THE
EMBO
JOURNAL

Manuscript EMBO-2012-84165

The nascent polypeptide associated complex is a key regulator of proteostasis

Janine Kirstein-Miles, Annika Scior, Elke Deuerling and Richard I Morimoto

Corresponding author: Richard I. Morimoto, Northwestern University

Review timeline:		
	Submission date:	04 April 2012
	Editorial Decision:	16 May 2012
	Resubmission:	09 December 2012
	Editorial Decision:	06 February 2013
	Revision received:	11 March 2013
	Accepted:	18 March 2013

Transaction Report:

(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.)

Editor: Isabel Arnold/Anne Nielsen

1st Editorial Decision	16 May 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it and their comments are shown below. All you will see, while referee 2 is considerably more positive and all three referees consider the study as interesting in principle, referees 1 and 3 are not convinced that the dataset is conclusive and complete enough at this point to justify your main conclusions. From the reports, it becomes clear that a substantial set of additional biochemical data that characterise the nature of the aggregates, directly demonstrate that NAC is sequestered away from the ribosomes into the aggregates and that this sequestration event is causal for translation inhibition would be required. Clearly, addressing these issues is a major task and effort, and the outcome of the additional experiments required cannot be predicted at this point. This goes well beyond the scope and time frame of a single revision, and it is our policy to allow for a single round of revision only. In this situation, we cannot invite a formal revision.

Still, given the interest expressed by the referees in principle, we would be happy to consider a new, substantially revised version of this manuscript as a new submission at a later stage that provides more direct biochemical evidence for the key aspects of your model along the lines suggested by the referees. This new version will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh (involving our original referees again if available at the time of resubmission), also with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful. Please do not hesitate to get back to me at any time in case you would like to consult on any aspect of a possible resubmission further.

REFEREE REPORTS

Referee #1

The manuscript by Kirstein-Miles and coworkers presents the idea that NAC is a key regulator of proteostasis in C. elegans. The authors suggest that NAC affects the aggregation of polyQ proteins as an active chaperone. The authors also suggest that natively folded NAC is recruited to aggregates formed upon aging or heat stress. Moreover, the authors suggest that NAC is a general attenuator of translation.

However, based on the data I am not convinced that NAC plays an important, active role in these processes. The data presented are compatible also with a model in which NAC behaves similar to other components of the translational apparatus (compare below). In principal, I feel that the experiments described are not suited to test if NAC passively co-aggregates or is specifically recruited to protein aggregates or stress granules that appear during aging or heat shock.

The term "translational attenuation" has been previously used to describe local discontinuous translation of specific regions in an mRNA or stalling of ribosomes on mRNA for different reasons. This question is not addressed in this study and the term is thus misleading.

Results:

related to Figure 1

The data suggest that upon NAC depletion, Q35-YFP accumulates in cellular bodies in 4 days old animals. This is an interesting finding, however, some additional controls are required to draw a valid conclusion.

- Q35-YFP "aggregation" is visible also in the 4 day control animal, though to a lesser extent. This is consistent with the previous finding that wild type "Q35 animals accumulated aggregates after a lag period of 4-5 days (Morley et al., 2002)". Thus, Q35-YFP starts to form "aggregates" in the NAC-knockdown only slightly earlier compared to control animals (compare also Fig. 6A and Fig. 4B). Moreover, the average number of 6 Q35-YFP bodies (Fig. 1B) is very low compared to what was found in previous studies. I feel that the experiment has to be performed at a polyQ length, at which the difference between control and NAC-knockdown is more significant. In addition, a time course for control and NAC-knockdown cells should be performed.

- It is surprising that both βNAC and αNAC seem to affect Q35-YFP (Fig. 1), but only RNAi against βNAC affects C. elegans offspring (Fig. S1). Please comment. In this context it might be worth mentioning that αNAC-independent roles for βNAC homologs have been suggested by many studies (include in the Introduction).

- A control expressing YFP lacking polyQ should be included.

- localization of the nuclei relative to the YFP-bodies should be shown e.g. using Normarski photographs.

- Whole animals should be shown.

"This increase in aggregation is similar to that observed upon knock-down of the cytosolic Hsp70 chaperones, hsp-1 and F44E5.4 (data not shown), consistent with our proposal that NAC is an important chaperone component of the cellular proteostasis network. "

This does not tell too much, because there is redundancy between Hsp70 chaperones and a single knockout is expected to display only a mild effect (as it seems to be the case for the NAC-knockdowns).

related to Figure 2

Fig. 2 A and B

a control protein, which remains soluble during the time course should be shown. Also, a ribosomal marker has to be included in the analysis. This is important because David et al. (David et al., 2010) found that many ribosomal components end up in the insoluble fraction upon aging. This might be the fate of NAC also.

Fig. 2C
Please improve the data. I cannot follow the conclusion that NAC is distributed between cytosol and nucleus.

Fig. 2D
Standard controls for subcellular fractionation should be included.

Fig. 2E
The quality of the data should be improved. It is not clear if single cells are shown. The localization of the nucleus (nuclei) is unclear.

Fig. 2F

"The analysis showed that the aggregated proteins did not sequester RPL-25, establishing that ribosomes remain soluble in our assay conditions. "

I cannot follow the argument. Not all ribosomal proteins end up in the insoluble pellet during aging, however, many do. Rpl25 is actually not one of them (David et al., 2010). However, Rpl31, which is the ribosomal attachment site of βNAC and thus interacts with βNAC directly (Pech et al., 2010) was found in the insoluble pellet fraction. Soluble NAC e.g. might interact with Rpl31 contained in the insoluble pellet and thus be recruited.

"This NAC signal most likely stems from residual NAC found in the insoluble fraction of day 16 old nematodes, that was not removed by detergent treatment during the aggregate isolation procedure."

It is an essential experiment in this context to establish conditions that allow for the isolation of "insoluble fraction" that contains NAC. Otherwise it remains an open question if NAC binds to insoluble material in living cells.

Fig. 2H and I
The whole profile should be shown to allow for an estimate of the loading. Some error bars would be helpful.

related to Figure S2 and S3

"NAC antibody also recognized a higher MW protein in day 2 old nematodes, which likely represents mono-ubiquitinated αNAC as shown by western blot using Ubiquitin-specific antibodies (Fig. S3)."

It is highly speculative that the band recognized by the ubiquitin antibody is one of the ubiquitinated NAC subunits. If this was the case, the shapes of the bands on the reprobed blot should match. However, this is not the case. Please note, that e.g. a number of ribosomal proteins are monoubiquitinated and might be recognized by the ubiquitin antibody. The authors might want to discuss the work of (Panasenko et al., 2009) in this context. The molecular mass of the bands on the Western blots should be indicated. It should be clear in all Figure Legends if an antibody directed α or βNAC was used.

related to Figure 3

"Depletion of NAC from its ribosomal function by sequestration to aggregates causes a decline in polysome formation (Figs. 2F, H+I) and hence the attenuation of protein synthesis."

A decline of polysomes is expected if ribosomes (or important ribosomal proteins) go to an insoluble pellet during aging (David et al., 2010). However, there is no evidence for a causative role of NAC in this process. One should also carefully distinguish between effects due to the number of

active ribosomes and "attenuation of protein synthesis" on a single ribosome.

related to Figure 5

Fig. 5D
It is not clear how exactly the experiment was performed. The text (middle of page 14) and the Figure Legend do not match. When exactly where the pictures taken? After recovery ?

"A similar response of a translational attenuation, albeit transiently, occurs upon exposure to acute stress such as heat shock (Lindquist 1980)."

Maybe the authors want to include some more recent literature on the effect of heat shock on the translation machinery e.g. summarized in (Buchan and Parker, 2009).

related to Figure 6
"This effect was not due to differences in the pre-existing levels of Q35-YFP (Fig. 6C), from which we conclude that a reduction in bulk protein synthesis is beneficial for cells exposed to protein folding stress. "

This conclusion is not clear to me.

Introduction and Discussion

There is ongoing discussion about the in vivo function of NAC. In the interest of a general readership, the most important models and hypothesis should be presented to give an unbiased view about the current status of the field.

"Both systems (NAC and RAC) bind transiently to the large ribosomal subunit and interact with nascent polypeptides during early protein biogenesis."

This is only partly correct. While NAC binds directly to nascent chains, the two subunits of RAC do not. RAC rather stimulates the binding of the ribosome-bound Hsp70 Ssb (Gautschi et al., 2002; Huang et al., 2005). Actually, the model shown in Fig. 2G shows this correctly.

"In yeast, however, deletion of NAC has no apparent phenotype, unless the cooperating RAC system is also deleted. Yeast cells lacking both ribosome-associated systems are highly sensitive to proteotoxic conditions, exhibit aggregation of newly synthesized proteins, and impairment in ribosome biogenesis along with a strongly reduced translation activity (Albanese et al. 2010; Koplin et al. 2010)."

The study by Albanese et al. 2010 investigated the interplay between the RAC subunit Zuo1 and the J-homolog Jjj1. NAC was not the topic in this study. The major role of the two J-homologs was on rRNA maturation. The study by Koplin et al. 2010 focused on the interplay between NAC and Ssb (please note, Ssb, in contrast to RAC is a yeast specific component and is not conserved in higher eukaryotes).

Bloss et al. 2003 report that C. elegans βNAC (ICD-1) localizes to mitochondria. How do the authors interpret this? Please also discuss the potential role of ICD-1 in repression of caspases and its effects on apoptosis (Bloss et al., 2003).
Markesich et al. 2000 suggest that loss of Drosophila βNAC function results in a release of translational repression. This is opposite to what is suggested by the authors, please discuss (Markesich et al., 2000).

Del Alamo et al. 2011 report that yeast NAC modulates SRP-specificity and -fidelity in vivo. These new data on the role of NAC should be discussed (Del Alamo et al., 2011).

Minor points:

page 5, line 5 from the bottom: Fig. 1A (not Fig.1B)

page 13, line 5 and line 9: Fig. 4F (not Fig. 4D)

Bloss, T.A., E.S. Witze, and J.H. Rothman. 2003. Suppression of CED-3-independent apoptosis by mitochondrial betaNAC in Caenorhabditis elegans. Nature. 424:1066-1071.

Buchan, J.R., and R. Parker. 2009. Eukaryotic stress granules: the ins and outs of translation. Mol Cell. 36:932-41.
David, D.C., N. Ollikainen, J.C. Trinidad, M.P. Cary, A.L. Burlingame, and C. Kenyon. 2010. Widespread protein aggregation as an inherent part of aging in C. elegans. PLoS Biol. 8:e1000450.

Del Alamo, M., D.J. Hogan, S. Pechmann, V. Albanese, P.O. Brown, and J. Frydman. 2011. Defining the Specificity of Cotranslationally Acting Chaperones by Systematic Analysis of mRNAs Associated with Ribosome-Nascent Chain Complexes. PLoS Biol. 9:e1001100.

Gautschi, M., A. Mun, S. Ross, and S. Rospert. 2002. A functional chaperone triad on the yeast ribosome. Proc. Natl. Acad. Sci. USA. 99:4209-4214.

Huang, P., M. Gautschi, W. Walter, S. Rospert, and E.A. Craig. 2005. The Hsp70 Ssz1 modulates the function of the ribosome-associated J-protein Zuo1. Nat. Struct. Mol. Biol. 12:497-504.

Markesich, D.C., K.M. Gajewski, M.E. Nazimiec, and K. Beckingham. 2000. bicaudal encodes the Drosophila beta NAC homolog, a component of the ribosomal translational machinery. Development. 127:559-572.

Morley, J.F., H.R. Brignull, J.J. Weyers, and R.I. Morimoto. 2002. The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U S A. 99:10417-10422.

Panasenko, O.O., F.P. David, and M.A. Collart. 2009. Ribosome association and stability of the nascent polypeptide-associated complex is dependent upon its own ubiquitination. Genetics. 181:447-460.

Pech, M., T. Spreter, R. Beckmann, and B. Beatrix. 2010. Dual binding mode of the nascent polypeptide-associated complex reveals a novel universal adapter site on the ribosome. J. Biol. Chem. 285:19679-19687.

Referee #2

In this manuscript, Kirstein-Miles and colleagues investigate the role of the Nascent polypeptide Associated Complex (NAC) in proteostasis using C. elegans as a model organism. The authors show that under normal conditions, NAC promotes translation and correct protein folding. In contrast, during ageing and under conditions that alter protein folding homeostasis, such as heat shock, expression of Aβ peptide or polyglutamine-expansion proteins, NAC is recruited to protein aggregates leading in depletion of NAC at the ribosome and a decrease in translational activity. This decline in translation is similar to the reduction observed upon RNAi- mediated knockdown of either αNAC or βNAC subunit. Based on these findings the authors suggest that NAC plays a key role not only in detecting proteotoxic stress but also in mediating the reduction of protein synthesis under proteotoxic conditions. The authors propose a model whereby NAC is primarily associated with the ribosome, assisting folding of nascent polypeptides. Upon proteotoxic conditions protein aggregates recruit NAC, depleting NAC available for ribosomal function, thus, attenuating the formation of polysomes and general protein synthesis. This is a thorough and convincing study, providing compelling evidence for a novel role of the NAC complex as a regulator of protein homeostasis, as well as, interesting insights into the dynamics of mRNA translation regulation. The manuscript comprises several solid elements, is well written and the data are well-presented. In my view it is worthy of publication in the EMBO Journal, provided the authors address the points below.

Comments:

The authors show that NAC is recruited to various protein aggregates caused by expression of Q35 (fig 1A,B), ageing (fig 2A,B,E), heat-shock (fig 5D) or expression of Aβ peptide (fig 4E). Are other ribosome-bound chaperones also recruited in a similar manner or is recruitment NAC-specific?

