A reference-based protein degradation assay without global translation inhibitors

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Although it is widely appreciated that the use of global translation inhibitors, such as cycloheximide, in protein degradation assays may result in artefacts, these inhibitors continue to be employed, owing to the absence of robust alternatives. We describe here the promoter reference technique (PRT), an assay for protein degradation with two advantageous features: a reference protein and a gene-specific inhibition of translation. In PRT assays, one measures, during a chase, the ratio of a test protein to a long-lived reference protein, a dihydrofolate reductase (DHFR). The test protein and DHFR are coexpressed, in the yeast Saccharomyces cerevisiae, on a low-copy plasmid from two identical PTDH3 promoters containing additional, previously developed DNA elements. Once transcribed, these elements form 5′-RNA aptamers that bind to the added tetracycline, which represses translation of aptamer-containing mRNAs. The selectivity of repression avoids a global inhibition of translation. This selectivity is particularly important if a component of a relevant proteolytic pathway (e.g. a specific ubiquitin ligase) is itself short-lived. We applied PRT to the Pro/N-end rule pathway, whose substrates include the short-lived Mdh2 malate dehydrogenase. Mdh2 is targeted for degradation by the Gid4 subunit of the GID ubiquitin ligase. Gid4 is also a metabolically unstable protein. Through analyses of short-lived Mdh2 as a target of short-lived Gid4, we illustrate the advantages of PRT over degradation assays that lack a reference and/or involve cycloheximide. In sum, PRT avoids the use of global translation inhibitors during a chase and also provides a “built-in” reference protein.

In vivo half-lives of intracellular proteins range from less than a minute to many days (1–6). The term “half-life” is, at best, an approximate descriptor of a protein’s degradation curve. The reasons for this include (i) a significant (and varying) probability of cotranslational degradation of a nascent (still being made) polypeptide chain (7–12); (ii) the process of conformational maturation, i.e. transitions from a newly formed, still partially unfolded (and often more vulnerable to proteolysis) monomer of a protein to its final state, either as a folded monomer or an oligomer of the homo or hetero kind (mature proteins are usually more resistant to proteolysis in part through steric shielding of their degradation signals (degrons)) (4, 5, 13–19); (iii) a variety of chemical (usually enzymatic) modifications of a protein that can change its metabolic stability either cotranslationally or posttranslationally (e.g. phosphorylation of a protein may either activate or inhibit its degron) (20–27); and (iv) posttranslational in vivo cleavage(s) of a protein by a nonprocessive protease, such as, for instance, a calpain or a caspase (28–30). The rates of subsequent degradation of the resulting protein fragments can be quite dissimilar. The in vivo destruction of a protein usually involves either its polyubiquitylation, followed by the proteasome-mediated degradation, or the entrapment of a protein in an autophagosome, followed by the lysosome-mediated proteolysis. Some proteins can be targeted through both of these routes concurrently, depending on the nature of a protein, its degron(s), and the physiological state of a cell (31).

These and other complexities of intracellular protein degradation make it particularly important to have the means for determining the in vivo decay curves of specific proteins with high accuracy and through methods that do not perturb the rates of proteolysis that these methods are designed to measure. A variety of protein degradation assays are currently in use, including pulse-chases as well as proteome-scale techniques based on mass spectrometry (19, 32–48).

One class of widely employed approaches, historically among the first to be introduced, are assays for in vivo protein degradation that involve the use of a global translation inhibitor to halt protein synthesis, followed by measurements of decreasing levels of a protein of interest (a “chase”) by SDS-PAGE and immunoblotting or by other means. The resulting decay curves are usually calibrated by measuring, in parallel, the levels of an abundant endogenous protein (e.g. tubulin or actin) that is presumed to be both long-lived and expressed at approximately equal levels under different physiological conditions and in varying genetic backgrounds.

