PROTEOSTASIS

CAT-tailing as a fail-safe mechanism for efficient degradation of stalled nascent polypeptides

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Ribosome stalling leads to recruitment of the ribosome quality control complex (RQC), which targets the partially synthesized polypeptide for proteasomal degradation through the action of the ubiquitin ligase Ltn1p. A second core RQC component, Rqc2p, modifies the nascent polypeptide by adding a carboxyl-terminal alanine and threonine (CAT) tail through a noncanonical elongation reaction. Here we examined the role of CAT-tailing in nascent-chain degradation in budding yeast. We found that Ltn1p efficiently accessed only nascent-chain lysines immediately proximal to the ribosome exit tunnel. For substrates without Ltn1p-accessible lysines, CAT-tailing enabled degradation by exposing lysines sequestered in the ribosome exit tunnel. Thus, CAT-tails do not serve as a degron, but rather provide a fail-safe mechanism that expands the range of RQC-degradable substrates.

In eukaryotic cells, when translation fails (e.g., due to a faulty mRNA or ribosome stalling), the incomplete nascent chain is targeted for proteasomal degradation (*1*) by a conserved ribosome-associated quality control pathway (*2*). This process involves disassociation of the mRNA and 40*S* subunit followed by recruitment of the ribosome quality control complex (RQC) (*3*–*7*) to the 60*S* subunit-nascent chain complex (Fig. 1A). The RQC in *Saccharomyces cerevisiae* comprises Rqc1p, Rqc2p, the E3 ubiquitin ligase Ltn1p, and Cdc48p. Rqc2p acts both to recruit Ltn1p and to facilitate tagging of partially synthesized polypeptides with a carboxyl-terminal alanine and threonine (CAT) tail in a noncanonical elongation reaction (CAT-tailing) (*8*). CAT-tails facilitate protein aggregation when RQC-mediated degradation is compromised, leading to chaperone sequestration and proteotoxic stress (*9*–*11*). However, an Rqc2p mutant defective in CAT-tailing ($Rqc2p^{mut}$) fully supports Ltn1p-dependent degradation of the limited number of stalling substrates studied so far (*8*–*11*). Thus, the biological role of CAT-tailing in the context of a functional RQC remains unclear.

To investigate the role of CAT-tailing in nascent polypeptide degradation, we designed two stalling constructs (GFP_{20Lys} and $GFP_{Lys\text{-}free}$; GFP, green fluorescent protein) that differed in whether they encoded lysines, the canonical ubiquitin acceptor (Fig. 1, B and C). Both constructs contained a