According to figure2A, already from day 10, there is detectable amount of NAC in the insoluble fraction. However in Figure 2F, the 16 day-old protein aggregates (sample 1) do not appear to contain NAC. NAC subunits were not detected among proteins that were found to aggregate with age, by mass spectrometry (Table S1). Why is this?

It would be interesting to examine the effects of NAC overexpression on polysome formation, protein synthesis, protein aggregation and longevity. In other words, does overexpression of NAC have a proteostatic effect?

Experiments presented in Fig.2F demonstrate the ability of soluble NAC to be recruited to aggregates (here in an ageing paradigm). The authors could also investigate the reverse: Whether insoluble/aggregated NAC can be readily re-solubilized. This could be examined in the already employed paradigm of cell stress, such by heat shock, which induces NAC aggregation. While there is indirect evidence provided in Fig. 5, it would be nice to complement these with direct biochemical data of NAC kinetics itself. Moreover, quantification of "global" aggregation as performed and presented in Fig. 5E is not easy to assess, especially in the absence of loading controls.

One possible explanation for lifespan extension by reduced protein synthesis could be the reduction of overall protein aggregation. Do the formation of polyQ aggregates, the localization of NAC to foci and the decline in translational activity coincide temporally?

The authors show that the translational capacity of WT animals decreases during ageing. What about the expression of NAC throughout the same experimental period?

One potential caveat of using polysome profiling to assess translation activity in whole-animal extracts of C. elegans is the bias of this methodology towards massive tissues, which would mask effects on translation (or lack thereof) in other less massive tissues (such as neurons or other minor cell populations for example). The authors should clarify that what they observe is the net outcome on translational activity over numerous different cell types. It remains possible that the situation might be different for a particular cell type. In addition, translation of certain mRNAs could even be favored / upregulated under conditions that decrease general mRNA polysome loading.

It would be useful if the authors could show co-localization of NAC with the protein aggregates Q-35, or the Ab peptide in vivo.

Figure 6: Q-35 aggregates do not necessarily contain only Q35-YFP but may contain other aggregation-prone proteins whose amount is reduced under these conditions. Although it is not clear to me, I suppose that the authors imply that by lowering protein expression NAC is translocated from ribosomes to protein aggregates and participates in their solubilization. Could the authors solidify this point by monitoring the redistribution of NAC from ribosomes to Q-35 aggregates under these conditions?

It would be interesting (although not necessary for publication of this study) to examine the role of NAC in translation in long-lived genetic backgrounds or in animals exhibiting enhanced activities of stress response factors. Could this mechanism contribute to the longevity of these mutants?

Minor comments

del Alamo et al, 2011: this reference is not included in the list although it is cited in the text.

Page 10: None of these gene knock-downs ....in day 3 old nematodes: substitute Fig. S3 with Fig. S4.

Page 13: Indeed, Aβ was detected only in the NAC protein-containing sample (Fig. 4D): substitute with Fig. 4F.

Page 14: 1st line of last paragraph: correct functional

Page 18: While Aβ peptide co-precipitated with NAC (Fig. 5F): change to Fig. 4F

Is the molecular nature of the foci observed after heat shock known?

Figure 3S: To verify that the higher MW band of aNAC is a ubiquitinated form it would be important to immune-precipitate it and analyze it by mass spectrometry.

The authors should discuss wether NAC, despite its absence of ATPase domain, could still have chaperoning activity.

The results from the in vitro assay for detecting the recruitment of NAC to aggregates should be interpreted with caution. I would expect that aggregates recruit other proteins with chaperoning activity as well.

It would benefit the discussion if authors elaborated further on how NAC might modulate translational activity.

Referee #3

This study uses a C. elegans model to examine the role of NAC as a potential chaperone and central regulator of cellular proteostasis. Convincing evidence is provided that proteotoxic stress (polyglutamine expansion, heat shock, and aging) result in NAC redistribution from a soluble cellular fraction to insoluble protein aggregates. NAC relocalization is temporally correlated with decreased polysome levels and thereby a reduction in global protein synthesis. In addition, NAC knockdown prevented recovery of polysome formation following heat shock and resolution of head shock-induced aggregates. The findings add substantially to our understanding of NAC function, which has remained a mystery for more than a decade. They also lead to a novel and exciting model in which NAC is proposed to function as a global regulator of proteostasis by influencing ribosome translational activity. As such, the results have broad implications for cellular regulation and organism homeostasis.

The experiments appear technically sound, and the data are generally convincing. My major concern is that most of the conclusions are derived from broad effects that lack sufficient mechanistic detail needed to adequately support the final model. This concern is exacerbated by the rather telegraphic writing style and limited discussion of the logic by which the data is used to reach specific conclusions. For example, there is no direct evidence that NAC is truly recruited away from the ribosomes, only that it is moves into poorly defined aggregates. Bochemical evidence is needed to more clearly delineate changes in ribosome binding. Are ribosomes also sequestered in aggregates? One would expect so, given the abundance of ribosomal proteins identified by MS. The nature of aggregates is also not well defined, as they are derived from different types of stress but discussed as though they are physiologically equivalent. Do all aggregates contain similar cohorts of aggregated protein? Second, there is no evidence that the recruitment of NAC to aggregates is the actual cause of translational attenuation. Some direct evidence is needed that ribosomes require NAC for translation. Third, there is no direct evidence that NAC actually exhibits chaperone activity, i.e. does it directly facilitate folding, prevent aggregation, etc. While the general effects of knockdown are convincing, the nature of these manipulations raise the possibility that other key factors, possibly regulated by NAC, might just as easily account for cellular changes. These concerns do not detract from the exciting findings, but significantly reduce confidence in the final model.

Specific concerns.

Page 9, Fig S3. Migration of NAC shown in polysome gradients (S2) does not support its presence

in the polysome fraction. In fact the NAC profile seems completely inconsistent with ribosomes, since NAC is found at similar levels in nearly all fractions from 1-11. I don't follow the arguments on page 9.

Figure 3. It is not clear why the authors attribute the gradual decline in polysomes from day 3-day 7 to NAC. What is the evidence that ribosomes in older organisms lack NAC? Accumulation of age-related aggregates or changes in protein expression patterns could just as well decrease translational by other mechanisms. The issue here is that the authors appear to interpret broad findings with a distinct bias towards their model, rather than building a strong case for their model through critically designed experiments.

Page 12. I do not understand the term "chaperone for protein aggregates". The data show that NAC localizes to aggregates, but I do not see any evidence that it 'chaperones' these structures. I am not even sure what such a designation might mean.

Page 13 lines 6-9. What figure panel is referred to here (4D or 4F)? Since this is an SDS gel, the nature of AB that binds to immobilized NAC (prior to denaturation) is difficult to interpret. I do not see how the authors conclude that NAC interacts with AB in a functional state. What is the evidence that this binding is due to chaperone activity as stated in lines 10-11? The statement that recruitment of NAC by AB leads to loss of ribosome binding, again, lacks direct evidence. Similarly, if the authors wish to conclude that NAC depletion from ribosomes (following heat shock) is the cause for attenuated translation, then this should be shown directly. Since ribosome proteins are also in aggregates, is it possible that there are simply fewer functional ribosome present? What is needed is some direct analysis of NAC-ribosome binding, and a functional readout for this interaction,

The conclusion that NAC depletion predisposes to polyQ aggregation and lack of heat shock recovery is convincing. However, it is not clear that this observation is due to a direct chaperone-like role of NAC as claimed, or an indirect effect of NAC on other cellular factors.

Minor points

Fig. S1. How do the authors explain the finding that b-NAC reduced offspring by 90% but no effect was observed for a-NAC. Does this imply a-NAC operates independently of b-NAC? Clearly these proteins have different functions if knock down is equivalent and should be discussed.

Page 6, Components isolated in NAC pull downs (Table 1) might interact with NAC as stated, but more likely reflect indirect interactions as well. The explanation here in the text is misleading and should be presented in a more balanced manner prior to its mention in the discussion.

Figure 2F. Description here is very confusing. Identity of each lane should be mentioned at the outset (page 7). A more detailed description should be provided for aggregate isolation. What fraction of the final insoluble pellet is solubilized in SDS? Why is NAC lost during this process (no NAC is present in aggregates, Lane 1) and why do the authors conclude that it is removed by detergent washing? How does mild detergent (NP-40) remove NAC from aggregates. Are the aggregates analyzed in 2F different from aggregates in 2A&B or aggregates analyzed by MS?

Legend of Figure S2 states that antibody signal of alpha and beta NAC are indicated on the right. Is this referring to blot identity or signal intensity? The band intensities bear almost no resemblance to the polysome fractions shown at top. What is the Y axis in the top graph? There is also a poor correlation between alpha and beta NAC bands in lanes 3 and 7. In addition, the blot shown in S2 looks completely different than blot shown in S3.

Page 8. Figure S3 shows non-cross reactivity of RPL-25, but I do not see how this establishes that aggregates do not sequester RPL25. No aggregates are shown in S3. Perhaps I am just missing something, but this discussion seems irrelevant to the point of the experiments. What is the relevance of yeast RPL-25?

Page 10, line 14. Data refer to S4, not S3.

MW markers should be specified on gels (5E, 2F)

Given the requirement of B-NAC for translational recovery and aggregate resolution from heat shock, one wonders whether the B-NAC knockdown decreases thermotolerance. Do these animals die more readily than WT?

A brief explanation of why Q35 is aggregated in Fig. 6 but not elsewhere (Fig. 1 & 4B) should be provided. Older organisms?

Figure 6, while interesting, has little to do with the main theme of NAC function and presents a purely circular argument. If less protein is synthesized, then a reduction in protein folding stress would be the inevitable and obvious outcome.

Resubmission   09 December 2012

Referee #1

The manuscript by Kirstein-Miles and coworkers presents the idea that NAC is a key regulator of proteostasis in C. elegans. The authors suggest that NAC affects the aggregation of polyQ proteins as an active chaperone. The authors also suggest that natively folded NAC is recruited to aggregates formed upon aging or heat stress. Moreover, the authors suggest that NAC is a general attenuator of translation. However, based on the data I am not convinced that NAC plays an important, active role in these processes. The data presented are compatible also with a model in which NAC behaves similar to other components of the translational apparatus (compare below).

In principal, I feel that the experiments described are not suited to test if NAC passively co-aggregates or is specifically recruited to protein aggregates or stress granules that appear during aging or heat shock.

> The reviewer expresses concern that the experimental design did not allow differentiation between a passive localization and active targeting of NAC to protein aggregates. We have addressed this concern by performing a substantial number of additional experiments with a different design. Specifically, we have carefully analyzed the change in NAC's subcellular localization from a soluble ribosome-bound state to the insoluble aggregated protein fraction. This was accomplished by using a combination of ex vivo and in vitro experiments to demonstrate that NAC does not passively co-aggregate (Figs. 2D and 5E-G). We now show that NAC can be washed off from aggregates formed during aging by using detergents, and that soluble NAC in a cell lysate can shift to aggregates upon their addition (Fig. 2D). Importantly, this is not the case for ribosomal proteins, thus demonstrating selectivity of this transition from a soluble to aggregated state (Fig. 2D). Moreover, we show that NAC is weakly associated with aggregates and not co-aggregated, consistent with its proposed role as a chaperone. Finally, we show in vivo that during heat shock, NAC rapidly forms foci that coincide with the appearance of aggregates, whereas aggregates of ribosomal proteins appear at a later time point. This further supports our argument that NAC does not shift passively into heat shock aggregates due to ribosome aggregation (Fig. 5E).
> In summary, we have shown by multiple state-of the art methods that NAC associates actively on demand with protein aggregates.

The term "translational attenuation" has been previously used to describe local discontinuous translation of specific regions in an mRNA or stalling of ribosomes on mRNA for different reasons. This question is not addressed in this study and the term is thus misleading.

> We have changed the terminology to translational reduction / decline in the text.

Results:

Related to Figure 1

The data suggest that upon NAC depletion, Q35-YFP accumulates in cellular bodies in 4 days old animals. This is an interesting finding, however, some additional controls are required to draw a valid conclusion.

> The reviewer recommends additional controls to support our conclusion of NAC's contribution to cellular proteostasis. We acknowledge this concern and have added a number of controls to further strengthen our data and conclusion in the revised manuscript. First, we added a negative control of only YFP (Q0) and analyzed the fluorescence distribution during aging and upon knockdown of NAC (Fig. S1H). Second, we have included as positive control a data set of Q35 aggregation propensity during aging by knockdown of hsp-1, a known modulator of polyQ aggregation (Figs S1C+D). And third, for all data we show the aggregation propensity during the progression of aging on day 4, 6, 7 and day 10 of life as whole animal images and with magnifications of the head region for better visualization of the Q35-YFP foci (Fig S1A-G).

Q35-YFP "aggregation" is visible also in the 4 day control animal, though to a lesser extent. This is consistent with the previous finding that wild type "Q35 animals accumulated aggregates after a lag period of 4-5 days (Morley et al., 2002)". Thus, Q35-YFP starts to form "aggregates" in the NAC-knockdown only slightly earlier compared to control animals (compare also Fig. 6A and Fig. 4B).

> The reviewer is concerned about differences in aggregation propensity of polyQ shown in different figures and also the literature. In response, the images shown in Figure 7A (former Figure 6A) are taken on day 5. A higher aggregation propensity for this later time point is expected as the aggregation of polyQ is age and polyQ length dependent (Morley et al 2002). The experiment shown in Figure 6B (right image) depicts an image of Q40-YFP, which has a higher aggregation propensity on day 4 due to the longer polyQ stretch, which has been demonstrated previously (Morley et al 2002).

