Figures and figure supplements

The Rqc2/Tae2 subunit of the ribosome-associated quality control (RQC) complex marks ribosome-stalled nascent polypeptide chains for aggregation

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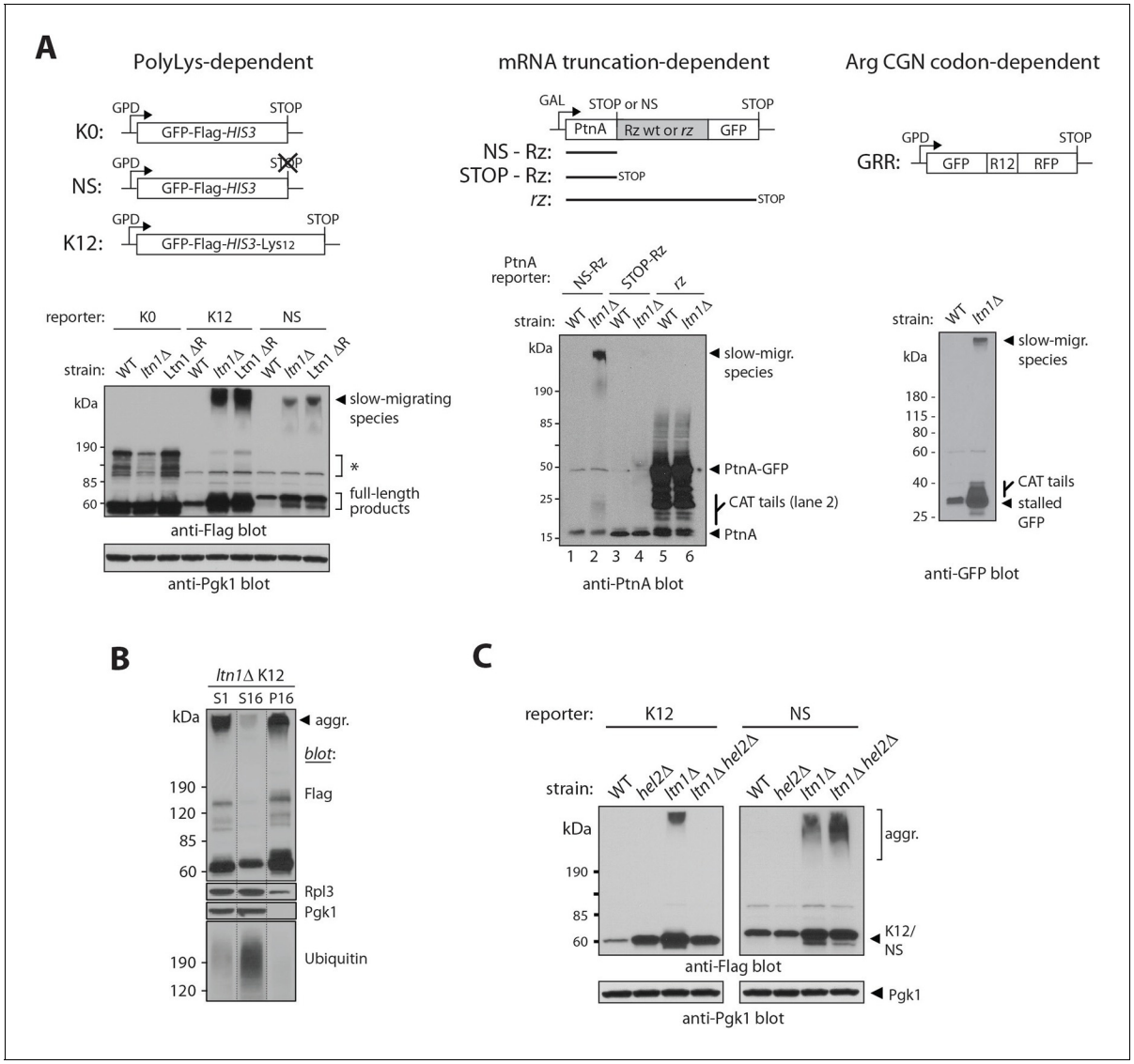

