Failure of RQC machinery causes protein aggregation and proteotoxicstress

Young-Jun Choe*, Sae-Hun Park*, Timm Hassemer, Roman Körner, Lisa Vincenz-Donnelly, Manajit Hayer-Hartland F. Ulrich Hartl

Department of Cellular Biochemistry,Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany

* These authors contributed equally to this work

Correspondence to uhartl@biochem.mpg.de

Abstract

Translation of mRNAs lacking a stop codon results in the addition of a C-terminal poly-lysine tract tothe nascent polypeptide,causing ribosome stalling. Non-stop proteins and other stalled nascent chains are recognized by the ribosome quality control (RQC) machinery and targeted for proteasomal degradation. Failure of this process leads to neurodegeneration by unknown mechanisms. Here, we show in a yeast model that deletion of the E3 ubiquitin ligase Ltn1p, a key RQC component, causesstalled proteins to formdetergent-resistantaggregates and inclusions. Aggregation is dependent ona C-terminal alanine/threonine tail that is added to the stalled polypeptides by the RQC componentRqc2p. Formation of inclusions additionally requires the poly-lysine tract present in non-stop proteins. The aggregates sequester multiple cytosolic chaperones and thereby interfere with general protein quality control pathways. These findings can explain the proteotoxicity of ribosome-stalled polypeptides and demonstrate the essential role of the RQC in maintaining proteostasis.

Eukaryotic cells have evolved quality control pathways to remove aberrant polypeptides from ribosomes that have stalled on mRNAs, due to mRNA truncation or the absence of a termination codon{Preissler, 2012 #58}{Pechmann, 2013 #55}{Lykke-Andersen, 2014 #54}{Frischmeyer, 2002 #19}{van Hoof, 2002 #57}. mRNAs typically contain a variable 3' untranslated region (UTR), followed by a poly(A) sequence of >60 nucleotides in mammals{Barrett, 2012 #52;Chang, 2014 #1;Subtelny, 2014 #73}. Translation of"non-stop" (NS) mRNAresults in the addition of a C-terminal poly-lysine tract,encoded by poly(A),whichcauses stalling of the NS-proteinin the negatively charged ribosomal exit tunnel{Lu, 2008 #10;Lykke-Andersen, 2014 #54;Koutmou, 2015 #72}. The ribosome quality control complex (RQC) recognizes NS-proteins and mediates their ubiquitylation and proteasomal degradation{Bengtson, 2010 #4;Brandman, 2012 #3;Defenouillere, 2013 #28;Shao, 2013 #27;Verma, 2013 #53;Shao, 2014 #35;Lyumkis, 2014 #8;Shao, 2015 #34;Shen, 2015 #5}. The RQC comprises the E3 ubiquitin ligase Listerin (Ltn1p), the proteins Rqc1p and Rqc2p, as well as the AAA^+ protein Cdc48p. Upon dissociation of the stalled ribosome{Shoemaker, 2010 #59;Shao, 2013 #27}, Rqc2p (also known as Tae2p) binds to the peptidyl-tRNA of the 60S subunit and recruits Ltn1p{Defenouillere, 2013 #28;Shao, 2015 #34}. The elongated Ltn1p curves around the 60S ribosome, positioning its ligase domain close to the nascent chain (NC) exit{Lyumkis, 2014 #8;Shen, 2015 #5;Shao, 2015 #34}.Rqc2p is a nucleotide binding protein that recruits $tRNA^{Ala}$ and $tRNA^{Thr}$ to the 60S peptidyl-tRNA complex. This resultsin the addition of a C-terminal Ala/Thr sequence (CAT-tail) tothe stalled NC in a mRNA independent manner{Shen, 2015 #5}. A possiblefunction of the CAT-tailis to help clear the ribosome tunnel of stalled polypeptides.

Mutation of Listerin results in neurodegeneration in a mouse model{Chu, 2009 #12}, presumably as a result of a chronic defect of neuronal cells in degradingaberrant translation

products. Here we investigated the consequences of RQC deficiency in yeast cells. We find that stalled polypeptides leave the ribosome and form SDS-resistant aggregates. Surprisingly, aggregation depends on addition of the CAT-tail to the stalled chains by Rqc2p, with the juxtaposed poly-lysine tractfacilitatingthe formation of cytoplasmic inclusions. The aggregates interfere with general protein quality control by sequesteringmultiple chaperones.These results explain the proteotoxicity of ribosome-stalled polypeptides and the essential role of the RQC in maintaining proteostasis.

Aggregation of non-stop proteins

To investigate the fate of NS-proteins upon RQC failure, we expressed green fluorescence protein (GFP) and firefly luciferase (Luc) from mRNAs with and without stop codon. Only small amounts of NS-proteinwere detected in wild-type (WT)yeast (Fig. 1a, lane 1 and Extended Data Fig. 1a),consistent with efficient ubiquitylation of NS-protein by Ltn1p (Extended Data Fig. 1b) and proteasomal degradation{Bengtson, 2010 #4}. In contrast, NS-protein accumulated in *ltn1*Δ cells, accompanied by the formation of SDS-resistant, high molecular weight (HMW)species (Fig. 1a, lane 2). This HMW protein represented aggregated NS-protein, which was not ubiquitylated (Extended Data Fig. 1b) and was dissolved by formic acid (Fig. 1a, lanes 5-6). Indeed, in ~17% of *ltn1*Δ cells NS-GFP accumulated incytosolic inclusions(Fig. 1b), independent of the *RNQ*prion state of the cells (Extended Data Fig. 1c, d). Thevast majority of NS-GFP was not associated with ribosomes (Extended Data Fig. 1e).SDS-resistant NS-GFP aggregates were substantially smaller than ribosomes, suggestingthat oligomeric aggregates coexist with visible inclusions.

Role of poly-basic tract and ribosome stalling in aggregation

The C-terminus of NS-proteins contains a poly-lysine (polyK) tract encoded by the variable poly(A) tail of the mRNA. To investigate the role of the C-terminal extension in aggregation, we expressed fusion proteinsconsisting eitherof GFP anda spacer of 134 amino acids(GFP-s) or an additional polyK tract of 12 or 20 residues(GFP-s-K12 and GFP-s-K20, respectively)(Fig. 2a). The spacer is the unstructured, aggregation-resistant M-domain of Sup35{Krishnan, 2005 #40;Mukhopadhyay, 2007 #26}. GFP-s was diffusely distributed in *ltn1*Δ cells, while GFP-s-K12 formed cytosolic inclusions in ~11% and GFP-s-K20 in ~55% of cells (Fig. 2a), independent of the sequence of the spacer(Extended Data Fig. 2a). Thus, aggregation depends on the length of thepolyK tract. Of note, the medianpoly(A) lengthin yeast is ~27 (~10 lysines){Subtelny, 2014 #73}, consistent with the inclusion frequency of ~17% observed with NS-GFP (Fig. 1b). Interestingly, GFP-s-K20 formedinclusions only in *ltn1*Δ cells, but notin WT cells(Fig. 2a), suggesting thatthe polyK tract may mediate aggregation indirectlyby causing NC stalling. These stalled chains would be degraded in WT cells.

