**Sequential recruitment of the mRNA decay machinery to the iron-regulated protein Cth2
in *Saccharomyces cerevisiae***

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ABSTRACT

Post-transcriptional factors importantly contribute to the rapid and coordinated expression of the multiple genes required for the adaptation of living organisms to environmental stresses. In the model eukaryote *Saccharomyces cerevisiae*, a conserved mRNA-binding protein, known as Cth2, modulates the metabolic response to iron deficiency. Cth2 is a tandem zinc-finger (TZF)-containing protein that co-transcriptionally binds to adenine/uracil-rich elements (ARE) present in the 3'-untranslated region of iron-related mRNAs to promote their turnover. The nuclear binding of Cth2 to mRNAs via its TZFs is indispensable for its export to the cytoplasm. Although Cth2 nucleocytoplasmic transport is essential for its regulatory function, little is known about the recruitment of the mRNA degradation machinery. Here, we investigate the sequential assembly of mRNA decay factors during Cth2 shuttling. By using an enzymatic *in vivo* proximity assay called M-track, we show that Cth2 associates to the RNA helicase Dhh1 and the deadenylase Pop2/Caf1 before binding to its target mRNAs. The recruitment of Dhh1 to Cth2 requires the integrity of the Ccr4-Pop2 deadenylase complex, whereas the interaction between Cth2 and Pop2 needs Ccr4 but not Dhh1. M-track assays also show that Cth2-binding to ARE-containing mRNAs is necessary for the interaction between Cth2 and the exonuclease Xrn1. The importance of these interactions is highlighted by the specific growth defect in iron-deficient conditions displayed by cells lacking Dhh1, Pop2, Ccr4 or Xrn1. These results exemplify the stepwise process of assembly of different mRNA decay factors onto an mRNA-binding protein during the mechanism of post-transcriptional regulation.

HIGHLIGHTS

- 1) Cth2 protein associates to Dhh1 and Pop2/Caf1 before binding to its target mRNAs.
- 2) The mRNA deadenylase complex Ccr4-Pop2/Caf1 is required for Cth2-Dhh1 interaction.
- 3) The interaction between Cth2 and Pop2/Caf1 requires Ccr4, but not Dhh1.
- 4) Cth2 interacts with the Xrn1 5' to 3' exonuclease only after binding to mRNAs.
- 5) Dhh1, Pop2 and Xrn1 contribute to the adaptation of yeast cells to iron deficiency.

Keywords: yeast, *Saccharomyces cerevisiae*, iron deficiency, gene expression, post-transcriptional regulation

Abbreviations: AMD, ARE-mediated decay; AU, adenosine and uridine; ARE, AU-rich element; BPS, bathophenanthroline disulfonic acid disodium; DIC, differential interference contrast; Gal4 binding domain, GFP, green fluorescent protein; HKMT, human histone lysine methyltransferase; HA, hemagglutinin; HU, hydroxyurea; MMS, methyl methanesulphonate; NES, nuclear export signal; NLS, nuclear localization signal; OD, optical density; P-bodies, processing-bodies; RT-qPCR, real time - quantitative polymerase chain reaction; r.u., relative units; SC, synthetic complete; SD, standard deviation; TTP, tristetraprolin; TZF, tandem zinc finger; UTR, untranslated region.

1. INTRODUCTION

In eukaryotic organisms, the coordinated expression of multiple genes in response to environmental changes can be achieved at the transcriptional and post-transcriptional level. Transcription factors bind to specific *cis*-acting elements within the promoter of particular genes to regulate their transcription by modulating the activity or the recruitment of the RNA polymerase. Post-transcription factors recognize specific sequences within the untranslated regions (UTRs) of mRNAs to enhance the recruitment of the machinery that controls translation and/or transcript stability, allowing rapid changes in mRNA expression. The first and rate-limiting step in the cytoplasmic turnover of mRNAs consists on the shortening of the 3' poly(A) tail, a conserved process referred to as deadenylation. In the model eukaryote *Saccharomyces cerevisiae*, the predominant deadenylase is the multifunctional Ccr4-Not complex, which is composed of two associated 3' to 5' exonucleases, Ccr4 and Pop2/Caf1, and seven additional subunits (Not1, Not2, Not3, Not4, Not5, Caf40 and Caf130) (reviewed in [1]). The Ccr4-Not complex participates in the regulation of all the stages of gene expression, from transcription elongation to mRNA quality control, export, translation and turnover (reviewed in [2, 3]). Following deadenylation, the transcript can undergo 3' to 5' turnover by the exosome complex, or be decapped by the Dcp1/Dcp2 decapping enzyme. In the latter case, the mRNA is subjected to 5' to 3' degradation by the Xrn1 exoribonuclease or its translation is inhibited at different steps (reviewed in [4]). Two multimeric complexes stimulate decapping upon assembly onto mRNAs: one complex consists of the scaffold protein Edc3, the DEAD-box RNA helicase Dhh1, Dcp1 and Dcp2; and the second set of proteins includes the Pat1 scaffold protein, the Lsm1-7 complex and Xrn1. Moreover, multiple components of the Ccr4-Not complex, including Ccr4, Pop2/Caf1 and Not1, have been shown to physically interact with Dhh1 [5-8]. The components of the mRNA decapping and decay machinery can accumulate into cytoplasmic ribonucleoproteic foci known as processing bodies or P-bodies, which contain untranslating mRNAs (reviewed in [9]). P-bodies are dynamic assemblies which abundance increases with the amount of mRNAs associated to the decapping machinery. Therefore, P-body assembly rises when

processes such as decapping or 5' to 3' mRNA turnover are defective, as it is the case with yeast *dcp1Δ* and *xrn1Δ* mutants, respectively. Most components of the major mRNA decay machinery, including Edc3, Dcp2, Dcp1, Pat1, Lsm1, Ccr4, Xrn1 and the poly(A)-binding protein Pab1, as well as other factors that associate to the RNA polymerase II, such as Rpb4 and Rpb7, shuttle between the nucleus and the cytoplasm to interconnect both transcription and mRNA stability [10, 11]. Thus, global changes in mRNA stability are compensated by alterations in bulk transcription rate that buffer total mRNA abundance (reviewed in [12, 13]). Xrn1, Pab1, the Ccr4-Not complex, Rpb4 and Rpb7 have been proposed as potential multifunctional coordinators of this circular gene expression process including transcription, mRNA stability, and translation [10, 11, 14-16]. However, in response to environmental cues, the abundance of particular subsets of transcripts needs to be altered for adaptation. The co-transcriptional imprinting of mRNAs with proteins that escort them to their ultimate destination plays an important role in gene expression, especially in response to stresses (reviewed in [17]).

Iron is an essential micronutrient for all eukaryotic organisms because it participates as a redox cofactor in many vital metabolic processes. Despite its abundance, iron bioavailability is highly restricted to living organisms due to the extremely low solubility of ferric iron (Fe^{3+}) at physiological pH. Therefore, cells have developed sophisticated regulatory strategies to maintain iron homeostasis. Eukaryotic cells respond to iron depletion by activating iron acquisition at the cell surface, mobilizing iron from intracellular stores, and promoting a global metabolic remodeling of iron-dependent processes directed to optimize cellular iron utilization [18, 19]. A central coordinator of this metabolic response is the conserved RNA-binding protein known as Cth2 in yeast, and its mammalian homologue tristetraprolin (TTP) [20]. Upon cellular iron deficiency, both proteins directly bind to adenosine and uridine (AU)-rich elements (ARE) within the 3'UTR of a set of mRNAs through two $\text{Cx}_8\text{Cx}_5\text{Cx}_3\text{H}$ tandem zinc fingers (TZFs), and promote ARE-targeted mRNA decay (AMD) and translation inhibition [21-29]. Cth2 and TTP targets include transcripts encoding for proteins that contain iron or participate in iron-dependent processes, such as mitochondrial respiration [24-26, 28]. Beyond iron

