Regulation of ARE transcript 3' end processing by the yeast Cth2 mRNA decay factor

Manoël Prouteau^{1,2,3},
Marie-Claire Daugeron^{1,2,3,4*}
and Bertrand Séraphin^{1,2,3,4,*}

¹Equipe Labellisée La Ligue, CNRS, Centre de Génétique Moléculaire, UPR 2167, Gif-sur-Yvette, France, ²Université Paris-Sud, Orsay, France and ³Université Pierre et Marie Curie, Paris, France

Regulation of mRNA decay is an important step modulating gene expression. The stability of numerous eukaryotic mRNAs is controlled by adenosine/uridine-rich elements (AREs) located in their 3'UTR. In *Saccharomyces cerevisiae*, the Cth2 protein stimulates the decay of target ARE mRNAs on iron starvation. Cth2, and its mammalian homologue tristetraprolin, contains a characteristic tandem CCCH zinc-finger essential for ARE binding and mRNA decay. We have performed a structure–function analysis of Cth2 to understand the mechanism(s) by which it destabilizes mRNAs. This indicated that a conserved N-terminal region of Cth2 is essential for its decay function but dispensable for RNA binding. Unexpectedly, Cth2 mutants lacking this domain blocked the normal 3' end processing of ARE mRNAs leading to the formation of extended transcripts. These can also be detected in mutant of the polyadenylation machinery. Consistently, Cth2 localization in the nucleus suggests that it may interfere with poly(A) site selection. Our analysis reveal that ARE-binding protein may affect mRNA 3' end processing and that this contributes to mRNA destabilization.

The EMBO Journal (2008) 27, 2966–2976. doi:10.1038/emboj.2008.212; Published online 16 October 2008

Subject Categories: RNA

Keywords: AU-rich elements; mRNA degradation; poly(A) tails; polyadenylation; tristetraprolin

Introduction

The regulation of mRNA stability is crucial to adapt gene expression to numerous stimuli. Various *cis*-acting elements modulate mRNA decay through interaction with specific RNA-binding factors. One of the most common elements in mammalian cells, first identified in particularly labile transcripts such as those encoding oncogenes and cytokines, is the adenosine/uridine-rich element (ARE) (Barreau *et al.*, 2005). Various families of ARE elements have been characterized, the most commonly found sequence being the non-amer UUAUUUAUU. Genome-wide analyses showed that

5–8% of mammalian mRNAs display AREs-containing 3'UTRs, indicating that ARE-dependent mRNA turnover is a widespread gene regulation mechanism (Bakheet *et al.*, 2006).

Among the numerous ARE-binding proteins stimulating or inhibiting mRNA decay, the human tristetraprolin (hTTP) is one of the more widely studied (Carrick *et al.*, 2004). hTTP and related proteins contain a characteristic tandem repeat of two CCCH zinc-fingers (TZFs). Each CCCH motif binds a UUAUU motif, two adjacent motifs cooperating to bind ARE (Hudson *et al.*, 2004). hTTP binds and destabilizes many mRNAs such as those encoding TNF α , GM-CSF or IL2 (Carrick *et al.*, 2004). Consistently, hTTP deficiency elicits various autoimmune pathologies. Other mammalian factors related to hTTP, such as BRF1 (Stoecklin *et al.*, 2002), also control ARE mRNA stability. Although hTTP family members have been intensively analysed, the mechanism by which they stimulate mRNA decay remains controversial: some studies suggest that ARE-stimulated decay proceeds in the 3'–5' direction, others suggest that it occurs in a 5'–3' direction, whereas implication of microRNA has also been proposed (Chen *et al.*, 2001; Lai and Blackshear, 2001; Mukherjee *et al.*, 2002; Fenger-Gron *et al.*, 2005; Jing *et al.*, 2005; Stoecklin *et al.*, 2006; Hau *et al.*, 2007).

More generally, hTTP homologues are present in many eukaryotes. However, strong sequence conservation is restricted to the TZF domain. In several species, these proteins were shown to control the stability of ARE-containing mRNAs. In *Saccharomyces cerevisiae*, two very similar proteins, Cth1 and Cth2, have been shown to be homologous to hTTP (Thompson *et al.*, 1996) and to have partially overlapping functions (Puig *et al.*, 2005). Cth2 expression is induced on iron starvation conditions (Foury and Talibi, 2001; Puig *et al.*, 2005) and genome-wide analyses demonstrated that it downregulates the mRNA encoding proteins involved in various iron-dependent pathways, such as the succinate dehydrogenase subunit 4 (*SDH4*). These transcripts contain ARE elements in their 3'UTR and these are required for Cth2-mediated mRNA decay (Puig *et al.*, 2005).