To distinguish direct and indirect rolesof the poly-basic tract in aggregation, we employed poly-arginine (polyR) stalling sequences{Dimitrova, 2009 #14;Brandman, 2012 #3} and modulated stalling efficiency by usingfrequent (AGA) or rare (CGA) Arg codons{Letzring, 2010 #13;Shen, 2015 #5}. We generatedGFP fusion proteinsin which polyRis followed by mCherry (Fig. 2b). A polyR tract of 20 residues encoded by frequent codons (GFP-s-R20$_{FREQ}$-mCh) allowed the efficient production of full-length protein (~95 kDa on SDS-PAGE), exhibiting both GFP and mCherry fluorescence, in WT and *ltn1*Δ cells (Fig. 2b, lanes 4 and Extended Data Fig. 2b). The protein did not form SDS-resistant aggregates and was diffusely distributed, with inclusions present in only ~5% of cells (Fig. 2b and Extended Data Fig.

2b).However, these inclusions were both GFP and mCherry fluorescent (data not shown) and thus wereunrelated to ribosome stalling. Stalling was observed when the polyR tract was encoded by rare codons ($R4_{RARE}$or $R20_{RARE}$), as indicated by reduced amounts of full-length protein(Fig. 2b, lanes 3 and 5,and Extended Data Fig. 2b).These constructs produced SDS-resistant aggregates in *ltn1*Δ cells (Fig. 2b, lanes 3 and 5), but visible inclusions were not detectable(Extended Data Fig. 2b). Interestingly, inclusionsoccurred in~23% of cellsexpressingthe protein$R20_{FREQ}R4_{RARE}$, in which20 Arg encoded by frequent codons are followed by 4 rare codonsto mediateefficient stalling ($R20_{FREQ}R4_{RARE}$) (Extended Data Fig. 2b). Thus,enhanced inclusion formation requires both ribosome stalling and the translation of a long poly-basic sequence. We note that while $R20_{FREQ}R4_{RARE}$formed inclusions,less SDS-resistant aggregateswere detectable (Fig. 2b, lanes 6), suggesting that thelong polyR tract modulated the aggregation behavior.Semi-denaturing detergent agarose gel electrophoresis revealed that cells expressing$R20_{FREQ}R4_{RARE}$ contained aggregates substantially larger in size than cells expressingeither $R4_{RARE}$ or $R20_{RARE}$(Extended Data Fig. 2c). These results support the notion that stalled polypeptides form distinctaggregate species, oligomers and inclusions,of which only the former are detectable by SDS-PAGE.

In summary, RQC failure causes stalled polypeptides to accumulate in SDS-resistant aggregates. Oligomeric aggregatesare already observed when the NC contains at most four arginines.NS-proteins with a poly-basic sequence exceeding a critical length of ~12 residues have anadditional propensity to form visible inclusions.

Rqc2p is required for aggregation of stalled chains

Rqc1p and Rqc2p are additional RQC components{Brandman, 2012 #3}. While the function of

Rqc1p is unclear,Rqc2p binds to 60S ribosomescarrying peptidyl-tRNA and recruits Ltn1p{Lyumkis, 2014 #8;Shao, 2015 #34;Shen, 2015 #5}. Deletion of *RQC1* or*RQC2*caused a marked stabilization of NS-GFP,similar to deletion of *LTN1* (Fig. 3a, lanes 1-4).*Rqc1*Δ cellsalso accumulated SDS-resistant NS-GFP aggregates. Surprisingly, we observed no aggregates in *rqc2*Δcells (Fig. 3a, lane 4), although the NS-GFPwas released from the ribosome (Extended Data Fig. 3a). Furthermore, deletion of*RQC2*ineither the *ltn1*Δor*rqc1*Δ background abolished aggregation (Fig. 3a, lanes 5-6), indicating an upstream role of Rqc2p.NS-GFP inclusions were also absent in *rqc2*Δ cells (Extended Data Fig. 3b), althoughother aggregation-prone proteins formed inclusions normally (Extended Data Fig. 3c).Interestingly, in all strains carrying the*RQC2*deletion,NS-GFP accumulated in the nucleus (Extended Data Fig. 3b), suggesting thatin the absence of aggregation the polyK tract functions as a nuclear localization or retention signal. These results demonstrate that Rqc2p is required, directly or indirectly, for the aggregation of stalled NCs when the downstream degradation pathway is blocked.

CAT-tailaddition by Rqc2p mediatesaggregation

We next tested the possibility that aggregation is mediated by the C-terminal Ala/Thr sequence tag (CAT-tail) added to stalled NCs by Rqc2p{Shen, 2015 #5}. CAT-tails of 5-19 residues have been characterized by mass spectrometry (MS){Shen, 2015 #5},but longer tags may exist.We observedCAT-tails with the stalled chains of the $R20_{FREQ}R4_{RARE}$ and $R20_{RARE}$ constructs, as revealed by comparing the band pattern of truncated chains in *ltn1*Δ and *ltn1*Δ*rqc2*Δ cells{Shen, 2015 #5} (Fig. 3b). While $R20_{FREQ}R4_{RARE}$ formed inclusions in *ltn1*Δ cells, no inclusions were detected in *ltn1*Δ*rqc2*Δ cells (Fig. 3c), suggesting that the CAT-tailis required both for the formation of SDS-resistant aggregates and inclusions. As a critical test of this possibility we

employed a variant of Rqc2p, $Rqc2_{aaa}$ (mutationsD9A, D98A, R99A),whichcan no longer synthesize CAT-tails butrecruits Ltn1p to 60S ribosomes{Shen, 2015 #5}.As expected, *ltn1*Δ*rqc2*Δ cells expressing WT *RQC2* added CAT-tails to stalled polypeptides, but cells expressing $rqc2_{aaa}$ did not (Extended Data Fig. 3d).However, $Rqc2_{aaa}$ restored the ability of *rqc2*Δ cells to degrade stalled chains, reflecting recruitment of Ltn1p (Fig. 3d, lanes 3 and 4). Importantly, only WT Rqc2p reestablished the formation of SDS-resistant aggregates and inclusions in *ltn1*Δ*rqc2*Δ cells (Fig. 3d, lanes 6 and 7; Extended Data Fig. 3e). Thus, the CAT-tail has an essential role in mediating aggregation.

To explore the role of the CAT-tail further, we generated a GFP fusion protein containing a C-terminal polyK tract of 20residues followed by 6 Ala-Thr repeats and a stop codon (GFP-s-K20-$(AT)_6$)(Extended Data Fig. 4a).We expressed this protein in cells lacking the ribosome associated protein Hel2p, previously implicated in ribosomal stalling{Brandman, 2012 #3}. In this strain, stalling of the K20 tract is reduced, allowing synthesis of the full construct (Extended Data Fig. 4b). K20-$(AT)_6$formed inclusions in ~54% of the cells, while proteins containing either only K20 or the $(AT)_6$tagdid not (Extended Data Fig. 4a). Substitution of the $(Ala\text{-}Thr)_6$ sequence with $(Gly\text{-}Ser)_6$strongly reduced visible inclusion, confirming that both the CAT-tail and the poly-basic tract are required for inclusion formation.

NS-protein aggregates sequester chaperones

Aggregates of neurodegenerative disease proteins often sequester molecular chaperones, resulting in proteostasis impairment{Olzscha, 2011 #16;Park, 2013 #21;Hipp, 2014 #17}. To identify the interactome of NS-proteins in *ltn1*Δ cells, we performed a quantitative proteomic analysis{Ong, 2006 #61}. Multiple chaperones and co-factors were specifically associated with

NS-GFP in *ltn1*Δ cells, prominently including the Hsp40 protein Sis1p, an essential co-chaperone of Hsp70{Yan, 1999 #18} (Fig. 4a and Extended Data Tables 1 and 2).