regulation, mammalian TTP is mostly implicated in diverse physiological and pathological processes including cancer and the inflammatory and immune responses (reviewed in [30-32]). Both Cth2 and TTP proteins interact with members of the mRNA decay and translation repression machineries. For instance, TTP associates to the Dhh1 homolog RCK/p54, the Ccr4-Not complex, the poly(A)-binding protein and the exosome [22, 33-35], whereas Cth2 has been reported to interact with Dhh1 [36]. Similarly to other components of the mRNA decay machinery, Cth2 and TTP accumulate in cytoplasmic P-bodies under certain circumstances [36, 37]. Both Cth2 and TTP proteins shuttle between the nucleus and the cytoplasm, and contain a specific nuclear localization signal (NLS) between the two TZFs [38-41]. However, opposite to TTP, yeast Cth2 does not contain any nuclear export signal (NES), instead it relies on binding to its target mRNAs to be exported to the cytoplasm [41]. Thus, mutations in the Cth2 TZFs that abrogate binding to its target mRNAs (e. g. Cth2-C190R or Cth2-C190A-C213A) prevent its exit from the nucleus. Moreover, Cth2 protein is also trapped in the nucleus in mutants defective in mRNA export or when bulk transcription is inhibited [41]. Additional data have shown that Cth2, and specially Cth2 proteins lacking its amino-terminal domain, interfere with the normal poly(A) site selection of ARE-containing mRNAs, leading to extended and highly unstable transcripts [42]. A mammalian TTP homologue denoted TIS11b also modulates the 3'-end processing of an ARE-containing mRNA [43]. These observations suggest that Cth2 and TTP co-transcriptionally associate to its target mRNAs and probably determine their nuclear and cytoplasmic fate during iron deficiency.

Despite Cth2 nucleocytoplasmic shuttling being required for its mRNA regulatory function, little is known about the molecular events that occur during this pathway. In this study, we have explored how the messenger ribonucleoprotein (mRNP) complex containing Cth2 protein and the nascent ARE-containing mRNAs assemble. Particularly, we have determined that Cth2 associates to Dhh1 helicase and Pop2 deadenylase before it binds to mRNAs, which supports that both factors perform important functions in the nucleus. However, Xrn1 exonuclease only associates to Cth2 after its mRNA interaction.

These results provide some light over the steps that mRNA-binding proteins follow to assemble mRNP complexes, which imprint a set of transcripts in response to stress.

2. MATERIALS AND METHODS

2.1. Yeast strains, growth conditions and plasmids

The yeast strains used in this study are listed in Supplemental Table S1. *SDH4* coding sequence was deleted from a *cth1Δcth2Δ* strain with a PCR-amplified *SDH4::loxP-LEU2-loxP* cassette from pUG73 plasmid [44] to obtain *cth1Δcth2Δsdh4Δ* strain. *LEU2* marker was removed by recombining the loxP sites with expression of the Cre recombinase from pSH47 plasmid, as previously described [44]. *XRN1* coding sequence was deleted from both *cth1Δcth2Δ* and *cth1Δcth2Δsdh4Δ* strains with a PCR-amplified *XRN1::hphMX4* cassette from pAG32 plasmid [45] to obtain *cth1Δcth2Δxrn1Δ* and *cth1Δcth2Δxrn1Δ sdh4Δ* strains, respectively. For M-track assays, BY4741 and AH109 cells were co-transformed with different plasmids (listed in Supplemental Table S2). Liquid yeast precultures were cultivated overnight at 30°C in a synthetic complete (SC) medium without the corresponding amino acids and reinoculated at an optical density at 600 nm (OD_{600}) of 0.25. To induce iron deficiency, 100 μ M of the Fe^{2+} -specific chelator bathophenanthroline disulfonic acid (BPS; Sigma) was added to the culture and cells were collected after 6 hours in continuous shaking at 30°C. For growth in 96-well plates, yeast cells were inoculated at an OD_{600} of 0.1 in SC-ura medium with or without 100 μ M BPS, and the OD_{600} was determined with a Spectrostar Nano absorbance microplate reader (BMG Labtech) every 30 minutes for 3 days at 28°C. For growth assays in solid media, yeast cells were incubated to exponential phase in liquid media, diluted to an OD_{600} of 0.1 and spotted on plates directly and after 1:10 and 1:100 dilutions in sterile water. To induce iron deficiency the Fe^{2+} -specific chelator Ferrozine (Sigma) was added at the indicated concentrations. YPD plates contained 2% glucose, and YPG plates contained 2% glycerol instead of glucose. For yeast two-hybrid assays, AH109 cells were co-transformed with GAD- and GBD-based plasmids (listed in Supplemental Table S2), grown in SC-leu-trp

(+His) and SC-leu-trp-his (-His; + 9 mM 3-amino-1,2,4-triazole), and incubated at 30°C for 3–5 days. For microscope assays, cells were reinoculated at an OD₆₀₀ of 0.03 at 30°C for 6 hours. Samples were collected for observation in an Eclipse 90i Nikon microscope (Nikon corporation) using a digital camera (Nikon DS-5Mc).

The plasmids used in this work are listed in Supplemental Table S2. The pRS416ΔNotI-P_{CTH2}-HKMT-CTH2 and pRS416ΔNotI-P_{CTH2}-HKMT-CTH2-C190/213A plasmids were made by the insertion in phase of the *Myc-HKMT* DNA fragment amplified from the YCp111-P_{ADH4}-3xMyc-HKMT plasmid into the NotI site located after the initiation codon of *CTH2* in pRS416ΔNotI-CTH2 and pRS416ΔNotI-CTH2-C190/213A plasmids, respectively [26]. The pRS416ΔNotI-P_{CTH2}-HKMT-T_{CTH2} plasmid was obtained by removing *CTH2* coding sequence from pRS416ΔNotI-P_{CTH2}-HKMT-CTH2 plasmid using specific phosphorylated oligonucleotides. The YEp195-XRN1-4xH3-3xHA and YEp195-DHH1-4xH3-3xHA plasmids were made by the insertion of the *P_{XRN1}-XRN1* and *P_{DHH1}-DHH1* DNA fragments amplified from genomic DNA into the PstI and XbaI sites of YEp195-4xH3-3xHA plasmid. The substitution of the *URA3* marker by *LEU2* in YEp195-XRN1-4xH3-3xHA and YEp195-DHH1-4xH3-3xHA plasmids was performed by yeast co-transformation with pUL9 digested with SmaI [46]. The pRS415-4xH3-3xHA-T_{TEF} plasmid was made by the insertion of the *4H3-3HA* DNA fragment from the YEp195-4xH3-3xHA into HindIII and SacI sites of pRS415-GCN4-T_{CTH1}. The pRS415-POP2-4xH3-3xHA-T_{TEF} plasmid was made by the insertion of the *P_{POP2}-POP2* DNA fragment, amplified from genomic DNA, into HindIII and XbaI sites of the pRS415-4xH3-3xHA-T_{TEF} plasmid. The pRS415-CCR4-4xH3-3xHA-T_{TEF} plasmid was made by the insertion of the *P_{CCR4}-CCR4* DNA fragment, amplified from genomic DNA, into SacI and XbaI sites of the pRS415-4xH3-3xHA-T_{TEF} plasmid. The pGBKT7-GAL4DBD-POP2 was made by the insertion of *POP2* amplified from genomic DNA into Sall and NotI sites of the pGBKT7 plasmid. All the plasmids constructed in this study were sequenced.

2.2. RNA analyses

Total yeast RNA isolation, as well as reverse transcription and quantitative PCR (RT-qPCR) were performed as previously described [47]. For the *SDH4* mRNA half-life determination, a *GAL1*-driven *SDH4*-expressing plasmid was used, and glucose was added to a galactose-containing medium to stop transcription as previously described [36]. Moreover, cDNA was obtained as described above but with the use of random primers instead of oligo-dT. The cDNA was then analyzed by RT-qPCR using specific primer pairs (listed in Supplemental Table S3). Data and error bars represent the relative average and standard deviations of three independent biological replicates.