A variety of RNA-binding proteins interact with 3'UTR to control the fate of mRNAs, including its 3' end processing, localization, translation and decay. 3'UTRs are followed, on their 3' side, by the poly(A) tail. The latter is added in a two-step process by the polyadenylation machinery. In yeast, this one is composed of cleavage factors I and II (CFI and CFII) and loaded co-transcriptionally onto the nascent transcript (Proudfoot and O'Sullivan, 2002). These factors catalyse the co-transcriptional RNA cleavage creating a site for poly(A) addition and contributing to transcription termination. Yeast polyadenylation signals appear relatively degenerate. Nevertheless, the position of strong poly(A) sites was shown to be defined, in addition to the poly(A) site itself, by two major elements: an UA repeat (consensus UUAUUA) and an A-rich sequence, usually located upstream of the cleavage site (Minvielle-Sebastia *et al.*, 1998; Proudfoot and O'Sullivan, 2002). The UA repeat has been proposed to

*Corresponding authors. M-C Daugeron or B Séraphin, Equipe Labellisée La Ligue, CNRS, Centre de Génétique Moléculaire, UPR 2167, Gif-sur-Yvette 91198, France. Tel.: +01 69 82 38 83; Fax: +01 69 82 36 70; E-mails: daugeron@cgm.cnrs-gif.fr or seraphin@cgm.cnrs-gif.fr

⁴These authors contributed equally to this work

Received: 10 June 2008; accepted: 12 September 2008; published online: 16 October 2008

function as a docking site of the Hrp1/Nab4, a CFI-associated subunit that has a crucial function in cleavage site choice (Minvielle-Sebastia *et al*, 1998; Perez-Canadillas, 2006).

Little is known about polyadenylation regulation in yeast. Yet, recently, some RNA-binding proteins have been reported to modulate alternative poly(A) site selection by competing with components of the polyadenylation machinery for 3'UTR binding. For example, Npl3 suppresses the recognition of the normal 3' end processing signals located in its own transcript. This feature creates an autoregulatory loop that controls Npl3 protein levels (Bucheli *et al*, 2007; Lund *et al*, 2008). Similarly, the nuclear poly(A)-binding protein Nab2 binds an A-rich track located in the 3'UTR of its own mRNA, leading again to autogenous regulation of protein production (Roth *et al*, 2005).

Similar to its mammalian counterparts, the mechanism(s) by which Cth2p destabilizes its targets remains unclear. The conserved TZF domain of Cth2 was shown to be essential for this function (Puig *et al*, 2005). However, the role of the other Cth2 regions in this process was not analysed. To decipher the mode of action of Cth2, we have undertaken a structure–function analysis of this factor. This identified a conserved N-terminal region of Cth2 (named conserved region 1 (CR1)) necessary to stimulate mRNA decay. Analysis of CR1 mutants revealed that Cth2 modulates poly(A) site selection in the yeast nucleus, leading to the production of extended transcripts that are unstable. Thus, our results reveal for the first time the implication of a TTP family member in polyadenylation control.

Results

SDH4 mRNA steady-state levels as an assay for the destabilization activity of Cth2

In addition to the TZF, essential for stimulating RNA degradation, Cth2 contains N- and C-terminal regions of unknown function. The corresponding amino acids are unlikely to catalyse mRNA degradation, but could interact with other factors mediating mRNA decay. To investigate the mechanism by which Cth2 stimulates mRNA degradation, we initiated a structure–function analysis of this factor.

SDH4 mRNA is one of the most affected targets of Cth2 (Puig *et al*, 2005), thus we tested whether variations of the steady-state levels of this mRNA could report the Cth2 mRNA decay activity. For this purpose, we compared SDH4 mRNA levels from wild-type cells grown under iron-rich condition (+Fe) or iron deprivation condition (–Fe) for 4 h. The latter state, artificially generated by the addition of Fe(II) chelator bathophenanthroline disulphonic acid (BPS) to the media, induces Cth2 expression (Puig *et al*, 2005). Consistent with previous results, northern blot analyses revealed that iron scarcity results in a loss of $51.8 \pm 5.4\%$ (average and standard deviation from four independent experiments) of the SDH4 mRNA steady-state amount normally present in iron-replete condition (Figure 1, the loss of SDH4 mRNA observed in low iron condition is hereafter used as a measure of mRNA decay). The SDH4 mRNA downregulation was not detected in strains lacking the *CTH1* and *CTH2* genes (Δ cth1 Δ cth2). Introduction, in the latter strain, of plasmids encoding Cth2, or a TAP–Cth2 fusion, under the control of the natural CTH2 promoter, fully restored the downregulation of SDH4 mRNA (Figure 1). Transcriptional chase experiments demonstrated

Figure 1 Steady-state SDH4 mRNA levels report Cth2 decay activity. Northern blot analysis of SDH4 mRNA steady-state levels from WT and Δ cth1 Δ cth2 cells transformed by empty vector (–), vector carrying Cth2 or TAP–Cth2. Cells were grown for 4 h in the presence or absence of iron. The latter condition induces Cth2 expression. SDH4 levels, normalized to SCR1 levels, was used to calculate the loss of SDH4 mRNA on Cth2 expression, referred to as % of decay. This value was calculated by subtracting SDH4 value from lane (–Fe) to that of lane (+Fe), dividing the resulting value by the SDH4 value from lane (+Fe) and expressing the result as a percentage. Percentages and standard deviations were calculated from at least three independent experiments.

that the Cth2-dependent reduction of SDH4 mRNA levels resulted from a faster decay (Supplementary Figure S1). Overall, these results indicate that Cth2 function can be monitored by assaying the SDH4 mRNA steady-state levels and that a Cth2 variant carrying an amino terminal TAP tag is fully functional.