A substantial fraction of the NS-GFP bound Sis1p was associated withthe SDS-resistant aggregatesin *ltn1*Δ and *rqc1*Δ cells (Fig. 4b, lanes 2-3; Extended Data Fig. 5a, b), andSis1p was also recruited to NS-GFP inclusions(Extended Data Fig. 5c). Notably, when *RQC2* was deleted, the association of Sis1p with NS-GFP was much reduced and no SDS-resistant co-aggregation occurred(Fig. 4b, lanes 4-6).Expression of WT *RQC2* but not of $rqc2_{aaa}$ restored Sis1p co-aggregation (Extended Data Fig. 5d). Rqc2p-dependent co-aggregation of Sis1p was also observed withthe stalling construct GFP-s-$R4_{RARE}$-mCh (Fig. 4c, lanes 3-4), which forms SDS-resistant aggregates but lacks the critical length of poly-basic sequence for inclusion formation (data not shown). These finding indicate that stalled NCs form Sis1p associated aggregates in a CAT-tail dependent manner, although the CAT-tailmay mediate Sis1p binding indirectly.

Cells may constantly generate aberrant polypeptides that must be removed by the RQC{Turner, 2000 #70;Duttler, 2013 #69}. We found that more than 40% of *ltn1*Δ cells contained Sis1p positive inclusions, even in the absence of recombinant NS-protein (Extended Data Fig. 5e). Blue native-PAGEof cell extracts showed that ~30% of total Sis1p was present inaggregates of ~700-1200 kDa (Extended Data Fig. 5f). Again expression of WT Rqc2p, but not $Rqc2_{aaa}$ mutant, restored the formation of Sis1p containing aggregates in *ltn1*Δ*rqc2*Δcells (Extended Data Fig. 5g). Thus, yeast cells accumulateconsiderable amounts of faultyNCs in aggregates when the RQC fails, withaggregationbeingCAT-taildependent.

To assess the consequences of RQC deficiency more broadly, we analyzed the spectrum of proteins associated with the SDS-resistant Sis1p aggregates by quantitative proteomics. GFP- or HA-tagged Sis1p (expressed under the *SIS1* promoter) was immunoprecipitated from WT and

*ltn1*Δ cells(Extended Data Fig. 5h). Approximately 400 proteins were reproducibly recovered inSis1p aggregates (Supplementary Information Table 1a-c). Among these were ~30 proteins categorized as chaperones or stress response proteins (Fig. 4d and Supplementary Information Table 1c), contributing ~12% to aggregate mass. Many of these chaperones were also NS-GFP interactors (~43% overlap) (Extended Data Table 1 and Supplementary Information Table 1c), suggesting that the recombinantly expressed NS-protein merged with the endogenous protein aggregates. The other proteins in the Sis1p aggregates are mostly localized in the cytosol and belong to various functional categories(Fig. 4d). They are typically of high abundance in the proteome{Wang, 2015 #79} (Extended Data Fig. 5i), which presumably facilitated the identification of aggregated NCs. These results indicate that RQC deficiency causes the formation of aggregates containing numerous endogenous proteins and proteostasis components.

RQC deficiency leads to proteostasis impairment

We next investigated whether the sequestration of multiple chaperones results in proteostasis impairment of *ltn1*Δ cells. Sis1p is critical for the proteasomal degradation of terminally misfolded proteins such as cytosolic carboxypeptidase Y* (CPY*){Heck, 2010 #62;Park, 2013 #21;Summers, 2013 #43}. Indeed, CPY* fused to mCherry (CmCh*) or to GFP (CG*) was markedly stabilized in *ltn1*Δ cells compared to WT(Fig. 5a), although CPY* was efficiently polyubiquitylated(Extended Data Fig. 6a). Overexpression of Sis1p rescued degradation (Extended Data Fig. 6b). Importantly, CmCh* degradation was also restored in *ltn1*Δ*rqc2*Δ cells (Fig. 5a), which have a normal Sis1p pool (Extended Data Fig. 5f). Thus, RQC deficient cells fail to support general quality control pathways due to Sis1p sequestration.

CmCh* aggregates when proteasome function is inhibited{Park, 2013 #21}. We also

observed Sis1p positive inclusions of CmCh* in *ltn1*Δ cells (Extended Data Fig. 6c),and these co-localized with NS-GFP inclusions (Extended Data Fig. 6d), suggesting that terminally misfolded proteins and NS-proteins follow similar pathways for aggregate deposition. We note, however, that Sis1p overexpression failed to suppress NS-protein aggregation (Extended Data Fig. 6e).

Deletion of *LTN1* did not result in a growth defect in yeast (Extended Data Fig. 7a), despite causing substantialSis1psequestration. However, upon exposure toproteostasis stress, such as CmCh*expression at 37°C, the *ltn1*Δ mutant showed slowgrowth (Extended Data Fig. 7a). Overexpression of Sis1p or *RQC2* deletion rescued this growth defect (Extended Data Fig. 7b, c), consistentwith CmCh* expression driving Sis1p sequestration beyond a critical level. Moreover, treatment with hygromycin B, an antibiotic that reduces translational fidelity, also caused a severe growth defect of *ltn1*Δ and *rqc1*Δ cells (Extended Data Fig. 7d), accompanied by enhanced formation of Sis1p positive inclusions (Extended Data Fig. 7e). Again this growth defect was partially rescued by *RQC2* deletion (Extended Data Fig. 7d), suggesting that it was caused by aggregation of faulty NCs. Together these results demonstrate that RQC deficiency markedly impairs cellular proteostasis capacity.

Conclusions

Failure of ribosomal quality control, a highly evolved rescue mechanism for the removal of aberrant polypeptides, results in proteotoxic stress.We have shown that stalled nascent polypeptides aggregate when their degradation is inhibited (Fig. 5b, c). Surprisingly, the Ala-Thr sequence (CAT-tail) that is added to the C-terminus of stalled chains by Rqc2p{Shen, 2015 #5}is critical in this process and is probably the major driver of aggregation of stalled

polypeptidesoriginating from truncated mRNAs{Lykke-Andersen, 2014 #54}(Fig. 5b). The aggregation process of NS-proteins is more complex (Fig. 5c). In this case, read-through into the poly(A) tail of the mRNA results in the translation of a basic polyK tract that causes stalling and participates in aggregation. Our data indicate that the CAT-tail, following after the polyK tract, initiates the assembly of the NS-chains to SDS-resistant oligomers, while the polyK tract mediatesthe formation of visible inclusions (Fig. 5c). The aggregates interact with multiple chaperones,and thereby interfere with general protein quality control. The exact mechanism of chaperone sequestration and the role of the CAT-tail in this process remains to be explored(Fig. 5b, c).

The SDS insolubility of the aggregatesformed bystalled polypeptides suggests thatthe CAT-tailsequences act in a manner comparable to the poly-alanine expansions of certain disease proteins{Amiel, 2004 #64;Forood, 1995 #65}. The polyK tract present in NS-proteins probably contributes to aggregate formation, consistent with poly-lysine forming fibrils when charge repulsion effects are reduced at high pH{Fandrich, 2002 #2}. Aggregate ‘nucleation’ bythe CAT-tail may serve to overcome this repulsion at physiological pH (Fig. 5c), perhaps in cooperation with negatively-charged agents, such as poly-phosphate{Gray, 2014 #66} or nucleic acids. Poly-basic sequences have a pronounced potential to form toxic aggregates, as exemplified by the Gly-Arg or Pro-Arg dipeptide repeat sequencesencoded by*C9orf72* mutant genes, whichcause amyotrophic lateral sclerosis and frontotemporal dementia{Mori, 2013 #32;Zu, 2013 #67}.

