Quantification of mRNA stability of stress-responsive yeast genes following conditional excision of open reading frames

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Abbreviations: CEO, conditional excision of open reading frames; TORC1, target of rapamycin complex I; RNA, ribonucleic acid; mRNA, messenger RNA; Tet, tetracycline; 4-tU, 4-thiouracil; 4-sU, 4-thiouridine; DTA, dynamic transcriptome analysis; loxP, locus of X-over P1; Cre, causes recombination; PP2A, protein phosphatase type 2A; YPD, yeast peptone dextrose; SD, synthetic defined; bp, base pair; ORF, open reading frame; PCR, polymerase chain reaction; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription PCR; DNA, deoxyribonucleic acid; cDNA, complementary DNA; EBD, estradiol-binding; UTR, untranslated region

Eukaryotic cells rapidly adjust the levels of mRNAs in response to environmental stress primarily by controlling transcription and mRNA turnover. How different stress conditions influence the fate of stress-responsive mRNAs, however, is relatively poorly understood. This is largely due to the fact that mRNA half-life assays are traditionally based on interventions (e.g., temperature-shifts using temperature-sensitive RNA polymerase II alleles or treatment with general transcription inhibitory drugs), which, rather than blocking, specifically induce transcription of stress-responsive genes. To study the half-lives of the latter suite of mRNAs, we developed and describe here a minimally perturbing alternative method, coined CEO, which is based on discontinuance of transcription following the conditional excision of open reading frames. Using CEO, we confirm that the target of rapamycin complex I (TORC1), a nutrient-activated, central stimulator of eukaryotic cell growth, favors the decay of mRNAs that depend on the stress- and/or nutrient-regulated transcription factors Msn2/4 and Gis1 for their transcription. We further demonstrate that TORC1 controls the stability of these mRNAs via the Rim15-lox151-lox152-PP2Acdc55 effector branch, which reportedly also controls Gis1 promoter recruitment. These data pinpoint PP2Acdc55 as a central node in homo-directional coordination of transcription and post-transcriptional mRNA stabilization of a specific array of nutrient-regulated genes.

Introduction

mRNA turnover is a key factor in the regulation of eukaryotic gene expression, which, in parallel to transcriptional activation/repression, allows cells to rapidly adjust their levels of specific transcripts in response to environmental stress conditions. Information on how different stress conditions impact on the post-transcriptional fate of stress-responsive mRNAs, however, is still quite limited. Studies in Saccharomyces cerevisiae for instance suggest that heat shock favors the stabilization of heat-shock inducible mRNAs, while pre-existing mRNAs may suffer from accelerated degradation via the 5'-3' mRNA decay pathway or be shifted from polysomes toward a repressing mRNA state within P bodies.1–3 Similarly, nutrient limitation or inactivation of the nutrient-regulated target of rapamycin complex 1 (TORC1) broadly stimulates 5'-3' mRNA decay,4 while specifically endorsing the protection of newly expressed mRNAs of genes (e.g., HSP26) that are controlled by the stress-responsive and/or nutrient-regulated transcription factors Msn2, Msn4 and Gis1.5 The limited amount of information on mRNA half-lives of stress-responsive genes is primarily due to the fact that most of the currently available methods to assess mRNA half-lives suffer from caveats that specifically affect stress-responsive mRNAs. For instance, assays that allow determination of mRNA half-lives are traditionally based on inhibition of general transcription using either a temperature-sensitive allele of the catalytic subunit of RNA polymerase II (RNA Pol II; i.e., rpb1-1),6 or a variety of transcription inhibitors (e.g., thiolutin and 1,10-phenanthroline).7 However, both interventions per se, namely temperature-shift (when using rpb1-1 strains) and thiolutin/1,10-phenanthroline

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transcriptome analysis (DTA), which combines metabolic mRNA labeling with dynamic kinetic modeling, is applicable on a genome-wide level and may currently be best suited to estimate mRNA half-lives under stress conditions.\textsuperscript{13,16} A theoretical concern of this method, which is technically challenging,\textsuperscript{15} may be the fact that it is built on the assumption that mRNA synthesis and decay rates remain constant during the labeling time.