Moreover, the average number of 6 Q35-YFP bodies (Fig. 1B) is very low compared to what was found in previous studies.

> The number of aggregates scored in the vector control and upon knockdown of known modulators is very similar to data reported in previous screens (Nollen et al., 2004; Silva et al., 2011).

I feel that the experiment has to be performed at a polyQ length, at which the difference between control and NAC-knockdown is more significant.

> The reviewer is concerned about the threshold model of Q35-YFP, yet it is this length of polyQ in which even human disease shifts from unaffected to affected. Furthermore, in the C. elegans models generated in our laboratory, Q35 has been demonstrated to be the ideal threshold length to screen for both genetic suppressors and enhancers (Nollen et al., 2004; Silva et al., 2011) and as a basis to test the effects of small molecule proteostasis regulators (Calamini et al., 2011). Finally, the reason why NAC had not been identified in previous genetic screens is because of the severe developmental defect resulting from RNAi knockdown, which is performed in the L1 stage of development to assess effects on polyQ aggregation and toxicity.

In addition, a time course for control and NAC-knockdown cells should be performed.

> We agree with the reviewer that a time course would add information on the effect of NAC to the cellular proteostasis during the lifespan of the animal. In response, we have included a time course of the threshold Q35-YFP sensor (Figs. S1A-G).

It is surprising that both alphaNAC and betaNAC seem to affect Q35-YFP (Fig. 1), but only RNAi against βNAC affects C. elegans offspring (Fig. S1). Please comment. In this context it might be worth mentioning that αNAC-independent roles for βNAX homologs have been suggested by many studies (include in the Introduction).

The reviewer points out a discrepancy between the different phenotypes upon knockdown of the single NAC subunits. We agree that these differences need to be discussed. We have repeated the offspring analysis depicted in Figure S2 (former S1) and observed only a moderate effect of an aNAC knockdown on the number of progeny. We do not understand this yet, whether both aNAC and bNAC exhibit distinct functions or whether they always cooperate in a heterodimeric functional complex. We are aware of studies indicating individual roles for aNAC and bNAC and have included the references in the introduction. In addition, there is no knowledge of how the knockdown of one component affects the level, subcellular localization and function of the other, which might explain the observations.

A control expressing YFP lacking polyQ should be included.

We included this control (Fig. S1H).

(Images of) Whole animals should be shown.

In response, we have added images of the entire animal (see Fig S1C+F+H). We show figures of a magnification of the head region for a better resolution and detection of the Q35 aggregates as well (see Figs. S1A, B, E+H).

"This increase in aggregation is similar to that observed upon knock-down of the cytosolic Hsp70 chaperones, hsp-1 and F44E5.4 (data not shown), consistent with our proposal that NAC is an important chaperone component of the cellular proteostasis network."

This does not tell too much, because there is redundancy between Hsp70 chaperones and a single knockout is expected to display only a mild effect (as it seems to be the case for the NAC-knockdowns).

The reviewer expresses concerns about the validity of our comparison of a NAC knockdown with the knockdown of hsp70 genes. We have addressed this concern by including a data set (in the original manuscript described as data not shown) on the analysis of the Q35-YFP aggregation propensity upon knockdown of individual cytosolic Hsp70 members to demonstrate that the effect on the aggregation propensity of polyQ proteins is specific and not redundant among the Hsp70 members. As shown in Figures S1E-G the knockdown of hsp-1, F44E5.4 and F11F1.1 causes an increase in aggregation, whereas knockdown of stc-1 and C12C8.1 have a mild effect, which could be explained by a redundancy as suggested by the reviewer. Furthermore, the increase in aggregation observed for hsp-1 knockdown, which was the strongest modulator among the Hsp70s, was only slightly higher compared to NAC-knockdown. This finding strengthens our argument that NAC is an important component of the cellular proteostasis network.

Related to Figure 2

Fig. 2 A and B
a control protein, which remains soluble during the time course should be shown.

We agree with the reviewer and present YFP as a control, representing a very stable protein (Figs. 2A+B). For this, we extracted and subsequently fractionated the total, soluble and insoluble protein fractions from a C. elegans line expressing YFP (AM134 [rmIs126[Punc-54::q0::yfp]]). YFP remains almost entirely soluble during the progression of aging (day 4-10) as can also be observed in the fluorescence images in Figure S1H.

Also, a ribosomal marker has to be included in the analysis. This is important because David et al. (David et al., 2010) found that many ribosomal components end up in the insoluble fraction upon aging. This might be the fate of NAC also.

The reviewer asks to see data on ribosomal proteins and their subcellular localization during aging. We have performed LC-MS/MS analysis on protein aggregates formed during aging (Table S1) and can indeed verify the previous observations of David et al.

(2010) to validate the accumulation of ribosomal proteins in the insoluble fraction during aging. This is, as the reviewer speculated, also the case for NAC. Indeed, both aNAC and bNAC are found in the aggregated protein fraction during aging, upon heat shock and polyQ expression (Fig. 2A and Tables S1-3). However, we propose that the change in localization of NAC from a soluble to the insoluble protein fraction represents an active process and not a passive co-aggregation. We addressed this hypothesis in Figures 2D and 5 (see response above and below). In addition, we could identify many ribosomal proteins in the aggregated protein fraction (Tables S1-3) and have also included ribosomal antibodies as control in subsequent experiments (Figs. 2D, 5E+G and S5B).

Fig. 2C
Please improve the data. I cannot follow the conclusion that NAC is distributed between cytosol and nucleus.

We have edited the text and figure legends to clarify the data in former Fig. 2C (now Fig S4A). In brief, the data shows immunostaining of NAC and a co-stain with DAPI. The NAC immunostaining is depicted in the left column, the images of the DAPI channel in the middle and the merge of both channels in the right columns. The merge shows the clear co-localization of NAC and the DAPI stain demonstrating nuclear co-localization. NAC can also be visualized in the cytosol. We have observed this localization pattern for all tissues in C. elegans and have depicted the germ line (top row) and intestine (bottom row). The sub-cellular distribution of NAC in muscle tissue is shown in Figure 2C and shows the same cytosolic and nuclear localization of NAC.

Fig. 2D
Standard controls for subcellular fractionation should be included.

We added the controls in former Fig 2D (now Fig. S4B). We use Histone H3 as a marker of the nuclear fraction and alpha-tubulin as marker for the cytosolic fraction.

Fig. 2E
The quality of the data should be improved. It is not clear if single cells are shown. The localization of the nucleus (nuclei) is unclear.

The images in Fig. 2C (former Fig. 2E) shows single muscle cells. We have added a better description in the figure legends to indicate this point. Blue arrowheads show the localization of nuclei.

Fig. 2F (Figure 2 D in revised manuscript)

"The analysis showed that the aggregated proteins did not sequester RPL-25, establishing that ribosomes remain soluble in our assay conditions. "

I cannot follow the argument. Not all ribosomal proteins end up in the insoluble pellet during aging, however, many do. Rpl25 is actually not one of them (David et al., 2010). However, Rpl31, which is the ribosomal attachment site of betaNAC and thus interacts with βNAC directly (Pech et al., 2010) was found in the insoluble pellet fraction. Soluble NAC e.g. might interact with Rpl31 contained in the insoluble pellet and thus be recruited.

The reviewer raises an important point regarding the ribosomal control. The purpose of this control, using an antibody against a ribosomal protein, was to demonstrate that the presence of insoluble material does not cause aggregation of the whole ribosome (sample 2) during the incubation of insoluble (sample 1) and soluble fractions (sample 3). Unfortunately, there is no antibody commercially available for the C. elegans ortholog of yeast Rpl31. Therefore, we used two available antibodies against RPL-17 and RPL-25 (Fig. 2D, 5G+E and data not shown) to show that ribosomal proteins remain soluble even upon incubation with the aggregated fraction. We could identify RPL-17 and RPL-25 in our analyses of protein aggregates during aging, upon heat shock and polyQ expression (Tables S1-3). Therefore, we feel that the use of RPL-17 and RPL-25 antibodies is effective as a control. We have improved the text in the manuscript to clarify this point.

Moreover, we have also used antibodies targeted against an additional ribosomal protein (RPL-4) to analyze the behavior of a wider spectrum of ribosomal proteins (Fig. 5E).

"This NAC signal most likely stems from residual NAC found in the insoluble fraction of day 16 old nematodes, that was not removed by detergent treatment during the aggregate isolation procedure."

It is an essential experiment in this context to establish conditions that allow for the isolation of "insoluble fraction" that contains NAC. Otherwise it remains an open question if NAC binds to insoluble material in living cells.

We agree with the reviewer that it is essential to demonstrate that NAC can be found in the aggregated protein fraction and indeed, this can be observed in Figures 2A+C and Tables S1-3. This is further demonstrated by a new experimental design (Figure 2D) to show that NAC is first found in the aggregated fraction before washing with detergents in agreement to what we observed in Figs. 2A and 2C. However, upon a washing step with detergents, to remove proteins that are only loosely bound to the aggregate, but not part of the aggregate themselves, NAC is found in the wash fraction and almost no NAC remains in the aggregate fraction, which represents the pure aggregates (post-wash). After incubation of the aggregates with lysate, NAC then re-binds from the lysate to the aggregates. This experiment clarifies that NAC has a strong affinity to aggregated proteins, but is not a constituent of the aggregate itself.

Fig. 2H and I
The whole profile should be shown to allow for an estimate of the loading. Some error bars would be helpful.

We added the error bars (now Fig. 2G). We only observed differences in the formation of polysomes upon knockdown of a/bNAC compared to the control. Therefore, we decided to only show the 80S peaks and polysomes to keep the graph clear and uncluttered for ready interpretation (Fig. 2F). For the polysome analyses, we have always loaded equal absorption units, which allows for comparison and quantification of the individual ribosomal peaks.

Related to Figure S2 and S3

"NAC antibody also recognized a higher MW protein in day 2 old nematodes, which likely represents mono-ubiquitinated αNAC as shown by western blot using Ubiquitin-specific antibodies (Fig. S3)."

It is highly speculative that the band recognized by the ubiquitin antibody is one of the ubiquitinated NAC subunits. If this was the case, the shapes of the bands on the re-probed blot should match. However, this is not the case. Please note, that e.g. a number of ribosomal proteins are monoubiquitinated and might be recognized by the ubiquitin antibody. The authors might want to discuss the work of (Panasenko et al., 2009) in this context. The molecular mass of the bands on the Western blots should be indicated. It should be clear in all Figure Legends if an antibody directed α or βNAC was used.

We agree with the sentiment of this comment that the ubiquination of the NAC subunits is not central to this paper and have deleted this figure.

Related to Figure 3

"Depletion of NAC from its ribosomal function by sequestration to aggregates causes a decline in polysome formation (Figs. 2F, H+I) and hence the attenuation of protein synthesis."

A decline of polysomes is expected if ribosomes (or important ribosomal proteins) go to an insoluble pellet during aging (David et al., 2010). However, there is no evidence for a causative role of NAC in this process. One should also carefully distinguish between effects due to the number of active ribosomes and "attenuation of protein synthesis" on a single ribosome.

The reviewer raises a critical point regarding the causative role of NAC. We interpreted our data based on the previous findings that NAC is required for ribosomal activity in yeast (Koplin et al., 2010). A depletion of NAC is associated with a decline in polysome formation. We addressed the causative role of NAC in Figures 2D and 5 and have discussed the data accordingly. In brief, we show two experiments (Fig. 2D and Fig. 5) where we mix aggregates with lysates or ribosomes, respectively. In both experiments, we demonstrate that "ribosome aggregation" does not occur, as we did not observe a shift of ribosomal proteins from a soluble lysate or intact ribosomes towards pre-existing aggregates, whereas NAC does shift from the soluble to aggregated state. These findings strengthen our proposal of a causative role of NAC in the decline of translation.

Several scenarios could explain our observations: NAC could be aiding in the biogenesis of the ribosome or at the initiation of translation or even at pausing events. All these translational events and checkpoints might be affected upon imbalances of proteostasis. We have added a discussion addressing mechanistic aspects of NAC in the revised manuscript.

The reviewer is correct in that we did not analyze the attenuation of protein synthesis on a single ribosome and we have clarified this in the text.

Related to Figure 5

Fig. 5D
It is not clear how exactly the experiment was performed. The text (middle of page 14) and the Figure Legend do not match. When exactly where the pictures taken? After recovery?

We apologize for the missing information in the figure legends. We now added more information on the experimental procedure in Material and methods as well as in the figure legends.

"A similar response of a translational attenuation, albeit transiently, occurs upon exposure to acute stress such as heat shock (Lindquist 1980)."

Maybe the authors want to include some more recent literature on the effect of heat shock on the translation machinery e.g. summarized in (Buchan and Parker, 2009).

We cited the first observation of this phenomenon and thank the reviewer for pointing out the Buchan and Parker review article which is now included in the text.

Related to Figure 6

"This effect was not due to differences in the pre-existing levels of Q35-YFP (Fig. 6C), from which we conclude that a reduction in bulk protein synthesis is beneficial for cells exposed to protein folding stress. "

This conclusion is not clear to me.

The reviewer states that the conclusion from the data depicted in Figure 6C (now Figure 7C) is not clear. In this experiment, protein synthesis was reduced by a knockdown of two elongation initiation factors, eIF4G and eIF4E, at the L4 stage of development. This knockdown thus reduced bulk protein synthesis, but did not affect the already existing pool of proteins including Q35-YFP. We used western blot analysis to demonstrate that Q35-YFP remained stable and thus the reduction in aggregation propensity was not due to a lower protein level of Q35-YFP itself, but rather due to a reduction in newly synthesized proteins and thus potential chaperone clients.