Defective RQC surveillance results in the aggregation of a wide range of endogenous proteins and the sequestration of critical proteostasis components,(Fig. 5b, c). The aggregates become highly toxic under conditions of mild conformational stress or when translational fidelity is reduced. Even in the absence of additional proteostasis pressure, the sequestration of Sis1p and

other chaperones potently interferes with cytosolic protein quality control–a positive feedback loop with the potential to cause chronic proteotoxic stress{Hipp, 2014 #17;Roth, 2014 #50}. Future studies will investigate, whether aggregate formation by ribosome stalled polypeptides and proteostasis impairment also underlies the age-dependent neurodegenerative phenotype of the *listerin* mouse{Chu, 2009 #12}.

References

Supplementary Informationis linked to the online version of the paper at www.nature.com/nature.

Extended Dataincludes 6 Extended Data Figures and 4 Extended Data Tables.

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Author Contributions:

Y-J.C. and S-H.P. designed and performed most of the biochemical and functional experiments. T.H. performed the sucrose gradient and SDD-AGE analyses. R.K. performed the MS analysis and proteomics and L.V.-Dthe bioinformatics. F.U.H. and M.H.-H. supervised the experimental design and wrote the manuscript with contributions from Y-J.C and the other authors.

Author Information:

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Correspondence and requests for materials should be addressed to uhartl@biochem.mpg.de

Figure legends

Figure 1 | NS-protein aggregates in *ltn1*Δcells.a,GFP or NS-GFP was immunoprecipitated (IP)from cell extracts of WT or *ltn1*Δ yeast cells with GFP antibody, followed by anti-GFP immunoblotting (IB) (lanes 1-4). SDS-res., SDS-resistant. Pgk1pwas used as loading control. EV, empty vector.NS-GFP was incubated with formic acid (FA) and analyzed byIB (lanes 5, 6).**b,** Fluorescence microscopy of cellsexpressing GFP or NS-GFP. Nuclei stained with Hoechst 33342. *ltn1*Δcellswere exposed for a shorter time. The fraction of cells with visible inclusions is indicated (s.d. from 3 experiments). DIC, differential interference contrast.

Figure 2 | Effect of poly-basic sequence and ribosomal stalling on NC aggregation.a, GFP fusion proteins containing an unstructured spacer (s)and poly-lysine sequence (top). Fluorescence images of *ltn1*Δor WTcells expressing the proteins indicated (bottom). Cells with visible inclusions were quantified as in Fig. 1b.**b,**GFP-s fusion proteins containing Arg residues encoded by rare or frequent codons followed by mCherry (mCh) (top).The proteins were expressed in WT or *ltn1*Δ cells and cell extracts analyzed as in Fig. 1a. Arrow head, position of full-length protein; asterisk, stalled truncation products and proteolytic fragments.

Figure 3 | Role of Rqc2p in aggregation of stalled NCs.a, NS-GFP was expressed in WT and RQC mutant cells,and analyzed as in Fig. 1a. **b,**Extracts from *ltn1*Δ or *ltn1*Δ*rqc2*Δ cells expressing GFP-s-$R20_{FREQ}R4_{RARE}$-mCh or GFP-s-$R20_{RARE}$-mCh (see Fig. 2b) were analyzed by IB with anti-GFP antibody. CAT-tails are indicated.**c,** Fluorescence images of*ltn1*Δ or *ltn1*Δ*rqc2*Δ cells expressing GFP-s-$R20_{FREQ}R4_{RARE}$-mCh.Cells with visible inclusions were quantified as in Fig. 1b.**d,** NS-GFP was expressed in WT, *rqc2*Δ or *ltn1*Δ*rqc2*Δ cells. When indicated, the cells

expressed WT Rqc2p or $Rqc2_{aaa}$. NS-GFP was analyzed as in Fig. 1a.

Figure 4 | Aggregates of stalled polypeptide sequester chaperones.a, Chaperone proteins interacting with NS-GFP. Black bars, fold enrichment overGFP in *ltn1*Δcells and gray bars, over NS-GFP in WT cells (see Extended Data Tables 1 and 2 and Methods).**b,**NS-GFP expressed in WT or RQC mutant cells was analyzed by anti-GFP IP and anti-Sis1p IB. SDS-sol., SDS-soluble. **c,**GFP-s-mCh or GFP-s-$R4_{RARE}$-mCh (see Fig. 2b) in WT, *ltn1*Δ or *ltn1*Δ*rqc2*Δ cells. Cell extracts were analyzed by IP with anti-GFP, followed by IB with anti-GFP (left) or anti-Sis1p (right). Arrow head, stalled truncation products; asterisk, full-length protein and proteolytic fragments. Dashed box, CAT-tails.**d,** Category enrichment of proteins in SDS-resistant Sis1p aggregates (Benjamini-Hochberg FDR≤0.02) (see Extended Data Fig. 5h and Methods). The keyword category chaperones is highlighted in black.

Figure 5 | Failure of RQC results in impairment of cytosolic quality control.a, CmCh* was expressed in WT, *ltn1*Δ and *ltn1*Δ*rqc2*Δ cells and degradation followed by cycloheximide chase. CmCh* was detected by IB with anti-mCherry (top) and quantified by densitometry (bottom). Error bars indicate s.d. from three independent experiments.**c-d**, Models for the aggregation of stalled NCs (b) and NS-proteins (c), resulting in chaperone sequestration and proteostasis impairment. Stalled NCswithout poly-basic tract are generated from truncated mRNAs.

METHODS

Yeast Strains. Yeast genetic experiments were carried out using standard methods. Strain

BY4741 was used as thewild-type (WT) parental strain. All yeast strains used in this study are listed in Extended Data Table 3.*ltn1*Δ, *rqc1*Δ and*rqc2*Δ single deletion mutants were obtained from EUROSCARF. To delete *HEL2*and *RQC2*in the*ltn1*Δ strain, PCR amplified marker gene expression cassettes{Gueldener, 2002 #39}with overhangs complementary to upstream and downstream sequences of each gene were transformed. Addition of aC-terminal mCherry tag to *SIS1* was performed as described{Young, 2012 #42}.

Plasmids. All NS-proteins, polyK and polyR expression vectors were constructed in the plasmid pRS416. The SacI-EcoRI fragment containing the*GAL1* promoter but without the *CYC1* terminator from p423GAL1{Mumberg, 1994 #37} was ligated into pRS416. The BamHI-EcoRI fragment from pSA158 or pSA159{Ito-Harashima, 2007 #31} was inserted into this pRS416-*GAL1* promoter plasmid to clone the*HIS3* terminator with or without a stop codon. The PCR amplified GFP gene (including the following mutations to enhance stability and brightness:F64L, S65T, F99S, M153T, V163A, S208L{Ito, 1999 #38}) was inserted using XbaI-BamHI restriction sites to generate GFP and NS-GFP expression vectors.2myc-Luc was also ligated into XbaI-BamHI sites to generate Luc and NS-Luc expression vectors. To generate the GFP-K12/K20 vectors, the*HIS3* terminator wasfirst PCR amplified using long primerswith an upstream overhang containing a $(AAG)_{12}$-stop codon or $(AAG)_{20}$-stop codon sequence. These were cloned as BamHI-EcoRI fragments. GFP was inserted using XbaI-SpeI sites and the middle domain of Sup35p (amino acid residues 124 to 253, referred to as M-domain) or a flexible region of Hsp82p (amino acid residues 210 to 263)was inserted as a natively unstructured linker{Scheibel, 1999 #75;Krishnan, 2005 #40;Mukhopadhyay, 2007 #26}using SpeI-BamHI sites.