A shortcoming, in the context of these assays, of a global translation inhibitor such as, for example, cycloheximide (CHX),3 is not only the inhibitor’s cytotoxicity but also the fact that a proteolytic pathway under study may itself involve a short-lived protein(s). Naturally unstable components of specific proteolytic systems continue to be identified (49–53). In all such cases, a global halt to the synthesis of cellular proteins

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3 The abbreviations used are: CHX, cycloheximide; DHFR, dihydrofolate reductase; PRT, promoter reference technique; Tc, tetracycline; Ub, ubiquitin; SC, synthetic complete.
would perturb the very process that a degradation assay is meant to measure, because the activity of a relevant proteolytic pathway would start to decrease as soon as its short-lived component is no longer produced. In addition and independently, it can be problematic to calibrate chase-based degradation assays by measuring the relative amounts of endogenous “marker” proteins, inasmuch as their levels often change in response to altered physiological conditions or genetic backgrounds.

Two of our 2017 studies described and employed a degradation assay termed the promoter reference technique (PRT) (54, 55). Key features of PRT are (i) a coexpression, from identical transcriptional promoters, of a test protein and a long-lived reference protein; and (ii) a gene-specific (i.e., not global) inhibition of translation during a chase (Fig. 1). In the present work, this technique is described in detail (Figs. 1–4), was further optimized, and was also used, in specific PRT-based degradation assays, to demonstrate a significant downside of using a global translation inhibitor, in comparison with a gene-specific inhibition of translation (Fig. 4).

Results and discussion

**PRT vis-à-vis the Pro/N-end rule pathway**

PRT is described below through its applications to one of the N-end rule pathways (Fig. 1 and Fig. S1). These pathways are a set of proteolytic systems whose unifying feature is their ability to recognize proteins containing N-terminal degradation signals called N-degrons, thereby causing the degradation of these proteins, largely by the proteasome (and also by autophagy) in eukaryotes and by the proteasome-like ClpAP protease in bacteria (4, 54–64).

The main determinant of an N-degron is a destabilizing N-terminal residue of a protein. Initially, most N-degrons are pro-N-degrons. They are converted to active N-degrons either constitutively (e.g., during the emergence of a nascent protein from a ribosome) or conditionally, via regulated steps. A pro-N-degron can become an N-degron through a site-specific cleavage of a protein by a protease and/or through enzymatic acetylation, formylation, arginylation, or leucylation of specific proteins at the α-amino group of their N-terminal residues. Studies over the last 3 decades have shown that all 20 amino acids of the genetic code can act, in cognate sequence contexts, as destabilizing N-terminal residues (Fig. S1). Consequently, many, possibly most, proteins in a cell are conditionally short-lived N-end rule substrates, either as full-length proteins or as protease-generated natural protein fragments (54, 55, 65). Recognition components of N-end rule pathways are called N-recognins. Regulated degradation of proteins and their fragments by the N-end rule pathways has been shown to mediate a remarkably wide range of biological processes (Fig. S1) (4, 54–77).

Eukaryotes contain three N-end rule pathways (see Fig. S1 and its legend). One of these proteolytic systems, termed the
A reference-based degradation assay

Pro/N-end rule pathway, is mediated, in \textit{Saccharomyces cerevisiae}, by the multisubunit GID Ub ligase. Gid4, a subunit of GID that functions as the Pro/N-recognin, targets proteins that bear the N-terminal Pro residue or a Pro at position 2, in addition to adjoining and also required sequence motifs (54) (Fig. S1C). Physiological substrates of the \textit{S. cerevisiae} Pro/N-end rule pathway include the gluconeogenic enzymes Fbp1, Mdh2, Icl1, and Pck1, which are long-lived in cells deprived of glucose but are selectively destroyed upon return to glucose-replete conditions (Fig. S1C) (54, 78, 79). Degradation assays of the present study focus on Mdh2, a physiological substrate of the Pro/N-end rule pathway (Fig. S1C).

The promoter reference technique

Fig. 1 illustrates PRT. A plasmid, based on the low-copy \textit{S. cerevisiae}–\textit{Escherichia coli} pJO629 vector (see “Experimental procedures”), contains the required PRT elements, including an ORF encoding an epitope-tagged protein of interest (Fig. 1C, \textit{test protein-flag}). The test protein and a long-lived reference protein (an epitope-tagged mouse DHFR) are coexpressed, in \textit{Escherichia coli} and \textit{S. cerevisiae}, from a pJO629-based plasmid and its two identical promoters and/or aptamer-mediated regulatory elements specific for small compounds other than Tc.