Introduction and Discussion

There is ongoing discussion about the in vivo function of NAC. In the interest of a general readership, the most important models and hypothesis should be presented to give an unbiased view about the current status of the field.

We thank the reviewer and have revised the introduction to be more comprehensive.

"Both systems (NAC and RAC) bind transiently to the large ribosomal subunit and interact with nascent polypeptides during early protein biogenesis."

This is only partly correct. While NAC binds directly to nascent chains, the two subunits of RAC do not. RAC rather stimulates the binding of the ribosome-bound Hsp70 Ssb (Gautschi et al., 2002; Huang et al., 2005). Actually, the model shown in Fig. 2G shows this correctly.

We thank the reviewer for making this clarification and have revised the introduction accordingly.

"In yeast, however, deletion of NAC has no apparent phenotype, unless the cooperating RAC system is also deleted. Yeast cells lacking both ribosome-associated systems are highly sensitive to proteotoxic conditions, exhibit aggregation of newly synthesized proteins, and impairment in ribosome biogenesis along with a strongly reduced translation activity (Albanese et al. 2010; Koplin et al. 2010)."

The study by Albanese et al. 2010 investigated the interplay between the RAC subunit Zuo1 and the J-homolog Jjj1. NAC was not the topic in this study. The major role of the two J-homologs was on rRNA maturation. The study by Koplin et al. 2010 focused on the interplay between NAC and Ssb (please note, Ssb, in contrast to RAC is a yeast specific component and is not conserved in higher eukaryotes).

We thank the reviewer for this comment. We now no longer cite the study by Albanese et al. 2010 when we discuss the interplay between NAC and the second ribosome-associated chaperone system in the introduction.

Bloss et al. 2003 report that C. elegans βNAC (ICD-1) localizes to mitochondria. How do the authors interpret this?

The reviewer points out a previous observation on mitochondrial localization of NAC (Bloss et al., 2003). We had addressed this using Mitotracker to co-stain mitochondria in our NAC localization and did not observe co-localization. Because these are negative results, we had not included them in the original manuscript, however this is now indicated in the revised version to address this previous statement in the literature (Fig. S4C).

Please also discuss the potential role of ICD-1 in repression of caspases and its effects on apoptosis (Bloss et al., 2003).

We thank the reviewer for raising this point. We have not discussed these data as they are not related to our observations, but we have added this point in the introduction of the revised manuscript and included the respective reference.

Markesich et al. 2000 suggest that loss of Drosophila βNAC function results in a release of translational repression. This is opposite to what is suggested by the authors, please discuss (Markesich et al., 2000).
Del Alamo et al. 2011 report that yeast NAC modulates SRP-specificity and -fidelity in vivo. These new data on the role of NAC should be discussed (Del Alamo et al., 2011).

We have included this reference in the introduction of the revised manuscript.

Minor points:

page 5, line 5 from the bottom: Fig. 1A (not Fig.1B)
page 13, line 5 and line 9: Fig. 4F (not Fig. 4D)

We have corrected these points accordingly in the revised manuscript.

Referee #2

In this manuscript, Kirstein-Miles and colleagues investigate the role of the Nascent polypeptide Associated Complex (NAC) in proteostasis using C. elegans as a model organism. The authors show that under normal conditions, NAC promotes translation and correct protein folding. In contrast, during ageing and under conditions that alter protein folding homeostasis, such as heat shock, expression of Aβ peptide or polyglutamine-expansion proteins, NAC is recruited to protein aggregates leading in depletion of NAC at the ribosome and a decrease in translational activity. This decline in translation is similar to the reduction observed upon RNAi- mediated knockdown of either αNAC or βNAC subunit. Based on these findings the authors suggest that NAC plays a key role not only in detecting proteotoxic stress but also in mediating the reduction of protein synthesis under proteotoxic conditions. The authors propose a model whereby NAC is primarily associated with the ribosome, assisting folding of nascent polypeptides. Upon proteotoxic conditions protein aggregates recruit NAC, depleting NAC available for ribosomal function, thus, attenuating the formation of polysomes and general protein synthesis. This is a thorough and convincing study, providing compelling evidence for a novel role of the NAC complex as a regulator of protein homeostasis, as well as, interesting insights into the dynamics of mRNA translation regulation. The manuscript comprises several solid elements, is well written and the data are well-presented. In my view it is worthy of publication in the EMBO Journal, provided the authors address the points below.

We thank the reviewer for the enthusiastic review.

Comments:

The authors show that NAC is recruited to various protein aggregates caused by expression of Q35 (fig 1A,B), ageing (fig 2A,B,E), heat-shock (fig 5D) or expression of Aβ peptide (fig 4E). Are other ribosome-bound chaperones also recruited in a similar manner or is recruitment NAC-specific?

> The reviewer raises the question whether other ribosomal chaperones are also recruited to protein aggregates similar to NAC. Our preliminary data shows that a knockdown of the C. elegans ortholog of the Hsp40 component of the RAC complex, dnj-11 also affects polysome formation. However, dnj-11 is the only identified component of the RAC complex and we feel that it is insufficient to draw any conclusions about the role of this ribosome-associated complex based on a knockdown of a single component of the complex. However, we have added a paragraph in the discussion speculating about the contribution and interplay of both ribosomal chaperone complexes to cellular proteostasis and ribosomal control.

According to figure2A, already from day 10, there is detectable amount of NAC in the insoluble fraction. However in Figure 2F, the 16 day-old protein aggregates (sample 1) do not appear to contain NAC.

> The reviewer's concern addresses the subcellular localization of NAC in different experiments. We have used a different, more stringent method to isolate aggregates in the experimental set-up of the original Figure 2F (now Fig. 2D). We altered the design of this experiment to resolve the concerns also brought forth by Reviewer 1 and now show that NAC is present in the unwashed (pre-wash) aggregate fraction (in agreement with Figs. 2A-C), but that this interaction is not detected in aggregates treated with detergents. This shows that NAC is only associated with aggregates in a detergent-sensitive manner, but that it is not a constituent of the aggregate. We have clarified this difference in the text, accordingly. In addition we have identified NAC in the aggregated protein fraction during aging, upon heat shock and polyQ expression (Tables S1-3).

NAC subunits were not detected among proteins that were found to aggregate with age, by mass spectrometry (Table S1). Why is this?

> Actually, these NAC subunits were identified by MS. Both aNAC and bNAC are listed in the C. elegans nomenclature: aNAC (Y65B4BR5, now re-named icd-2) and bNAC (icd-1). We have added their NAC nomenclature in the revised tables S1-3.

It would be interesting to examine the effects of NAC overexpression on polysome formation, protein synthesis, protein aggregation and longevity. In other words, does overexpression of NAC have a proteostatic effect?

> This is a very interesting question. However, so far we have failed to generate a transgenic C. elegans line for the co-overexpression of both, aNAC and bNAC with equal stoichiometry. It is possible that the protein levels of NAC need to be carefully adjusted in vivo and that an imbalance has toxic effects during development. The observation that NAC plays an essential role during embryonic development supports this assumption (Bloss et al., 2001; Fig. S2).

Experiments presented in Fig.2F demonstrate the ability of soluble NAC to be recruited to aggregates (here in an ageing paradigm). The authors could also investigate the reverse: Whether insoluble/aggregated NAC can be readily re-solubilized. This could be examined in the already employed paradigm of cell stress, such by heat shock, which induces NAC aggregation. While there is indirect evidence provided in Fig. 5, it would be nice to complement these with direct biochemical data of NAC kinetics itself. Moreover, quantification of "global" aggregation as performed and presented in Fig. 5E is not easy to assess, especially in the absence of loading controls.

> The reviewer indicates that it would strengthen our argument to show that NAC can be re-solubilized with cessation of e.g. heat shock conditions. In response, we have performed such an experiment and show that the localization of NAC is dynamic as the foci formation of NAC upon heat shock could be reversed during recovery period at 20°C (Fig. 4D+G).

One possible explanation for lifespan extension by reduced protein synthesis could be the reduction of overall protein aggregation. Do the formation of polyQ aggregates, the localization of NAC to foci and the decline in translational activity coincide temporally?

> The reviewer points out an important connection of protein aggregation, the subcellular localization of NAC, and translational activity. Indeed, the appearance of polyQ aggregates, change in the localization of NAC from a soluble ribosome-associated state to the insoluble aggregated protein faction and the decline in protein synthesis all occur at the same time period starting at the peak of fecundity on day 4 of life. We have discussed this connection in more detail in the discussion of the revised manuscript.

The authors show that the translational capacity of WT animals decreases during ageing. What about the expression of NAC throughout the same experimental period?

> The reviewer asks whether the expression of NAC changes during aging. We can show in Figures 2A+B that the protein levels of NAC do not change between day 3 and day 10. We have extended the time frame of this analysis to include time points from day 1 to day 14 of life (Fig. S3). Both, aNAC and bNAC, protein levels remain constant throughout the mean lifespan of the nematode. This observation supports our model that NAC can act as proteostasis sensor, in particular as NAC levels do not change during aging or stress conditions. What changes, however, is NAC sub-cellular localization, which we propose triggers the switch to translational repression in response to proteotoxic conditions (Figs. 2A-C, 4D and 5E).

One potential caveat of using polysome profiling to assess translation activity in whole-animal extracts of C. elegans is the bias of this methodology towards massive tissues, which would mask effects on translation (or lack thereof) in other less massive tissues (such as neurons or other minor cell populations for example). The authors should clarify that what they observe is the net outcome on translational activity over numerous different cell types. It remains possible that the situation might be different for a particular cell type. In addition, translation of certain mRNAs could even be favored / upregulated under conditions that decrease general mRNA polysome loading.

> The reviewer is correct to point out that not all tissues are equally represented in a global polysome analysis. Due to size and abundance of the intestinal and muscle tissue, these cells will dominate the polysome analysis. We also agree with the reviewer that certain

mRNAs can escape the repression or are upregulated under conditions that cause a global translational decline. In our discussion we mention that in future studies we will employ polysome-profiling experiments to address whether there is discrimination in the translation of specific mRNAs during aging, as it is known to occur during ER stress.

It would be useful if the authors could show co-localization of NAC with the protein aggregates Q-35, or the Ab peptide in vivo.

The reviewer suggests testing for co-localization of Q35/Ab aggregates and NAC in vivo. Unfortunately, we could not detect a clear co-localization of NAC with polyQ or Ab aggregates in vivo using immunofluorescence. The failure of detection of a co-localization could be due to technical issues and detection limitations. We therefore used mass spectrometry (LC-MS/MS) to analyze the composition of polyQ aggregates and could indeed detect NAC in aggregates formed by polyQ (Table S3).

Figure 6: Q-35 aggregates do not necessarily contain only Q35-YFP but may contain other aggregation-prone proteins whose amount is reduced under these conditions. Although it is not clear to me, I suppose that the authors imply that by lowering protein expression NAC is translocated from ribosomes to protein aggregates and participates in their solubilization. Could the authors solidify this point by monitoring the redistribution of NAC from ribosomes to Q-35 aggregates under these conditions?

The reviewer raises a valid point of a potential re-localization of NAC towards polyQ aggregates when protein synthesis rates are lowered. We addressed this by isolating polyQ aggregates and analyzing their composition by mass spectrometry (see above). Indeed, we have identified NAC as a co-aggregating protein (Table S3). However, it is not feasible to test whether NAC re-localizes to polyQ aggregates under conditions of decreased protein synthesis rates, as polyQ expression, itself, results in a decrease of translation.

It would be interesting (although not necessary for publication of this study) to examine the role of NAC in translation in long-lived genetic backgrounds or in animals exhibiting enhanced activities of stress response factors. Could this mechanism contribute to the longevity of these mutants?

This is a very interesting question. It is known that translational repression contributes to lifespan extension. To analyze the function of NAC and its role in the modulation of ribosomal activity in long-lived mutants is indeed an interesting question. We agree however with the reviewer that this would be of interest for future studies.

Minor comments

del Alamo et al, 2011: this reference is not included in the list although it is cited in the text.

We thank the reviewer for pointing out that error. We have included the citation in the reference list.

Page 10: None of these gene knock-downs ....in day 3 old nematodes: substitute Fig. S3 with Fig. S4.

Page 13: Indeed, Aβ was detected only in the NAC protein-containing sample (Fig. 4D): substitute with Fig. 4F.

Page 14: 1st line of last paragraph: correct functional

Page 18: While Aβ peptide co-precipitated with NAC (Fig. 5F): change to Fig. 4F

We have corrected these points accordingly.

Is the molecular nature of the foci observed after heat shock known?

> The reviewer raises an important question regarding the nature of the foci formed after heat shock. We addressed this question by performing immunofluorescence using NAC antibodies on animals expressing the metastable protein luciferase, which forms rapidly aggregates upon heat shock. We observed that the foci formed by NAC during aging or upon heat shock overlap with the luciferase aggregates (Fig. 4D). This observation suggests that the NAC foci are indeed composed of aggregated protein. We isolated heat shock aggregates and analyzed their composition by mass spectrometry (Table S2 and Fig. S10). The foci themselves however are fixated due to the experimental conditions of the immunostaining and are therefore unfortunately not biochemically accessible to characterize them further.

Figure 3S: To verify that the higher MW band of aNAC is a ubiquitinated form it would be important to immune-precipitate it and analyze it by mass spectrometry.

> We have deleted this figure in the revised manuscript (please see our response above).

The authors should discuss wether NAC, despite its absence of ATPase domain, could still have chaperoning activity.