To generate polyR vectors, GFP-SUP35M was PCR amplified without overhang

sequence or with overhang sequences bearing $(CGA)_4$, $(AGA)_{20}$, $(CGA)_{20}$ or $(AGA)_{20}(CGA)_4$ sequences without a stop codon. These were ligated as XbaI-BamHI fragments. PCR amplified mCherry and the stop codon was inserted into a BamHI site.

CAT-tail constructs were generated by PCR amplifying the*HIS3* terminator using primers with $(Ala\text{-}Thr)_6$ or $(Gly\text{-}Ser)_6$ overhang sequences. TheCAT-tail-*HIS3* terminator was cloned into BamHI-EcoRI sites and GFP-SUP35M or GFP-SUP35M-$Lys_{20}$was cloned into XbaI-BamHI sites.

To generate *RQC2* expression constructs, the *RQC2* promoter was first cloned in p413GAL1 using SacI-BamHI sites. WT *RQC2* was PCR amplified from genomic DNA and cloned into BamHI-XhoI sites. Residues D9, D98 and R99 were mutated to alanine, resulting in$rqc2_{aaa}$[Shen, 2015 #5], using Q5 site-directed mutagenesis (NEB).

To generate the Rnq1-GFP expression construct, the *CUP1* promoter and *CYC1* terminator were cloned into pRS316 by using SacI-BamHI and XhoI-KpnI sites, respectively. *RNQ1* and *GFP* were cloned into BamHI-EcoRI and EcoRI-XhoI sites, respectively. The internal EcoRI site of *RNQ1* was removed by using modified PCR primers.

All plasmids used in this study are listed in Extended Data Table 4.

Immunoprecipitation of GFP and NS-GFP proteins. Cells with GFP and NS-GFP expression vector were pre-cultured in raffinose medium and then transferred to galactose/raffinose medium for ~16 h (~5 generations) at 30°C to induce expression.Unless stated otherwise, all recombinant protein expression in this study was driven by the*GAL1* promoter under these conditions. Yeast cells were lysed with glass beads in lysis buffer A (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 5% glycerol, complete protease inhibitors; Roche) using a FastPrep-24 homogenizer

with a CoolPrep adapter (MP Biomedicals). After clearing lysates by repeated centrifugation at 2,000 x g for 5 min, lysates were adjusted to 2 mg/ml protein with lysis buffer containing 0.5% NP-40. 50 μl of anti-GFP MicroBeads (Miltenyi Biotec) were added to 1 ml of final lysate. After incubation for 1 h at 4°C, lysates with anti-GFP MicroBeads were applied to aμ column (Miltenyi Biotec). The beads were washed four times with 200 μl of lysis buffer followed by elution of bound proteins with 50 μl of HU buffer (8 M urea, 200mM Tris-HCl pH 6.8, 1 mM EDTA, 100mM DTT, 5% SDS, 0.01% bromophenol blue). After heating at 70°C for 10 min, 15 μl of eluate was separated on 4-12% Bis-Tris NuPAGE gel (Invitrogen).

Immunoprecipitation of Luc and NS-Luc under denaturing condition. To preserve the ubiquitylation status of the proteins, immunoprecipitation was performed under denaturing conditions, essentially as previously reported{Kragt, 2005 #41}. Cells were treated with 95 μM MG132 for 1.5 h before the harvesting. 400 μl of 5% trichloroacetic acid (TCA) was added to a cell pellet from 20 OD of cells, followed by glass bead lysis. After incubation for 1 h on ice, protein was precipitated by centrifugation and the pellet resuspended in 200 μl of 2% SDS containing 20 mM NEM (N-ethylmaleimide), 100 μM MG-132, complete protease inhibitors, and bromophenol blue. 1 M Tris base was added until the solution turned blue. Samples were heated at 95°C for 5 min and undissolved material was removed by centrifugation at 13,000 g for 5 min. 180 μl of supernatant was diluted with 800 μl of buffer (1.2% Triton X-100, 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% BSA, 20 mM NEM, complete protease inhibitors). 50 μl of anti-myc MicroBeads (Miltenyi Biotec) were added, followed by incubation for 1.5 h at 4°C. The beads were processed as above and eluates analyzed by immunoblotting

with anti-Luc and anti-ubiquitin antibodies.

Polysome gradient analysis.Experiments were carried out as previously described{Bengtson, 2010 #4} with minor modifications. Yeast cultures were grown to mid-log phase (OD_{600} 0.8 – 1.0) at 30°C. Cycloheximide (CHX, final 0.1 mg/ml) was added 10 min before cell harvest. Cell lysates were prepared in lysis buffer B (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 30 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml CHX and complete protease inhibitors) using glass beads. Cell debris was removed by centrifugation at 400 x g for 5 min. An amount of lysate corresponding to 40 A_{260} units was layered on a continuous 7-47% sucrose gradient prepared in 40 mM Tris-acetate pH 7.0, 50 mM NH_4Cl, 12 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml CHX. Gradients were centrifuged at 40,000 rpm for 2 h at 4°C using a SW41 rotor (Beckman) and fractionated using a piston gradient fractionator coupled to an A_{254nm} spectrophotometer (Biocomp). Fractions were subjected to TCA precipitation. Briefly, sodium deoxycholate was added to a final concentration of 0.02% and fractions were incubated for 15 min on ice. TCA was added to a final concentration of 10% and fractions were further incubated for 1 h on ice. Samples were then centrifuged for 30 min at 16,000 xg. Pellets were washed with -20°C cold acetone and air dried. Pellets were resuspended in HU buffer and equal amounts of each fraction were loaded on a 4-12% Bis-Tris NuPAGE gel. Immunoblot analysis was carried out using anti-GFP and anti-Rpl3p antibodies.

Formic acid treatment of SDS-resistant aggregates. NS-GFP was expressed under the *GAL1* promoter in *ltn1*Δ cells and immunoprecipitated using µMACS GFP isolation kits (Miltenyi Biotec). Proteins bound to antibody beads were eluted using 100 mM triethylamine buffer (pH

11.8). After neutralizing the eluates with 1 M MES (pH 3), proteins were TCA precipitated. Thepellets were washed with cold acetone and then treated with 100% formic acid at 37°C for 1h, followed by drying in a vacuum centrifuge concentrator. Dried proteins were re-suspended in HU buffer and heated with vigorous shaking at 65°C for 30 min, followed by SDS-PAGE and anti-GFP or anti-Sis1p immunoblotting.

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE). SDD-AGE was performed as described previously{Kryndushkin, 2003 #76}. Briefly, a 1.5% agarose gel was prepared with TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) including 0.1% SDS. Yeast cell lysates were normalized to 4 mg/ml and mixed with an equal volume of 2x SDD-AGE sample buffer (2x TAE, 10% glycerol, 4% SDS and 0.002% bromophenol blue) and incubated at room temperature for 10 min. 20 μg of total protein was loaded and electrophoresis was performed at 4°C for 3 h at 75 V in TAE buffer with 0.1% SDS. After electrophoresis, proteins were transferred on a nitrocellulose membrane overnight at room temperature using the capillary transfer method with 50 mM Tris-HCl pH 7.5, 150 mM NaCl as transfer buffer, followed by immunodetection.