2.3. Protein analyses

Yeast proteins were extracted and analyzed by Western blot as previously reported [48]. The primary antibodies used in this study include anti-Myc (1:2000 dilution; clone 9E10, Roche), anti-HA (1:5000 dilution; 3F10, Roche-Sigma), anti-Histone H3 trimethyl-K9 (anti-me3K9H3) (1:2000 dilution; clone 6F12-H4, Novus Biologicals), anti-Dhh1 (1:1000 dilution; sc-51481, Santa Cruz Biotechnology) and anti-Xrn1 (1:5000 dilution; provided by Dr. Arlen Johnson, University of Texas). The secondary antibodies used included anti-mouse (1:50000 dilution; NA931, Amersham), anti-goat (1:2000; sc-2350, Santa Cruz Biotechnology) and anti-rabbit (1:10.000; sc-2313, Santa Cruz Biotechnology). Immunoblot images were obtained with an ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Life Sciences), and protein levels were quantified with ImageQuant TL analysis software (GE Healthcare Life Sciences) or Image J 1.42q software (<http://rsb.info.nih.gov/ij>).

2.4. Statistical analyses

The statistical analysis of the relative gene expression was performed by the pairwise fixed reallocation randomization test [49]. For the remaining parameters, the analysis

was carried out using two-way ANOVA with the means compared by the Duncan test or a Kruskal-Wallis (p -value < 0.05 and p -value < 0.01) test for non-parametric measurements using the InfoStat software, version 2010 (<http://www.infostat.com.ar>).

3. RESULTS

3.1. *Cth2* protein associates to *Dhh1* before binding to its target mRNAs

We have previously used the yeast two-hybrid system to show that the RNA-binding protein Cth2 interacts with the DEAD-box RNA helicase Dhh1 [36]. However, this experimental approach forces both proteins to localize to the nucleus, masking the effect of their constitutive nucleus-cytoplasmic shuttling. To further investigate the dynamics of Cth2-Dhh1 interaction *in vivo*, we decided to use the M-track approach [50, 51]. According to the M-track method, when a bait protein tagged with a mutant version of the human histone lysine methyltransferase (HKMT) enzyme interacts with a prey protein fused to various copies of a small piece (amino acids 1–21) of histone H3 (H3 tag), the HKMT enzyme trimethylates histone H3 at lysine 9. Trimethylation can be detected by Western blot with a specific anti-me3K9H3 monoclonal antibody (Fig. 1A). Therefore, we tagged Cth2 bait protein at its amino-terminus with HKMT and the Myc epitope tag (Fig. 1A). To test the functionality of the Cth2-tagged protein, we first expressed *MYC-HKMT-CTH2* in the *cth1Δcth2Δ* strain under the control of *CTH2* native promoter, and assayed growth and targeted mRNA down-regulation under iron-deficient conditions. We observed that the expression of *MYC-HKMT-CTH2* fully complemented the growth defect of a *cth1Δcth2Δ* strain under iron-deficient conditions (Supplemental Fig. S1A). We have previously demonstrated that Cth2 promotes the degradation of the ARE-containing mRNAs *SDH4* (subunit four of succinate dehydrogenase), *ACO1* (aconitase) and *HEM15* (ferrochelatase) in response to iron depletion [26]. As a consequence, *SDH4* and *HEM15* mRNA levels decrease under low iron conditions, whereas *ACO1* transcript abundance only slightly increases (Supplemental Fig. S1B). In the absence of *CTH2* (vector), all three transcripts increase their abundance when iron availability is limited. Importantly, expression of Myc-

HKMT-Cth2 brings the mRNA levels of these three Cth2 targets to wild-type levels (Supplemental Fig. S1B). Both observations indicate that the Myc-HKMT-Cth2 fusion protein is fully functional. The prey protein for this M-track assay, Dhh1, was tagged at its carboxyl-terminus with four tandemly arranged copies of the H3 tag followed by three copies of hemagglutinin (HA) epitope to obtain Dhh1-H3-HA (Fig. 1A). By using a specific Dhh1 antibody, we first compared Dhh1-H3-HA protein levels with the endogenous Dhh1 expression in both a wild-type and a *dhh1Δ* mutant strain, and concluded that Dhh1-H3-HA is expressed at levels similar to those of the untagged Dhh1 protein (Supplemental Fig. S2A). Second, the levels of *EDC1* mRNA, which are up-regulated in *dhh1Δ* mutant cells [52], were restored in the presence of either Dhh1 or Dhh1-H3-HA (Supplemental Fig. S2B). And third, the growth defect generated by the treatment of *dhh1Δ* cells with the DNA alkylating agent methyl methanesulphonate (MMS) or the chemical agent hydroxyurea (HU) [53, 54] was reverted to wild-type levels with Dhh1-H3-HA (Supplemental Fig. S2C). These results demonstrate that both Myc-HKMT-Cth2 and Dhh1-H3-HA proteins are fully functional.

To study the dynamics of Cth2-Dhh1 interaction *in vivo*, yeast cells co-expressing both Myc-HKMT-Cth2 and Dhh1-H3-HA proteins were cultivated under iron-deficient conditions to induce *CTH2* expression. Both Myc-HKMT-Cth2 and Dhh1-H3-HA proteins were expressed under iron-deficient conditions as determined by Western blot with anti-Myc and anti-HA antibodies, respectively (Fig. 1B). The interaction between Myc-HKMT-Cth2 and Dhh1-H3-HA proteins would lead to the specific trimethylation of Dhh1-H3-HA protein. To thoroughly quantify the Cth2-Dhh1 interaction, we performed three biologically independent replicates, quantified the Western blot signals, and normalized the me3K9H3 signal with HA values (Fig. 1C). Importantly, a Dhh1 trimethylation signal was detected in cells co-expressing both tagged proteins, which is indicative of Cth2-Dhh1 association (Figs. 1B and 1C). To assess the specificity of the Dhh1 trimethylation signal, we co-expressed Dhh1-H3-HA with a Myc-HKMT protein lacking Cth2 amino acid sequence, but under the control of the iron-regulated *CTH2* promoter. Despite Myc-HKMT was expressed at high levels under iron-deficient conditions, no Dhh1-H3-HA

trimethylation signal was observed in this case (Figs. 1B and 1C, Myc-HKMT). Moreover, no trimethylation signal was detected in cells that did not express HKMT (Figs. 1B and 1C, vector). Collectively, these results confirm that Cth2 and Dhh1 proteins interact during the growth of yeast cells under iron-deficient conditions. Given that Dhh1 interacts with Cth2 protein by yeast-two hybrid and M-track ([36]; Figs. 1B and 1C), we ascertained whether Dhh1 was important for growth under iron-deficient conditions. We observed that cells lacking Dhh1 displayed an exacerbated growth defect under iron deplete conditions (Fig. 1D), which highlights its relevance when iron availability is low. Again, Dhh1-H3-HA protein rescued *dhh1Δ* growth defect (Fig. 1D), reinforcing the functionality of Dhh1 tagged protein under low iron conditions.

Fig. 1. Cth2 interacts *in vivo* with Dhh1 RNA helicase in a TZF-independent manner. (A) Representative cartoon of the M-track assay. Cth2 protein is fused to the carboxyl-

terminus of the histone methyltransferase (HKMT) preceded by the Myc epitope (Myc), and the prey protein is fused to the amino-terminus of histone 3 (H3) and the hemagglutinin epitope tag (HA). Upon interaction, the prey protein is stably labeled by trimethylation (M). (B) Western blot showing the interaction between Myc–HKMT–Cth2 and Dhh1-H3-HA. The *cth1Δcth2Δ* strain co-transformed with pSP1020 (Dhh1-H3-HA) and either pRS416 (vector), pSP951 (Myc-HKMT), pSP942 (Myc-HKMT-Cth2) or pSP921 (Myc-HKMT-Cth2-TZFmt) plasmids was cultivated in SC-leu-ura with 100 μM BPS for 6 hours to exponential phase. Immunoblots were developed with anti-HA and anti-Myc antibodies to determine Dhh1-H3-HA and Myc-tagged protein levels. Trimethylation of the Myc-HKMT-tagged proteins was detected with anti-me3K9H3. (C) Relative quantification of the methylation (anti-me3K9H3) signals normalized against HA (anti-HA) signal. The average and standard deviations (SD) of three biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.01). (D) BY4741 (WT) cells transformed with pRS416 (vector), and *dhh1Δ* mutant transformed with pRS416 (vector) and pSP962 (Dhh1-H3-HA) were assayed for growth on SC-ura (+Fe) and SC-ura plus 900 μM Ferrozine (-Fe).