> We agree with the reviewer and have experimentally tested potential chaperone functions of NAC. We performed two chaperone assays: prevention of aggregation and association with aggregates using two model chaperone substrates, luciferase and malate dehydrogenase. NAC did not associate with aggregates of either model substrate, nor did it prevent their heat-induced aggregation using in vitro assays. However using ex vivo assays as shown in Figures 2D + 5G, we detected association of NAC with endogenous protein aggregates formed during aging and upon heat shock. Moreover we can also detect an interaction of NAC with Ab peptide in a cell extract (Fig. 6F). The knockdown of NAC causes an imbalance of proteostasis as shown using the polyQ aggregation model and recovery from heat shock (Figs. 1+4) and greatly affects survival after prolonged heat shock (Fig. 4I). We conclude from these findings that NAC probably cooperates with other cellular chaperones as a component of the proteostasis network. This assumption is further supported by our data of the co-immunoprecipitation of the major chaperone families such as Hsp70, Hsp90, Hsp110 and sHsps using NAC antibodies (Table 1).

The results from the in vitro assay for detecting the recruitment of NAC to aggregates should be interpreted with caution. I would expect that aggregates recruit other proteins with chaperoning activity as well.

> The author is correct that NAC could also be sequestered by the aggregates via another chaperone. We have changed the text accordingly.

It would benefit the discussion if authors elaborated further on how NAC might modulate translational activity.

> We agree and have extended the discussion on that point.

Referee #3

This study uses a C. elegans model to examine the role of NAC as a potential chaperone and central regulator of cellular proteostasis. Convincing evidence is provided that proteotoxic stress (polyglutamine expansion, heat shock, and aging) result in NAC redistribution from a soluble cellular fraction to insoluble protein aggregates. NAC relocalization is temporally correlated with decreased polysome levels and thereby a reduction in global protein synthesis. In addition, NAC knockdown prevented recovery of polysome formation following heat shock and resolution of head shock-induced aggregates. The findings add substantially to our understanding of NAC function, which has remained a mystery for more than a decade. They also lead to a novel and exciting

model in which NAC is proposed to function as a global regulator of proteostasis by influencing ribosome translational activity. As such, the results have broad implications for cellular regulation and organism homeostasis.

We thank the reviewer for this positive feedback.

The experiments appear technically sound, and the data are generally convincing.

My major concern is that most of the conclusions are derived from broad effects that lack sufficient mechanistic detail needed to adequately support the final model.

The reviewer is concerned about the lack of mechanistic detail. We acknowledge these concerns that we have addressed by performing a large number of carefully designed experiments to analyze the role of the NAC complex in modulating translation.

This concern is exacerbated by the rather telegraphic writing style and limited discussion of the logic by which the data is used to reach specific conclusions.

We have thoroughly edited the manuscript, which is now much improved.

For example, there is no direct evidence that NAC is truly recruited away from the ribosomes, only that it is moves into poorly defined aggregates. Biochemical evidence is needed to more clearly delineate changes in ribosome binding.

The reviewers concern of the lack of biochemical data on the re-localization of NAC is valid. In the revised manuscript, we have added experiments to address the change in subcellular localization of NAC from the ribosome to the insoluble protein fraction (Figs. 2D and 5G+E). We addressed the causative role of NAC in Figures 2D and 5G. In Fig. 5G we mixed ribosomes carrying NAC with heat shock aggregates and subsequently isolated aggregates and ribosomes. Upon immunoblotting of NAC, we could observe that the amount of NAC bound to the ribosomes decreased about 50% in the presence of aggregates. This experiment clearly demonstrates that the presence of aggregates diminishes the ribosome binding of NAC. These findings strengthen our argument of a causative role of NAC in the decline of translation.

We have also characterized all of the protein aggregates used in our experiments by mass spectrometry (LC-MS/MS) and now report the molecular composition of the proteins in these aggregates. Moreover, we have added data on co-localization of NAC with the aggregation-prone Luciferase-YFP protein in vivo. These results provide further support for the immunofluorescence data and demonstrate that the foci formed by NAC represent protein aggregates.

Are ribosomes also sequestered in aggregates? One would expect so, given the abundance of ribosomal proteins identified by MS. The nature of aggregates is also not well defined, as they are derived from different types of stress but discussed as though they are physiologically equivalent. Do all aggregates contain similar cohorts of aggregated protein?

The reviewer raises important point on the nature of the aggregates (see above). We have analyzed the aggregates formed during aging, upon heat shock and upon expression of polyQ by LC-MS/MS (Tables S1-3). We have also improved the text to clearly state which aggregate we use in each respective experiment.

Second, there is no evidence that the recruitment of NAC to aggregates is the actual cause of translational attenuation. Some direct evidence is needed that ribosomes require NAC for translation.

The reviewer addresses a very important point on the causative role of NAC that was also noted by Reviewer 1. We have carefully adjusted our experimental approach to address the role of NAC in modulating translation. Please see above for a description of the experiments shown in Figure 2D and 5.

Third, there is no direct evidence that NAC actually exhibits chaperone activity, i.e. does it directly facilitate folding, prevent aggregation, etc.

> We have addressed this question and performed in vitro chaperone assays with purified recombinant NAC protein. Please see below for a more detailed description of the experiments. In brief, we did not detect any "holdase" or "foldase" chaperone activity of NAC. This is not surprising, as there is no evidence that NAC has an ATPase activity or is dependent upon ATP to direct its remodeling functions. We propose that NAC acts together in a chaperone network, consequently the analysis of individual contributions to chaperone activity would be challenging.

While the general effects of knockdown are convincing, the nature of these manipulations raise the possibility that other key factors, possibly regulated by NAC, might just as easily account for cellular changes. These concerns do not detract from the exciting findings, but significantly reduce confidence in the final model.

> We thank the reviewer for pointing out open questions and acknowledge the overall positive impression, but also the constructive criticism.

Specific concerns.

Page 9, Fig S3. Migration of NAC shown in polysome gradients (S2) does not support its presence in the polysome fraction. In fact the NAC profile seems completely inconsistent with ribosomes, since NAC is found at similar levels in nearly all fractions from 1-11. I don't follow the arguments on page 9.

> The reviewer requests more data supporting a specific ribosome association. To address this concern, we performed a salt wash of the polysome profile and analyzed the association with NAC at low and high salt conditions and could observe a dissociation of NAC from the ribosome to the non-bound fraction under high salt conditions (Fig. S5B). This finding supports our argument that NAC associates with polysomes in a salt-sensitive manner.

Figure 3. It is not clear why the authors attribute the gradual decline in polysomes from day 3-day 7 to NAC. What is the evidence that ribosomes in older organisms lack NAC? Accumulation of age-related aggregates or changes in protein expression patterns could just as well decrease translational by other mechanisms. The issue here is that the authors appear to interpret broad findings with a distinct bias towards their model, rather than building a strong case for their model through critically designed experiments.

> The reviewer is concerned with the potential bias in the interpretation of our data on the causative role of NAC in the decline of polysomes. We acknowledge that there are many regulatory pathways controlling ribosomal activity. We did not intend to give the impression that the regulation via ribosomal chaperones is the only or most important one. The additional data provided in the revision adds further support that NAC modulates ribosomal activity in response to an imbalance in protein folding conditions in the cytosol and thus represent a novel mechanism to adjust protein synthesis in response to proteotoxic conditions. We added experiments to address how the association/disassociation of NAC to/from the ribosome in response to cellular proteostasis conditions affects translational capacity. We now show that NAC has an affinity for protein aggregates (endogenous aggregates formed during aging and upon heat shock as well as Aβ and polyQ aggregates; Figs 2C, 4D, 5E+G, 6F, Table S1-3) and is probably recruited to those aggregates by other chaperones. This affinity of NAC for aggregates results in a dissociation from the ribosome (Fig. 5G). Furthermore, we also show that NAC is the first component of the ribosome to localize to the insoluble protein fraction upon stress (Fig. 5E). It is known that ribosomal proteins are prone to aggregation. However using a kinetic assessment of aggregation we can show that NAC shifts from a soluble to the insoluble state before the ribosomal proteins, thus supporting our model that NAC can act as a sensor for proteotoxic stress (Fig. 5E).

Page 12. I do not understand the term "chaperone for protein aggregates". The data show that NAC localizes to aggregates, but I do not see any evidence that it 'chaperones' these structures. I am not even sure what such a designation might mean.

> The reviewer is correct and we deleted this statement.

Page 13 lines 6-9. What figure panel is referred to here (4D or 4F)? Since this is an SDS gel, the nature of AB that binds to immobilized NAC (prior to denaturation) is difficult to interpret. I do not see how the authors conclude that NAC interacts with AB in a functional state.

> The reviewer addresses the interaction of Ab and NAC. To address this, we have employed an ex vivo/in vitro system to demonstrate active targeting (as opposed to a passive co-aggregation of NAC in the presence of pre-existing aggregates such as formed by Ab). These experiments used purified recombinant active NAC protein and thus could control for the activity of NAC in this recruitment. The reviewer is correct however that we cannot distinguish whether NAC associates with the Ab aggregates, oligomeric or monomeric species as we apply denaturing conditions (SDS-PAGE) before analyzing the interaction via western blot. We have corrected our description and discussions of these data in the text accordingly.

What is the evidence that this binding is due to chaperone activity as stated in lines 10-11? The statement that recruitment of NAC by AB leads to loss of ribosome binding, again, lacks direct evidence. Similarly, if the authors wish to conclude that NAC depletion from ribosomes (following heat shock) is the cause for attenuated translation, then this should be shown directly. Since ribosome proteins are also in aggregates, is it possible that there are simply fewer functional ribosome present? What is needed is some direct analysis of NAC-ribosome binding, and a functional readout for this interaction,

> The reviewer is correct that there is more experimental evidence required to support our conclusions of a causative role of NAC in mediating a translational decline in response to its binding to Ab and upon heat shock. We have addressed this experimentally in the revised manuscript and the data are shown in Fig. 5 (please also see our response to previous questions above).

The conclusion that NAC depletion predisposes to polyQ aggregation and lack of heat shock recovery is convincing. However, it is not clear that this observation is due to a direct chaperone-like role of NAC as claimed, or an indirect effect of NAC on other cellular factors.

> The reviewer points out a central question whether NAC itself is an active chaperone or if NAC cooperates with other chaperones (please see our response above). In brief, we performed in vitro chaperone assays and did not detect a chaperone activity by NAC alone this is not surprising as NAC does not have an ATPase domain/activity to support protein-remodeling functions. However, all in vivo and ex vivo data point to a central role for NAC in maintaining proteostasis (Figs. 1, S1, 4, 5). We think that NAC cooperates with other chaperones and this assumption is supported by our data of a co-immunoprecipitation of the major cellular chaperones by NAC (Table 1).

Minor points

Fig. S1. How do the authors explain the finding that b-NAC reduced offspring by 90% but no effect was observed for a-NAC. Does this imply a-NAC operates independently of b-NAC? Clearly these proteins have different functions if knock down is equivalent and should be discussed.

> The reviewer is correct and we have added a reference in the introduction that addresses the individual functions of aNAC and bNAC.

Page 6, Components isolated in NAC pull downs (Table 1) might interact with NAC as stated, but more likely reflect indirect interactions as well. The explanation here in the text is misleading and should be presented in a more balanced manner prior to its mention in the discussion.

We thank the reviewer for pointing this out and we changed the text accordingly.

Figure 2F. Description here is very confusing. Identity of each lane should be mentioned at the outset (page 7). A more detailed description should be provided for aggregate isolation. What fraction of the final insoluble pellet is solubilized in SDS? Why is NAC lost during this process (no NAC is present in aggregates, Lane 1) and why do the authors conclude that it is removed by detergent washing? How does mild detergent (NP-40) remove NAC from aggregates. Are the aggregates analyzed in 2F different from aggregates in 2A&B or aggregates analyzed by MS?

The reviewer points out that Figure 2 D (former Fig. 2F) requires more clarity. This concern was also noted by Reviewer 1, and we have therefore expanded the data in this experiment, improved the figure presentation, and provided the requested clarity in the figure legends.

Legend of Figure S2 states that antibody signal of alpha and beta NAC are indicated on the right. Is this referring to blot identity or signal intensity? The band intensities bear almost no resemblance to the polysome fractions shown at top. What is the Y axis in the top graph? There is also a poor correlation between alpha and beta NAC bands in lanes 3 and 7. In addition, the blot shown in S2 looks completely different than blot shown in S3.

The reviewers concern addresses the presentation and description of Figure S2 (now figure S5). The signal refers to the blot identity and we have improved the figure and figure legends for more clarity and easier interpretation of the data.

Page 8. Figure S3 shows non-cross reactivity of RPL-25, but I do not see how this establishes that aggregates do not sequester RPL25. No aggregates are shown in S3. Perhaps I am just missing something, but this discussion seems irrelevant to the point of the experiments. What is the relevance of yeast RPL-25?

We agree with the reviewer that the ribosomal interaction of RPL-25 is misplaced in this figure and have deleted this data set. We show instead now the cross-reactivity of all yeast ribosomal antibodies for C. elegans in Figure S5B.

Page 10, line 14. Data refer to S4, not S3.

We thank the reviewer for pointing out this error.

MW markers should be specified on gels (5E, 2F)

We have added the MW marker in the gels.

Given the requirement of B-NAC for translational recovery and aggregate resolution from heat shock, one wonders whether the B-NAC knockdown decreases thermotolerance. Do these animals die more readily than WT?

The reviewer brings forward an excellent question on the contribution of NAC to sustaining prolonged heat shock. In response, we have performed a thermotolerance experiment to address this point and observed that animals treated with RNAi against NAC are more sensitive to prolonged heat shock conditions and show lower survival rates compared to the wild type. This phenotype is comparable to a knockdown of the major hsp70 gene, hsp-1 (Fig. 4I).

A brief explanation of why Q35 is aggregated in Fig. 6 but not elsewhere (Fig. 1 & 4B) should be provided. Older organisms?