SILAC labeling and preparation of samples for proteomic analysis. Yeast cells were grown in synthetic complete medium with 2% raffinose without uracil and labeled with L-lysine isotopes. L-lysine-$^{13}C_6$,$^{15}N_2$ was used as heavy lysine (H) and L-lysine D4 was used as medium lysine (M) (Cambridge Isotope Laboratories). The final concentration of lysine in the medium was 150 μg/ml. WT cells expressing NS-GFP and *ltn1*Δ cells expressing GFP were labeled with (H) and (M) lysine, respectively. *ltn1*Δ cells expressing NS-GFP were grown with normal lysine

(light, L). GFP and NS-GFP expression from the *GAL1* promoter was induced by inoculating cells into the respective media containing 2% galactose and 1% raffinose. Cells were grown for at least five generations to an OD600 of 0.7-0.8. Immunoprecipitation of GFP and NS-GFP was carried out as described above. (H), (M) and (L) samples were mixed at 1:1:1 ratio and loaded onto 4-12% Bis-Tris NuPAGE gels. Preparation of gel slices, reduction, alkylation, and in-gel protein digestion were carried out essentially as previously reported{Shevchenko, 1996 #47}. Peptides were desalted, filtered, and enriched as described{Rappsilber, 2003 #48}.

NS-GFP interactome analysis by LC-MS/MS. Tryptic peptides were dissolved in 6 µl of 5% formic acid and analyzed by nanoLC-MS/MS using an EASY-nLC 1000 nano liquid chromatography system (Thermo) coupled to a Q-Exactive mass spectrometer (Thermo). Samples were injected onto a home-made 25 cm silica reversed-phase capillary column (New Objective) packed with 1.9-µm ReproSil-Pur C18-AQ (Dr. Maisch GmbH). Samples were loaded on the column by the nLC autosampler at a flow rate of 0.5 µl/min. No trap column was used. Peptides were separated by a stepwise 120-min gradient of 0–95% between buffer A (0.2% formic acid in water) and buffer B (0.2% formic acid in acetonitrile) at a flow rate of 250 nL/min. MS/MS analysis was performed with standard settings using cycles of 1 high resolution (70000 FWHM setting) MS scan followed by MS/MS scans of the 10 most intense ions with charge states of 2 or higher at a resolution setting of 17500 FWHM. Protein identification and SILAC based quantitation was performed with MaxQuant (version 1.3.0.5) using default settings. The UNIPROT *Saccharomyces cerevisiae* database (version 2013-12-05) was used for protein identification. MaxQuant uses a decoy version of the specified UNIPROT database to adjust the false discovery rates for proteins and peptides to below 1%.

Analysis of SDS-resistant Sis1p aggregates by LC-MS/MS. Yeast cells were grown in synthetic complete medium with 2% glucose and SILAC labeled as described above. Chromosomal *SIS1* was replaced by *SIS1*-HA or *SIS1*-GFP in WT and *ltn1*Δ cells. WT cells were isotope labeled with L-lysine-$^{13}C_6$,$^{15}N_2$ (H, heavy) and *ltn1*Δ cells were grown in normal L-lysine (L, light). Sis1p was immunoprecipitated with anti-HA or anti-GFP MicroBeads (Miltenyi Biotec). The beads from WT and *ltn1*Δ cells were eluted and the eluates mixed at a 1:1 ratio, followed by electrophoresis on 4-12% Bis-Tris NuPAGE gels. Proteins migrating above 170 kDa size were subjected to in-gel digestion and LC-MS/MS analysis (see Extended Data Fig. 4g). Proteins that were enriched ≥2-fold in at least two out of three experiments each from Sis1-HA and Sis1-GFP cells (403 proteins) were defined as Sis1p aggregate interactors. The category enrichment of keywords in the set of Sis1p aggregate interactors was calculated using the Fisher exact test with a cut-off Benjamini-Hochberg FDR≤0.02 after annotation using Perseus (1.5.2.12). Relative abundances of proteins were estimated based on iBAQ (intensity-based absolute quantification) values (MaxQuant).

Native-PAGE analysis of cells lysates. Spheroplasts were lysed in lysis buffer C (25 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 0.5% Triton X-100, complete protease inhibitors) using a Dounce tissue grinder. Total lysates were centrifuged at 500 x g for 5 min at 4°C to remove unbroken cells. An aliquot of lysate (40µg protein) was loaded on a3-12% Bis-Tris native PAGE gel (Invitrogen), followed by immunoblotting with anti-Sis1 and anti-Pgk1 antibodies. Native Protein Standard (Life Technologies) was used to estimate the molecular weight of Sis1 and its HMW form.

Cycloheximide chase. Cells grown in SC medium containing 2% glucose were transferred tomedium containing 2% raffinose and 2% galactose instead of glucose. After 15-18 h of induction, CHXwas added to 0.5 mg/mland 2.5 OD600 of cells were removed at the indicated time points. Cell extracts were prepared by alkaline lysis of cell pellets{Park, 2007 #22}, followed by immunoblotting as above.

Isolation of His_6-Ub conjugated proteins.ΔssCPY* fused to mCherry (CmCh*) under the *GAL1* promoter was expressed in galactose medium for 15 h at 30ºC, followed by expression of His_6-ubiquitin (His_6-Ub){Scazzari, 2015 #71} by addition of 100 µM $CuSO_4$ for 4h. Cells were harvestedand lysed with glass beads in denaturing buffer (6M GdmCl, 100mM NaH_2PO_4, 10 mM Tris-HCl, pH 7.0, 10 mM imidazole, 1% Triton X-100) using a FastPrep-24 homogenizer (MP biomedical). After removing cell debris (16,000 x g, 10 min at 4°C), lysate corresponding to 2 mg protein was incubated with 100 µl of TALON magnetic beads (Clontech) for 2h at 4ºC. Bound protein was washed three times with denaturing wash buffer (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-HCl, pH 7.8, 10 mM imidazole, 1% Triton X-100). His_6-Ub conjugated proteins were eluted withHUbuffer containing 250 mMimidazoleand heated for 5 min at 95°C. Eluates were separated on 4-12% Bis-Tris NuPAGE gel, followed by immunoblotting with anti-CPY antibody.

Fluorescence microscopy and image analysis. Fluorescence imaging was performed using a Zeiss Axiovert 200M inverted fluorescence microscope. Image J and AxioVision 4.7.1 were used for image analysis. For nuclear staining, 1.5 ml of cells were collected and re-suspended in 1 ml

of wash buffer (10mM Tris-HCl pH 8.0, 10mM $MgCl_2$). Cells were stained for 45 min in the dark by addition of Hoechst 33342 (final concentration 2 µg/ml) and washed three times with wash buffer prior to fluorescence microscopy. Cells with visible inclusions were quantified by analyzing >200 cells per condition in at least three independent experiments.

Antibodies. Anti-mCherry (Life Technologies, M11217), anti-CPY (Life Technologies, A-6428), anti-GFP (Roche, 11814460001), anti-HA (Roche, 11867423001), anti-Luciferase (Promega, G7451), anti-c-Myc (Santa Cruz Biotechnology Inc., sc- 40), anti-PGK (Life Technologies, 459250), anti-Rpl3 (Developmental Studies Hybridoma Bank), anti-Sis1p (Cosmo Bio, cop-080051) and anti-ubiquitin (Santa Cruz Biotechnology Inc., sc-8017) were used for immunoblot analyses. Anti-Sis1p was a kind gift from D. Cyr. Anti-goat IgG-HRP (Sigma, A5420), anti-mouse IgG-HRP (Dako, P044), anti-rabbit IgG-HRP (Sigma, A6154) and anti-rat IgG-HRP (Sigma, A9037) were used as secondary antibodies for immunoblot analysis.