We have previously demonstrated that Cth2 C₈C₅C₃H TZFs are essential for binding to its ARE-containing target mRNAs, which occurs co-transcriptionally in the nucleus [26, 41, 42]. Moreover, Cth2 does not possess any nuclear export signal, and relies on its binding to its mRNA targets for being exported from the nucleus to the cytoplasm [41]. Thus, Cth2 mutants in its TZFs, such as Cth2-C190A-C213A (Cth2-TZFmt), are trapped in the nucleus at a stage previous to mRNA binding [41]. To ascertain whether Cth2 protein interacted with Dhh1 before binding to its mRNA targets in the nucleus, we studied the *in vivo* interaction between Myc-HKMT-Cth2-TZFmt and Dhh1-H3-HA by M-track. The expression levels of both Myc-HKMT-Cth2-TZFmt and Dhh1-H3-HA proteins were similar to those previously observed for cells expressing Myc-HKMT-Cth2 (Fig. 1B). More important, the interaction between Myc-HKMT-Cth2-TZFmt and Dhh1-H3-HA, as assessed by the Dhh1-H3-HA trimethylation signal, was indistinguishable of the one observed with wild-type Myc-HKMT-Cth2 (Figs. 1B and 1C). These results demonstrate

that Cth2-TZFmt and Dhh1 proteins interact *in vivo*, and strongly suggest that Cth2 recruits Dhh1 helicase before binding its target mRNAs.

3.2. The mRNA deadenylase complex Ccr4-Pop2 is required for Cth2-Dhh1 interaction under iron-deficient conditions

To further characterize how Dhh1 recruitment to Cth2 protein occurs, we used M-track to investigate the interaction between Cth2 and Dhh1 protein in yeast strains lacking components of the mRNA decay machinery. No defect in Cth2-Dhh1 interaction was observed in *pat1Δ* and *lsm1Δ* mutant cells by M-track (Figs. 2A and 2B). Interestingly, Cth2-Dhh1 interaction slightly decreased in *xrn1Δ* mutant cells (Figs. 2A and 2B), in which both Cth2 and Dhh1 proteins accumulate in P-bodies [36, 55]. Then, we assessed Cth2-Dhh1 interaction in the absence of components of the Ccr4-Pop2 mRNA deadenylase complex, which also shuttles between the nucleus and the cytoplasm [10]. As observed for *lsm1Δ* cells, the expression levels of Myc-HKMT-Cth2 were slightly lower in *ccr4Δ* and *pat1Δ* than in wild-type cells (Fig. 2A). Remarkably, according to the K9H3 trimethylation signal, the interaction between Cth2 and Dhh1 was strongly diminished in cells lacking either *CCR4* or *POP2* (Figs. 2A and 2B). Given that Ccr4 and Pop2 proteins assemble in a conserved multimeric complex, denoted Ccr4-Not, that regulates gene expression at multiple levels [2, 3], we decided to evaluate Cth2-Dhh1 association in cells lacking any single component of this complex. We observed that the interaction between Cth2 and Dhh1 was not abolished in *not3Δ*, *not4Δ*, *caf40Δ*, or *caf130Δ* cells (Supplemental Fig. S3). The Not1, Not2 and Not5 subunits of the Ccr4-Not complex were not assayed because they either were essential genes or their corresponding mutant displayed very slow growth. Altogether, these results strongly suggest that the mRNA deadenylase complex Ccr4-Pop2 is required for Cth2-Dhh1 interaction in low iron conditions.

Figure 2. The interaction between Cth2 and Dhh1 depends on the Ccr4-Pop2 deadenylase complex. (A) Representative Western blots showing the interaction of Myc-HKMT-Cth2 and Dhh1-H3-HA. Wild-type BY4741 (WT), *ccr4Δ*, *pop2Δ*, *pat1Δ*, *lsm1Δ* and *xrn1Δ* cells co-transformed with pSP1020 (Dhh1-H3-HA) and pSP942 (Myc-HKMT-Cth2) were cultivated and analyzed as described in Figure 1. (B) Relative quantification of anti-me3K9H3/anti-HA signals. The average and SD of at least three biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.01).

3.3. Cth2 protein interacts with Pop2 before binding its target mRNAs

Given that both Ccr4 and Pop2 are necessary for the interaction between Cth2 and Dhh1 during iron depletion, we wondered whether Cth2 also interacted with Ccr4 and/or

Pop2 proteins. We focused on Pop2, since a genome-wide study reported that *pop2Δ* mutants display a growth defect in low iron conditions [56]. Since our previous yeast two-hybrid assay was able to reveal the interaction of Dhh1 with Cth2 and its homolog Cth1 protein [36], we decided to use this experimental approach to test Cth1/2-Pop2 protein interaction. The full-length *POP2* coding sequence fused to the Gal4 DNA-binding domain (GBD) was co-expressed with either Cth1 or Cth2 fused to the Gal4 activation domain (GAD-Cth1 and GAD-Cth2, respectively). According to the yeast two-hybrid system, if both proteins interact, the transcription of the *HIS3* reporter gene within the *his3*-deficient AH109 host strain would be activated, and the cells would grow in the absence of histidine (-His). As shown in Figure 3A, cells co-expressing GBD-Pop2 with either GAD-Cth1 (Pop2 + Cth1) or GAD-Cth2 (Pop2 + Cth2) grew in minus histidine medium, whereas cells expressing GBD-Pop2 (Pop2 + vector), GAD-Cth1 (vector + Cth1), or GAD-Cth2 (vector + Cth2) alone did not grow in these conditions. These results indicate that Pop2 interacts with both Cth1 and Cth2 proteins.

To further characterize this interaction, we focused on Cth2 protein and the M-track assay. First, we fused the H3-HA tag to the carboxyl-terminus of Pop2 protein and assayed for Pop2-H3-HA functionality. In this case, we used the previously reported growth defect of *pop2Δ* mutants in the presence of caffeine or, more interesting, in iron-deficient conditions [56, 57]. In both cases, the expression of Pop2-H3-HA fully rescued *pop2Δ* growth defect (Supplemental Fig. S4A). Consistent with the yeast two-hybrid results, the Pop2-H3-HA trimethylation signal obtained with the M-track assay corroborated that Cth2 and Pop2 associate *in vivo* (Figs. 3B and 3C). Moreover, the M-track interaction between Cth2 and Pop2 was still present upon mutation of Cth2 TZF, as shown for cells expressing Myc-HKMT-Cth2-TZFmt instead of Myc-HKMT-Cth2 (Figs. 3B and 3C). Remarkably, the interaction between Cth2 and Pop2 decreased when the TZFs were mutated probably due to a destabilization of the complex. In any case, these results strongly suggest that, similarly to the Cth2-Dhh1, the interaction between Cth2 and Pop2 deadenylase under iron-deficient conditions is already established when Cth2 associates to its target mRNAs.

A

B

C

Figure 3. Cth2 interacts *in vivo* with Pop2 deadenylase. (A) Yeast two-hybrid assay between Cth1/Cth2 and Pop2 proteins. AH109 cells containing either pGBK-T7 (vector) or pGBK-Pop2 (Pop2) were co-transformed with pGAD-T7 (vector), pGAD-Cth1 (Cth1) or pGAD-Cth2 (Cth2), and spotted on SC-leu-trp (+His) and SC-leu-trp-his with 9 mM 3-aminotriazol (-His). (B) Western blot showing the interaction between Myc-HKMT-Cth2 and Pop2-H3-HA. The *cth1Δcth2Δ* strain co-transformed with pSP1041 (Pop2-H3-HA) and either pRS416 (vector), pSP951 (Myc-HKMT), pSP942 (Myc-HKMT-Cth2) or pSP921 (Myc-HKMT-Cth2-TZFmt) plasmids were cultivated and analyzed as described in Figure 1. (C) Relative quantification of anti-me3K9H3/anti-HA signals. The average and SD of three biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.05).