Figure 1. Stalled translation can lead to the formation of nascent chain aggregates. (**A**) *Top panels*, diagrams of reporter constructs encoding stalling-prone nascent chains and respective controls. PolyLys-dependent stalling (*left*): GFP-Flag-*HIS3* fusion protein control (K0), its *bona fide* nonstop (NS) protein derivative, and a derivative fused to 12 lysines (K12). Endonucleolytic mRNA cleavage-dependent stalling (*middle*): Protein A ZZ domain-Ribozyme-GFP fusion constructs. A self-cleaving ribozyme (Rz) within coding sequence generates a nonstop (NS) mRNA encoding stalled Protein A (PtnA). Controls are constructs with a cleavage-defective ribozyme generating a full-length PtnA-GFP fusion (*rz*), or with a stop codon preceding the Rz cleavage site (STOP-Rz), such that nascent PtnA is not expected to become stalled in ribosomes. Horizontal lines represent the encoded polypeptides. Arg CGN codon-dependent stalling (*right*): GFP-R12-RFP (GRR), where R12 is encoded by unpreferred Arg codons. *Lower panels*, reporter protein expression in a wild type strain (WT; BY4741), a *LTN1*-deleted strain (*ltn1*△) or a strain whose endogenous Ltn1 lacks the RING domain (Ltn1 △R). Immunoblots of SDS-boiled cell extracts: anti-Flag, anti-PtnA, or anti-GFP to monitor reporter expression, and anti-Pgk1 as loading control. The migration of CATylated species is indicated. Asterisks indicate bands of unknown identity. (**B**) Stalling reporter slow-migrating species are pelleted upon high speed centrifugation. The extract of a K12 reporter-expressing *ltn1*△ strain was pre-cleared by centrifugation at 1000 x *g* for 5 min and its supernatant (S1) was then subjected to 16,000 x *g* for 10 min. The resulting supernatant (S16) and pellet (P16) were analyzed by western blot against Flag tag (K12), Rpl3 (a 60S ribosomal protein), Pgk1 (phosphoglycerate kinase 1, a soluble protein) and ubiquitin (high-molecular weight conjugates migrating above 120 kDa are shown). (**C**) Translational stalling is required for reporter aggregation. NS and K12 reporter protein expression in strains lacking Ltn1 and/or the translational stalling factor, Hel2.

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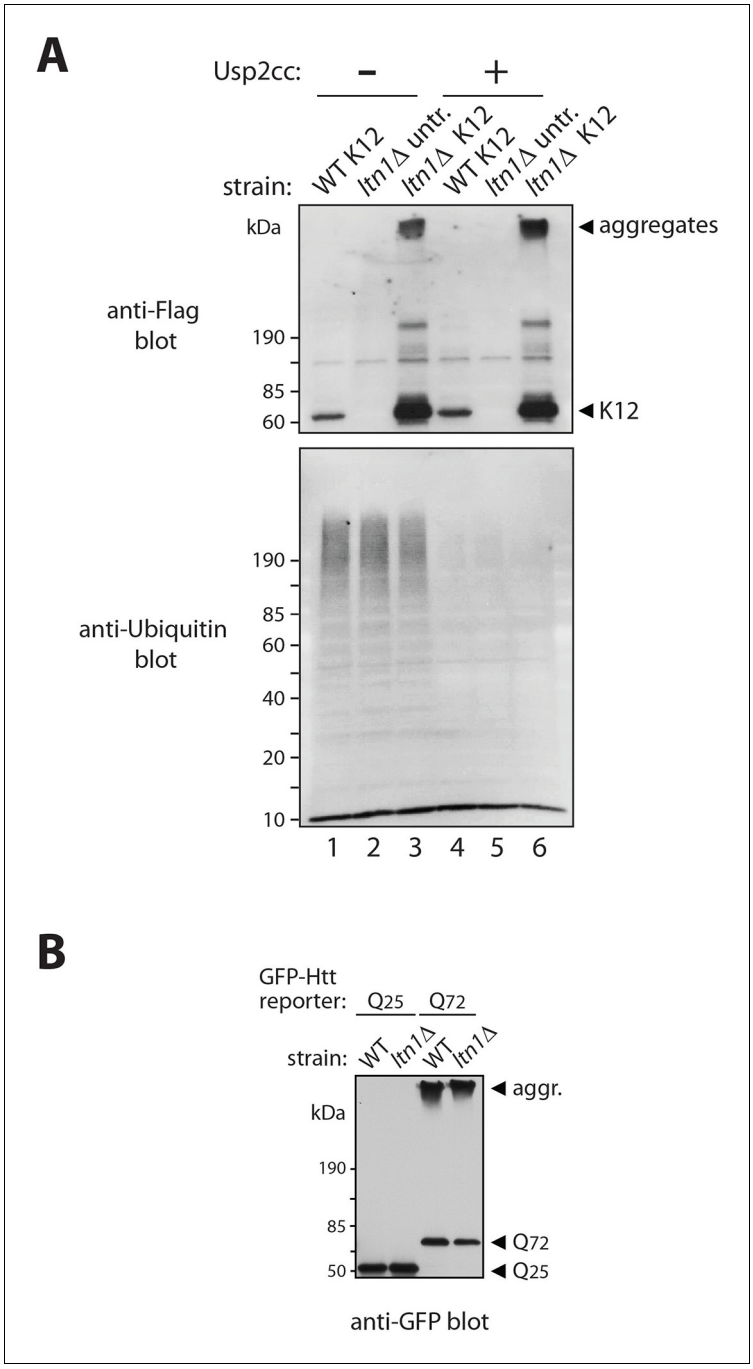

