Dissecting mRNA decay and translation inhibition during iron deficiency

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Abstract

Iron participates as a vital cofactor in multiple metabolic pathways. Despite its abundance, iron bioavailability is highly restricted in aerobic and alkaline environments. Therefore, living organisms have evolved multiple adaptive mechanisms to respond to iron scarcity. These strategies include a global remodeling of iron metabolism directed to optimize iron utilization. In the baker's yeast *Saccharomyces cerevisiae*, this metabolic reorganization is accomplished to a large extent by an mRNA-binding protein called Cth2. Yeast Cth2 belongs to a conserved family of tandem zinc finger containing proteins that specifically bind to transcripts containing AU-rich elements and promote their turnover. A recent study has revealed that Cth2 also inhibits the translation of its target mRNAs. Interestingly, the mammalian Cth2 ortholog known as tristetraprolin (aka TTP/TIS11/ZFP36), which is also implicated in controlling iron metabolism, promotes the decay and prevents the translation of its regulated transcripts. These observations open the possibility to study the relative contribution of altering mRNA stability and translation to the physiological adaptation to iron deficiency, the function played by the different domains within the mRNA-binding protein, and the potential factors implicated in coordinating both post-transcriptional events.

Keywords: Protein translation, mRNA degradation, iron deficiency, yeast, tristetraprolin, Cth2.

Introduction

Iron is an essential element for all eukaryotic organisms because it participates as a redox cofactor in many biological processes, including the generation of energy by oxidative phosphorylation and the biosynthesis of the major cellular components: proteins, lipids and DNA. When present in excess, iron participates in Fenton-type reactions that generate reactive oxygen species (ROS) that can damage lipids, proteins and nucleic acids (Meneghini 1997). The extremely low solubility of ferric iron at physiological conditions frequently leads in humans to the development of iron deficiency anemia, which is considered the most widespread nutritional disorder, with large impact on children and pregnant women (Baynes and Bothwell 1990, Sanvisens and Puig 2011). Moreover, iron imbalances lead to several human diseases including hereditary hemochromatosis, aceruloplasminemia and Friedreich's ataxia (Muckenthaler, et al. 2017).

Living organisms, ranging from bacteria to mammals, have evolved intricate regulatory networks to appropriately respond to iron starvation (Martins, et al. 2018, Muckenthaler, et al. 2017, Salvail and Masse 2012, Sanvisens and Puig 2011). In particular, the post-transcriptional regulation of gene expression plays a central role in controlling iron homeostasis. In several bacterial species, iron starvation activates the expression of the RyhB-like small noncoding RNAs, which degrade transcripts encoding proteins involved in iron metabolism and storage (Salvail and Masse 2012). Another family of small regulatory RNAs, called IsaR1 in the cyanobacterium *Synechocystis*, post-transcriptionally represses the photosynthetic electron transport chain in response to iron starvation by directly targeting certain

mRNAs encoding components of the chain, maintaining the regulation of iron-sulfur cluster biogenesis, and via the inhibition of the tetrapyrrole biosynthesis pathway (Carrier, et al. 2017, Georg, et al. 2017). In mammals, the iron regulatory proteins IRP1 and IRP2 bind to specific stem-loop RNA structures, known as iron responsive elements (IREs), located in the non-coding regions of a set of transcripts implicated in cellular iron homeostasis, to modulate its stability and translation efficiency (Muckenthaler, et al. 2017, Thompson and Bruick 2012). Finally, mammals express various proteins characterized by the presence of two tandem zinc fingers (TZFs) of the Cx₈Cx₅Cx₃H type that specifically interact with AU-rich elements (AREs: 5'-UUAUUUAUU-3') present in the 3'-untranslated region (3'-UTR) of many mRNAs, promoting their degradation and inhibiting their translation (Carballo, et al. 1998). Human tristetraprolin (TTP/TIS11/ZFP36) is the most studied member of this TZF-containing family of RNA-binding proteins (Brooks and Blackshear 2013, Wells, et al. 2017). In addition to its anti-inflammatory and tumor suppressor function (Clark and Dean 2016, Guo, et al. 2017, Patial and Blackshear 2016), TTP is highly expressed in response to iron deficiency (Bayeva, et al. 2012). Under these conditions, TTP controls the expression of mRNAs critical for mammalian iron metabolism, such as the transferrin receptor, and influences cell survival (Bayeva, et al. 2012). Recent data have demonstrated that TTP is also necessary for maintaining cardiac function during iron starvation by promoting the turnover of mRNAs of mitochondrial iron-containing proteins, and thus avoiding electron leakage and oxidative damage (Sato, et al. 2018).