3.4. The interaction between Cth2 and Pop2 requires Ccr4 but not Dhh1

To determine the factors that contribute to the interaction between Cth2 and Pop2, we performed the Cth2-Pop2 M-track assay in multiple mutant strains. We first used the assay in cells lacking either *DHH1* or *LSM1*. Despite the decrease in Myc-HKMT-Cth2 protein levels observed in the *dhh1Δ* mutant, no defect in Pop2-H3-HA trimethylation was observed, which indicates that Dhh1 is not necessary for Pop2 association to Cth2 protein (Figs. 4A and 4B). Moreover, these M-track results also showed that the interaction between Cth2 and Pop2 was not altered in the absence of *LSM1* (Figs. 4A and 4B). Interestingly, Pop2-H3-HA trimethylation was fully abolished in the absence of Ccr4 (Figs. 4C and 4D). These results indicate that the integrity of the Ccr4-Pop2 deadenylase is indispensable for the association of Pop2 to Cth2, and suggest that Ccr4 could bridge the interaction between Cth2 and Pop2. To address whether Cth2 interacted with Ccr4, we fused the 4xH3-3xHA tag to Ccr4 carboxyl-terminus. Similarly to *pop2Δ*, we observed that *ccr4Δ* cells display a growth defect in media containing caffeine and in iron-deficient conditions (Supplemental Fig S4A). Importantly, *CCR4-4xH3-3xHA* expression fully complemented the growth defect of *ccr4Δ* cells in iron-deficient conditions, but not in the presence of caffeine (Supplemental Fig S4A). Since these experiments are performed in iron-limited conditions, we proceeded with the M-track

assay under these conditions. However, we did not observe any specific trimethylation signal when HKTM-Cth2 and Ccr4-4xH3-3xHA proteins were coexpressed (Supplemental Fig S5). These results indicate that Cth2 does not interact with Ccr4 by M-track probably because Ccr4 is not closer enough to Cth2 to become methylated. These results suggest that Cth2 would associate to Pop2 only when the Pop2-Ccr4 complex can properly assemble.

Figure 4. Ccr4 is required for the interaction between Cth2 and Pop2. Western blots showing the interaction between Myc-HKMT-Cth2 and Pop2-H3-HA. (A) BY4741 (WT), *dhh1Δ* and *lsm1Δ* strains co-transformed with pSP1041 (Pop2-H3-HA) and pSP942 (Myc-HKMT-Cth2). (B) Relative quantification of anti-me3K9H3/anti-HA signals from panel A. (C) BY4741 (WT) co-transformed with pSP1041 (Pop2-H3-HA) and pSP951 (Myc-HKMT),

and BY4741 (WT) and *ccr4Δ* strains co-transformed with pSP1041 (Pop2-H3-HA) and pSP942 (Myc-HKMT-Cth2). (D) Relative quantification of anti-me3K9H3/anti-HA signals from panel C. Yeast cells were cultivated and analyzed as described in Figure 1. The average and SD of at least four biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.01).

Given that both Pop2 and Ccr4 are required for growth in iron-deficient conditions, we decided to check the importance of other components of the Ccr4-Not complex in the adaptation to iron starvation. For this purpose, we assayed the growth of cells lacking non-essential components of the Ccr4-Not complex, such as *NOT3*, *NOT4*, *CAF40* and *CAF130*, in low iron conditions (Supplemental Fig. S4B). None of these mutants displayed any growth defect when cultivated in iron limitation, which suggests that, opposite to Pop2 and Ccr4, their contribution to the adaption to iron deprivation is not important.

3.5. The Xrn1 exonuclease contributes to the adaptation to iron deficiency.

By studying the mRNA decay intermediates trapped during the degradation of the *SDH4* transcript under iron-depleted conditions, we previously concluded that Cth2 promotes 5' to 3' mRNA turnover [36]. However, the molecular mechanisms that provide RNA degradation directionality to Cth2 protein have not been elucidated. Yeast cells express the shuttling 5' to 3' evolutionary conserved ribonuclease Xrn1. Given that Cth2 protein interacts with Dhh1 and Pop2 (Figs. 1 and 3), and that Cth2 accumulates in cytoplasmic P-bodies in *dcp1Δ* and *xrn1Δ* mutants [36], we hypothesized that Cth2-mediated mRNA decay would occur via Xrn1. To investigate the involvement of Xrn1 in Cth2-mediated mRNA degradation during iron starvation, we determined the mRNA half-life of *SDH4* transcript in wild-type cells, and in cells lacking *CTH2*, *XRN1* or both genes. As previously reported [36], *SDH4* mRNA half-life increased in cells lacking Cth2 protein (Fig. 5A). We observed that *SDH4* mRNA was more stable in *xrn1Δ* cells than in wild-type or *cth2Δ* mutant cells (Fig. 5A), which is consistent with a function of Xrn1 exonuclease in

global mRNA decay. Importantly, the decay rate of *SDH4* transcript did not increase when *CTH2* was deleted from the *xrn1Δ* strain (Fig. 5A), which suggests that Cth2 is only able to promote *SDH4* mRNA turnover via Xrn1.

Cells lacking *CTH2* or expressing a non-functional *CTH2* allele (*CTH2-TZFmt*) display a growth defect under iron-deficient conditions [26, 41]. Since Xrn1 is required for Cth2-mediated mRNA turnover (Figure 5A), we hypothesized that Xrn1 could contribute to the adaptation of yeast cells to iron starvation. Therefore, we cultivated both wild-type and *xrn1Δ* mutant cells under iron-sufficient and iron-deficient conditions, and made the following observations. First, *xrn1Δ* cells already displayed a slight growth defect under normal conditions (Figs. 5B and 5C). Second, a small growth defect was also obtained when wild-type cells were cultivated in iron-limited conditions (Figs. 5B and 5C). Third, and more important, *xrn1Δ* cells exhibited a growth defect more severe than wild-type cells when iron availability was limited (Figs. 5B-5D). These results suggest that Xrn1 is important for growth under iron starvation, probably because it is necessary for Cth2-mediated mRNA decay.

Figure 5. The Xrn1 exonuclease is required for optimal growth under iron deficiency. (A) *SDH4* mRNA half-life. *cth1Δcth2Δsdh4Δ* cells were co-transformed with either pRS416-CTH2 plus p415GAL-*SDH4* plasmids (WT) or pRS416 plus p415GAL-*SDH4* plasmids (*cth2Δ*), and *cth1Δcth2Δxrn1Δsdh4Δ* cells were co-transformed with either pRS416-CTH2 plus p415GAL-*SDH4* plasmids (*xrn1Δ*) or pRS416 plus p415GAL-*SDH4* plasmids (*xrn1Δcth2Δ*). Transformants were cultivated under in SC-leu-ura with 100 μ M BPS for 6 hours to exponential phase. A medium containing 2% galactose was used to induce *SDH4* expression and glucose to a final 2% concentration was added to stop its transcription. Total RNA was extracted and analyzed by RT-qPCR. *ACT1* was used as housekeeping

reference gene. The average and SD of two biological replicates are shown. (B) BY4741 (WT or Xrn1) cells transformed with pRS416, and *xrn1Δ* mutant transformed with pRS416 (*xrn1Δ* + vector) or pSP934 (Xrn1-H3-HA) were assayed for growth on solid SC-ura (+Fe) and SC-ura plus 900 μ M Ferrozine (-Fe) media. (C) and (D) Growth in liquid SC-ura (+Fe) and SC-ura plus 100 μ M BPS (-Fe) media. The average and SD of the $\mu_{\max}$ (panel C) and final OD₆₀₀ (panel D) of four biological replicates is shown. Different letters indicate statistically significant differences (*p* value < 0.01).