Extended Data

Extended Data Figure 1 | Properties of NS-proteins in *ltn1*Δ cells. **a,** Firefly luciferase (Luc) or NS-Luc was expressed under the *GAL1* promoter in WT or *ltn1*Δ yeast cells for ~16 h (~5 generations) at 30°C. Proteins were immunoprecipitated (IP) from cell extracts with anti-myc antibody, followed by anti-Luc immunoblotting (IB). SDS-res., SDS-resistant aggregates. The cell extracts used as input were analyzed by immunoblotting against phosphoglycerate kinase 1 (Pgk1p) as a loading control. EV, empty vector.**b,** SDS-resistant HMW forms of NS-Luc do not represent polyubiquitylated protein. Myc-tagged NS-Lucwas expressed under the *GAL1* promoter in *pdr5*Δ or *ltn1*Δ yeast cells. *pdr5*Δ cells were incubated with DMSO or with MG132 (95 μM in DMSO) for 1.5 h. Cell lysates were prepared under denaturing conditions (see Methods), followed by NS-Luc immunoprecipitation (IP) with anti-myc antibody and immunoblotting (IB) with anti-Luc antibody (left panel) or anti-Ub antibody (right panel). The positions of SDS-resistant NS-Luc, polyUb-Luc and IgG are indicated. NS-Luc and Pgk1p in input fractions were analyzed. **c,** TheWT yeast strain used in this study (BY4741) and its *LTN1* deletion strain were in the [*RNQ*$^{+}$] state. To cure [*RNQ*$^{+}$], cells were grown on YPD plates containing 3 mM guanidinium chloride (GdmCl) and subsequently streaked on YPD plates without GdmCl to isolate single colonies. The [*RNQ*$^{+}$] prion state was confirmed by Rnq1-GFP inclusion body formation upon expression of Rnq1-GFP from *CUP1* promoter by induction for 4 h with 50 μM $CuSO_4$ during exponential growth. Live cells were analyzed by fluorescence microscopy. Scale bar, 5 μm. **d,** NS-GFP was expressed under the *GAL1* promoter in *ltn1*Δ cells in the [*RNQ*$^{+}$] or [*rnq*$^{-}$] state. Cell extracts were analyzed by IP and IB with anti-GFP antibody.**e,** Sucrose density gradient fractionation of*ltn1*Δ cells expressing NS-GFP for 16-18h. Absorbance at 254 nm indicates the position of ribosomes and polysomes (top panel). Gradient fractions were immunoblotted for the 60S protein Rpl3p with anti-Rpl3p antibody (middle panel) or anti-GFP antibody (bottom panel). SDS-resistant (SDS-res.) material was incompletely recovered, presumably due to the use of 10% TCA to precipitate the fractions prior to IB. Note that the immunoblot was overexposed to visualize the fractionation of SDS-resistant NS-GFP.

Extended Data Figure 2 | Inclusion formation by stalled poly-basic proteins in *ltn1*Δ cells. **a,** The disordered region from Hsp82p (residues 210 to 263) was used as an alternative spacer sequence (s*) in the stalling construct GFP-s*-K20, using GFP-s* as control. Representative live cell fluorescence images are shown and cells with visible inclusions were quantified as in Fig. 1b. GFP-s-K20 (Fig. 2a) and GFP-s*-K20 showed a similar frequency of inclusion formation. **b,** Live cell fluorescence microscopy of *ltn1*Δ cells expressing the GFP-s-polyR-mCh proteins indicated (see Fig. 2b). Cells were analyzed for GFP and mCherry fluorescence. Cells with visible inclusions were quantified as in Fig. 1b. Scale bar, 5μm.**c,**The GFP-s-polyR-mCh proteins shown on the left were expressed in *ltn1*Δ cells. Cell extracts were analyzed by SDD-AGE, followed by IB with anti-GFP antibody. Note that constructs $R4_{RARE}$ (3) and $R20_{RARE}$ (5) form SDS-resistant aggregates detectable by SDS-PAGE (Fig. 2b), while $R20_{FREQ}R4_{RARE}$ (6) forms inclusions but little SDS-resistant aggregates by SDS-PAGE (Fig. 2b and b). In lane 1 only 25% of cell lysate was applied to avoid overloading.

Extended Data Figure 3 | Rqc2p-dependent aggregation of stalled polypeptides. **a,** NS-GFPis released from ribosomes in *rqc2*Δcells. Sucrose density gradient fractionation of*rqc2*Δ cells expressing NS-GFP for 16-18 h. Analysis was performed as in Extended Data Fig. 1e. **b,** Live cell fluorescence microscopy of RQC mutant cells expressing NS-GFP. Hoechst 33342 was used for nuclear staining. Cells with visible inclusions were quantified as in Fig. 1b. Scale bar, 5μm.**c,***rqc2*Δ cells preserve the ability to deposit aggregated protein in inclusions. The *rqc2*Δ and *ltn1*Δ*rqc2*Δ strains used in this study were derived from the [RNQ^+] WT strain. Rnq1-GFP was expressed as in Extended Data Fig. 1c to confirm inclusion formation in the*RQC2* deletion strain. WT [rnq^-] cells were isolated from WT [RNQ^+] cells by GdmCl treatment as in Extended Data Fig. 1d. Inclusion formation was analyzed by fluorescence microscopy. Scale bar, 5 μm. **d,** *RQC2* or $rqc2_{aaa}$ was expressed under the *RQC2* promoter in *ltn1*Δ*rqc2*Δ cells expressingGFP-s-$R20_{RARE}$-mCh. Cell extracts were analyzed by IB with anti-GFP antibody. Pgk1p was used as a loading control. CAT-tails are indicated. **e,***RQC2* deletion prevents inclusion formation of stalled polypeptides in *ltn1*Δ cells. GFP-s-K20 was expressed in *ltn1*Δ*rqc2*Δ cells under the *GAL1* promoter. WT Rqc2p or $Rqc2_{aaa}$ was co-expressed under the *RQC2* promoter in a single copy plasmid. Inclusion formation was analyzed by fluorescence microscopy. Scale bar, 5 μm.

Extended Data Figure 4 | Engineered CAT-tails mediate aggregation.a,Schematic representation of GFP-s fusion proteins with stop codon containing 20 Lys residues or a $(Ala-Thr)_6$ sequence, or 20 Lys residues followed by a $(Ala-Thr)_6$ or $(Gly-Ser)_6$ sequence (top). Live cell fluorescence microscopy of *ltn1*Δ*hel2*Δ cells expressing the proteins indicated. The fraction of cells with visible inclusions is indicated (quantified as in Fig. 1b). Scale bar, 5μm.**b,** Deletion of *HEL2* increases read-through efficiency through a 20 Lys tract (encoded by AAG codons). The fusion proteins indicated and shown schematically in the top panel were expressed in *ltn1*Δ or *ltn1*Δ*hel2*Δ cells. Cell extracts were analyzed by IB with anti-GFP antibody (bottom panel). Arrow head indicates position of full-length GFP-s-K20-mCh.