A central role for the Cth2 mRNA-binding protein in the response of yeast cells to iron deficiency.

The baker's yeast *Saccharomyces cerevisiae* has developed sophisticated mechanisms to adapt to altered levels of iron (Kaplan and Kaplan 2009, Li and Ward 2018, Philpott and Protchenko 2008). Upon iron scarcity, yeast cells activate the transcription of a set of genes collectively known as the iron regulon, which encode the high-affinity iron uptake machinery at the plasma membrane, proteins implicated in the mobilization of iron from the vacuole and two mRNA-binding proteins known as Cth1 and Cth2 (Philpott and Protchenko 2008, Sanvisens and Puig 2011). Cth1 and Cth2 belong to the TTP-like family of mRNA-binding proteins (Puig, et al. 2005, Puig, et al. 2008, Thompson, et al. 1996, Vergara, et al. 2011). Genome-wide studies with yeast *cth2Δ* cells cultivated in iron-depleted conditions and mRNA half-life experiments have shown that Cth2 promotes the destabilization of more than a hundred transcripts, which mainly encode iron-dependent proteins and proteins that participate in highly iron-consuming pathways (Puig, et al. 2005, Puig, et al. 2008). Specifically, Cth2-regulated processes include the mitochondrial electron transport chain, the tricarboxylic acid cycle, and key steps in the biosynthesis of various amino acids, lipids, deoxyribonucleoside triphosphates (dNTPs), heme, biotin and lipoic acid cofactors (Puig, et al. 2005, Puig, et al. 2008). Thus, Cth2 seems to optimize iron utilization during iron depletion by repressing dispensable iron-requiring pathways, such as mitochondrial respiration, and activating essential iron-dependent processes like the biosynthesis of dNTPs via ribonucleotide reductase (Sanvisens, et al. 2011, Sato, et al. 2018). The physiological relevance of Cth2 is highlighted by the

significant growth defects associated to cells lacking a functional Cth2 protein when cultivated under iron-limiting conditions (Puig, et al. 2005). The function of Cth1, the second TTP-family yeast homolog, partially overlaps with Cth2. However, Cth1 performs a secondary role since *cth1Δ* cells do not display a growth defect under iron starvation (Puig, et al. 2005, Puig, et al. 2008). The contribution of Cth1 to iron-deficiency is shown by the more severe growth defect of the *cth1Δcth2Δ* double mutant as compared to the *cth2Δ* single mutant in these conditions (Puig, et al. 2005). Therefore, the experiments to address Cth2 function are usually performed in a *cth1Δ* genetic background in order to exclude any interference of Cth1 (Puig, et al. 2005). *CTH2* is also transiently activated in the response to oxidative stress (Castells-Roca, et al. 2016). In coordination to its Cth1 counterpart, Cth2 contributes to the resistance to ROS by decreasing the activity of mitochondrial respiration and by limiting the acquisition of oxidant-promoting iron through the reductive high-affinity uptake system (Castells-Roca, et al. 2016, Matsuo, et al. 2017, Sato, et al. 2018).

Although many changes in mRNA levels have been reported for yeast cells lacking *CTH2*, much less is known about its final effect on protein abundance. Previous studies have shown that Cth2 expression leads to a decrease in the protein levels of the succinate dehydrogenase subunits Sdh2 and Sdh4 (Martinez-Pastor M, et al. 2013, Ramos-Alonso, et al. 2018). By using specific antibodies, we have now addressed how Cth2 influences the levels of other iron-containing proteins when iron is scarce. Specifically, we have observed that the levels of ferrochelatase (Hem15), biotin synthase (Bio2), and to a minor extent aconitase (Aco1) increase in the absence of Cth2 (Figure 1). This result reinforces the notion

that Cth2 limits the expression of multiple iron-dependent proteins when iron is scarce.

Contribution of yeast Cth2 protein domains to mRNA decay and translational inhibition.