Fig. 2 illustrates the use of Tc-based PRT to characterize the degradation of Mdh2, a gluconeogenic enzyme. When glucose is low or absent, cells synthesize it through gluconeogenesis, an ATP-consuming process (81). When \textit{S. cerevisiae} grow on ethanol as a carbon source, gluconeogenic enzymes, such as Mdh2, are expressed and long-lived. Transition to a medium containing glucose inhibits the synthesis of gluconeogenic enzymes and induces their degradation, which is mediated by the multisubunit GID Ub ligase (54, 78, 79). Gid4, a subunit of GID, was recently identified as the N-recognin of a distinct N-end rule pathway, termed the Pro/N-end rule pathway (Fig. S1C) (54). Gid4 recognizes the N-terminal Pro residue of Mdh2 in the context of an also required adjoining sequence motif (54). Previous work showed that Gid4, which is absent in yeast growing on ethanol, is an expressed but short-lived protein in cells that grow in a glucose-containing medium (82).

Our 2017 study of the Pro/N-end rule pathway (54) involved Tc chases with the gluconeogenic enzymes Mdh2, Fbp1, and Pck1. To avoid repetition, Fig. 2 shows a previously unpublished set of PRT-based Mdh2 results. These assays involved using a Tc-mediated, gene-specific (\textit{i.e.} non-global and therefore largely nontoxic) repression of translation (Fig. 1, A and C). The logic of PRT (Fig. 1A) is not confined to Tc as a genespecific translation inhibitor. It should also be possible to develop PRT assays that employ, for example, other transcriptional promoters and/or aptamer-mediated regulatory elements specific for small compounds other than Tc.

Figure 2. PRT-based chase assays with Mdh2. A, \textit{S. cerevisiae} Mdh2 is the cytosolic malate dehydrogenase and a substrate of the GID-mediated Pro/N-end rule pathway (54). Lane 1, kDa markers. Tc-based chases were performed at 30 °C during the transition from ethanol to glucose medium (see “Experimental procedures”) with wild-type (lanes 2–5) or gid2Δ \textit{S. cerevisiae} (lanes 6–9 and 14–17) expressing the DHFR<sub>ha</sub> reference and either wild-type P-Mdh2<sub>3f</sub> (bearing N-terminal Pro; lanes 2–9) or S-Mdh2<sub>3f</sub> (bearing N-terminal Ser; lanes 10–17). At the indicated times of a chase, proteins in cell extracts were fractionated by SDS-PAGE, followed by immunoblotting with anti-flag antibody. The bands of X-Mdh2<sub>3f</sub> test proteins and the DHFR<sub>ha</sub> protein are indicated on the right. B, quantification of the data in A. The time 0 (before-chase) level of S-Mdh2<sub>3f</sub> in gid2Δ cells was taken as 100%. Note the much lower time 0 level (40% of the short-lived, wild-type P-Mdh2<sub>3f</sub> in wild-type cells, Its half-life (the time it took for the level of P-Mdh2<sub>3f</sub> to decrease from the initial 40% to 20%) was ~20 min. All chases in this study were performed at least twice and yielded results that differed by <10%.
wild-type and gid2Δ S. cerevisiae carrying PRT-based plasmids (Fig. 1C) that expressed either the 377-residue, C-terminally flag-tagged P-Mdh2$_{3f}$ (bearing N-terminal Pro) or its otherwise identical mutant S-Mdh2$_{3f}$ bearing N-terminal Ser. gid2Δ cells lack an essential subunit of the GID Ub ligase and therefore lack the Pro/N-end rule pathway (Fig. S1C). After 18 h in ethanol medium at 30 °C, cells were shifted to a glucose medium while the synthesis of both the Tc (Fig. 3), to identical samples of JOY379 cells carried a PRT-based plasmid (Fig. 1C) expressing the epitope-tagged Chk1$_{3f}$ protein and the fDHFR$_{ha}$ reference protein (55).