The reviewer is correct that these images correspond to older animals (day 5). Figure 1 depicts day 4 old animals and Figure 4B depicts Q40-YFP expressing nematodes, which have a higher aggregation propensity than Q35-YFP (Morley et al., 2002).

Figure 6, while interesting, has little to do with the main theme of NAC function and presents a purely circular argument. If less protein is synthesized, then a reduction in protein folding stress would be the inevitable and obvious outcome.

While we appreciate this reviewer's comment regarding the value of showing the correlation of reduced translation and enhanced proteostasis (Figure 7), we feel that these data are important to emphasize the biological significance of a reduction of protein synthesis. These observations strengthen our proposal on chaperone mediated translational control in response to proteotoxicity.

2nd Editorial Decision   06 February 2013

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the unusually long reviewing period in this case. Your manuscript has now been seen by three referees, whose comments are shown below.

As you will see from the reports, while referees #1 and #3 express great interest in your findings and emphasize that the experimental data support the conclusions made, referee #2 is more critical and raises a number of serious concerns, which could potentially preclude your manuscript from publication. However, in light of the apparent discrepancy between the reports, I consulted with the other two referees on the points raised by ref #2 and the outcome of these discussions is that we have decided to pursue publication of your work. I would therefore invite you to submit a revised version of your manuscript where you discuss the criticisms raised by ref#2 and provide additional control data, if available, as well as conduct the experiment to address NAC recruitment from young animals to salt-washed protein aggregates, if this can be done within a reasonable time-frame. In addition, you will need to discuss the minor points raised by ref #3.
Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-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://www.nature.com/emboj/about/process.html

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.

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

REFEREE REPORTS

Referee #1

I have now read the revised manuscript, as well as, the authors' responses to the comments from the previous round of review. The authors have done a commendable job addressing my criticisms, with multiple additional experiments and data. I have no further points to make and I believe that the

manuscript is now suitable for publication in the EMBO Journal.
Referee #2

General

The study by Kirstein-Miles and co-workers suggests a role of the nascent polypeptide associated complex (NAC) in the regulation of cellular proteostasis. Based on in vivo and in vitro experiments using C. elegans as a model the authors suggest that i) depletion of NAC from ribosomes leads to a decline in translational activity, ii) NAC can bind to protein aggregates that form under stress conditions like e.g. heat shock. These two observations are assembled into a model suggesting a central and active role of NAC in the regulation of translation during stress: because NAC is depleted from ribosomes when aggregates accumulate in the cell, translation comes to a stall.

I feel that the data presented do not justify this conclusion. A direct correlation between in vivo and in vitro data remains uncertain. Also, as detailed below, the quality of the data should be improved.

A general concern is that the authors fail to put their data into the context of our current understanding of the different research fields touching this topic. Several relevant papers from the last years related to NAC function are omitted. Foci observed in vivo are referred to as "aggregates". I am missing an understanding that P-bodies, stress granules, and other "bodies" can form upon stress and that these intracellular microdomains (which play an important role in translational repression and can also contain ribosomal proteins) may look very similar to what the authors observe in this study. One review from the Parker lab is cited, however, its subject is not referred to.

Two more recent findings also question the central role for NAC in translational regulation suggested in this work.

First, two papers (Liu et al, 2013; Shalgi et al, 2012) suggest that Hsc70/Hsp70 is the major sensor for heat stress and regulates translation. In brief sequestration of Hsp70 to protein aggregates results in depletion of Hsp70 from the ribosomes and this then causes translational pausing. I find the combined evidence from this work quite convincing, however, I do not understand (from the work presented here) how NAC could be connected to the process.

Second, recently the Bloss lab found (Arsenovic et al, 2012), also using RNAi depletion experiments in embryonic and adult C. elegans, that alpha- and beta-NAC (termed ICD1 and ICD2) play a role in UPR. Specifically, the data showed that NAC-depletion modulated the response to heat stress and resulted in the up-regulation of ER-localized Hsp70. Worms with a partially defective Ire-1 pathway were significantly more sensitive to depletion of NAC. Also, the work observed an increase in lysosomal structures (again some "bodies" that can form) containing aggregates of protein and lipids. I feel in many aspects the data shown by Kirstein-Miles could be related to these processes.

Introduction

Please inform the reader what is known about the ribosomal binding site of NAC (Pech et al, 2010; Wegrzyn et al, 2006; Zhang et al, 2012).

Discussion:

"Despite the extensive analysis of NAC and its interactions with the ribosome ... " References are missing, compare above.

"NAC is involved in the co-translational translocation of ER proteins (Wiedmann & Prehn 1999)." The authors may know that in the meantime the story has developed quite a bit. There is some unexpected new aspects on the role of NAC during ER translocation, which explains also the connection to the UPR. Please discuss: (Arsenovic et al, 2012; Del Alamo et al, 2011; Zhang et al, 2012).

Figures:

It would have been very helpful if the Figures had been assembled, or at least correctly labelled on each page.

Results general:

Please show the effect of RNAi depletion on alpha/beta-NAC levels in the animals using Western blots.

Please show specificity of the NAC antibodies using whole cell extract and a control for the NAC immunolocalization data using NAC-depleted cells. The data differ from previously reported NAC localization data and also in some of the Figures localization remains unclear (compare also below).

Fig.1 and Fig. S1
Colocalization of NAC and Q35 aggregates has to be tested directly, best coexpression of Q35-YFP and a fluorescently tagged NAC subunit (if not possible: NAC immunofluorescence, but compare general comment above).

The experiments should be repeated with a reporter fused to a longer polyQ-stretch to enhance the difference between control and NAC-depleted cells.

One comment in respect to the Q35 aggregation in general. I have difficulties to follow the counting of "aggregates" in the Q35 assay. Looking at S1C, for example, the control and knockdown animals look very similar to me. Also, e.g. the S1C control animal at day 4 contains significantly more dot-like structures than the quantification in Fig. S1D suggests. Also, it is not clear to me how the Q35 "aggregates" and the "aggregates" formed by Luc-YFP or A-beta relate to each other. E.g. there is between 2 - 50 aggregates (e.g. Fig. 1B or 7B) of Q35 in a whole animal and an (estimated) number of 50 aggregates (Fig. 4D) of Luc-YFP in a single cell. It seems "aggregates" and "aggregates" are very different entities. Do the authors expect that NAC acts in a similar manner on all of these structures ?

How many animals were used for the quantifications shown in Fig. 1B and S1D, S1G ? Please provide info on the statistics.

Could the authors comment on why depletion of alpha-NAC affects offspring much less severely (Fig. S2), but Q35-aggregation to the same extent as beta-NAC depletion ?

Heading page 5: NAC does not prevent Q35 aggregation, rather the aggregation seems slightly delayed (Fig. S1D).

Fig. 2A
The Western blots used for quantifications are partly overexposed, the bands run funny, alpha-NAC is sometimes a double band sometimes a single band. The quality of the data should be improved.

A ribosomal marker protein and a nuclear marker (e.g. Histone H3) should be included in the Western Blot analysis. Intact nuclei might be present in the insoluble material. Compare Fig. S4, NAC in the nuclei.

Fig. 2C
It is not clear to me how dots are selected for labeling with the white arrow. There is no direct evidence that the NAC-dots in the immunostaining represent NAC bound to aggregates. One cannot not correlate NAC-localization in Fig. 2C with the Q35 data shown in Fig. 1 and S1.

Fig. 2D
page 9 "this suggests that NAC most likely binds loosely to the surface of aggregates":
As ribosomal proteins are major component of the insoluble pellet (MS data), this is not surprising. Reportedly NAC binds to Rpl25, Rpl13, and Rpl17. NAC-binding might not be related to

"aggregation" but to the fact that the "aggregates" contain ribosomes or ribosomal proteins.

page 10, first paragraph "Thus the interaction of NAC with insoluble proteins cannot primarily stem from the aggregation-propensity of ribosomes":
I do not understand the argument. The lysate from the 3 days old animal should contain little aggregation prone material (compare Materials and Methods). Why then does the amount of Rpl17 in the "post-washed aggregates" increase 3-fold upon incubation with lysate? Can the authors exclude that ribosomes are spun down (partly)? Of note, the ratio of NAC to Rpl17 in 'post-wash aggregates + lysate' (lane 2) appears to be quite similar to the ratio NAC/Rpl17 in the lysate of the day 3 worms, this would fit to a partial sedimentation of intact ribosome-NAC complexes. Another possibility is that NAC interacts with its ribosomal binding partner (Rpl25, 17, or 31) contained in the "post-washed aggregates" (compare also above).

What is meant by "NAC is associated with aggregates in a detergent-dependent manner" ?

Fig. 2E
The subunits of C. elegans RAC complex cannot be "identified" via sequence alignments. Please rephrase. References for the RAC subunits in higher eukaryotes should be included.

Fig. 2F-H
Complete ribosome profiles have to be recorded and presented (like in Fig. 3). This is essential for the interpretation of this experiment. Corresponding amounts of ribosomes have to be loaded to the gradients. For this reason the a total (e.g. 10% of the load) and the individual fractions should be tested in a Western Blot using at least one ribosomal protein. It is not a reliable method to determine the total RNA (A260 ?) in the different samples to perform the quantification shown in Fig. 2G.

One more concern: In the aging animals the decline of polysomes goes along with an increase of the 80S ribosomes (Fig. 3C). In the NAC depleted strains (Fig. 2F) this is not the case. I understand that 3 days old animals were used in this experiment. This is before aggregation processes should start to be a problem, whether or not NAC is present (Fig. S1D). As mentioned above: the amount of ribosomes in these profiles has to be determined more precisely.

It is not clear to me what "% polysomes" in quantifications refers to.

page 11
what is the data indicating that NAC is "quantitatively" sequestered by protein aggregates ?

Fig. 4A and B
complete ribosome profiles have to be shown (compare above).

Fig. 4D
Luc-YFP images (yellow ? green ?) and NAC images (red) have to be shown separately not only in the overlay (two lower panels). Please enhance the intensity of the NAC images in the two lower panels (compare upper 3 panels). As presented the images do not clearly show whether or not Luc-YFP and NAC colocalize or not.

Fig. 5 A-D
The timing of events (first formation of aggregates, than decline of translational activity) is inconsistent with the observation that ribosomal proteins are a major constituent of the isolated aggregates. The authors write "[...] the disassembly and collapse of the whole ribosome." What is the evidence that ribosomes disassemble?

Fig. 5 F and G
A general problem with this type of experiment is that both, aggregates and ribosomes, are easily isolated via centrifugation. This is critical because the aggregates of interest contain ribosomal proteins (or maybe even ribosomal subunits, ribosomes, polysomes ?) and thus a ribosomal marker protein cannot distinguish between ribosomes and aggregates. For this reason the outcome of the experiment is not conclusive. An experiment that might more convincingly test the idea that NAC redistributes from ribosomes to aggregates is the following. Use an extract of a young animals to which "salt-washed heat shock aggregates" are added. Run a ribosome profile, the aggregates will

end up in the pellet fraction of a ribosome profile. Perform a Western Blot of the fractions (ribosomal protein and NAC) and determine if NAC goes to the pellet fraction depending on the presences of aggregates.

Referee #3

This interesting study addresses the role of NAC (nascent polypeptide associated complex) in the maintenance of protein homeostasis (proteostasis) in a C. elegans model. NAC is comprised of two subunits, alpha and beta. NAC interacts with ribosomes and emerging nascent polypeptides. There have been many speculations about the function of this intriguing complex but its role in protein biogenesis/proteostasis has remained enigmatic. Thus, this is a timely study.

The authors found that NAC delays aggregate formation of a polyglutamine construct (Q35-YFP) and is required for efficient protein translation. They also observed that NAC is recruited to protein aggregates during proteotoxic stress and aging, and facilitates the resolubilisation of aggregates. Relocalization of NAC from polysomes to protein aggregates results in the reduction of translational capacity, and thus reduces the pressure on the proteostasis system. These results characterize NAC as a molecular chaperone with important regulatory function in the proteostasis network.

I find that the experiments in this study are well designed and performed with proper controls. The paper should become suitable for publication with relatively minor revisions.

Major Comments:

1. The authors found that NAC depletion enhances Q35-YFP aggregation. Was the effect of NAC overexpression on aggregation also tested? A positive result would support the notion that NAC acts as a chaperone.

2. The authors observed that NAC is required for survival of prolonged heat stress (page 14-15). This observation seems to contradict a recent study by Arsenovic et al PlosOne 2012 (i.e., NAC depleted worms are not more sensitive to heat stress relative to wild-type controls.). The discrepancy may be explained by differences in the age at which worms were examined. The authors should comment.

3. Page 15: Authors stated that they did not observe induction of hsps upon knockdown of NAC. Please clarify whether this relates to induction with or without additional heat shock.

1st Revision - authors' response   11 March 2013

Point-by-point response

Referee #1

I have now read the revised manuscript, as well as, the authors' responses to the comments from the previous round of review. The authors have done a commendable job addressing my criticisms, with multiple additional experiments and data. I have no further points to make and I believe that the manuscript is now suitable for publication in the EMBO Journal.

> *We thank the reviewer for the very positive review.*

Referee #2

General

The study by Kirstein-Miles and co-workers suggests a role of the nascent polypeptide associated complex (NAC) in the regulation of cellular proteostasis. Based on in vivo and in vitro experiments using C. elegans as a model the authors suggest that i) depletion of NAC from ribosomes leads to a decline in translational activity, ii) NAC can bind to protein aggregates that form under stress conditions like e.g. heat shock. These two observations are assembled into a model suggesting a central and active role of NAC in the regulation of translation during stress: because NAC is depleted from ribosomes when aggregates accumulate in the cell, translation comes to a stall.
I feel that the data presented do not justify this conclusion. A direct correlation between in vivo and in vitro data remains uncertain. Also, as detailed below, the quality of the data should be improved.
A general concern is that the authors fail to put their data into the context of our current understanding of the different research fields touching this topic. Several relevant papers from the last years related to NAC function are omitted.