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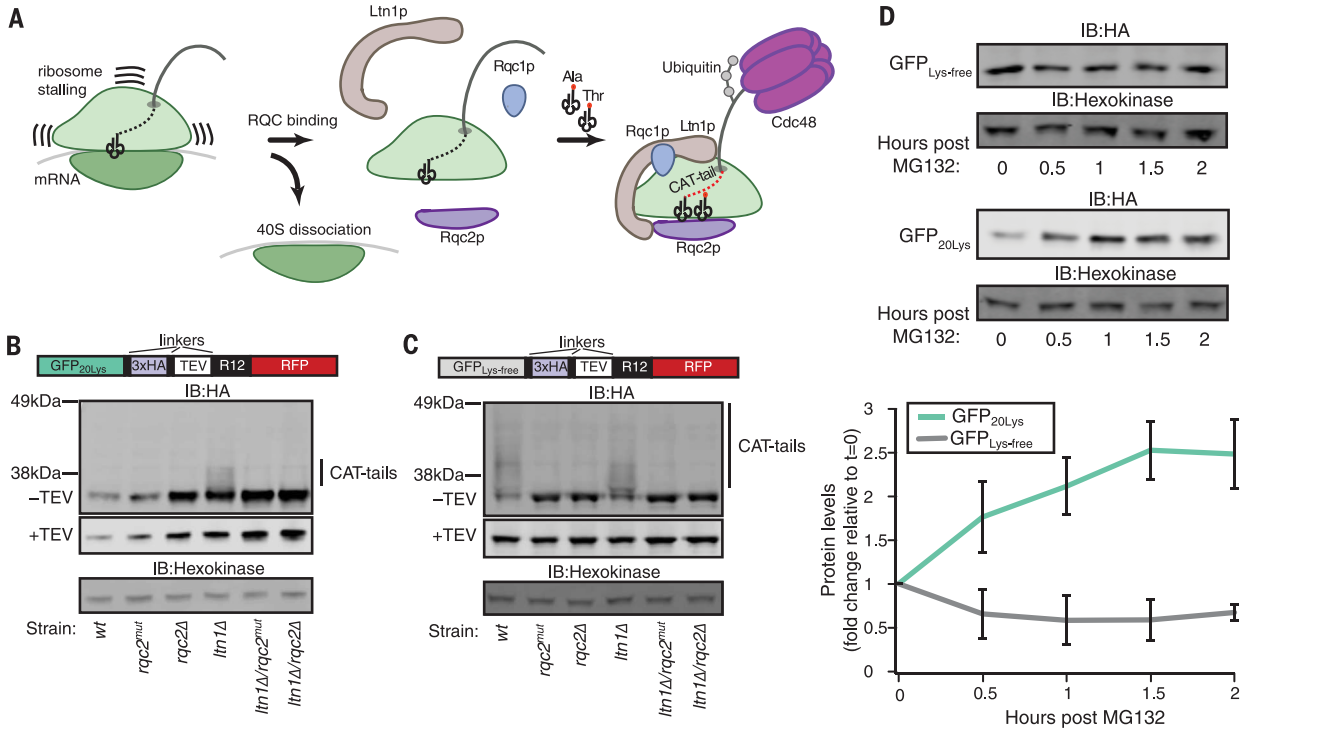

Fig. 1. Degradation of RQC substrates requires lysines. (**A**) Model for RQC-mediated degradation of nascent polypeptides. (**B** and **C**) Immunoblots (IBs) of stalling reporters with or without lysines in RQC deletion strains. (**D**) Proteasome inhibition time course for stalling constructs expressed in *pdr5*Δ cells. The relative amount of stalling substrate (mean ± SD, *N* = 3) accumulating over time was visualized by IB (top) and quantified by densitometry (bottom).

polyarginine track (R12) that efficiently blocked translation (*12*, *13*) (fig. S1). The resulting GFP_{20Lys} nascent polypeptide was degraded in an RQC-dependent manner (Fig. 1B and fig. S2A). In *ltn1Δ* cells, we detected higher molecular weight CAT-tailed species, which collapsed into a discrete band upon removal of C-terminal extensions by tobacco etch virus (TEV) protease treatment (Fig. 1B, middle panel). GFP_{20Lys} was not further stabilized in *ltn1Δ* cells upon proteasomal inhibition (fig. S2, C and D). Finally, as expected (*8*), GFP_{20Lys} was efficiently degraded in the CAT-tailing–deficient $rqc2^{mut}$ strain, confirming that CAT-tails are not necessary for degradation of this substrate (Fig. 1B).

In contrast to GFP_{20Lys}, $GFP_{Lys\text{-}free}$ was heavily CAT-tailed even in wild-type (*wt*) cells (Fig. 1C), as expected (*10*). However, these CAT-tails did not trigger degradation, because comparable amounts of $GFP_{Lys\text{-}free}$ accumulated in *wt* cells and RQC-deletion strains (Fig. 1C and fig. S2B). Proteasome inhibition did not lead to additional substrate stabilization (Fig. 1D), providing evidence that CAT-tails do not serve as a degron and that the presence of lysines is a key requirement for nascent polypeptide degradation.