Tc was added, to different final levels (varying from 0.1 to 0.5 mM, as indicated in Fig. 3), to identical samples of JOY379 S. cerevisiae. After a time interval in the presence of Tc that varied from 0 to 20 min, cell suspensions were pulse-labeled for 3 min with $^{35}$S-Met/Cys (see "Experimental procedures"), followed by extraction of proteins, immunoprecipitation of Chk1$_{3f}$ and fDHFR$_{ha}$ with anti-flag antibody, SDS-PAGE, and autoradiography. Fig. 3, lanes 1–9 show the SDS-PAGE results with total $^{35}$S-pulse-labeled proteins, before anti-flag immunoprecipitation. Fig. 3 (lanes 10–18) shows the same samples from $^{35}$S-pulse-labeled cells but after anti-flag immunoprecipitation.

None of the examined concentrations of Tc, including 0.5 mM, caused significant changes in the levels (or patterns) of toxic and high enough to result in a non-leaky gene-specific repression of translation. We found that 0.5 mM Tc was preferable to the also acceptable but slightly "leaky" 0.2 mM Tc (Fig. 3).

In contrast to the assays in Figs. 2 and 4, which involved Mdh2, the Tc-optimization experiments described in Fig. 3 involved S. cerevisiae Chk1, a mitotic checkpoint kinase. Chk1 and several other yeast proteins were recently identified as conditionally short-lived physiological substrates of the Ubr1-mediated Arg/N-end rule pathway (55). The Ubr1-containing targeting complex of this pathway (Fig. S1A) was shown to recognize a C terminus–proximal degron of the Chk1 protein (55). Our sole aim, in the experiments of Fig. 3, was to determine a level of Tc that would be optimal for PRT assays. Therefore, we used the ubr1Δ JOY379 S. cerevisiae strain (lacking the Arg/N-end rule pathway), in which Chk1 was long-lived. JOY379 cells carried a PRT-based plasmid (Fig. 1C) expressing the epitope-tagged Chk1$_{3f}$ protein and the Tc (Fig. 3), to identical samples of JOY379 S. cerevisiae. After a time interval in the presence of Tc that varied from 0 to 20 min, cell suspensions were pulse-labeled for 3 min with $^{35}$S-Met/Cys (see "Experimental procedures"), followed by extraction of proteins, immunoprecipitation of Chk1$_{3f}$ and fDHFR$_{ha}$ with anti-flag antibody, SDS-PAGE, and autoradiography. Fig. 3, lanes 1–9 show the SDS-PAGE results with total $^{35}$S-pulse-labeled proteins, before anti-flag immunoprecipitation. Fig. 3 (lanes 10–18) shows the same samples from $^{35}$S-pulse-labeled cells but after anti-flag immunoprecipitation.

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expressed from mRNAs whose translation was repressed by Tc. In particular, lanes 10–15 show 35S-labeled Chk13f and fDHFRα in cells that were incubated with 0.1 mM Tc for either 0, 2, 4, 8, 15, or 20 min before the beginning of a 3-min [35S]Met/Cys pulse. The 35S-labeling of Chk13f and fDHFRα “flattened out” by 15 min after the addition of Tc (to a final concentration of 0.1 mM) and was much lower than the 35S-labeling immediately after the addition of Tc (Fig. 3, lane 10; compare with lane 15), indicating a gene-specific inhibition of translation by Tc. The levels of 3-min 35S-labeling of Chk13f and fDHFRα 20 min after the addition of Tc decreased to essentially undetectable (at this and significantly longer autoradiographic exposures) at a Tc concentration of 0.5 mM (Fig. 3, lane 18) and to low but still detectable levels at a Tc concentration of 0.2 mM (Fig. 3, lane 16; compare with lane 18). We concluded that 0.5 mM Tc yielded an approximately optimal level of gene-specific inhibition of translation in PRT assays, as this concentration of Tc was sufficiently “non-leaky” while still without effects on either the level or pattern of total protein synthesis (Fig. 3, lane 9).

Comparing a PRT-based cycloheximide chase with a PRT-based tetracycline chase

Global translation inhibitors, such as CHX, continue to be widely used in studies that involve chase-based degradation assays. As mentioned in the Introduction, a major shortcoming of global translation inhibitors, in the context of chases, stems from the fact that a proteolytic pathway under study may itself involve a short-lived protein(s) (49–53). In such cases, a halt to the synthesis of cellular proteins would perturb the very process that a degradation assay is meant to measure, because the activity of a relevant proteolytic pathway would start to decrease as soon as its unstable component is no longer produced. Inasmuch as the Gid4 Pro/N-recognin of the Pro/N-end rule pathway that targets P-Mdh23f for degradation (Fig. 2 and Fig. S1C) is known to be a short-lived protein (82), we asked whether results of a PRT-based Tc chase with P-Mdh23f would significantly differ from those of an otherwise identical PRT-based CHX chase.