3.6. Cth2 interacts with Xrn1 exonuclease only after binding to mRNAs

Xrn1 protein shuttles between the nucleus and the cytoplasm, connecting multiple steps of gene expression including transcription, mRNA stability and translation [10]. Since Cth2 interacts with Dhh1 and Pop2 before binding to its target mRNAs in the nucleus, it is especially relevant to decipher whether, and at which step of shuttling, Cth2 and Xrn1 associate. To study the potential interaction between Cth2 and Xrn1 by the M-track technique, we epitope tagged Xrn1 at its carboxyl terminus with the H3-HA tag, and assayed for functionality. By using an antibody specific to Xrn1 protein, we showed that the expression levels achieved with the Xrn1-H3-HA fusion protein were similar to those obtained by the endogenous Xrn1 (Supplemental Fig. S6A). Three additional data supported XRN1-H3-HA functionality. First, *XRN1-H3-HA* fully complemented the growth defect of *xrn1Δ* cells under low iron (Figs. 5B-5D). Second, *XRN1-H3-HA* rescued the growth defect exhibited by *xrn1Δ* cells under fermentative (glucose) and, especially, under respiratory conditions (glycerol) (Supplemental Fig. S6B). And third, the accumulation of Cth2 protein in the P-bodies that assemble in *xrn1Δ* cells was relieved by *XRN1-H3-HA* expression (Supplemental Figs. S6C and S6D). Therefore, we proceeded to analyze the interaction between Myc-HKMT-Cth2 and Xrn1-H3-HA proteins by M-track under iron-deficient conditions. A strong Xrn1-H3-HA trimethylation signal demonstrated the Cth2-Xrn1 interaction *in vivo* (Fig. 6). Remarkably, this association was strongly reduced when Cth2 could not bind mRNAs due to TZF mutagenesis (Fig. 6, Cth2-TZFmt). These results strongly suggest that Cth2 only interacts with Xrn1 after binding to its target mRNAs.

Figure 6. Cth2 interacts *in vivo* with Xrn1 exonuclease in a TZF-dependent manner. (A) Western blot showing the interaction of Myc-HKMT-Cth2 and Xrn1-H3-HA proteins. The *cth1Δcth2Δxrn1Δ* strain co-transformed with pSP1019 (Xrn1-H3-HA) and either pRS416 (vector), pSP951 (Myc-HKMT), pSP942 (Myc-HKMT-Cth2) or pSP921 (Myc-HKMT-Cth2-TZFmt) plasmids was cultivated and analyzed as described in Figure 1. (B) Relative quantification of anti-me3K9H3/anti-HA signals. The average and SD of three biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.01).

4. DISCUSSION

Post-transcriptional factors are mRNA-binding proteins that simultaneously regulate the expression of multiple mRNAs in response to environmental stresses. It has been widely accepted that these factors recognize specific *cis*-acting elements within a set of mRNAs and modulate the recruitment of the regulatory machinery that controls mRNA expression. The conserved TZF-containing family of proteins constitutes an excellent model to study how 3'UTR mRNA-binding factors post-transcriptionally regulate gene expression. Human TTP has been shown to promote the shortening of the poly(A) tail of ARE-containing mRNAs followed by both 3' to 5' and 5' to 3' exonucleolytic turnover (reviewed in [58, 59]). Co-immunoprecipitation experiments support that TTP recruits

multiple mRNA decay enzymes including the CCR4/CAF1 deadenylase, components of the exosome, the decapping enzyme, and XRN1 exonuclease [33, 34, 58, 60-62]. In yeast, the detection of decay intermediates of an ARE-containing mRNA suggests that the yeast Cth2 TZF-family member preferentially promotes 5' to 3' mRNA destabilization [36]. Consistent with 5' to 3' directionality, we have shown here that Cth2 interacts *in vivo* with the Pop2/Caf1 deadenylase and the Xrn1 exonuclease (Figs. 3 and 6). Both TTP and Cth2 also interact with the conserved RNA helicase RCK/p54 and Dhh1, respectively, which promotes decapping and favors both mRNA turnover and translation repression [22, 23, 28, 36]. Therefore, TZF-containing proteins in both yeast and humans interact with the machinery necessary to post-transcriptionally inhibit gene expression by enhancing the decay and inhibiting the translation of ARE-containing mRNAs.

The co-transcriptional imprinting of mRNAs with proteins that modulate its progression and final fate is considered to play a central role in connecting multiple steps of gene expression (reviewed in [17]). However, little is known about the stepwise assembly of the different regulatory factors that determine the destination of a transcript. The nucleocytoplasmic movement of both TTP and Cth2 prompted us to hypothesize that, during its transit through the nucleus, they could be recruiting certain regulatory factors to assemble an mRNP complex that would pre-determine the destination of its target transcripts. While human TTP contains a NES that facilitates its export to the cytoplasm [39, 40], Cth2 relies on its ability to bind to ARE-containing mRNAs to exit the nucleus [41]. In fact, the inhibition of global transcription by either adding the RNA polymerase inhibitor thiolutin or by using a strain expressing the temperature sensitive RNA polymerase *rpb1-1^{ts}* leads to the accumulation of Cth2 protein into the nucleus [41]. Here, we have investigated the stepwise assembly of mRNA decay and translation factors onto Cth2 protein by using the Cth2-TZFmt, which is trapped in the nucleus in a step preceding mRNA binding. We have observed that the Pop2/Caf1 deadenylation enzyme and the Dhh1 enhancer of decapping play important functions in the nucleus by associating to Cth2 protein even before Cth2 recognizes any nascent ARE-containing mRNA (Figs. 1 and 3). This result strongly suggests that Cth2 is already charged with the decay machinery

when it binds emerging ARE-containing mRNAs, pre-determining at the very beginning post-transcriptional stages of regulation. Moreover, the interaction with the Cth2 TZF mutant is also consistent with an RNA-independent association between Cth2 and both Dhh1 and Pop2/Caf1. The M-track experiments also show that the interaction between Cth2 and Pop2 does not occur in a *ccr4Δ* mutant (Fig. 4), but no interaction was observed between Cth2 and Ccr4 (Supplemental Fig. S5). These results suggest that Cth2 primarily interacts with Pop2 and that the integrity of the Ccr4-Pop2 deadenylase complex is essential for this association *in vivo*. However, the role of other components of the Ccr4-Not complex, such as Not3, Not4, Caf40 and Caf130, does not seem to be important during the adaptation of yeast cells to low iron conditions. First, the interaction between Cth2 and Pop2 was not altered in mutants in these Ccr4-Not components (Supplemental Fig. S3), and second, they did not display any growth defect in iron-deficient conditions (Supplemental Fig. S4B). Altogether, these data point to the Ccr4-Pop2 deadenylase as an essential player in the adaptation to iron deficiency mediated by Cth2, whereas other non-essential components of the complex are not required. Further studies would be necessary to ascertain the role played by the Not1 scaffold protein.

The association of Cth2 to Dhh1 does not take place in cells lacking either *CCR4* or *POP2* (Fig. 2), whereas Pop2 associates to Cth2 even in *dhh1Δ* mutant cells (Fig. 4). These results suggest a gradual association of the decay/translation machinery to Cth2, in which Ccr4-Pop2 deadenylase complex is recruited to Cth2 in the first place, and Dhh1 binding to the Cth2-Ccr4-Pop2 complex occurs later on (Fig. 7). The previously reported physical interaction between Dhh1 and the Ccr4-Pop2 deadenylase supports their simultaneous association to Cth2 protein [5-7]. We postulate that the interaction between Cth2 and Pop2 would be rather direct, whereas Pop2 would be necessary to link Dhh1 to Cth2. However, we have been unable to purify these proteins in order to assess protein-protein interaction *in vitro*. Remarkably, despite Xrn1 also shuttles between the nucleus and the cytoplasm [10], only wild-type Cth2, but not Cth2-TZFmt, associates to the 5' to 3' exonuclease (Fig. 6). This result indicates that the initial Cth2 complex does not contain Xrn1, which would join the mRNP complex only after Cth2 binds its mRNA target. Since

Xrn1 is a nucleocytoplasmatic protein, it is possible that it associates co-transcriptionally to the mRNA via Cth2, or otherwise that a previous deadenylation and decapping of the target transcript within the cytoplasm was required for recruitment (Fig. 7). Of course, this model does not discard that Xrn1 association to Cth2 is RNA dependent. Moreover, we have to accommodate the relatively short half-life of Cth2 protein, which has been recently estimated in ~15 minutes under iron-deficient conditions [63]. A recruitment mechanism has been previously described for the yeast PUF family of mRNA-binding proteins [7]. Particularly, Puf5/Mpt5 promotes mRNA deadenylation, decay and inhibits the translation of specific mRNAs by directly binding Pop2 and recruiting a complex containing Ccr4, Dhh1 and the decapping component Dcp1 [7]. A more recent study has shown that Ccr4 and Dhh1 are recruited to many common nutrient-regulated mRNAs in response to changing environmental conditions, probably via RNA-binding proteins such as Puf5, Puf3 and others implicated in mRNA decay and/or translational repression [64]. Altogether, these results suggest that the Cth2 TZF-protein sequentially and rapidly assembles different members of the mRNA decay and translational regulatory machinery, including Dhh1 and Pop2, onto nascent ARE-containing transcripts to deliver them to the cytoplasm for its degradation and/or inhibiting its translation.