Cryo–electron microscopy structures of Ltn1p bound to the 60*S* subunit have revealed that the RING domain of Ltn1p, which is required for ubiquitin transfer, is held in close proximity to the ribosome exit tunnel (*14*, *15*). Thus, the position of lysines along the nascent polypeptide may determine the ability of Ltn1p to ubiquitinate them. Indeed, comparing three stalling constructs that differed only in the number of amino acids between the stalling site and the most C-terminal lysine of GFP (Fig. 2A) revealed that the construct with a short (37 amino acids) linker was robustly degraded, but constructs with extended linkers (51 and 75 amino acids) were increasingly resistant to RQC-mediated degradation.

These results suggest that the efficient RQC-dependent degradation of nascent polypeptides requires the presence of lysines in proximity to the Ltn1p RING domain. To further explore this idea, we designed stalling constructs with four lysines in an unstructured region (3xFLAG tag) that were systematically displaced from the R12 stalling site by 0, 10, 20, 40, or 80 amino acids of neutral XTEN linker (*16*) (XTEN0-80, respectively) (Fig. 2B and fig. S3). For these constructs, the four lysines will be either sequestered in (XTEN0), emerging from (XTEN10, XTEN20), or past (XTEN40, XTEN80) the ribosome exit tunnel (fig. S5A). Initially, we used the relative degradation of the constructs as a proxy for Ltn1p's ability to ubiquitinate the lysines.

In contrast to previously studied RQC substrates (*8*), XTEN0 was efficiently degraded only in cells capable of CAT-tailing (Fig. 2B and fig. S5B), in an Ltn1p-dependent manner (fig. S4A). $Rqc2p^{mut}$ did not rescue degradation even when overexpressed (fig. S4B), suggesting that the defect was caused by the lack of CAT-tailing. Similar behaviors were seen for analogous constructs based on yeast His3p (fig. S6). These data provide evidence that that CAT-tail elongation pushes sequestered lysines out of the exit tunnel, making them available for Ltn1p-mediated ubiquitination, as hypothesized (*2*, *17*).