These assays were carried out identically to the ones described in Fig. 2, except that the concentration of Tc during chase was 0.5 mM (instead of 0.2 mM Tc in the assays of Fig. 2), and cells were grown in a glucose-containing medium before and during chases. Both PRT-based Tc chase and PRT-based CHX chase assays with P-Mdh23f detected the in vivo degradation of P-Mdh23f, in contrast to the metabolic stability and much higher time 0 (before-chase) levels of S-Mdh23f (Fig. 4).

Remarkably, however, the actual decay curves of P-Mdh23f were quite different when determined using the Tc chase, in comparison with the CHX chase. Specifically, the time 0 levels of P-Mdh23f at the beginning of both chases, were nearly identical to each other (Fig. 4, A (lane

Figure 4. Comparing a PRT-based tetracycline chase with a cycloheximide chase. PRT-based Tc chases and CHX chases were carried out with wild-type S. cerevisiae growing in SC medium and expressing either P-Mdh23f (bearing N-terminal Pro) or S-Mdh23f (bearing N-terminal Ser). A, lane 1, kDa markers. Lanes 2–5, CHX chase, for 0, 0.5, 1, and 2 h, with P-Mdh23f. Lanes 6–9, same as lanes 2–5 but with S-Mdh23f. Lanes 10–13, same as lanes 2–5 but a Tc chase. Lanes 14–17, same as lanes 6–9, but a Tc chase. The bands of X-Mdh23f test proteins and the fDHFRα protein are indicated on the right. B, quantification of the data in A. The time 0 (before-chase) level of S-Mdh23f in the Tc chase was taken as 100%. Note the time 0, before-chase degradation of ~85% of P-Mdh23f, relative to S-Mdh23f, and the similar time 0 levels (in contrast to subsequent levels) of P-Mdh23f in the Tc chase versus the CHX chase. Note, also, the lower rate of P-Mdh23f decrease (and the subsequent flattening of the decay curve) in the CHX chase, in comparison with the Tc chase. All chases in this study were performed at least twice and yielded results that differed by <10%. Also see “Results and discussion.”
A reference-based degradation assay

2 versus lane 10) and B). However, whereas the rapid degradation of P-Mdh23f continued throughout 2 h of Tc chase, the degradation of P-Mdh23f during an otherwise identical CHX chase was not as fast. Moreover, the degradation curve of the CHX chase “flattened out” after 30 min, in contrast to the results with the Tc chase (Fig. 4, A (lanes 2–5 versus lanes 10–13) and B).

To the best of our knowledge, these results are the first direct and rigorously controlled evidence for a significant distortion of an in vivo decay curve of a protein in a PRT-based chase that involves a global translation inhibitor, in comparison with the otherwise identical PRT-based chase that utilizes Tc (Fig. 4). Because Gid4, the N-recognin of the Pro/N-end rule pathway (Fig. S1C), is a short-lived protein (82), the above results were in agreement with the a priori expectation of a difference between these PRT-based chase patterns (Fig. 4). Thus, at least in the setting of the Pro/N-end rule pathway, a chase that employs a global translation inhibitor, such as CHX, would underestimate the actual rate of degradation of a protein of interest, due to the pathway inhibition effect detected and described above (Fig. 4).

Concluding remarks

The Tc-based PRT (Figs. 1–4) has two independently advantageous features: a “built-in” reference protein and a gene-specific inhibition of translation during a chase. A long-lived reference protein, the epitope-tagged DHFR in the present version of PRT (Figs. 1C and 4A), addresses the problem of data scatter in reference-lacking pulse-chase assays and immunoblotting-based chase assays. The presence of a reference protein makes it possible to measure the level of a test protein during a chase as the ratio of its level to that of the reference at a given chase time. Consequently, in both immunoblotting-mediated chases and radioactive labeling–mediated pulse-chases, a reference-based and ratio-based measurement can compensate for the scatter of labeling efficiencies, scatter of immunoprecipitation (and/or immunoblotting) yields, imprecisions in sample volumes, and other sources of sample-to-sample variation. The resulting robustness and higher accuracy of reference-based degradation assays also makes it easier to detect and measure, at the start of a chase, the “early,” before-chase degradation of a test protein (Figs. 2 and 4).