> *The reviewer has expressed concern that we have not been exhaustive in our description on the roles of NAC in the manuscript. Although we feel that the manuscript contained all necessary information to understand the presented data, we are happy to fulfill the request of this referee. The revision now includes the requested references and relevant commentary to provide a broad description of multiple potential properties of NAC.*

Foci observed in vivo are referred to as "aggregates". I am missing an understanding that P-bodies, stress granules, and other "bodies" can form upon stress and that these intracellular microdomains (which play an important role in translational repression and can also contain ribosomal proteins) may look very similar to what the authors observe in this study. A review from the Parker lab is cited, however, its subject is not referred to.

1

The reviewer points out that cells contain a diverse collection of intracellular bodies formed in response to stress conditions including P-bodies and stress granules. The reviewer indicates that the foci observed upon heat shock or during aging are not sufficiently characterized to conclude that they represent protein aggregates. To restate, our evidence is the following:

(i) We employed immunofluorescence to show the subcellular relocalization of NAC from a soluble to aggregated state during aging (Fig. 2C).

(ii) We complemented this finding with biochemical approaches using subcellular fractionation of soluble and insoluble proteins during the progression of aging (Fig. 2A).

(iii) We isolated protein aggregates and identified aggregated proteins subsequently by mass spectrometry analysis (Tables S1-4).

(iv) We also showed that aggregation-prone luciferase co-localizes with the foci formed by NAC upon heat shock. This result strongly supports our assumption that NAC foci correspond to an aggregated protein state (Fig. 4B).

(v) We show biochemically and by immunofluorescence that the loss of NAC activity hampers the re-solublization of heat-denatured luciferase and endogenous protein aggregates (Fig. 4C+D).

Two more recent findings also question the central role for NAC in translational regulation suggested in this work. First, two papers (Liu et al, 2013; Shalgi et al, 2012) suggest that Hsc70/Hsp70 is the major sensor for heat stress and regulates translation. In brief sequestration of Hsp70 to protein aggregates results in depletion of Hsp70 from the ribosomes and this then causes translational pausing. I find the combined evidence from this work quite convincing, however, I do not understand (from the work presented here) how NAC could be connected to the process.

The work by Liu et al and Shalgi et al both appeared while our manuscript was in review and we now include both references in our revision. Both studies emphasized the role of Hsp70 in translational regulation by ribosome pausing events in response to either heat shock or by a combined treatment with an amino acid analog and proteasome inhibitor. Their findings do not contradict our model nor does our model of a translational regulation by ribosome-associated chaperones contradict with their studies. Together, these studies reveal that ribosomal activity is controlled at multiple levels and expand our knowledge on the regulation of protein synthesis in response to imbalances of proteostasis. We would like to point out however that while both, Liu et al and Shalgi et al carried out their studies in cell culture, our studies have gone further within an animal and with highly relevant biological processes such as aging and other proteotoxic challenges such as heat shock, expression of aggregation-prone disease-associated proteins such as polyglutamine and Aβ.

Second, recently the Bloss lab found (Arsenovic et al, 2012), also using RNAi depletion experiments in embryonic and adult C. elegans, that alpha- and beta-NAC (termed ICD1 and ICD2) play a role in UPR. Specifically, the data showed that NAC-depletion

modulated the response to heat stress and resulted in the up-regulation of ER-localized Hsp70. Worms with a partially defective Ire-1 pathway were significantly more sensitive to depletion of NAC. Also, the work observed an increase in lysosomal structures (again some "bodies" that can form) containing aggregates of protein and lipids. I feel in many aspects the data shown by Kirstein-Miles could be related to these processes.

"NAC is involved in the co-translational translocation of ER proteins (Wiedmann & Prehn 1999)." The authors may know that in the meantime the story has developed quite a bit. There is some unexpected new aspects on the role of NAC during ER translocation, which explains also the connection to the UPR. Please discuss: (Arsenovic et al, 2012; Del Alamo et al, 2011; Zhang et al, 2012).

> *The reviewer points out a connection between NACs function and the unfolded protein response in the ER. We have made similar observations of an induction of ER-localized Hsp70 upon depletion of NAC and have discussed this and the findings of a connection of NAC, SRP and ER-targeting in yeast (Zhang et al., 2012 and del Alamo et al., 2011) as follows in our discussion to provide a comprehensive discussion on the current literature:*
>
> *"Interestingly, a knockdown of NAC leads to an induction of ER Hsp70 ((Arsenovic et al, 2012) and data not shown), which is not unexpected as it has been shown that NAC is involved in the co-translational translocation of ER proteins (Wiedmann & Prehn, 1999b). In yeast, NAC is required for the early recruitment of SRP to ribosome-nascent chain complexes (RNCs) (Zhang et al, 2012). In addition, NAC has been proposed to modulate the binding specificity of SRP, thus indicating a dynamic interplay of NAC and the SRP for the co-translational translocation of RNCs to the ER (del Alamo et al, 2011). These observations indicate a connection between the UPR and NAC's function in translational control. However, changes in the phosphorylation of eIF2α have not been observed during aging of C. elegans (Fig. S9) and have also not been detected upon cytosolic protein misfolding stress (Liu et al, 2012). NAC therefore likely provides a new pathway to regulate protein synthesis upon both acute and chronic proteotoxic stress conditions. "*

Introduction
Please inform the reader what is known about the ribosomal binding site of NAC (Pech et al, 2010; Wegrzyn et al, 2006; Zhang et al, 2012).
Discussion:
"Despite the extensive analysis of NAC and its interactions with the ribosome ... "
References are missing, compare above.

> *The exact binding site of NAC on the ribosome is not subject of our study. Based on biochemical analyses, several binding sites have been proposed for the interaction of NAC to the ribosome, but structural information of NAC bound to the ribosome are still missing.*

Figures:

3

It would have been very helpful if the Figures had been assembled, or at least correctly labeled on each page.

> *We have gone over the text with attention to the figures and they are correctly assembled and labeled. In the absence of details on this point, we are sorry but we cannot follow this statement.*

Results general:
Please show the effect of RNAi depletion on alpha/beta-NAC levels in the animals using Western blots.

> *We have added a control showing the RNAi efficiency of a double knockdown of $\alpha+\beta NAC$ on NAC protein level using Western blot analysis in Fig. S3B (left panel).*

Please show specificity of the NAC antibodies using whole cell extract and a control for the NAC immunolocalization data using NAC-depleted cells. The data differ from previously reported NAC localization data and also in some of the Figures localization remains unclear (compare also below).

> *We have added a control showing the immunostaining of NAC in C. elegans muscle cells treated with $\alpha+\beta NAC$ RNAi in Fig. S3B (right panel).*

Fig.1 and Fig. S1
Colocalization of NAC and Q35 aggregates has to be tested directly, best coexpression of Q35-YFP and a fluorescently tagged NAC subunit (if not possible: NAC immunofluorescence, but compare general comment above).

> *The reviewer asks to see co-localization data of NAC and Q35-YFP aggregates. The experiment shown in Fig. 1 addresses a potential chaperone function of NAC and we used the polyQ threshold model Q35-YFP as proteostasis sensor. We do not analyze or state here whether NAC interacts with the Q35-YFP aggregates. To answer the question of the reviewer whether NAC interacts with Q35-YFP aggregates, we have shown that Q35 aggregates contain NAC as demonstrated by the mass spectrometry analysis of Q35-YFP protein aggregates (Table S3).*

The experiments should be repeated with a reporter fused to a longer polyQ-stretch to enhance the difference between control and NAC-depleted cells.

> *The reviewer raises the concern whether the polyglutamine model we have used is of suitable length for the analysis shown in Figs. 1 and S1. We have extensively employed the threshold length Q35 as the polyQ model to monitor the effects of potential modulators of protein aggregation as this represents a highly sensitized state (Morley et al., 2001). We believe that the differences in aggregation propensity are significant and we want to point out that the phenotype of a NAC knockdown is similar to that observed of a depletion of the hsp70 genes (Fig. S1).*

4

One comment in respect to the Q35 aggregation in general. I have difficulties to follow the counting of "aggregates" in the Q35 assay. Looking at S1C, for example, the control and knockdown animals look very similar to me.

> *We provide higher magnifications of the nematode images for all samples so that the foci can be better visualized. The magnification of images shown in Fig. S1C are displayed in Fig. S1B. Here, the differences between the different genetic backgrounds are easier to compare.*

Also, e.g. the S1C control animal at day 4 contains significantly more dot-like structures than the quantification in Fig. S1D suggests. Also, it is not clear to me how the Q35 "aggregates" and the "aggregates" formed by Luc-YFP or A-beta relate to each other. E.g. there is between 2 - 50 aggregates (e.g. Fig. 1B or 7B) of Q35 in a whole animal and an (estimated) number of 50 aggregates (Fig. 4D) of Luc-YFP in a single cell. It seems "aggregates" and "aggregates" are very different entities. Do the authors expect that NAC acts in a similar manner on all of these structures ?

> *The aggregates formed by different proteins exhibit distinct morphology and foci numbers. For example, luciferase forms amorphous aggregates, whereas polyQ and Aβ form amyloids. Both aggregate types also differ in terms of aggregation kinetics and their effects on the cellular proteostasis. Thus, it is not possible or particularly useful to compare the number of aggregates formed by different models. The aggregation propensity of these models with regards to aging, proteotoxicity and genetic background (e.g. depletion of NAC) is similar and thus further supports our conclusion that NAC is an important member of the cellular proteostasis network.*

How many animals were used for the quantifications shown in Fig. 1B and S1D, S1G ? Please provide info on the statistics.

> *For each experiment 50 animals were analyzed. We thank the reviewer for pointing out this missing information and have added it in the Materials and Methods section.*

Could the authors comment on why depletion of alpha-NAC affects offspring much less severely (Fig. S2), but Q35-aggregation to the same extent as beta-NAC depletion ?

> *The reviewer points out the different phenotypes upon knockdown of the single NAC subunits. This is an interesting observation and we do not understand this yet, whether both αNAC and βNAC exhibit distinct functions, whether loss of one subunit may have a gain of function effect or whether they always cooperate in a heterodimeric functional complex. We are aware of studies indicating individual roles for αNAC and βNAC and have included the references in the introduction. In addition, there is no knowledge of how the knockdown of one component affects the level, subcellular localization and function of the other, which might*

explain the observations.

Heading page 5: NAC does not prevent Q35 aggregation, rather the aggregation seems slightly delayed (Fig. S1D).

> *We have changed the heading of the paragraph accordingly.*

Fig. 2A
The Western blots used for quantifications are partly overexposed, the bands run funny, alpha-NAC is sometimes a double band sometimes a single band. The quality of the data should be improved.

> *We have repeated the experiment shown in Figs. 2A+B and the resulting western blot is much improved.*

A ribosomal marker protein and a nuclear marker (e.g. Histone H3) should be included in the Western Blot analysis. Intact nuclei might be present in the insoluble material. Compare Fig. S4, NAC in the nuclei.

> *The reviewer expresses concern with the fractionation protocol of soluble and insoluble protein and whether nuclei are included in the insoluble fraction. We have used a well-established protocol for the isolation of the total, soluble and insoluble material (David et al., 2010). Moreover, we have included a control for a soluble protein, YFP, and feel that our methodology is sound. We have identified ribosomal proteins and histones as aggregation prone proteins (see Table S1). Thus, an additional Western blot analysis using antibodies targeted against ribosomal proteins and histones would not answer the question whether intact nuclei are present in the insoluble fraction as the respective proteins might be simply present due to their aggregation propensity during aging.*

Fig. 2C
It is not clear to me how dots are selected for labeling with the white arrow. There is no direct evidence that the NAC-dots in the immunostaining represent NAC bound to aggregates. One cannot not correlate NAC-localization in Fig. 2C with the Q35 data shown in Fig. 1 and S1.

> *The white arrowheads used in Fig. 2C point to NAC foci. In Figs. 1 and S1 we use white arrowheads to indicate Q35-YFP foci. For both figures we have indicated the labeling in the respective figure legends. We did not intend to give the impression that white arrowheads generally label the same protein moiety throughout the manuscript.*

Fig. 2D
page 9 "this suggests that NAC most likely binds loosely to the surface of aggregates": As ribosomal proteins are major component of the insoluble pellet (MS data), this is not surprising. Reportedly NAC binds to Rpl25, Rpl13, and Rpl17. NAC-binding might not be

related to "aggregation" but to the fact that the "aggregates" contain ribosomes or ribosomal proteins.

>*It might be the case that ribosomal proteins represent a subclass of chaperone substrates for NAC as has been proposed (del Alamo et al., 2011; Koplin et al., 2010; Tables S1-4). However, we also show that NAC is sequestered by aggregates formed by Aβ peptides in Fig. 6E. In addition, we show in Fig. 5D that NAC is recruited to heat shock aggregates that were formed in a post-ribosomal lysate. As described in the Materials and Methods section, we pelleted all ribosomal particles and then heat-shocked the remaining ribosome-depleted lysate. From this experiment we can exclude that NAC interaction with aggregates is mediated only by the presence of ribosomal proteins.*

page 10, first paragraph "Thus the interaction of NAC with insoluble proteins cannot primarily stem from the aggregation-propensity of ribosomes":
I do not understand the argument. The lysate from the 3 days old animal should contain little aggregation prone material (compare Materials and Methods). Why then does the amount of Rpl17 in the "post-washed aggregates" increase 3-fold upon incubation with lysate? Can the authors exclude that ribosomes are spun down (partly)? Of note, the ratio of NAC to Rpl17 in 'post-wash aggregates + lysate' (lane 2) appears to be quite similar to the ratio NAC/Rpl17 in the lysate of the day 3 worms, this would fit to a partial sedimentation of intact ribosome-NAC complexes. Another possibility is that NAC interacts with its ribosomal binding partner (Rpl25, 17, or 31) contained in the "post-washed aggregates" (compare also above).