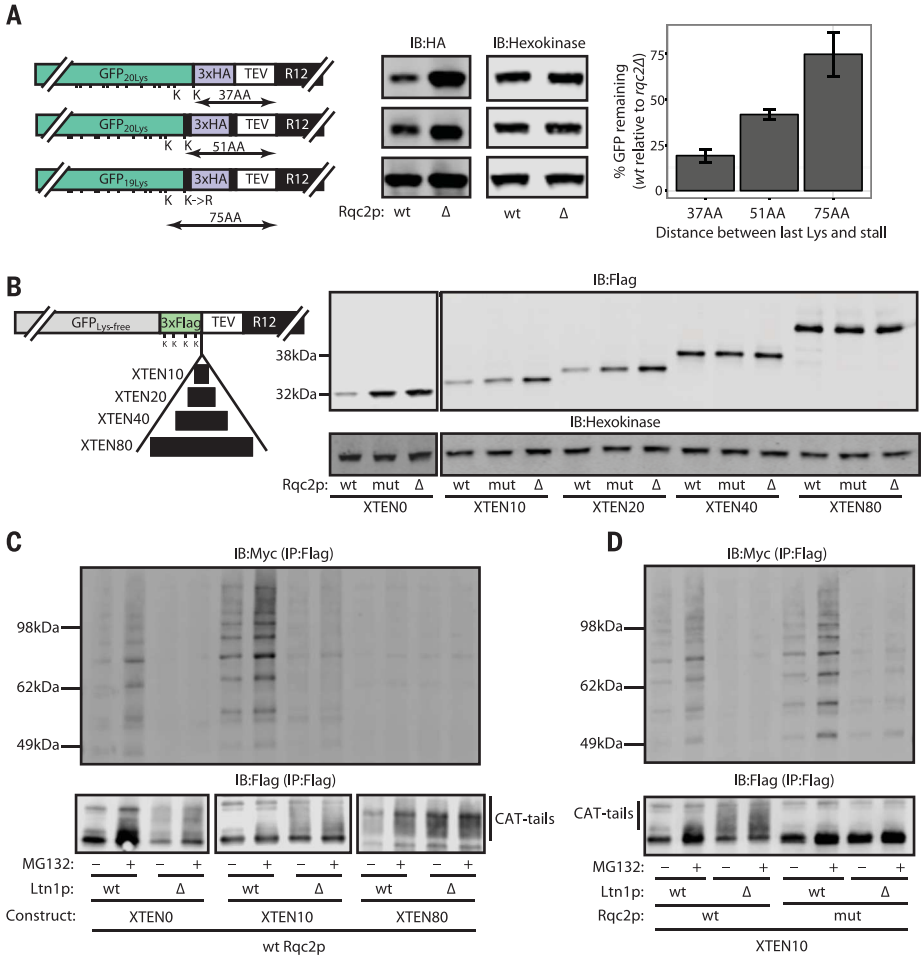

Fig. 2. Lysine positioning is critical for Ltn1p-mediated ubiquitination and proteasomal degradation. (**A**) IB of stalling constructs with a variable number of amino acids (AA) between the most C-terminal lysine of GFP and the first arginine of the stall site. The relative amount of substrate remaining in *wt* cells relative to *rqc2Δ* cells was quantified and normalized to a loading control (bar graph) (mean ± SD, *N* = 3). (**B**) IB of TEV-treated XTEN constructs. (**C** and **D**) Denaturing immunoprecipitation (IP:Flag) of XTEN 0, 10, and 80 stalling constructs from cells expressing Myc-tagged ubiquitin.

Compared to XTEN0, degradation of XTEN10 and XTEN20 became less dependent on CAT-tailing (Fig. 2B). Indeed, when the lysines were positioned past a certain distance (XTEN40 and XTEN80), the substrates were completely resistant to RQC-mediated degradation. Consistent with these degradation results, we observed Ltn1p-dependent ubiquitination when lysines were positioned in the exit tunnel or within the Ltn1p-accessible region, and no ubiquitination was observed when the lysines were past the Ltn1p-accessible window (Fig. 2C and figs. S7 and S8). In addition, we observed similar levels of ubiquitination in cells expressing wild-type or mutant Rqc2p (Fig. 2D). These data support a model in which Ltn1p can only access a limited window of amino acids on the nascent polypeptide. Furthermore, the process of CAT-tailing enables Ltn1p to gain access to lysines sequestered in the exit tunnel.

To determine the effect of sequence and structural context on Ltn1p's ability to ubiquitinate lysines, we generated $GFP_{Lys\text{-}free}$ constructs with a 3xHA tag (HA, hemagglutinin) upstream of a single lysine positioned at various distances from the R12 stalling site (Fig. 3A and fig. S9A). These constructs were degraded in a CAT-tail–dependent manner when the lysine was within or near the ribosome exit tunnel, but not when the lysine was 49 amino acids away. The inability of the RQC to degrade this latter substrate was not caused by steric hindrance, because positioning the lysine between two unstructured regions did not rescue degradation (Fig. 3B). The discrete position of the lysines for these constructs allowed us to refine the Ltn1p-accessible window to ~12 amino acids outside the ribosome exit tunnel (Fig. 3, C and D). As observed with the FLAG-based constructs, the differences in degradation were