To specifically study the mRNA half-life of individual stress-responsive mRNAs, we developed a minimally perturbing method, which is not susceptible to any of the caveats of the classical mRNA half-life studies outlined above. Our method is based on the excision from the genome of a given \textit{loxP}-flanked open reading frame, and hence discontinuance of transcription, following conditional nuclear targeting of the Cre recombinase. Using this assay, which we coin CEO (for conditional excision of the open reading frame [ORF]), we confirm our previous data, which indicated that inactivation of TORC1 results in stabilization of newly expressed mRNAs of Msn2-, Msn4-, and Gis1-controlled genes.\textsuperscript{5,17-19} Using CEO, we extend our earlier studies and demonstrate that TORC1 controls mRNA stability via the Cdc55-protein phosphatase 2A (PP2ACdc55), which we recently found to be also implicated in direct regulation of the transcription factor Gis1.\textsuperscript{18} Thus, our data indicate the existence of a mechanism that allows cells to tightly coordinate transcription with the post-transcriptional fate of specific mRNAs under nutrient stress conditions.

**Results**

The experimental system. To design our method, we used a Cre recombinase that is fused to the estradiol-binding domain (EBD) of the murine estrogen receptor (Cre-EBD78),\textsuperscript{20,21} which mediates constitutive expression of the respective mRNAs. Most recent mRNA half-life assays are based on metabolic labeling of newly transcribed mRNAs with 4-thiouracil (4-tU) or 4-thiouridine (4-sU), which requires the heterologous expression of a human nucleoside transporter (hENT1) to enable 4-sU uptake by yeast cells.\textsuperscript{13,14} Potential concerns regarding the applicability of 4-tU-pulse/uracil-chase experiments to assess the half-lives of newly expressed mRNAs under dynamic stress conditions, however, include the required long pulse/labeling period (i.e. > 4 h) and the possibility of uncontrolled 4-tU recycling within cells.\textsuperscript{15} Finally, dynamic metabolic mRNA labeling with dynamic kinetic modeling, is applicable on a genome-wide level and may currently be best suited to estimate mRNA half-lives under stress conditions.\textsuperscript{13,16} A theoretical concern of this method, which is technically challenging,\textsuperscript{15} may be the fact that it is built on the assumption that mRNA synthesis and decay rates remain constant during the labeling time.

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estradiol to yeast cells containing both reporter constructs should therefore trigger nuclear transfer of the Cre-EBD78 fusion protein and consequent excision of the HSP26 ORF (including the hphNT1 cassette from the genome (as illustrated). The efficacy of HSP26 excision was assessed by qPCR using the indicated primers (P1 and P2) and genomic DNA as template. Bar graphs show the mean levels of three independent experiments (± S.D.) of PCR-amplified HSP26 (normalized to 1.0 for exponentially growing, untreated cells) in cells treated with either vehicle alone (v; on the left) or with estradiol (on the right) for the times indicated. Samples to determine the half-life of HSP26 mRNA were collected (at 10 min intervals) 70 min following vehicle or estradiol treatment and the respective data are presented in Figure 3A. (B) qRT-PCR analysis of HSP26 mRNA induction in wild-type (WT) and loxP-HSP26-loxP reporter cells (same as in (A)) treated for the times indicated with 200 ng ml⁻¹ rapamycin. Mean HSP26 mRNA levels of three independent experiments (± S.D.) normalized to the values at 60 min, are shown. Notably, in terms of absolute levels, the rapamycin-induced accumulation of HSP26 mRNA (and of Hsp26 protein) did not significantly differ in loxP-HSP26-loxP reporter cells when compared with wild-type cells (data not shown). (C) Estradiol per se (added at time 60 min), like vehicle alone, does not interfere with rapamycin-induced HSP26 expression (quantified by qRT-PCR as in (B)) in wild-type cells.
mRNAs of Msn2-, Msn4- and Gis1-controlled genes via a
activation of TORC1 results in stabilization of newly expressed
cells (half-lives of 34 and 38 min, respectively, in rapamycin-treated
PP2Acdc55 signaling branch also regulates transcription (in part via Gis1) of at time point 0 (corresponding to the time point 130 min of the rapamycin
Figure 2A
construct (see Table 1
mRNA levels in rapamycin-treated cells. mRNA samples were harvested from cells, which harbored the Cre-EBD78 and an appropriate loxP-ORF-loxP reporter
process that depends on the greatwall protein kinase ortholog Rim15 and its targets Igo1/2.5 In this study, the experimental caveats of mRNA half-life assays (outlined above) were circumvented by rendering HSP26 transcription doxycycline-repressible due to the insertion of a series of doxycycline-responsive tetO elements in the HSP26 promoter. Notably, identification of sites in the HSP26 promoter that tolerated the respective tetO insertions without interfering with normal transcriptional regulation of HSP26 was challenging and time-consuming. Using the much easier applicable CEO assay, we confirm here our earlier results that loss of Rim15 or of Igo1/2 reduced the HSP26 mRNA half-life in rapamycin-treated cells about 2-fold (Fig. 3A).
To further extend these data, we also studied the half-lives of two additional mRNAs, which were encoded by genes that either depended (i.e., RTN2), or did not depend (i.e., CIT2), on the presence of Rim15 and Igo1/2 for normal expression following TORC1 inactivation.5 In line with our expectations, we found that the half-life of the RTN2 mRNA, but not the one of CIT2 mRNA, was reduced 2-fold in rapamycin treated rim15Δ and igo1Δ igo2Δ cells when compared with wild-type cells (Fig. 3B and C). These data not only demonstrate the validity of CEO for mRNA half-life studies, but also corroborate our earlier conclusion that TORC1 controls the stability of a specific set of mRNAs via Rim15-Igo1/2.5,17,24,25