Figure 1—figure supplement 1. Stalled translation can lead to the formation of nascent chain aggregates. (**A**) The slow migration of stalling reporters expressed in *ltn1*△ cells is not due to poly-ubiquitylation. Strains utilized were wild type (WT), *ltn1*△ cells, and *ltn1*△ cells expressing the K12 reporter. Cell extracts were treated with the recombinant catalytic core of Usp2 (Usp2cc), which has general deubiquitylase activity, for 1h at room temperature, as described (***Kaiser et al., 2011***). The anti-Flag blot shows a lack of effect of Usp2cc on the migration of high molecular weight stalling reporter species (compare lanes 3 and 6) while the anti-ubiquitin blot confirms that the enzyme was able to efficiently disassemble ubiquitin chains linked to proteins in the extract. (**B**) Loss of *LTN1* is not generally associated with increased formation of protein aggregates. Expression of GFP-Huntingtin exon 1 polyglutamine reporters (Htt-Q25 and Htt-Q72) in WT and *ltn1*△ strain extracts revealed by anti-GFP immunoblot.

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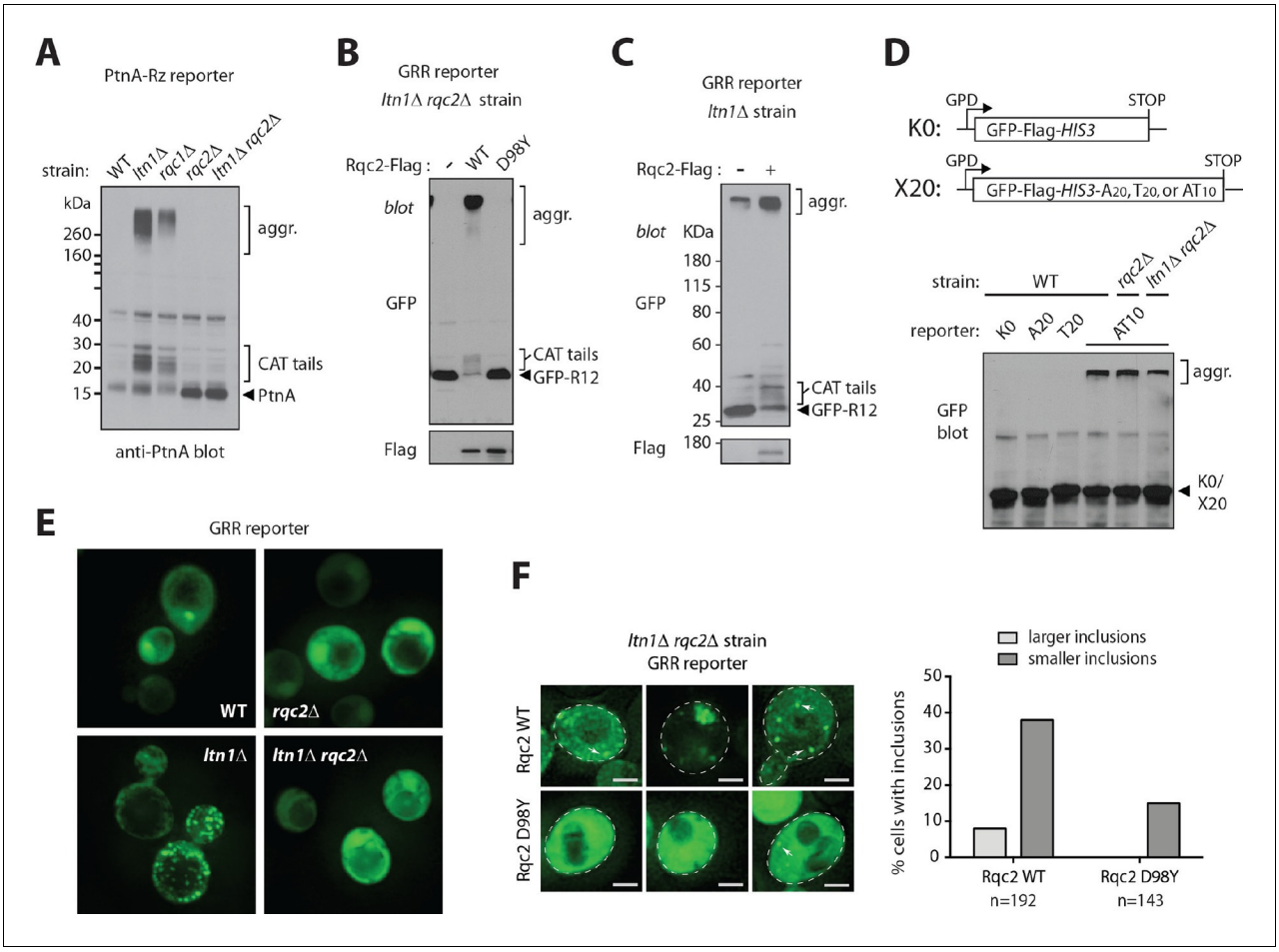