The mechanism proposed for Cth2-mediated mRNA decay initiates with the cotranscriptional binding of Cth2 to its target mRNAs in the nucleus (Martinez-Pastor MT, et al. 2013, Prouteau, et al. 2008, Vergara, et al. 2011). Then, Cth2 either interferes with the polyadenylation machinery, leading to extended transcripts that are processed in the nucleus, or translocates to the cytoplasm where the mRNA is degraded from 5' to 3' (Prouteau, et al. 2008, Vergara, et al. 2011). Binding to its target mRNAs is required for Cth2 export to the cytoplasm, since Cth2 proteins mutated in its TZFs, which do not associate to transcripts, are trapped in the nucleus and do not promote mRNA turnover (Vergara, et al. 2011).

The most conserved region in the TTP family of proteins is the TZF motif. Thus, the TZF of the fission yeast *Schizosaccharomyces pombe* family member is functionally interchangeable with the analogous domain of other distantly related organisms including humans, insects, plants and pathogenic yeasts (Wells, et al. 2015). To date, the overall structure of TTP-type proteins has not been elucidated. However, the structure of the human TIS11d TZF domain associated to the single stranded RNA oligonucleotide 5'-UUAUUUAUU-3' has been solved, showing two highly similar Cx₈Cx₅Cx₃H zinc fingers bound to separate 5'-UAUU-3' motifs (Hudson, et al. 2004). By using this sequence as a template, we have predicted now

a potential structure for the yeast Cth2 TZF domain interacting with the mRNA (Figure 2). Beyond the TZF, the primary sequence conservation of this family of proteins decreases. Bioinformatic analyses of the mammalian and *Drosophila melanogaster* TIS11/TTP suggest that both proteins lack a stable tertiary structure, showing highly unstructured amino-terminal and carboxy-terminal regions (NTD and CTD) aside of the TZFs and short stretches at both ends of the protein (Ngoc, et al. 2014). These intrinsically disordered regions could facilitate the exposure of motifs implicated in the recruitment of other proteins. In fact, deletion of either the NTD or the CTD of TTP abrogates function (Rigby, et al. 2005). In addition to its TZFs, *S. cerevisiae* Cth2 protein contains three regions that are conserved among the genus *Saccharomyces*: CR1 and CR2, which localize to its NTD region, and CR3, which is present in its CTD end (Prouteau, et al. 2008). Similarly to other TTP family members, the bioinformatic analysis of the Cth2 NTD with the IUPred2A algorithm (Meszaros, et al. 2018) suggests that the NTD of yeast Cth2 protein acquires an unstructured conformation. Interestingly, in the structural model of Cth2 CTD, CR3 folds back into the TZFs (Figure 2). This is in agreement with the lack of functionality of any CTD-tagged Cth2 protein, as the tag likely interferes with the TZFs function (Prouteau, et al. 2008, Puig, et al. 2005, Vergara, et al. 2011). None of these three conserved domains (CR1, CR2 and CR3) is necessary for Cth2 nucleocytoplasmic shuttling and for binding to ARE-containing mRNAs, which depend on the integrity of the TZFs (Prouteau, et al. 2008, Ramos-Alonso, et al. 2018, Vergara, et al. 2011). Importantly, the deletion of CR1 alone, or in combination with CR2, leads to an increase in the steady-state levels of Cth2 mRNA targets and to the appearance of new extended transcript species during iron depletion; whereas no changes are observed for a Cth2 protein lacking CR3

(Prouteau, et al. 2008, Ramos-Alonso, et al. 2018). These results suggest that Cth2 NTD is implicated in mRNA decay and transcription termination by probably recruiting the mRNA decay and termination machineries, whereas the CTD does not participate in these processes. Consistent with this, recent mRNA half-life measurements have demonstrated that the CR3 is dispensable for the turnover of *SDH4* mRNA in response to iron-depleted conditions (Ramos-Alonso, et al. 2018). By using protein/mRNA ratios and polysome profiles, we have shown that, in addition to promoting mRNA decay, Cth2 inhibits the translation of its target mRNAs in a process that depends on the integrity of the ARE within the transcript and of Cth2 TZFs (Ramos-Alonso, et al. 2018). Remarkably, we have observed that not only the NTD but also Cth2 CTD contribute to repress the translation of the target mRNAs (Ramos-Alonso, et al. 2018). The fact that CR3 is only necessary to inhibit mRNA translation, but does not alter the stability of the transcript, opened the possibility to study the contribution of translation repression to cell growth and to the regulatory function played by Cth2 during iron starvation. Cells expressing a Cth2 protein lacking the CR3 (Cth2 Δ C52) displayed a growth defect under iron-depleted conditions, emphasizing the importance of Cth2 CTD (Ramos-Alonso, et al. 2018). The role played by the Cth2-mediated regulation of mRNA translation is stressed by the striking increase in the protein levels of various iron-using enzymes displayed in Cth2 Δ C52-expressing cells (Figure 1). Altogether these data strongly suggest a central contribution of Cth2 CTD to the adaptation of yeast cells to iron depletion via translation repression.