In addition to optimizing Tc concentration in PRT (Fig. 3), we also demonstrated, through specific PRT-based degradation assays, the significant downside of using a global translation inhibitor, in comparison with a gene-specific inhibition of translation (Fig. 4). An extension of Tc-based PRT from its described use in S. cerevisiae (Figs. 1–4) to multicellular eukaryotes, including mammalian cells, is highly desirable and should be feasible but is not technically straightforward, because the set of RNA aptamers that works in yeast (Figs. 1B, 2, and 4) did not suffice, so far, with mammalian cells (80).

Experimental procedures

Antibodies and other reagents

The antibodies used were anti-flag mouse M2 monoclonal antibody (Sigma, F1804) and anti-ha rabbit monoclonal antibody (Sigma, H6908). Fluorescence of immunoblots was quantified using an Odyssey 9120 imaging system (LI-COR, Lincoln, NE). Secondary antibodies for immunoblotting were LI-COR IRDye-conjugated goat anti-mouse 800CW (LI-COR, C60405-05) or anti-rabbit 680RD (LI-COR, C51104-08). Other reagents included “complete protease inhibitor mixture” tablets (Roche Applied Science, 11697498001); protease inhibitor mixture “for use with fungal and yeast extracts” (Sigma, P8215); phenylmethylsulfonyl fluoride (Sigma, P7626); CHX (Sigma, C7698); and Tc (Sigma, T3383).

Genetic techniques, media, yeast strains, and plasmids

Standard techniques were used for strain construction and transformation (54, 55, 83, 84). S. cerevisiae media included YPD (1% yeast extract, 2% peptone, 2% glucose; only the most relevant components are cited); SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose); and synthetic complete (SC) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose), plus a drop-out mixture of compounds required by specific auxotrophic strains. The S. cerevisiae strains used in this work were BY4741 (MATa his3-1 leu2-0 Met15-0 ura3-0), BY4742 (MATa his3-1 leu2-0 lys2-0 ura3-0 can1-100), and JOY379 (an ubr1Δ:HphNT1 derivative of the BY4742 strain) (54, 55). The plasmids pJO629 (a parent of the “generic” PRT plasmid encoding a C-terminally epitope-tagged test protein and the DHFRha reference [Fig. 1C]), pCSJ125 (a PRT-based plasmid encoding P-Mdh23f and the DHFRha reference), pCSJ126 (an otherwise identical plasmid encoding S-Mdh23f and the DHFRha reference), and pJO630 (a PRT-based plasmid encoding Chk13f and the DHFRha reference) were constructed and described previously (54, 55). pJO629 and plasmids derived from it contained two direct repeats of a relatively long nucleotide sequence (P_{TDH3} promoter and the adjoining aptamer-forming DNA segment) (Fig. 1), a recombinogenic configuration. Therefore, a small fraction of S. cerevisiae that received a pJO629-derived plasmid (about 5% of transformants, in our experience) contained recombination-produced derivatives of that plasmid. This minor complication could be dealt with by discarding transformants that failed to produce the correctly sized DHFRha reference and/or a test protein.

PRT-based degradation assays

These assays used low-copy pJO629-based plasmids that expressed a C-terminally triple-flag-tagged test protein and the long-lived, also tagged DHFRha reference protein from a pair of identical P_{TDH3}-based promoters. In this setting, the synthesis of both proteins could be selectively extinguished by the addition of Tc (Fig. 1). Initially, the final concentration of Tc was 0.2 mM (Fig. 2). It became 0.5 mM in later PRT assays (Figs. 3 and 4). When CHX, a global translation inhibitor, was used instead of Tc, it was at 0.36 mM (0.1 mg/ml). As described under “Results” (Fig. 3), 0.5 mM Tc was found to be preferable to the also acceptable but slightly “leaky” 0.2 mM Tc, the level used for Tc chases in our earlier work (54, 55) and also, initially, during the present study. Neither 0.2 nor 0.5 mM Tc detectably altered the growth rate of S. cerevisiae.