Figure 2. Rqc2-mediated modification of stalled nascent chains with CAT tails results in their aggregation. (**A**) NC CATylation correlates with aggregation—effects of *RQC1* and *RQC2* deletion. The indicated strains were transformed with the PtnA NS-Rz reporter. Reporter expression was monitored by immunoblot anti-PtnA. The migration of CATylated species is indicated. (**B**) An Rqc2 mutant defective in CAT tail synthesis fails to promote aggregation of stalled NCs. The *ltn1*△ *rqc2*△ strain expressing the GRR reporter was transformed with plasmids encoding Rqc2-Flag wild type (WT) or D98Y mutant. (**C**) Endogenous Rqc2 is limiting for NC CATylation and aggregation in *ltn1*△ cells. The *ltn1*△ strain expressing the GRR reporter was transformed or not with plasmid encoding Rqc2-Flag wild type (WT). Reporter expression was monitored by immunoblot anti-GFP. (**D**) Fusion of a CAT tail-mimetic sequence to the C-terminus of the K0 reporter protein suffices to promote aggregation independently of stalling or Rqc2. *Top panel*, diagram of constructs. *Lower panel*, as in 'a'. The indicated strains were transformed with plasmids encoding the parental reporter (K0, as described in 1a) or its derivatives fused to a C-terminal tail of 20 Ala, 20 Thr, or 10 Ala-Thr repeats, as indicated. (**E**) *Punctae* formed by stalling reporters in intact cells correlate with aggregates observed in WCE. Fluorescence microscopy imaging of indicated strains expressing the GRR reporter. GFP-positive *punctae* can be observed in the *ltn1*△ strain. (**F**) CAT tail-dependent incorporation of the GRR stalling reporter into *punctae*. *Left*, The *ltn1*△ *rqc2*△ strain was transformed with plasmids encoding Rqc2-Flag wild type (WT) or D98Y mutant as in panel 'B' and examined by fluorescence microscopy. Three different distribution patterns of the GFP signal that are representative for each strain are shown. Arrows point to selected *punctae*. Scale bar, 2 μm. *Right*, Quantification of cells harboring GFP-positive inclusions in the *ltn1*△ *rqc2*△ strains expressing Rqc2 WT or D98Y mutant.
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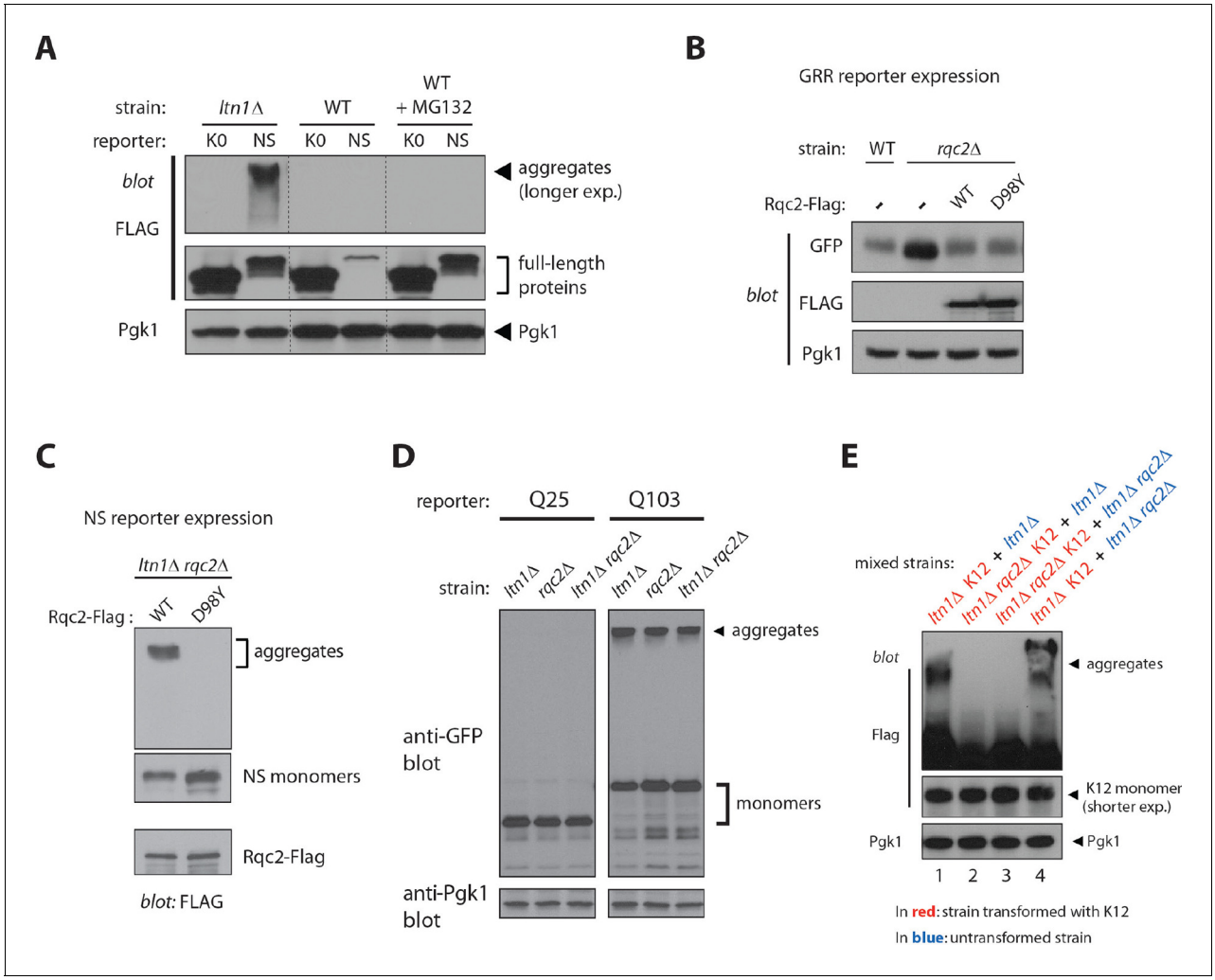