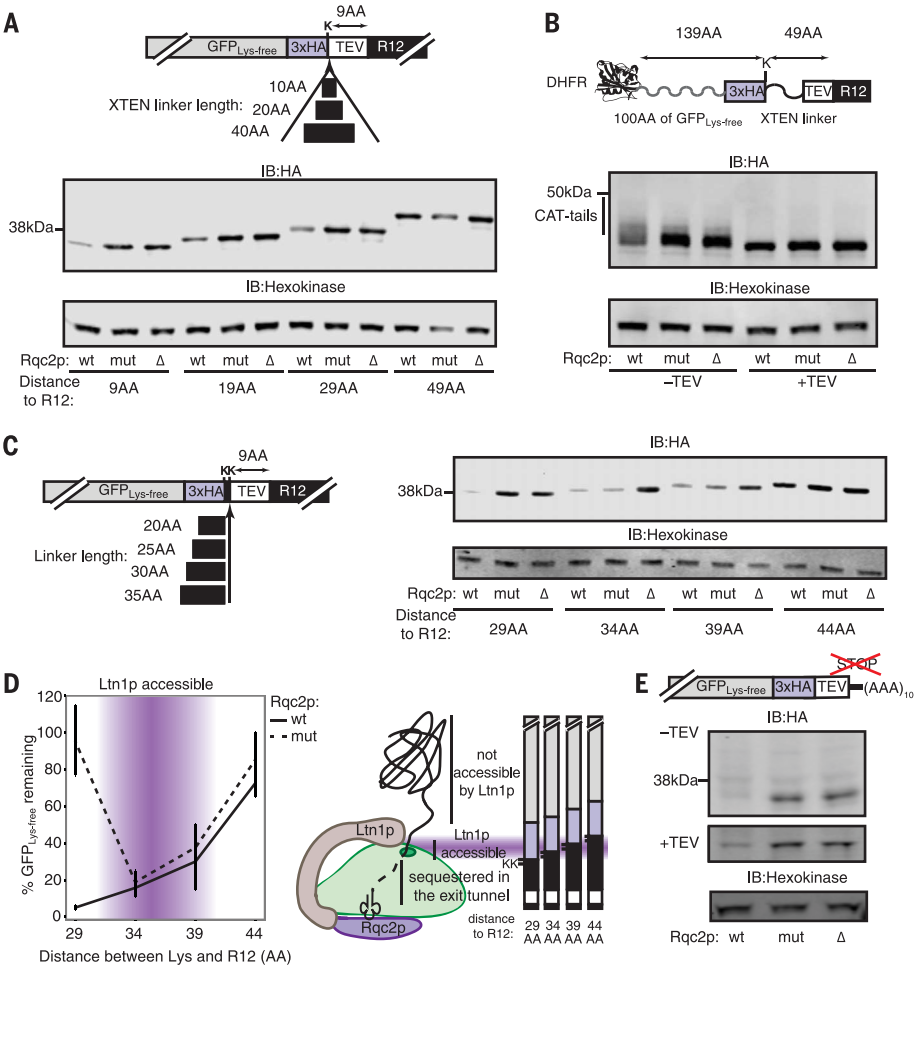

Fig. 3. The ability of Ltn1p to access lysines outside the exit tunnel is primarily determined by the distance of the lysines from the C terminus. (**A** and **C**) IB of TEV-treated $GFP_{Lys\text{-}free}$ stalling constructs with XTEN linkers of the indicated length inserted after the lysines. (**B**) IB of a stalling construct with a lysine positioned between two unstructured sequences. DHFR was used to stabilize the N terminus of the construct. (**D**) Quantification of protein levels from the stalling constructs in (C) (left). Shown is the percentage of stalling construct remaining in *wt* or $rqc2^{mut}$ cells relative to *rqc2*Δ cells as a function of the distance between the lysines and the R12 stalling site (mean ± SD, $N = 3$). Schematic of Ltn1p's accessible region (shaded area) (right). (**E**) IB of non-stop $GFP_{Lys\text{-}free}$ construct.