Extended Data Figure 5 | Chaperone sequestration by aggregates of stalled polypeptides.a, SDS-resistant co-aggregates of NS-GFP with Sis1p are solubilized with formic acid. NS-GFP was expressed in *ltn1*Δ cells and immunoprecipitated from cell extracts with anti-GFP antibody. The precipitate was incubated without or with formic acid (FA) as in Fig. 1a and analyzed by IB with anti-Sis1p antibody. SDS-res., SDS-resistant aggregates. **b,** Formation of SDS-resistant NS-GFP aggregates and co-aggregation with Sis1p are independent of prion state.NS-GFP was expressed under the *GAL1* promoter in *ltn1*Δ [RNQ^+], *ltn1*Δ [rnq^-] or *ltn1*Δ*rqc2*Δ [RNQ^+] cells. Cell extracts were analyzed by IP with anti-GFP, followed by IB with anti-GFP antibody (left panel) or anti-Sis1p antibody (right panel). Interaction of Sis1p and NS-GFP in [rnq^-] state indicates that their interaction was not mediated by Rnq1p aggregates. **c,** Sis1-mCh co-localizes with NS-GFP inclusions in *ltn1*Δ cells. NS-GFP was expressed under the *GAL1* promoter at 30°C in cells with *SIS1*-mCh integrated into the *SIS1* locus in the chromosome. Live cells were analyzed by fluorescence microscopy. Scale bar, 5μm. **d,** NS-GFP aggregation and co-aggregation with Sis1p in *ltn1*Δ*rqc2*Δ cells is restored by expression of WT *RQC2* but not $rqc2_{aaa}$. NS-GFP was co-expressed with WT Rqc2p or $Rqc2_{aaa}$ in *ltn1*Δ*rqc2*Δ cells. NS-GFP was

expressed under the *GAL1* promoter and WT Rqc2p and Rqc2$_{aaa}$ were expressed under the *RQC2* promoter. Cell lysates were analyzed by IP with anti-GFP, followed by IB with anti-GFP antibody (left panel) or anti-Sis1p antibody (right panel). **e,**Formation of Sis1p positive inclusions in *ltn1*Δ cells not expressing recombinant NS-protein.WT or *ltn1*Δ cells expressing Sis1-GFP from the genomic *SIS1* locus were grown in YPD media at 30°C. Cells with ≥2 Sis1-GFP inclusions were quantified by analyzing >200 cells per condition in four independent experiments. Scale bar, 5μm. **f,** Sis1p in *ltnl*Δ cells in high molecular weight (HMW) aggregates. Cell extracts from WT, *ltnl*Δ and *ltn1*Δ*rqc2*Δ cells not expressing recombinant NS-protein were analyzed by Blue native-PAGE and IB with anti-Sis1p antibody. Pgk1p was used as a loading control. The positions of the native Sis1p dimer and of HMW forms are indicated. The amount of HMW Sis1p was quantified by densitometry and expressed as percent of total. Error bars s.d. from three independent experiments. p-values from Student's t-test. **g,**Aggregation of Sis1p in *ltn1*Δ*rqc2*Δ cells is restored by expression of WT *RQC2* but not *rqc2*$_{aaa}$. Extracts of *ltn1*Δ*rqc2*Δcells expressing WT Rqc2p or Rqc2$_{aaa}$ under the *RQC2* promoter were analyzed as in (f) without expression of recombinant NS-protein. **h,**Formation of SDS-resistant aggregates in *ltn1*Δ cells observed with Sis1-HA or Sis1-GFP.*SIS1* was chromosomally replaced by *SIS1*-HA or *SIS1*-GFP in WT or *ltn1*Δ cells. Tagged Sis1 proteins wereimmunoprecipitated with anti-HA antibody or anti-GFP antibody, followed by IB with anti-Sis1p antibody (right panel). Input fraction was analyzed with anti-Sis1p antibody (left panel). SDS-res., SDS-resistant aggregates.Gel slices corresponding to the position of SDS-resistant Sis1p aggregates were excised from gels and subjected to MS-analysis to identify proteins interacting with the aggregates (see Online Methods). **i,**Proteins in SDS-resistant Sis1p aggregates are of relatively high abundance in the total yeast proteome. Abundance values measured in total proteome in ppm are plotted[35].

Extended Data Figure 6 | Impairment of cytosolic protein quality control in *ltn1*Δ cells. **a,**CmCh* ubiquitylation is preserved in *ltn1*Δ cells. Followingexpression of CmCh* from *GAL1* promoter, His$_6$-tagged ubiquitin (His$_6$-Ub) expression from *CUP1* promoter was induced with $CuSO_4$ for 4 h beforeharvesting cells. Ubiquitylated proteins were isolated by His$_6$pull-down from cell lysates prepared in 6 M GdmCl to preserve polyubiquitylation. Eluates were analyzed by IB with anti-CPY antibody. The positions of CmCh* and of polyubiquitylated CmCh* (CmCh*-Ub$_n$) are indicated. **b,**Inhibition of degradation of CPY* in *ltn1*Δ cells is rescued by overexpression of Sis1p. CPY* fused to GFP (CG*) and N-terminally HA-tagged Sis1p (HA-Sis1p) were expressed from the *GAL1* promoter. The degradation of CG* was analyzed after inhibition of protein synthesis with cycloheximide as in Fig. 5a. CG* was detected by IB with anti-GFP antibody and Sis1p with anti-HA antibody (top panel). Pgk1p was used as a loading control. Data were quantified by densitometry (bottom panel). Error bars s.d. from three independent experiments. **c,** Sis1p co-aggregates with CmCh* in *ltn1*Δ cells. CPY*-mCherry (CmCh*) was expressed under the *GAL1* promoter at 30°C in *ltn1*Δ cells expressing Sis1-GFP from the genomic *SIS1* locus. Live cells were analyzed by fluorescence microscopy. Scale bar, 5 μm.**d,** Live cell fluorescence microscopy of WT and *ltn1*Δ cells co-expressing NS-GFP with CmCh* for 18 h at 30°C. Nuclei were counterstained with Hoechst 33342. DIC, differential interference contrast. Scale bar, 5μm.**e,** Sis1p overexpression does not suppress the formation of SDS-resistant NS-GFP aggregates in *ltn1*Δcells. NS-GFP and N-terminally HA-tagged Sis1p (HA-Sis1p) was expressed under the *GAL1* promoter in WT or *ltn1*Δ cells. Empty vector was

used as a control for HA-Sis1p. SDS-resistant aggregates were analyzed by IP with anti-GFP, followed by IB with anti-GFP antibody (left panel) or anti-Sis1p antibody (right panel).

Extended Data Figure 7 | Additional proteostasis stress causes growth defect of *ltn1*Δ cells. **a-c,** Growth phenotype of RQC mutant strains.Cells from WT and RQC mutant strains indicated were transformed with CmCh* expression vector under the *GAL1* promoter and were serially 5-fold diluted before spotting onto glucose medium (-Induction) and galactose/raffinose medium (+Induction). Plates were incubated for 3 days at 37°C. In (b) galactose inducible HA-tagged Sis1p was expressed. EV, empty vector. **d,** Hygromycin B (HygB) sensitivity of RQC mutant strains. Cells from WT and RQC mutant strains indicated were grown to exponential phase in liquid YPD medium, serially 5-fold diluted and spotted onto YPD plates with or without HygB (18 μg/ml). Plates without HygB were incubated for 2 days and with HygB for 3 daysat 37°C. **e,** Formation of Sis1p positive inclusions in *ltn1*Δ cells is enhanced under proteotoxic stress**.** *ltn1*Δ cells expressing Sis1-GFP replacing chromosomal *SIS1* were grown in YPD media at 30°C without or with hygromycin B (400 μg/ml) for 18 h. Live cell fluorescence microscopy was performed. Cells with Sis1-GFP inclusions were quantified by analyzing >200 cells per condition in four independent experiments. Data shown are an extension of the experiment shown in Extended Data Fig. 5e. Scale bar, 5μm.