Figure 2—figure supplement 1. Rqc2-mediated modification of stalled nascent chains with CAT tails results in their aggregation. (**A**) Accumulation of stalling reporters by proteasome inhibition is not sufficient to result in aggregation. Immunoblot analysis of K0 and NS reporter expression in wild type or *ltn1*△ strains, after treatment (+) or not (-) with the proteasome inhibitor MG132 for 2 hr. (**B**) The Rqc2 D98Y mutant is competent to support Ltn1 function. Stalling reporter expression in wild type or *rqc2*△ strains transformed with empty vector, wild type Rqc2-Flag, or Rqc2-Flag D98Y. (**C**) CATylation is required for aggregation of the NS reporter. The *ltn1*△ *rqc2*△ strain expressing the NS reporter was transformed with plasmids encoding Rqc2-Flag wild type (WT) or D98Y mutant. Expression of NS monomers, NS aggregates, and Rqc2-Flag revealed by anti-Flag immunoblot. Panels with different exposure times shown. (**D**) Loss of *RQC2* is not generally associated with the failure to form protein aggregates. Expression of GFP-Huntingtin exon 1 polyglutamine reporters (Htt-Q25 and Htt-Q103) in the indicated strains revealed by anti-GFP immunoblot. (**E**) Stalling reporter aggregates do not form post-lysis. K12 reporter-expressing strains (labeled in red) were mixed 1:1 with a second, untransformed strain (labeled in blue) before lysis, and extracts were analyzed for aggregate formation by anti-Flag immunoblot. The presence of the *ltn1*△ strain constituents during lysis was not sufficient to promote aggregation of the K12 reporter expressed in a *ltn1*△ *rqc2*△ strain (lane 2). Conversely, the presence of constituents of the *ltn1*△ *rqc2*△ strain did not interfere with aggregates formed by K12 reporter-expressing *ltn1*△ strain (compare lane 4 to lane 1).