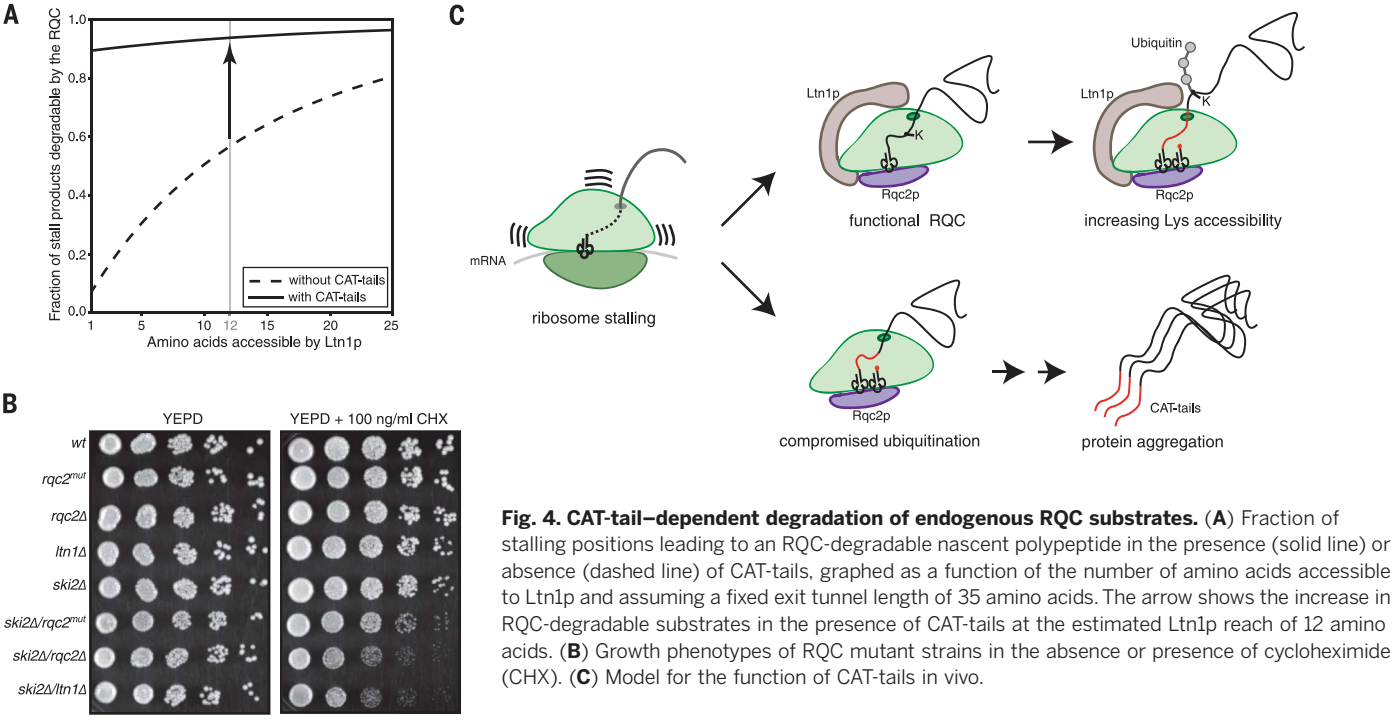

Fig. 4. CAT-tail–dependent degradation of endogenous RQC substrates. (**A**) Fraction of stalling positions leading to an RQC-degradable nascent polypeptide in the presence (solid line) or absence (dashed line) of CAT-tails, graphed as a function of the number of amino acids accessible to Ltn1p and assuming a fixed exit tunnel length of 35 amino acids. The arrow shows the increase in RQC-degradable substrates in the presence of CAT-tails at the estimated Ltn1p reach of 12 amino acids. (**B**) Growth phenotypes of RQC mutant strains in the absence or presence of cycloheximide (CHX). (**C**) Model for the function of CAT-tails in vivo.

consistent with differences in Ltn1p-mediated ubiquitination of the substrates (figs. S10 and S11). Notably, the Ltn1p-accessible window defined using four lysines (Fig. 2B) was fully consistent with the refined window (fig. S9B). Thus, the ability of Ltn1p to effectively access residues in a limited window proximal to the exit tunnel is primarily determined by the distance of the lysines from the C terminus, rather than local sequences or structure.

mRNAs lacking a stop codon (non-stop mRNAs) are an important source of natural RQC substrates with lysines sequestered in the exit tunnel (*2*). For such messages, the ribosome translates the polyadenine [poly(A)] tail, which appends AAA-encoded lysines to the C terminus. The resulting lysines are likely to be sequestered in the ribosome exit tunnel, owing to the short length of yeast poly(A) tails (median 27 nucleotides) (*18*, *19*). Indeed, degradation of non-stop $GFP_{Lys\text{-}free}$ with a defined poly(A) tail of 30 nucleotides was dependent on CAT-tails (Fig. 3E). Thus, CAT-tailing can expose lysines that result from translation through the poly(A) tract, allowing efficient ubiquitination and degradation of non-stop decay substrates.