S. cerevisiae were grown to A_{600} of ~1.0 in selective liquid media at 30°C, followed by treatment with 0.2 mM Tc (and in later assays, with the optimized Tc concentration of 0.5 mM; see
and the supernatants were centrifuged (identically) again. The pellet was resuspended in 0.8 ml of ice-cold water for 5 min, followed by centrifugation for 1 min at 3,000 rpm as above and resuspension in 0.8 ml of 2 M NaOH for 5 min on ice. The resulting supernatant was centrifuged for 5 min at 21,130 × g. The pellet was resuspended in 50 μl of HU buffer (8 M urea, 5% SDS, 1 M EDTA, 0.1 M DTT, 0.005% bromophenol blue, 0.2 M Tris-HCl, pH 6.8) containing 1X protease inhibitor mixture (Roche Applied Science) and 1× protease inhibitor mixture “for use with fungal and yeast extracts” (Sigma) and heated for 10 min at 70 °C. After centrifugation for 3 min at 21,130 × g, 15 μl of each supernatant was used to carry out SDS–4–12% NuPAGE (Invitrogen), followed by immunoblotting, performed as described previously (14, 54, 55, 75), using a mixture of anti-ha (1:2,000) and anti-flag (1:2,000) antibodies. Immunoblots were processed using secondary antibodies labeled with different fluorophores. Visualized protein bands were quantified using the Odyssey 9120 imaging system (LI-COR). The near-infrared fluorescence range and other features of the Odyssey scanner facilitate quantification of immunoblots.

Optimizing the level of tetracycline during chase

pJO630, a PRT-based plasmid (Fig. 1C) that expressed the flag epitope-tagged Chk1sr protein and the DHFRsr reference protein, was described previously (55). See above for a brief introduction of Chk1, a kinase and a physiological substrate of the Arg/N-end rule pathway (55) (Fig. S1A). S. cerevisiae JOY379 cells (ubr1Δ::HphNT1) in the BY4742 strain background that lacked the Arg/N-end rule pathway and carried pJO630 were grown at 30 °C to A600 of ~1 in 50 ml of SC medium without histidine (SC–His). Nine 15-ml tubes were filled, each with 5 ml of cell suspension, followed by pelleting of cells by a low-speed centrifugation and washing of the pellets in 0.8 ml of SC medium (see above) supplemented with Lys, Trp, Leu, and Ura (83).

Cells were pelleted again and resuspended in 0.4 ml of fresh SD medium, followed by incubation at 30 °C for 20 min. Tc was added to cell suspensions, to varying final concentrations of 0.1, 0.2, 0.3, or 0.5 mM, either at the start of the 20-min incubation, during it, or immediately after it (see the legend to Fig. 3 for details). Thereafter (at the end of the 20-min incubation), cell suspensions were labeled for 3 min at 30 °C with 0.16 mCi of 35S-EXPRESS Met/Cys (PerkinElmer Life Sciences), followed by centrifugation to pellet the cells, their resuspension in 0.1 ml of amino acid–supplemented SD medium, and their freezing, immediately afterward, in liquid nitrogen. To process the samples further, 0.8 ml of lysis buffer (10% glycerol, 0.5% Nonidet P-40, 0.2 M KCl, 1 M PMSF, 5 mM β-mercaptoethanol, 50 mM HEPES, pH 7.5) was added to each frozen sample, and extracts were prepared using Mini-Beadbeater-16 (BioSpec) (four times for 20 s each at maximum speed, with 5-min intervals on ice). The extracts were centrifuged at 21,000 × g for 15 min at 4 °C, and the supernatants were centrifuged (identically) again. 15 μl of each sample was taken and later analyzed as input samples.

The rest of the supernatants were processed for immunoprecipitation, using 8 μl of a suspension of anti-flag antibody immobilized on magnetic beads (Sigma). Immunoprecipitates were analyzed by SDS–4–12% NuPAGE (Invitrogen) and autoradiography.

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