**Figure 3.** TORC1 controls mRNA stability via the PP2Acdc55-inhibitory endosulfines. (A–E) qRT-PCR analysis of HSP26 (A and D), RTN2 (B and E) and CIT2 (C) mRNA levels in rapamycin-treated cells. mRNA samples were harvested from cells, which harbored the Cre-EBD78 and an appropriate loxP-ORF-loxP reporter construct (see Table 1) and that were treated with rapamycin and estradiol following the protocol outlined in Figure 2A. The values for the reference samples at time point 0 (corresponding to the time point 130 min of the rapamycin treatment) were normalized to 1.0 for each strain. Notably, the Rim15-Igo1/2-PP2Acdc55 signaling branch also regulates transcription (in part via Gis1) of HSP26 and RTN2, but not of CIT2 (see also the model in Fig. 4). Before their normalization to 1.0, the relative levels of HSP26 and RTN2 mRNAs, but not the ones of CIT2, therefore differed significantly between the various mutant strains. Accordingly, the HSP26 mRNA levels in rim15Δ, igo1/2Δ, cdc55Δ, cdc55Δ rim15Δ, and cdc55Δ igo1/2Δ cells were 23%, 13%, 225%, 170%, and 196%, respectively, when compared with those in wild-type cells (in A and D). Similarly, RTN2 mRNA levels in rim15Δ, igo1/2Δ, cdc55Δ, cdc55Δ rim15Δ, and cdc55Δ igo1/2Δ cells were 17%, 25%, 155%, 150% and 132%, respectively, when compared with those in wild-type cells (in B and E). Estradiol-induced excision of the loxP-ORF-loxP loci was verified independently and found to be at least 85% complete for each strain at time point 0 (i.e. 70 min following estradiol addition). Data points represent means ± S.D. of three independent experiments.

half-lives of 34 and 38 min, respectively, in rapamycin-treated cells (Fig. 3B and C).

**Validation of CEO.** We have previously reported that inactivation of TORC1 results in stabilization of newly expressed mRNAs of Msn2-, Msn4- and Gis1-controlled genes via a

Reported in the context of the situation in wild-type cells, loss of Rim15 or of Igo1/2 had no significant impact on the observed HSP26 and RTN2 mRNA half-lives in cdc55Δ cells under the same conditions (Fig. 3D and E). Thus, the enhanced turnover of HSP26 and RTN2 mRNAs in rapamycin-treated rim15Δ and igo1/2Δ cells can be suppressed by loss of Cdc55.
These data extend our previous model and suggest that TORC1 controls in fact both transcriptional activation of Gis1-dependent genes and post-transcriptional stability of the respective mRNAs via the Rim15-Igo1/2-PP2A<sub>Cdc55</sub> effector branch (Fig. 4).