Figure 7. A model for Cth2-mediated post-transcriptional regulation of gene expression in response to iron deficiency. Cth2 is a nucleocytoplasmic protein that is expressed in response to iron limitation to post-transcriptionally modulate yeast iron metabolism. Data included in this and previous studies suggest that Cth2 recruits the Ccr4-Pop2 deadenylase complex and the Dhh1 RNA helicase before its co-transcriptional binding to ARE-containing transcripts, whereas the Xrn1 5' to 3' exonuclease is later recruited to the mRNP complex to promote mRNA turnover.

Many data support a coupling between the different stages of gene expression ranging from the biogenesis of transcripts to its decay. The RNA polymerase II subunits Rpb4 and Rpb7, the poly(A)-binding protein, and the Ccr4-Not complex have been proposed as general *trans*-acting coordinators of transcription, mRNA decay and translation [11, 14, 65]. Multiple observations suggest that TTP and Cth2 could constitute another class of mRNA coordinators specific for ARE-containing transcripts. First, both proteins shuttle between the nucleus and the cytoplasm, which is essential for Cth2, and probably TTP, mediated AMD [40, 41, 66]. Second, both proteins bind co-transcriptionally to its target mRNAs, occasionally altering the 3' end processing of transcripts when its site of binding is embedded within the poly(A) signal [42, 43]. In the case of yeast cells, a small

portion of Cth2 target mRNAs is extended and destabilized within the nucleus [42]. Third, Cth2 AMD activity depends on active transcription and bulk mRNA export, since it accumulates in the nucleus upon thiolutin treatment or in mutants in mRNA export such as *xpo1-1* and *mex67-5* [41]. Fourth, initial studies suggested that both TTP and Cth2 could function as transcription factors because they activate transcription when artificially recruited to a promoter by the Gal4 DNA-binding domain [67, 68]. Fifth, *in vitro* studies with plant TZF-proteins have revealed their elevated avidity for double strand DNA binding [69-71]. And sixth, both TTP and Cth2 have been localized to cytosolic P-bodies [36, 37]. In the case of TTP, *in vivo* mRNA localization experiments have demonstrated that it can direct ARE-containing mRNAs to P-bodies and stress granules, where it could contribute to mRNA decay and translation repression, respectively [37]. Altogether, these observations suggest that, during TTP and Cth2 nucleocytoplasmic shuttling, different mRNP complexes assemble in a dynamic and stepwise manner to determine the post-transcriptional regulation of the ARE transcripts according to the environmental conditions. Current data have demonstrated their role in mRNA transport, turnover and translation repression. However, no demonstration for a natural role of TZF-proteins in transcription has been reported to date. Further studies will determine whether TZF-proteins represent multifunctional regulatory proteins that connect the different steps of mRNA expression including transcription.

The main function of human TTP has been attributed to the regulation of the immune and inflammatory responses, whereas Cth2 is almost fully devoted to iron metabolism. However, the expression of both TTP and Cth2 increases in response to iron depletion to repress the expression of ARE-containing mRNAs which function is directly related to iron metabolism [24-26]. Both proteins repress respiration, which is a highly iron-consuming process, and modulate iron utilization in other metabolic pathways [24-26, 29, 72]. Importantly, both human cells knocked out for *TTP* and yeast cells lacking *CTH2* exhibit an important growth defect under iron-limited conditions [25, 26]. The interaction of Dhh1, Pop2/Caf1 and Xrn1 with Cth2 (and TTP) points to a relevant function of the mRNA decay machinery in the adaptation of cells to iron deficiency. We have

shown here that *dhh1Δ*, *pop2Δ* and *xrn1Δ* mutants display a growth defect when cultivated in iron-limited conditions (Figs. 1 and 5, Supplemental Fig. S4; [56]). Probably, both Cth2-related and independent mechanisms contribute to the iron deficiency phenotype of mutants in the mRNA decay machinery.

We have used the M-track method to shed some light on how the iron-regulated Cth2 protein associates different mRNA decay factors to assemble an mRNP complex competent to promote targeted mRNA decay when iron is scarce. The results obtained here indicate that this is a sequential process that takes place during the nucleocytoplasmic transport of the mRNA-binding protein. Remarkably, Cth2 protein early acquires the mRNA-decay machinery that will determine the final fate of its targeted transcripts.

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SUPPLEMENTAL TABLES

Supplemental Table S1. List of *Saccharomyces cerevisiae* strains used in this work.

Strain	Description	Source
AH109	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80 URA3::MEL1UAS-MEL1TATALacZLYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2</i>	[73]
BY4741 (WT)	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</i>	Research Genetics
<i>caf130Δ</i>	<i>BY4741 caf130::KanMX4</i>	Research Genetics
<i>caf40Δ</i>	<i>BY4741 caf40::KanMX4</i>	Research Genetics
<i>ccr4Δ</i>	<i>BY4741 ccr4::KanMX4</i>	Research Genetics
<i>cth1Δcth2Δ</i>	<i>BY4741 cth1::KanMX4, cth2::HisMX6</i>	[26]
<i>cth1Δcth2Δxrn1Δ</i>	<i>BY4741 cth1::KanMX4, cth2::HisMX6 xrn1::hphMX4</i>	This study
<i>cth1Δcth2Δsdh4Δ</i>	<i>BY4741 cth1::KanMX4, cth2::HisMX6, sdh4::loxP</i>	This study
<i>cth1Δcth2Δxrn1Δsdh4Δ</i>	<i>BY4741 cth1::KanMX4, cth2::HisMX6 xrn1::hphMX4 sdh4::loxP</i>	This study
<i>dhh1Δ</i>	<i>BY4741 dhh1::KanMX4</i>	Research Genetics
<i>edc1Δ</i>	<i>BY4741 edc1::KanMX4</i>	Research Genetics
<i>lsm1Δ</i>	<i>BY4741 lsm1::KanMX4</i>	Research

		Genetics
<i>not3Δ</i>	<i>BY4741 not3::KanMX4</i>	Research Genetics
<i>not4Δ</i>	<i>BY4741 not4::KanMX4</i>	Research Genetics
<i>pat1Δ</i>	<i>BY4741 pat1::KanMX4</i>	Research Genetics
<i>pop2Δ</i>	<i>BY4741 pop2::KanMX4</i>	Research Genetics

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Supplemental Table S2. List of plasmids used in this work