Mechanism for Cth2-mediated repression of protein translation

Eukaryotic mRNA decay and protein translation are closely related processes. In general, mRNA turnover occurs as a consequence of translational repression (Huch and Nissan 2014). For instance, the yeast decapping activators Dhh1 and Pat1 promote decapping and mRNA turnover after inhibiting translation (Parker 2012, Roy and Jacobson 2013). However, this intimate interconnection is far from being straightforward. There are unstable mRNAs that are translated efficiently, while other highly stable transcripts remain untranslated (Roy and Jacobson 2013). In fact, degradation of actively translated mRNAs occurs in some instances (Hu, et al. 2009). Polysome loading is even a prerequisite to the mRNA decay exerted by some RNases (Mino, et al. 2015). There are also examples in which both processes have been separated, such as the mouse TTP homolog Zfp3611, which represses the translation the *VEGF* mRNA without altering its mRNA stability (Bell, et al. 2006).

After establishing that Cth2 plays an important role in regulating the translation of many iron-containing proteins under iron deficiency, several questions arise. We do not know yet if the Cth2 translational repression function comes first or after its role as an activator of mRNA decay. Our studies with truncated Cth2 proteins suggest that Cth2 CTD could preferentially interact with factors that control translation, while proteins implicated in both mRNA turnover and translation would associate to its NTD (Ramos-Alonso, et al. 2018). Many conserved mRNA decay and translational repression regulatory factors interact with TTP, the mammalian Cth2 ortholog, including the RNA helicase RCK/p54, the CCR4-NOT complex, the cap-binding factor eIF4E2 or the Poly-A-Binding Protein (Fu, et al. 2016, Lykke-Andersen and Wagner 2005, Qi, et al. 2012, Tao and Gao 2015, Zhang, et al. 2017). The identification of the machinery that allows Cth2 to

post-transcriptionally regulate gene expression could contribute to understand the connections between mRNA decay and translation. Dhh1, the yeast RCK/p54 homolog that functions as an activator of decapping and a repressor of translation under stress conditions, is a plausible candidate to mediate Cth2 translational repression for several reasons. First, we observed a genetic interaction between *CTH2* and *DHH1* genes, since the deletion of *DHH1* rescues the growth defect of Cth2-overexpressing cells. Secondly, the Cth2 target mRNA *SDH4* stabilizes in iron-deficient *dhh1Δ* cells. And thirdly, yeast two hybrid experiments indicate that Cth2 interacts with the CTD of Dhh1 (Pedro-Segura, et al. 2008). However, the domains of Cth2 involved in this interaction have not yet been determined. Dhh1 has been shown to act as a general translational repressor when overexpressed (Coller and Parker 2005). Although initially thought to act at the level of translation initiation, it has been recently postulated that Dhh1 slows down the movement of ribosomes on the mRNAs and, as a consequence, promotes decapping and decay (Sweet, et al. 2012). Therefore, the repression of protein translation during the elongation phase would occur prior to the activation of decapping and independently of it (Carroll, et al. 2011, Sweet, et al. 2012). Nonetheless, many details about the molecular mechanism of Dhh1-mediated repression of translation are still unknown, and an additional role of Dhh1 at the initiation step cannot yet be excluded. More information on the mode of action of Dhh1 as well as on the domains of Cth2 involved in the interaction with Dhh1 would be necessary to fully understand the particular role of both proteins in ARE-mediated translational inhibition and mRNA decay under iron deficiency.