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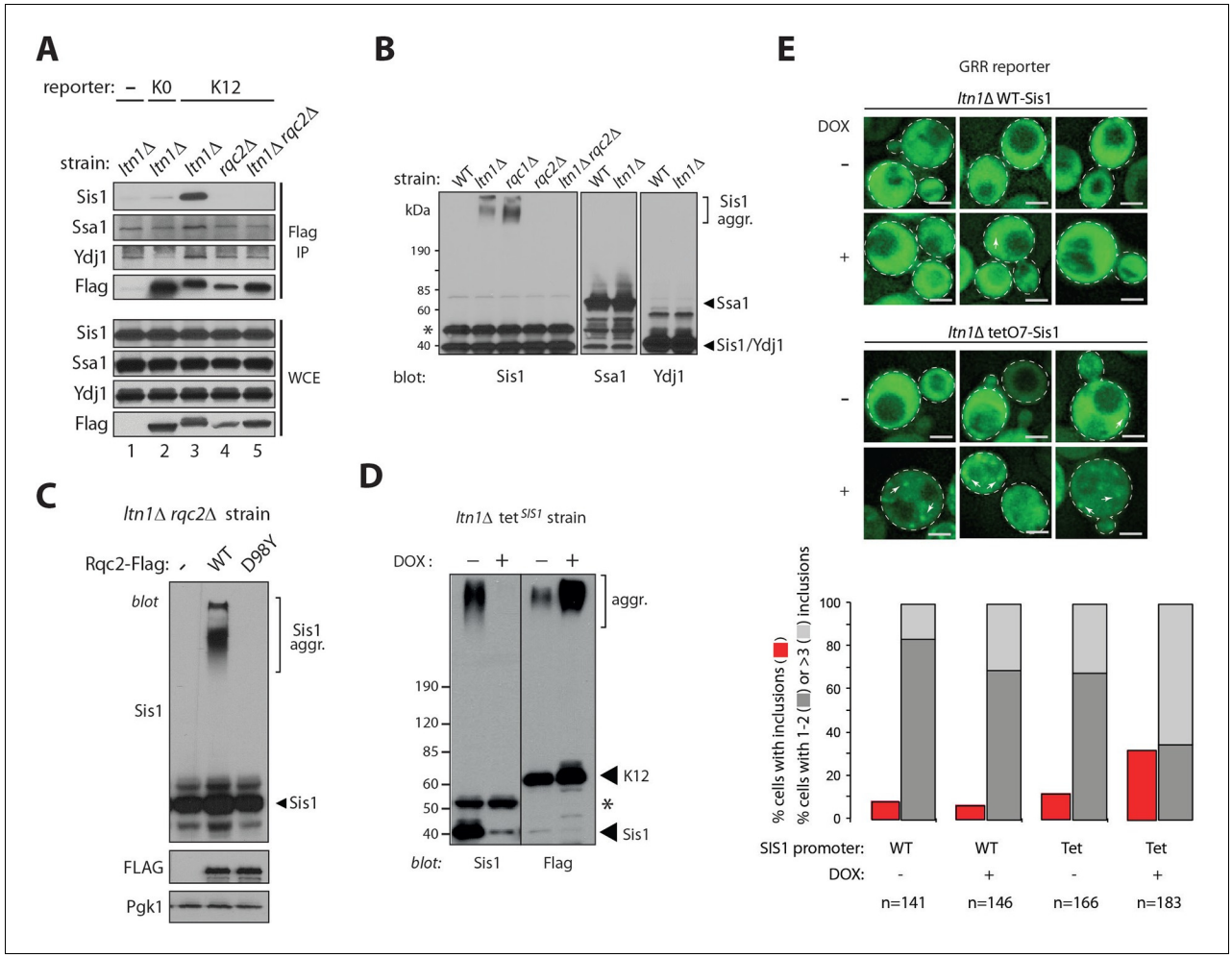

Figure 3. Sis1 association reveals endogenous stalled nascent chain aggregates. (**A**) Stalling reporters co-IP with Sis1 in an Rqc2-dependent manner. Whole cell extracts (WCE) of the indicated strains were Flag IP'ed (to pull down K0 or K12 reporters), followed by immunoblotting as indicated to the left of the panels. (**B**) Sis1 associates tightly with Rqc2-dependent aggregates formed by endogenous proteins in cells deficient for Ltn1 or Rqc1. WCE of the indicated strains were immunoblotted against Sis1, Ssa1, and Ydj1, as indicated. The ~47 kDa band in the Sis1 blot (asterisk) is nonspecific. (**C**) The formation of slow-migrating Sis1 species is dependent on Rqc2's ability to synthesize CAT tails. WCE of the *ltn1*△ *rqc2*△ strain expressing Rqc2-Flag wild type or D98Y mutant were analyzed by immunoblotting. (**D**) Sis1 depletion increases NC aggregation. *ltn1*Δ tetO7-Sis1 cells expressing the K12 stalling reporter were treated or not with doxycycline (DOX). WCE were analyzed by immunoblot against Sis1 (*left panel*) or Flag (*right panel*; for K12 detection). *Asterisk*, cross-reacting band. (**E**) Sis1 depletion increases GFP *punctae* formation in *ltn1*Δ cells. *ltn1*Δ WT-Sis1 or *ltn1*Δ tetO7-Sis1 cells expressing the GRR stalling reporter were grown for 24 hr in the presence (+) or absence (-) of doxycycline (DOX). *Top*, Three representative images are presented for each strain and treatment condition. Scale bar, 2 μm. Arrows point to selected *punctae*. *Bottom*, Quantification of cells harboring GFP *punctae* is represented by red bars; among those, the fraction of cells with 1 or 2 *punctae* is represented in dark gray, and the fraction of cells with 3 or more *punctae*, in light gray.