>*We show that the aggregate signal for NAC increases 16x whereas the RPL-17 signal only increases 3x in the mixed samples. We interpret this result to mean that the increase of NAC in the insoluble fraction is ~5x stronger. Therefore, we feel that the statement: "Thus the interaction of NAC with insoluble proteins cannot primarily stem from the aggregation-propensity of ribosomes" is supported by our experimental data.*

What is meant by "NAC is associated with aggregates in a detergent-dependent manner" ?

>*We have re-phrased this statement to now read, "Taken together, we conclude that NAC is associated with aggregates and that it can cycle from a soluble ribosome-bound state of a lysate of young animals to aging-induced aggregates.".*

Fig. 2E
The subunits of C. elegans RAC complex cannot be "identified" via sequence alignments. Please rephrase.

>*The reviewer feels that the terminology is not correct. We do not think that the statement is misleading.*

References for the RAC subunits in higher eukaryotes should be included.

> *Although the RAC complex is not subject of our work presented here, we have added the references for the subunits of the RAC complex in higher eukaryotes in the introduction: Jaiswal et al., 1011; Otto et al., 2005 and Hundley et al., 2005*

Fig. 2F-H
Complete ribosome profiles have to be recorded and presented (like in Fig. 3). This is essential for the interpretation of this experiment. Corresponding amounts of ribosomes have to be loaded to the gradients. For this reason the a total (e.g. 10% of the load) and the individual fractions should be tested in a Western Blot using at least one ribosomal protein. It is not a reliable method to determine the total RNA (A260 ?) in the different samples to perform the quantification shown in Fig. 2G.

> *The reviewer is concerned about the controls for the polysome analysis and presentation. We think that a calibration of all samples by analyzing the A260 nm values is a standard and valid procedure (Masek et al., 2011).*

One more concern: In the aging animals the decline of polysomes goes along with an increase of the 80S ribosomes (Fig. 3C). In the NAC depleted strains (Fig. 2F) this is not the case. I understand that 3 days old animals were used in this experiment. This is before aggregation processes should start to be a problem, whether or not NAC is present (Fig. S1D).

> *The data depicted in Figs. 3C (+S7) and 2G (former 2F) are very different experiments, therefore it is not possible to compare these results.*

As mentioned above: the amount of ribosomes in these profiles has to be determined more precisely.

> *We were very careful to adjust the A260 reading to the same level in each experiment (see above). We display the amount of the polysomes relative to the total amount of RNA. Thus, even differences in the loading of the gradient would not affect the data as the relative amounts of polysomes (ratios polysomes vs total RNA) do not change.*

It is not clear to me what "% polysomes" in quantifications refers to.

> *See above*

page 11
what is the data indicating that NAC is "quantitatively" sequestered by protein aggregates ?

> *The term quantitatively refers to our observations in Figs. 2A+B, where we show that up to 90% of NAC is found in the insoluble fraction during aging. In addition,*

the immunostaining of NAC shows a strong re-localization of soluble NAC into foci upon heat shock (Fig. 5C).

Fig. 4A and B
complete ribosome profiles have to be shown (compare above).

> *We had shown the 80S peaks and polysomes to keep the graph clear and uncluttered for ready interpretation. For the polysome analyses, we have always loaded equal absorption units, which allows for comparison and quantification of the individual ribosomal peaks.*

Fig. 4D
Luc-YFP images (yellow ? green ?) and NAC images (red) have to be shown separately not only in the overlay (two lower panels). Please enhance the intensity of the NAC images in the two lower panels (compare upper 3 panels). As presented the images do not clearly show whether or not Luc-YFP and NAC colocalize or not.

> *We have added the separate images of Luc-YFP (in green) and NAC (in red) images for the overlay images depicting the immunostaining of NAC in Luc-YFP expressing nematodes at 20°C and upon heat-shock (30 min 35°C) in Fig. 4B.*

Fig. 5 A-D
The timing of events (first formation of aggregates, than decline of translational activity) is inconsistent with the observation that ribosomal proteins are a major constituent of the isolated aggregates.

> *The reviewer feels that our data on the kinetics of aggregation of ribosomal proteins upon heat shock is inconsistent with the observations that ribosomal proteins are major constituents of protein aggregates. We do not see this discrepancy and think our data are consistent: the protein aggregates formed upon heat shock (that were analyzed by mass spectrometry (Table S2)) were heat-shocked for 1 hour. At that time point, we also detect aggregation of ribosomal proteins via immunostaining (Fig. 5C).*

The authors write "[...] the disassembly and collapse of the whole ribosome." What is the evidence that ribosomes disassemble?

> *We have removed this statement.*

Fig. 5 F and G
A general problem with this type of experiment is that both, aggregates and ribosomes, are easily isolated via centrifugation. This is critical because the aggregates of interest contain ribosomal proteins (or maybe even ribosomal subunits, ribosomes, polysomes ?) and thus a ribosomal marker protein cannot distinguish between ribosomes and aggregates.

The reviewer is concerned about our experimental protocol. We can distinguish between ribosomes and aggregates as their protein band patterns are distinct on a Coomassie stained SDS-PAGE gel. In addition, in sample 1, where only aggregates are present, we only isolate aggregates, as lane 4 is completely clear in the Coomassie stain (Fig. 5D). The same is true for sample 3, where only ribosomes are present and lane 3 remains clear by Coomassie staining.
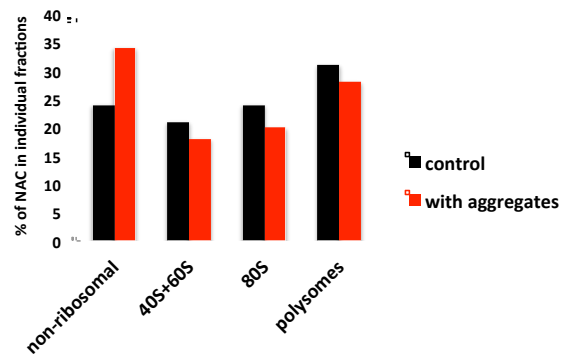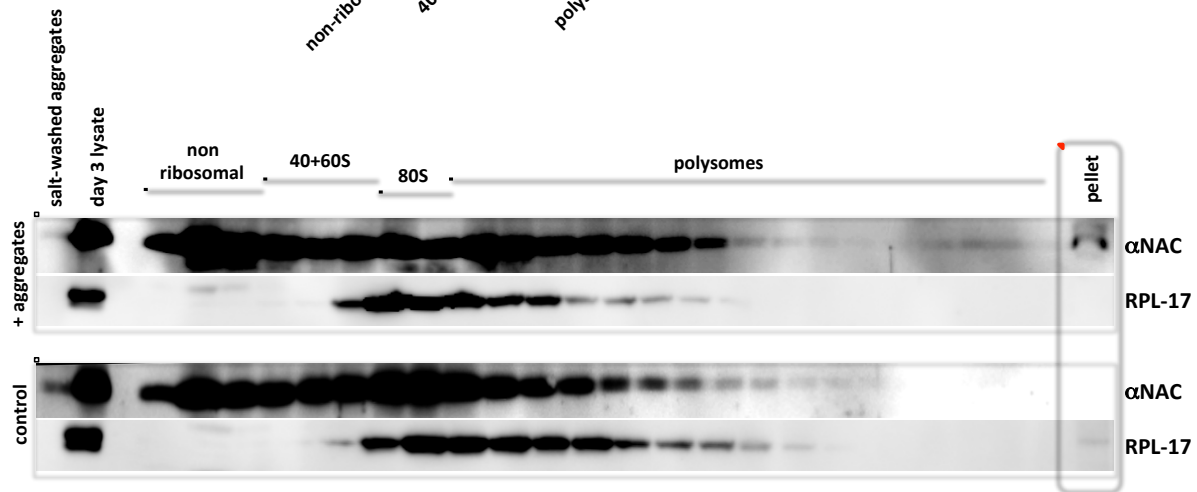
For this reason the outcome of the experiment is not conclusive. An experiment that might more convincingly test the idea that NAC redistributes from ribosomes to aggregates is the following. Use an extract of a young animals to which "salt-washed heat shock aggregates" are added. Run a ribosome profile, the aggregates will end up in the pellet fraction of a ribosome profile. Perform a Western Blot of the fractions (ribosomal protein and NAC) and determine if NAC goes to the pellet fraction depending on the presences of aggregates.

The reviewer suggests an alternative experimental set-up to test the dynamic localization of NAC. This experiment has now been performed. We generated protein aggregates by first depleting a lysate of ribosomes and then subjected the ribosome-depleted supernatant to 42°C for 1 h to generate heat-induced aggregates. These aggregates were then mixed with a lysate from young animals (day 3 of age) that was treated with cyclohexamide to stabilize the polysomes. In parallel, the lysate was also incubated with buffer alone. Both samples were then loaded onto a sucrose gradient and $A_{260\ nm}$ was monitored to analyze the ribosome profile. The individual fractions (non-ribosomal fraction, 40 + 60S, 80S and polysomes) were then analyzed using SDS-PAGE and subsequent Western blot with antibodies raised against NAC and RPL-17 (see Fig. 1B below).

Indeed, we could observe a shift of NAC towards the pellet fraction in the sample where the lysate was incubated with the aggregates (upper panel). We did not detect a signal in the pellet fraction in the control (lysate and buffer; bottom panel). We also observed a reduction of NAC association with the individual ribosomal fractions (40S+60S, 80S and polysomes) (see Fig. 1A). As expected the RPL-17 signals did not change in both samples along the ribosome profile.

We believe we have sufficiently addressed the concern of the reviewer, but also propose that the titration experiments described in Figs. 2D+E and 5D are better suited to demonstrate the dynamic localization of NAC from a ribosome-associated state towards the insoluble protein fraction. We therefore include Fig. 1 (below) to show that we have done the experiment but do not include it in the manuscript.

Figure 1

A

B

Referee #3

This interesting study addresses the role of NAC (nascent polypeptide associated complex) in the maintenance of protein homeostasis (proteostasis) in a C. elegans model. NAC is comprised of two subunits, alpha and beta. NAC interacts with ribosomes and emerging nascent polypeptides. There have been many speculations about the function of this intriguing complex but its role in protein biogenesis/proteostasis has remained enigmatic. Thus, this is a timely study.

The authors found that NAC delays aggregate formation of a polyglutamine construct (Q35-YFP) and is required for efficient protein translation. They also observed that NAC is recruited to protein aggregates during proteotoxic stress and aging, and facilitates the resolubilisation of aggregates. Relocalization of NAC from polysomes to protein aggregates results in the reduction of translational capacity, and thus reduces the pressure on the proteostasis system. These results characterize NAC as a molecular chaperone with important regulatory function in the proteostasis network.

I find that the experiments in this study are well designed and performed with proper controls. The paper should become suitable for publication with relatively minor revisions.

We thank the reviewer for the positive and constructive feedback.

Major Comments:

1. The authors found that NAC depletion enhances Q35-YFP aggregation. Was the effect of NAC overexpression on aggregation also tested? A positive result would support the notion that NAC acts as a chaperone.

> *We agree with the referee that this would be an interesting experiment, but unfortunately all of our efforts to generate a stable transgenic line over-expressing both NAC subunits have been unsuccessful that far. This may be due to challenges to obtain the proper 1:1 stoichiometry of both subunits and that any imbalance of the NAC subunits could affect the temporal and spatial functions of the NAC complex and thus organismal viability.*

2. The authors observed that NAC is required for survival of prolonged heat stress (page 14-15). This observation seems to contradict a recent study by Arsenovic et al PlosOne 2012 (i.e., NAC depleted worms are not more sensitive to heat stress relative to wild-type controls.). The discrepancy may be explained by differences in the age at which worms were examined. The authors should comment.

> *The reviewer addresses discrepancies of the survival rates of animals subjected to heat shock upon knockdown of NAC in our manuscript and data reported earlier (Arsenovic et al., 2012). Arsenovic et al observed that animals depleted for αNAC (icd-2) displayed 100% survival rates. Animals depleted for βNAC displayed the same survival rates as the control of about 60%. Contrary to these results, we observed that a depletion of both NAC subunits leads to a decrease of survival after prolonged heat shock of about 50% whereas the control showed survival rates of about 70% (Fig. 4E).*

> *The reviewer speculates that different experimental conditions might explain the observed differences. Indeed, the experimental conditions vary quite tremendously. We subjected nematodes to a heat shock of 35°C for 6 h and subsequently shifted them to 20°C for a recovery period and scored the survival rates after 24 h (see Materials and Methods). In contrast, Arsenovic et al subjected the nematodes to a heat shock of 36°C for up to 14 h with no recovery period. The RNAi treatment conditions varied as well. These differences in the experimental conditions make it difficult to compare the two data sets.*

3. Page 15: Authors stated that they did not observe induction of hsps upon knockdown of NAC. Please clarify whether this relates to induction with or without additional heat shock.

> *We tested the expression levels of two Hsps at normal growth temperatures of 20°C and upon heat shock of 35°C for 1 h and did not detect a higher induction of Hsp70 (C12C8.1) and a sHsp (hsp-16.2) for both conditions upon knockdown of NAC compared to the control (Fig. S8).*