Conclusions and perspectives

The post-transcriptional regulation of gene expression plays a central role in iron homeostasis, and the baker's yeast is not an exception. The RNA-binding protein Cth2 modulates iron metabolism by controlling both the stability and the translation of ARE-containing mRNAs during the adaptation to iron starvation (Puig, et al. 2005, Ramos-Alonso, et al. 2018). However, the relative contribution and the connections between Cth2-dependent mRNA turnover and translation have not been fully elucidated. Further studies on the sequential assembly of regulatory proteins onto the different Cth2 domains and onto the target mRNA could improve our current understanding of the mechanisms that post-transcriptionally regulate gene expression. We have previously shown that the conserved RNA helicase Dhh1 participates in the Cth2-mediated degradation of specific mRNAs under iron-deficient conditions (Pedro-Segura, et al. 2008). Since Dhh1 as a global regulator of mRNA translation, its involvement in controlling mRNA translation via Cth2 in response to low iron is a likely hypothesis that requires a thorough examination.

Materials and Methods

Yeast strains, plasmids and growth conditions. To determine protein levels, yeast *cth1 Δ cth2 Δ* (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 cth1::KanMX4 cth2::HisMX6*) cells transformed with pRS416-GFP-CTH2, pRS416 or pRS416-GFP-CTH2 Δ C52 plasmids were cultivated for 6 hours in synthetic complete medium without uracil (SC-ura) supplemented with 100 μ M of the Fe²⁺-specific chelator bathophenanthroline disulfonate (BPS) (Puig, et al. 2005, Ramos-Alonso, et al. 2018, Sikorski and Hieter 1989, Vergara, et al. 2011).

Protein analyses. Total protein extracts were obtained by using the alkali method (Kushnirov 2000). Equal amounts of protein were resolved in SDS-PAGE gels and transferred to nitrocellulose membranes. Ponceau staining was used to assess protein transfer. Aconitase (Aco1p), biotin synthase (Bio2p) and ferrochelatase (Hem15p) were detected using specific antibodies from rabbit kindly provided by Dr. Roland Lill (Philipps-Universität Marburg, Germany). We used HRP-labeled secondary anti-rabbit antibody from donkey (Santa Cruz Biotech) and ECL Select Western blotting detection kit (GE Healthcare Life Sciences). Immunoblot images were obtained in an ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Life Sciences) and specific signals were quantified with ImageQuant TL analysis software (GE Healthcare Life Sciences). Aco1, Bio2 and Hem15 protein levels were quantified, and the relative average and standard deviation of three independent biological samples are indicated. Tailed t-student tests were applied to evaluate statistical significance.

Bioinformatic analyses. The structural model for the TZFs and the CTD of the yeast Cth2 protein (from amino acid 160 to 285) has been obtained by using the I-TASSER server for protein structure and function prediction (Yang, et al. 2015). PDB structures 1RGO (Hudson, et al. 2004) and 2CQE, which corresponds to the zinc-finger domain of human protein KIA1046, were used as templates. The IUPred2A software (iupred2a.elte.hu) was used to predict potential intrinsically unstructured protein regions (Meszaros, et al. 2018).

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Figure legends

Figure 1. The deletion of the Cth2 carboxy-terminal conserved region CR3 alters the levels of various iron-dependent proteins. Yeast cells expressing *CTH2*, empty vector or *CTH2ΔC52* were grown in iron-deficient conditions to exponential phase. Total proteins were extracted and the levels of aconitase (Aco1p), biotin synthase (Bio2p) and ferrochelatase (Hem15p) determined. The average and standard deviation of three independent biological samples is shown. The asterisk (*) indicates a significant p-value (≤ 0.05) compared to *CTH2*-expressing cells.

Figure 2. Structural model for the carboxy-terminal region of the yeast Cth2 protein. A model for the TZFs and the CTD of the yeast Cth2 protein (from amino acid 160 to 285) bound to single stranded RNA is represented. The RNA is shown in red, whereas the protein backbone is indicated in gray except for the CR3 (from amino acid 263 to 285), which is depicted in green. The histidine and cysteine residues that coordinate the two Zn^{2+} ions (symbolized by dark spheres) are displayed in blue and yellow color, respectively. TZF1: TZF on the NTD side; TZF2: TZF on the CTD side.

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Figure 1

Figure 2