR via Sis1 relocalization. In fact, other studies have proposed diversity in the mechanisms by which different stressors activate the HSR.
(e.g. https://pubmed.ncbi.nlm.nih.gov/21085490/, https://pubmed.ncbi.nlm.nih.gov/14612437/,https://rupress.org/jcb/article/217/11/3809/120659/Quantification-of-Hsp90-availability-reveals, https://www.biorxiv.org/content/10.1101/2020.03.29.014845v1). It would be useful to make this distinction clear in the discussion.

We have added a sentence to the discussion stating that the generality by which Sis1 re-localization serves as a regulatory mechanism for the HSR in response to various stressors remains to be determined.

Minor comments:

Coloration of Fig 1C is confusing and inconsistent with the rest of the paper

We have changed the colors to make it more intuitive and to more clearly show the overlap and nuclear depletion.

Figure legends should say which fluorophores are being compared with MOC (I believe its YFP-mScarlet, but it's not explicitly stated)

Yes, it is YFP vs. mScarlet. We have added this to the figure legends.

Typo: Reference to "Fig 6 F,G" should be a reference to Fig 5F,G (p.20-21)

We have corrected this.

Reviewer #2

Major concerns

1. A central claim of this study is that Sis1 recruits Hsp70 to Hsf1. In support, depletion of Sis1 from the nucleus using anchor away results in activation of Hsf1 (Fig 1C-F) and also decreases the interaction with Ssa1/2 (Fig 2B). Yet, an alternative interpretation of these findings is that Sis1 depletion triggers instant and general protein misfolding in the nucleus via Hsp70 dysregulation.

According to this reasoning, the misfolded proteins titrate available Hsp70 and thus Hsf1 becomes indirectly liberated from its repressing chaperone. This scenario based on indirect effects can unfortunately readily be extended to also explain the Hsf1-repressive effect mediated by NLS-Sis1 (Fig 2C-E).

In our initial submission, we provided two lines of evidence to support the notion that anchoring away Sis1 has no immediate effects on general proteostasis. First, we showed that anchoring away Sis1 does not trigger formation of Hsp104-mKate foci, suggesting at least that cytosolic proteostasis remains uncompromised. Second, using RNA-seq, we showed that the only genes with altered expression in response to anchoring away Sis1 are Hsf1 target genes. This suggests that nuclear processes – most notably transcription – remain largely unperturbed, suggesting that proteostasis remains intact. We now provide more direct evidence that anchoring away Sis1 does not trigger proteostasis collapse. We performed a "total-supernatant-pellet" differential centrifugation assay to monitor and found that while heat shock triggers immediate recruitment of Hsp70 the pellet fraction, Hsp70 remains in the supernatant after Sis1 is anchored away (Figure S2C, D). Thus, depletion of Sis1 from the nucleus does not trigger bulk removal of Hsp70 from the soluble fraction, suggesting both that proteostasis remains largely intact and that there is no obvious reduction in the amount of Hsp70 available to repress Hsf1. Together, these data show that Sis1 nuclear depletion does not activate Hsf1 by triggering proteostasis collapse, at least not at the relevant early timepoints.

As for the NLS-Sis1 experiment, we agree that the early Hsf1 attenuation during heat shock could in principle be due to either direct Sis1-mediated deactivation or the indirect effects of Sis1 promoting more efficient restoration of proteostasis. However, the genetic rescue of NLS-Sis1 expression we observe with Hsf1ΔCE2 argues against the indirect effect. If NLS-Sis1 is reducing the load of misfolded proteins, then 1) why does its expression impair growth in the

first place? and 2) how does Hsf1ΔCE2 – which has even higher expression of the proteostasis machinery than wild type – rescue growth? The parsimonious explanation is that Sis1 directly represses Hsf1, which impairs proteostasis, so relieving Hsf1 repression by removing a binding site for Hsp70 restores proteostasis and rescues cell growth.

The key to distinguish a scenario in which Sis1 directly recruits Hsp70 to Hsf1 from the indirect alternative appears to be to establish a physical link between Sis1 and Hsf1. Since the described coIP approaches have failed to detect any interaction between Sis1 and Hsf1, perhaps more sensitive setups are required? For example, using a Sis1 mutant lacking a functional J-domain (deletion or HPD mutation) that cannot directly transfer its substrates to Hsp70. An alternative approach may involve assessing the interaction between purified Sis1 and Hsf1. In this regard it is worth noting that Masser et al 2019 eLife and Kmiecik, Le Breton & Mayer 2020 EMBO J include Sis1 and the related human JDP DnaJB1 when reconstituting the Hsf1-Hsp70 interaction in vitro.