Discussion

Here we describe an alternative method to study the mRNA half-life of stress-responsive genes in yeast that is based on the conditional excision of ORFs (CEO). CEO offers a distinct advantage over conventional mRNA half-life assays that build on the inactivation of transcription using either the temperature-sensitive <i>rpb1</i>-1 allele or a variety of specific transcription inhibitors (see Introduction). Accordingly, CEO relies on estradiol treatment (to induce cytoplasmic to nuclear transfer of the Cre-EBD78 fusion protein), which, unlike heat-shock (when using <i>rpb1</i>-1 mutants) or various treatments with transcription inhibitory drugs, per se does neither induce the expression nor interfere with the normal (rapamycin-mediated) regulation of stress-responsive genes such as <i>HSP26</i>, <i>RTN2</i>, and <i>CIT2</i> (Fig. 2C and data not shown). Based on our observation that estradiol-induced excision of various target ORFs was exerted to a large extent within a time frame of 10–20 min (following a lag period of 50 min), we infer that CEO represents a valid method to determine the stability of those mRNAs species that exhibit half-lives of at least 10–20 min. An additional advantage of CEO is that it preserves the endogenous structures of the 5’t/3’ UTRs and the promoters of the genes under study, which makes CEO less likely to alter the fate of mRNAs than traditional methods that are based on the use heterologous repressible promoters.26–30 Although it remains theoretically possible that the insertion of a <i>loxP</i> site within the ORF of a given target gene may affect its expression, our control experiments, in which we compared the level of <i>HSP26</i> mRNAs between rapamycin-treated wild-type and <i>loxP-HSP26-loxP</i> cells, indicated that the introduction of a <i>loxP</i> site within the <i>HSP26</i> ORF per se is minimally invasive with no significant effect on the expression and/or stability of <i>HSP26</i> mRNAs. Taken together, despite a few limitations (regarding the resolution of half-lives of very short-lived mRNAs and the unsuitability for genome-wide analyses), CEO offers a valid alternative to sample the mRNA half-life of stress-responsive genes, which, together with the toolbox offered here, only requires relatively easily applicable standard yeast genetic methods. Finally, although not specifically studied here, we would like to point out that CEO may in principle also be applied to the analysis of gene function as it will theoretically be possible that <i>PP2A<sub>Cdc55</sub></i> regulates, in addition to its role in transcription, also be implicated in post-transcriptional control of mRNA stability. Our data presented here indicate that this is the case. Thus, Igo1/2-dependent regulation of <i>PP2A</i> appears to represent a key node in nutrient-sensitive, homo-directional coordination of transcription and post-transcriptional mRNA stabilization of a specific array of genes (Fig. 4). Interestingly, loss of components of the 5’-3’ mRNA decay pathway, like loss of <i>Cdc55</i>, suppresses the defect in <i>HSP26</i> expression in rapamycin-treated <i>igo1/2Δ</i> cells.5,17,18 It is therefore possible that <i>PP2A<sub>Cdc55</sub></i> regulates, in addition to Gis1, a specific protein(s) within the 5’-3’ mRNA decay pathway. In this context, our previously published phosphoproteome studies pinpointed, among others, Vts1 as a potential <i>PP2A<sub>Cdc55</sub></i> target.18 Vts1 is a member of the Smaug family of proteins, which directly binds a defined RNA motif in target mRNAs of nutrient-regulated genes and controls their stability by interfering with the 5′-3′ mRNA decay pathway.33,34 It will therefore be interesting to explore in future studies whether parallel, temporally coordinated regulation by the Igo1/2-<i>PP2A<sub>Cdc55</sub></i> module of both Gis1 and Vts1 (or of other candidate proteins) may allow cells to coordinate transcription and posttranscriptional mRNA stability.

Materials and Methods

Strains and growth conditions. <i>S. cerevisiae</i> strains (Table 1) were grown at 30°C in standard rich medium (YPD) with 2% glucose or in synthetic defined (SD) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose) complemented with...
the appropriate nutrients for plasmid maintenance. Rapamycin and estradiol were used at a concentration of 200 ng ml$^{-1}$ and 1 μM, respectively.