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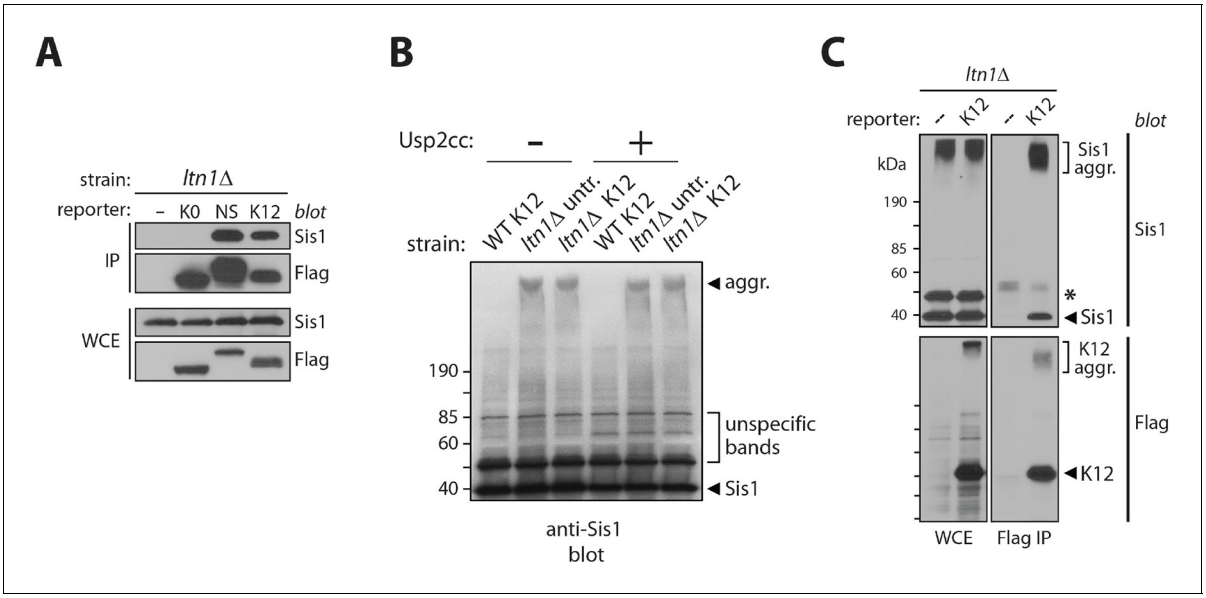

Figure 3—figure supplement 1. Sis1 association reveals endogenous stalled nascent chain aggregates. (**A**) The stalling reporters NS and K12, but not the parental K0, co-IP with Sis1. Whole cell extracts (WCE) of *ltn1Δ* strains expressing the indicated reporters were subjected to Flag IP, and analyzed by anti-Sis1 (or Flag control) immunoblot. (**B**) The migration of the high molecular weight Sis1 species present in *ltn1Δ* cells is not affected by treatment with a deubiquitylating enzyme (see ***Figure 1—figure supplement 1A***). Extracts from the indicated strains were treated with Usp2cc and analyzed by anti-Sis1 immunoblot. (**C**) Sis1 aggregates co-IP with stalling reporters. Extracts of Ltn1-deficient cells, untransformed or expressing the K12 reporter, were used for Flag IP (to pull down K12) and immunoblotted against Sis1 or Flag tag, as indicated. *Asterisk*, nonspecific band.

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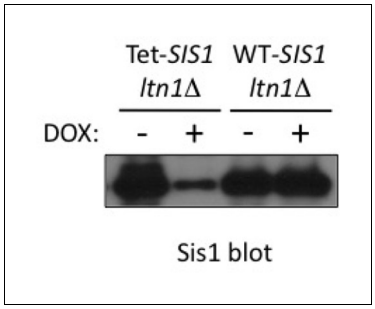