Having established lysine positioning as a critical determinant of CAT-tail–dependent degradation, we next computationally evaluated the frequency with which endogenous substrates would be expected to rely on CAT-tailing for degradation. Although the sites of endogenous stalling remain poorly defined, several processes such as mRNA fragmentation, oxidative damage (*20*), or stress from translation inhibitors can cause stalling at any position along a message. We therefore considered the nascent chains produced if ribosomes stall with uniform probability at each codon along every coding sequence in the yeast genome (*21*), and we calculated the fraction of potential stalling sites for which there are no lysines accessible to Ltn1p but at least one lysine "hidden" in the ribosome exit tunnel (fig. S12). Based on our experimental estimates of Ltn1p's reach (~12 amino acids) and previous measurements of the length of the ribosome exit tunnel (~35 amino acids) (*22*, *23*), we estimate that CAT-tailing would substantially increase the fraction of RQC-degradable substrates, from ~60% of possible nascent chains to ~95% (Fig. 4A).

This analysis suggests that if CAT-tailing is compromised, the accumulation of nondegradable endogenous substrates will lead to growth defects. Although yeast strains with deletions of RQC components do not exhibit a growth defect in rich media (*5*, *9*), such strains have increased sensitivity to the translation inhibitor cycloheximide (CHX) (*24*). Furthermore, deletion of mRNA-decay factors (e.g., *SKI2*) stabilizes defective or truncated mRNAs, increasing the stalling burden on the cell (*25*). Indeed, under conditions of widespread stochastic ribosome stalling induced by CHX treatment and mRNA stabilization in a *ski2*Δ background, $rqc2^{mut}$ cells exhibited a growth defect intermediate between that of *wt* and RQC-deletion cells, consistent with the hypothesis that a substantial fraction of the stalled nascent polypeptides depend on CAT-tailing for efficient degradation (Fig. 4B).

Collectively, our studies reveal an unanticipated feature of Ltn1p, the key ubiquitin ligase responsible for RQC-mediated degradation of incomplete nascent chains: Ltn1p can only efficiently access lysines that lie within a narrow window of the exit tunnel. This spatial specificity could protect the cell from collateral damage [e.g., degradation of ribosomal proteins or the translocation machinery, as well as unregulated quality control signaling (*26*, *27*)] caused by incidental ubiquitination by Ltn1p. However, the limited reach of Ltn1p presents a challenge to the RQC machinery that must deal with a diverse range of substrates: Without a mechanism to relieve this restriction on lysine positioning, many endogenous RQC substrates would be resistant to Ltn1p-mediated degradation. Our studies reveal that, in addition to its previously described role in promoting aggregation and inducing a heat shock response (*9*–*11*), CAT-tailing acts as a fail-safe mechanism that enables the degradation of a far broader range of substrates by exposing lysines sequestered in the ribosome exit tunnel (Fig. 4C).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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Tables S1 to S3
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A tale of CAT tails

When protein translation fails, the incomplete nascent polypeptide is targeted for degradation by the highly conserved ribosome-associated quality control complex (RQC). Mutations in RQC components lead to stress at the cellular level and neurodegeneration at the organismal level. Recent studies have shown that RQC tags partially synthesize proteins with C-terminal alanine and threonine (CAT) tails in an unusual elongation reaction. Working in yeast, Kostova *et al.* elucidated the role of this process. CAT-tailing is a fail-safe mechanism to ensure the degradation of partially synthesized proteins. The elongation process appears to "push" lysines out of the ribosome exit tunnel, which allows them to be marked by ubiquitin degradation signals.

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