**Plasmid constructions.** The plasmids used in this study are depicted in Figure 1. pNT081, containing part of the ADE3 gene (i.e., 600 bp upstream and 400 bp downstream of the ATG), is derived from pGPD1::Cre-EBD78.20,21 Digestion of pNT081 with NdeI and SphI generates a 10.4 kb fragment that drives integration of the GPD1::Cre-EBD78 cassette into the genomic ADE3 locus in yeast. Positive clones can be selected using the LEU2 marker that is also present on this fragment. pNT082 was constructed by inserting, via homologous recombination, elements of the HSP26 gene into the pFA6a-α3N4-ASK1-loxP-HphNT1 plasmid (kind gift of Yves Barral). Digestion of pNT082 with XbaI and SpeI generates two fragments (3686 and 3240 bp) of which the 3686 bp fragment can be inserted at the HSP26 locus by homologous recombination. As illustrated in Figure 1, this fragment contains sequentially the 347 bp upstream of and including the ATG plus the first 18 bp of the HSP26 ORF (HSP26-5'), a first loxP site that preserves the reading frame with respect to the preceding ATG and the following HSP26 ORF (encoding amino acids 2–214), the 428 bp downstream of and including the stop codon of HSP26 (HSP26-3'), the hphNT1 cassette, a second loxP site (in tandem orientation with the first loxP site) and the last 42 bp of the HSP26 ORF, including the stop codon followed by 543 bp of the 3′ region (HSP26-3′ [b]). The hphNT1 cassette allows expression of the hygromycin B resistance gene under the control of the TEF1 promoter. pNT083 is based on pRS316 and was designed to facilitate construction of reporter cassettes for use in CEN assays. To this end, a fragment containing the 300 bp upstream of and including the ATG plus the first 15 bp of the ORF of a given gene can be cloned at the Nael and NorI sites (or only at NorI) in pNT083 (in-frame with the subsequent loxP-encoded sequence indicated in Fig. 1). A second fragment coding for the entire ORF (except the ATG) plus at least 300 bp downstream of and including the stop codon can be cloned at the XbaI and BamHI sites (or only at XbaI; in frame with the preceding loxP-encoded sequence). Finally, a third fragment including at least the first 300 bp of the 3′ region following the stop codon can be cloned at the Sall and/or KpnI sites. For homologous recombination in yeast, the entire cassette can then be removed from the plasmid by digestion with Nael and KpnI. Both plasmids pNT084 and pNT085 were constructed using this approach and pNT083 as template. Accordingly, pNT084 contains sequentially the 300 bp upstream of and including the ATG plus the first 18 bp of the ORF (HSP26-5′), a first loxP site that preserves the reading frame with respect to the preceding ATG and the following HSP26 ORF (encoding amino acids 4–393), the 295 bp downstream of and including the stop codon of HSP26 (HSP26-3′), the hphNT1 cassette, a second loxP site in tandem orientation with the first loxP site, and the 295 bp downstream of the HSP26 ORF (HSP26-3′). Digestion of pNT084 with KpnI generates two fragments (3882 and 4482 bp) of which the 3882 bp fragment can be inserted at the HSP26 locus by homologous

| Strain    | Genotype                                      | Source      | Figure |
|-----------|-----------------------------------------------|-------------|--------|
| BY4741    | MATα; ura3Δ0, leu2Δ0, his3Δ1, met15Δ0         | Euroscarf   |        |
| NT393–1D  | MATα; HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′ | this study  | 2B     |
| NT393–6D  | MATα; HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 2A, 3A |
| NT394–1C  | MATα; rlm15Δ::kanMX, HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3A     |
| NT395–8A  | MATα; gao1Δ::kanMX, gao2Δ::kanMX, HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3A     |
| NT401–10C | MATα; cdc55Δ::kanMX, HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3D     |
| NT402–13C | MATα; cdc55Δ::kanMX, rim15Δ::kanMX, HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3D     |
| NT404–14D | MATα; cdc55Δ::kanMX, gao1Δ::kanMX, gao2Δ::kanMX, HSP26-5′-loxP-HSP26-HphNT1-loxP-HSP26-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3D     |
| NT418–10B | MATα; RTN2-5′-loxP-RTN2-HphNT1-loxP-RTN2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3B     |
| NT417–2B  | MATα; rlm15Δ::kanMX, RTN2-5′-loxP-RTN2-HphNT1-loxP-RTN2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3B     |
| NT416–17C | MATα; gao1Δ::kanMX, gao2Δ::kanMX, RTN2-5′-loxP-RTN2-HphNT1-loxP-RTN2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3B     |
| NT417–4C  | MATα; cdc55Δ::kanMX, RTN2-5′-loxP-RTN2-HphNT1-loxP-RTN2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3E     |
| NT417–17C | MATα; cdc55Δ::kanMX, rim15Δ::kanMX, RTN2-5′-loxP-RTN2-HphNT1-loxP-RTN2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3E     |
| NT425–3D  | MATα; cdc55Δ::kanMX, gao1Δ::kanMX, gao2Δ::kanMX, RTN2-5′-loxP-RTN2-HphNT1-loxP-RTN2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3E     |
| NT422–1C  | MATα; CIT2-5′-loxP-CIT2-HphNT1-loxP-CIT2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3C     |
| NT424–17D | MATα; rim15Δ::kanMX, CIT2-5′-loxP-CIT2-HphNT1-loxP-CIT2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3C     |
| NT423–1B  | MATα; gao1Δ::kanMX, gao2Δ::kanMX, CIT2-5′-loxP-CIT2-HphNT1-loxP-CIT2-3′, ADE3::GPD1::Cre-EBD78-LEU2 | this study  | 3C     |
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No potential conflicts of interest were disclosed.

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Author’s Contribution
NT designed and performed the experiments. All authors contributed to the data analysis and creation of figures and commented on the manuscript, which was written by CDV.
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