Plasmid	Description	Source
pRS415	CEN, <i>LEU2</i>	[74]
pRS416	CEN, <i>URA3</i>	[74]
pGBK-T7	2 μ m, <i>TRP1</i> , <i>P_{ADH1}-GBD-Myc</i>	BD Biosciences
pGAD-T7	2 μ m, <i>LEU2</i> , <i>P_{ADH1}-GAD-HA</i>	BD Biosciences
pUL9	<i>LEU2-kan^R</i>	[46]
pUG73	<i>loxP-LEU2-loxP</i>	[44]
pSH47	<i>P_{GAL1}-Cre</i> , CEN, <i>URA3</i>	[44]
pAG32	<i>hphMX4</i>	[45]
pSP410	<i>pRS416-CTH2</i>	[26]
pSP411	<i>pRS416-NotI-CTH2</i>	[26]
pSP434	<i>pRS416-NotI-CTH2-C190/213A</i>	[41]
pSP518	<i>pRS415-GCN4-T_{CTH1}</i>	[75]
pSP529	<i>p415GAL1-SDH4</i>	[26]

pSP539	<i>pGAD-T7-CTH2</i>	[36]
pSP542	<i>pGAD-T7-CTH1</i>	[36]
pSP753	<i>pRS415-GFP-CTH2</i>	[41]
pSP910	<i>YCp111-P_{ADH4}-3xMyc-HKMT</i>	A. Zuzuarregui
pSP911	<i>YEp195-4xH3-3xHA</i>	A. Zuzuarregui
pSP921	<i>pRS416ΔNotI-P_{CTH2}-3xMyc-HKMT-CTH2-C190/213A-T_{CTH2}</i>	This study
pSP934	<i>YEp195-XRN1-4xH3-3xHA</i>	This study
pSP942	<i>pRS416ΔNotI-P_{CTH2}-3xMyc-HKMT-CTH2-T_{CTH2}</i>	This study
pSP950	<i>pRS415-4xH3-3xHA-T_{TEF}</i>	This study
pSP951	<i>pRS416-P_{CTH2}-3xMyc-HKMT-T_{CTH2}</i>	This study
pSP958	<i>pRS415-DHH1</i>	This study
pSP962	<i>YEp195-DHH1-4xH3-3xHA (2 μM URA3)</i>	This study
pSP1019	<i>YEp195-XRN1-4xH3-3xHA (URA3::LEU2)</i>	This study
pSP1020	<i>YEp195-DHH1-4xH3-3xHA (URA3::LEU2)</i>	This study
pSP1041	<i>pRS415-POP2-4xH3-3xHA-T_{TEF}</i>	This study
pSP1051	<i>pRS415-CCR4-4xH3-3xHA-T_{TEF}</i>	This study
pSP1086	<i>pGBK-T7-POP2</i>	This study

Supplemental Table S3. List of oligonucleotides used for RT-qPCR in this work.

Name	Sequence (from 5' to 3')	
	Forward	Reverse
<i>ACO1</i>	GCCATCAAGAGACCCATTGT	ATCCAGCGTTTCCACATTCT
<i>EDC1</i>	AAACGCCACGACAGAACAAC	CACTGCCATTACAACCCATT
<i>HEM15</i>	CCAAAGTTGATGGCCTAATG	TATTCCGATTCCCAATGAC
<i>SDH4</i>	GCACTCCCAATGATGCCTAC	AATGGAACGACGGACAAGG
<i>ACT1</i>	TCGTTCCAATTTACGCTGGTT	CGGCCAAATCGATTCTCAA

SUPPLEMENTAL FIGURES

Supplemental Figure S1. Functionality of Myc-HKMT-CTH2. (A) *cth1Δcth2Δ* strain transformed with pRS416-CTH2 (Cth2), pRS416 (vector) and pSP951 (Myc-HKMT-Cth2)

were assayed for growth on SC-ura (+Fe) and SC-ura plus 600 μ M Ferrozine (-Fe) media. (B) The expression of *SDH4*, *ACO1* and *HEM15* genes was determined in the same conditions. Total RNA was extracted and analyzed by RT-qPCR. Values were normalized to *ACT1* mRNA levels. The average and SD of three biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.05). “r.u.” means “relative units”.

Supplemental Figure S2. Functionality of Dhh1-H3-HA. (A) Dhh1 protein levels. BY4741 (WT) and *dhh1Δ* cells were transformed with pSP962, which expresses Dhh1-H3-HA. Cells were cultivated in SC-ura to exponential phase, and Dhh1 and Dhh1-H3-HA protein levels were determined by Western blot with a Dhh1-specific antibody. (B) WT (BY4741) transformed with pRS415 (vector), and *dhh1Δ* cells transformed with pRS415 (vector), pRS415-DHH1 (Dhh1) or pSP962 (Dhh1-H3-HA) were cultivated as in panel A, and *EDC1/ACT1* mRNA levels were determined. The average and SD of three biological replicates are shown. Different letters indicate statistically significant differences (p value < 0.05). (C) BY4741 (WT) cells transformed with pRS416 (vector), and *dhh1Δ* mutant transformed with pRS416 (vector), pRS415-DHH1 (Dhh1) or pSP962 (Dhh1-H3-HA) were assayed for growth on SC media at 37°C, and 30°C without or with the addition of 0.04 % methyl methanesulfonate (MMS) or 50 mM hydroxyurea (HU).

Supplemental Figure S3. Other components of the Ccr4-Not complex are not necessary for the interaction between Cth2 and Dhh1. (A) Western blot showing the interaction of Myc-HKMT-Cth2 and Dhh1-H3-HA. BY4741 (WT) co-transformed with pSP1020 (Dhh1-H3-HA) and pSP951 (Myc-HKMT), and BY4741 (WT), *not3Δ*, *not4Δ*, *caf40Δ* and *caf130Δ* mutants co-transformed with pSP1020 (Dhh1-H3-HA) and pSP942 (Myc-HKMT-Cth2) were cultivated and analyzed as described in Figure 1. (B) Relative quantification of anti-me3K9H3/anti-HA signals.

Supplemental Figure S4. Growth of mutants in the components of Not-Ccr4 complex in iron-deficient conditions. (A) Functionality of Pop2-H3-HA and Ccr4-H3-HA. BY4741 (WT) transformed with pRS415 (vector), *pop2Δ* strain transformed with pRS415 (vector) or pSP1041 (Pop2-H3-HA), and *ccr4Δ* strain transformed with pRS415 (vector) or pSP1051 (Ccr4-H3-HA) were assayed for growth on SC-leu (+Fe), YPD + 8 mM caffeine, and SC-leu + 500 μ M Ferrozine (-Fe). (B) Pop2 and Ccr4, but not other components of the Ccr4-Not complex are required for growth in iron-deficient conditions. BY4741 (WT), *pop2Δ*, *ccr4Δ*, *not3Δ*, *not4Δ*, *caf40Δ* and *caf130Δ* were assayed for growth on SC (+Fe) and SC + 700 μ M Ferrozine (-Fe).

Supplemental Figure S5. Cth2 does not interact *in vivo* with Ccr4 deadenylase by M-track. Western blot to assay the interaction between Myc-HKMT-Cth2 and Ccr4-H3-HA. The *cth1Δcth2Δ* strain co-transformed with pSP1051 (Ccr4-H3-HA) and either pSP951 (Myc-HKMT), pSP942 (Myc-HKMT-Cth2) or pSP921 (Myc-HKMT-Cth2-TZFmt) plasmids were cultivated and analyzed as described in Figure 1. Dhh1-Cth2 and Pop2-Cth2 interactions were used as positive controls for anti-me3K9H3.

Supplemental Figure S6. Functionality of Xrn1-H3-HA. (A) Xrn1 protein levels. BY4741 (WT) and *xrn1Δ* cells were transformed with pSP934, which expresses Xrn1-H3-HA. Cells were cultivated in SC-ura to exponential phase, and Xrn1 and Xrn1-H3-HA protein levels were determined by Western blot with an Xrn1-specific antibody. (B) BY4741 (WT) transformed with pRS416 (vector), and *xrn1Δ* strain transformed with pRS416 (vector) or pSP962 (Xrn1-H3-HA) were assayed for growth on YPD (Glucose) and YPG (Glycerol). (C) GFP-Cth2 subcellular localization. BY4741 (WT) transformed with pRS415 and *xrn1Δ* strain transformed with pRS415 (*xrn1Δ*) or pSP962 (Xrn1-H3-HA) were co-transformed with pRS415-GFP-Cth2. Then, cells were cultivated in SC-ura-leu with 100 μM BPS for 6 hours and visualized under Nomarski (DIC) and GFP fluorescence optics. (D) Quantification of P-bodies from cells on panel C. The average and SD of at three biological replicates are shown. Different letters mean statistically significant differences (p value < 0.01).