As suggested by the reviewer, we utilized a mutant of Sis1 with a disrupted HPD motif (HPD>AAA). Indeed, unlike wild type Sis1, we were able to detect HPD>AAA following IP of Hsf1, thus establishing a direct link between Hsf1 and Sis1 (Figure 2C).

2. Upon heat shock, Sis1-YFP rapidly relocalizes to form a ring around the nucleolus and a continuous ER network together with other protein quality controls components. Yet it is unclear if this relocalization is a required part of the chaperone titration mechanism that activates Hsf1 or a downstream event involving association of the Hsp70 system with aggregated/phase-separated misfolded proteins. In quantitative analysis, is the relocalization of Sis1 from the nucleoplasm immediate (like Hsf1-dependent transcription) or does it require somewhat longer time and thus represents downstream events? On a similar note, does Sis1 form similar structures when more specific Hsf1 activating regimes than heat shock are applied? An informative approach may

involve activating Hsf1 using azetidine 2-carboxylic acid.

To quantify Sis1-YFP re-localization dynamics during the early phase of heat shock, we fixed cells at multiple time points following heat shock and imaged them on the lattice light sheet microsocope. We monitored localization to the nucleolar periphery by co-imaging Nsr1-mScarlet (a nucleolar protein that came down with Sis1 in our IP/MS experiment) and we cytosolic colocalization with Hsp104. We found that Sis1 re-localizes to form nucleolar rings in less than 2 minutes following heat shock, however it takes more than 5 minutes for Sis1 and Hsp104 to form cytosolic foci (new Figure S5). Thus, the Sis1 subnuclear re-localization represents an immediate event following heat shock that coincides with the earliest detected Hsf1 activation. We are currently investigating Sis1 localization patterns under other stress conditions, including AZC, that we will report in a future manuscript.

Minor concerns

3. Fig 1A: The results in this panel do not add much to the literature or to this study. It is well established that inactivation of SSA1, SSA2, HSC82 or YDJ1 result in Hsf1 activation. For readability, I suggest removing the data from the manuscript or alternatively keep it as supplementary data for the purpose of validation of the reporter and strain.

We removed this panel from the main figure and left the series of ssa deletions in the supplement.

4. Fig 2B: The decreased interaction between Hsf1 and Ssa1/2 upon Sis1 depletion is a key observation in this study. It would be nice to see the ratios for each of the three replicates in the figure.

We have included this (new Figure 2D).

5. Fig 1 C-D/Fig S1B: As the authors rightly point out, the Ssa1/2-AA-GFP are impaired proteins

and the anchor away setup is therefore unmeaningful for these constructs. Thus, the data probably should be removed from the manuscript not to confuse readers.

We have removed these data.

6. Table S1: Since many strains are used in the manuscript, it would be helpful to link the strains to the data in the figure, for example by including the relevant strain names in the figure legends or by including information about the relevant figure panels in the strain list. The list also includes strains that do not appear to be part of the manuscript, for example strains expressing NES-Sis1.

We have added the figure information to the strain list and removed extraneous strains.

7. Discussion, end of 2nd paragraph: For clarity I suggest that the two sentences are rephrased, "Second, the requirement for Sis1 [...] Hsp70-ADP near Hsf1 would be expected to drop, thereby repressing Hsf1". Hsp70-ADP is the regulatory species that binds Hsf1 but it is unlikely to engage Hsf1 (it probably has another substrate occupying its substrate binding site). Hsp70-ATP is the regulatory species that Sis1 can bind and potentially recruit to Hsf1.

We have rephrased these sentences.

October 29, 2020

RE: JCB Manuscript #202005165R

Dr. David Pincus
University of Chicago
929 E 57th Street
GCIS W522
Cambridge, Il 60637

Dear Dr. Pincus,

Thank you for submitting your revised manuscript entitled "Subcellular localization of the J-protein Sis1 regulates the heat shock response." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

3) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

4) Make sure to indicate in the methods the source, species, and catalog numbers (where

appropriate) for all of your antibodies and other reagents.

5) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

6) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

7) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

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B. FINAL FILES:

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Judith Frydman, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors revisions have strengthened an already very interesting paper. I recommend publication.

Reviewer #2 (Comments to the Authors (Required)):

All my concerns have been adequately addressed.