Figure 3—figure supplement 2. *tetO7* promoter-dependent Sis1 depletion. *ltn1Δ tetO7-SIS1* and *ltn1Δ* WT-*SIS1* cells were treated with doxycycline as indicated, and analyzed by western blot using antibodies against Sis1.
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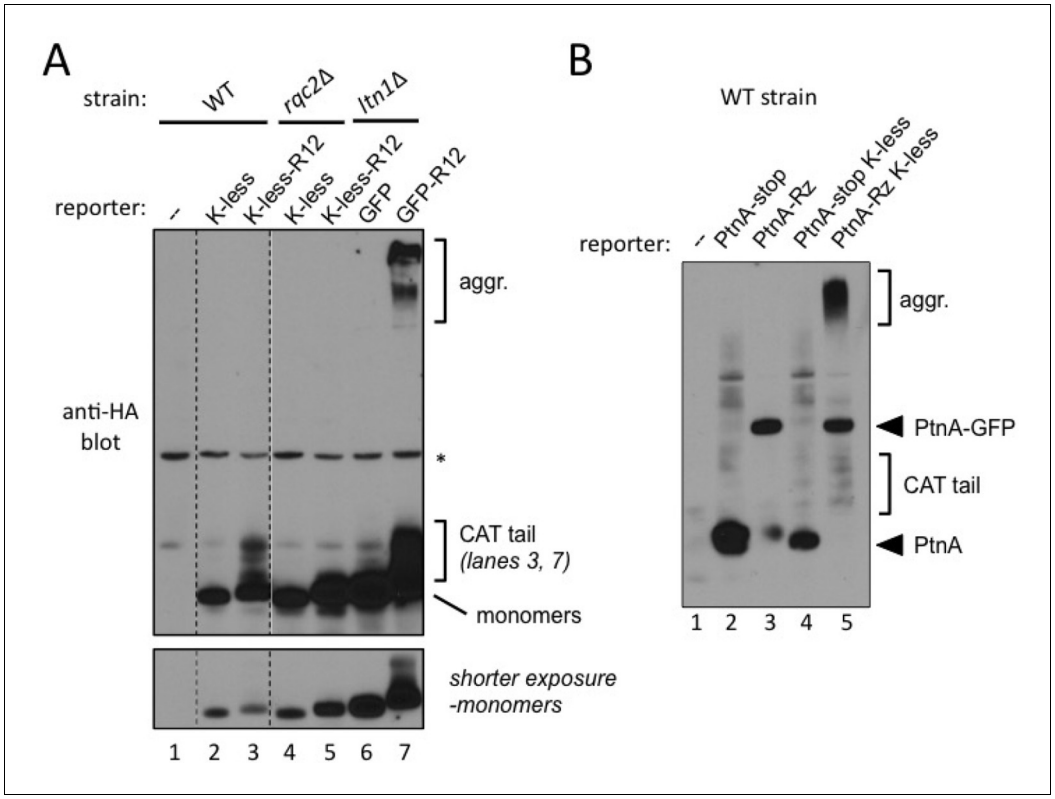

Figure 4. Evidence for stalled nascent chain modification with CAT tails and aggregation in wild type cells. (**A**) Stalling Lys-less reporter modification with CAT tails in wild type yeast. All constructs were HA-tagged. Expression of GFP, GFP-R12, GFP K-less ('K-less'), or GFP K-less R12 ('K-less-R12') reporter proteins in the indicated strains, revealed by anti-HA immunoblot. 'R12' is the stalling signal, consisting of 12 suboptimal Arg CGN codons. GFP-R12 expression in *ltn1*△ cells is used as a control for aggregate formation. *Lower panel*, shorter exposure to reveal relative steady-state levels of monomeric reporter species. (**B**) Stalling PtnA-Rz reporter CATylation and aggregation in wild type yeast. All constructs were HA-tagged. Expression of PtnA-STOP-Rz, PtnA-Rz, PtnA-STOP-Rz K-less, and PtnA-Rz K-less reporter proteins in the wild type strain, revealed by anti-HA immunoblot.
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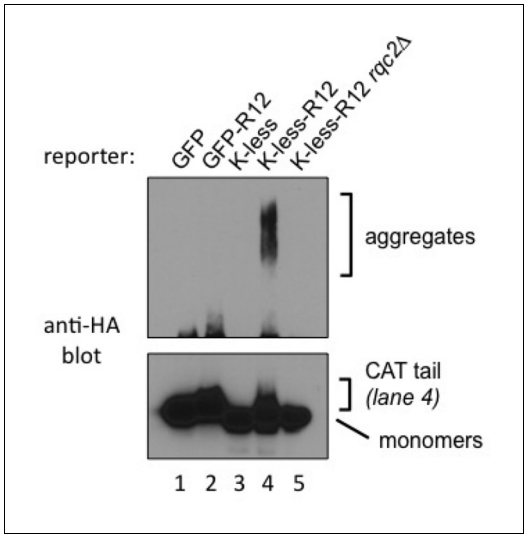

Figure 4—figure supplement 1. Stalling NC reporter aggregation in wild type yeast. HA-tagged GFP, GFP-R12, GFP K-less ('K-less'), or GFP K-less R12 ('K-less R12') reporters expressed in the MG132-treated wild type strain (or MG132 treated *rqc2*△ strain as a control, lane 5) were concentrated by anti-HA IP and analyzed by anti-HA immunoblot. *Lower panel*, the monomeric forms are shown in a shorter exposure to reveal relative steady-state levels of monomeric reporter species, as well as CAT tails in lane 4.
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