Identification of a Cth2 conserved region important for its mRNA decay-promoting activity

The TZF is the only highly conserved feature of all tristetraprolin protein family members from fungi, plants and animals. Thus, to identify more moderately conserved regions of Cth2, we performed a multiple sequence alignment of Cth2 homologues and the related Cth1 proteins from five *Saccharomyces* species (*bayanus*, *cerevisiae*, *kudriavzevii*, *mikatae* and *paradoxus*). Plotting the percentage of identity along the Cth2 sequence (Figure 2A) revealed the presence of three additional conserved regions, named CR1, CR2 and CR3, that span respectively amino acids 37–55, 106–133 and 268–285 and show 81, 78 and 86% of identity between *Saccharomyces* Cth proteins.

To investigate the functions of the various Cth2 domains in its mRNA destabilization activity, we constructed 12 *cth2* alleles with deletions encompassing combinations of the conserved regions and non-conserved linkers (Figure 2A). All Cth2 variants were fused to an N-terminal TAP tag to monitor their expression, and expressed under the control of the CTH2 promoter from centromeric plasmids. These plasmids were introduced in Δ cth1 Δ cth2 cells, in parallel with controls encoding full-length Cth2 or no protein. The resulting cells were grown in either iron-rich (+Fe) or iron-deprivation conditions (–Fe) for 4 h and the SDH4 mRNA levels and Cth2 protein expression were then assayed.

Protein analysis demonstrated that the expression of all Cth2 variants was induced in low iron conditions and that the cognate polypeptides were of the expected size (Figure 2B).

Figure 2 The conserved region 1 (CR1) and TZF domains of Cth2 are essential for mRNA destabilization. (A) Structure of the Cth2 deletion mutants. Plot of the percentages of identity along the Cth2 sequence determined from a ClustalX alignment of Cth1 and Cth2 proteins from *Saccharomyces bayanus*, *cerevisiae*, *kudriavzevii*, *mikatae* and *paradoxus*. The highly conserved repeat of two CCCH zinc-fingers is shown as a green box and the three conserved regions (CR1, CR2 and CR3) as blue boxes. The structures and names of the 12 Cth2 deletion mutants designed on the basis of the sequence conservation are depicted below. (B) Deletion mutants lacking CR1 or TZF are unable to destabilize the SDH4 mRNA. Northern blot analysis of SDH4 mRNA and SCR1 (loading control) from cells expressing the various *cth2* mutants, or wild-type control, was performed and analysed as described in Figure 1. Decay values were calculated from at least two independent experiments. The black arrowheads mark the location of the extended transcripts. The amount of TAP-Cth2 in the same cell samples was analysed by western blotting. Stm1 was used as a loading control. Position of the molecular weight marker is indicated on the left. (C) Cth2 Δ1-86 binds the SDH4 mRNA. SDH4 mRNA levels in cells expressing TAP-Pop2, TAP-Pop2-Cth2 or TAP-Pop2-Cth2Δ1-86 was analysed by northern blot as described for Figure 1. Reduced SDH4 mRNA levels in cells expressing TAP-Pop2-Cth2Δ1-86 demonstrate that Cth2Δ1-86 is sufficient to target Pop2 to the SDH4 mRNA.

Remarkably, in many cases doublet bands, possibly arising from post-translational modifications, were detected. Analysis of the SDH4 mRNA levels indicated that some Cth2 mutant forms were unable to induce mRNA decay. This included, expectedly, deletion of the TZF alone (Δ159-250; Figure 2B). Interestingly, expression of the TZF alone (Δ1-157/Δ252-285) was not sufficient to destabilize the SDH4 mRNA, indicating that at least another Cth2 region

is required for this function. This region is located in the protein N-terminal part, as deletion of the first 86 residues of Cth2 (Δ1-86) nearly abolished its activity (4% decay; Figure 2B). This is confirmed by analysis of a larger deletion encompassing this region (Δ1-157). Shorter deletions had only partial phenotypes, suggesting possibly that several elements in this region contribute to mRNA decay. Nevertheless, CR1 appeared to have a major contribution as

its removal ($\Delta 36$ –57) induced only a low decay (35% as compared with more than 51% for all active alleles) and the other deletion encompassing CR1 was also clearly defective ($\Delta 1$ –57, 28% decay). In contrast, deleting the region N-terminal to CR1 ($\Delta 1$ –34), or regions encompassing CR2 ($\Delta 106$ –134), CR3 ($\Delta 264$ –285 and $\Delta 252$ –285) and non-conserved linkers ($\Delta 59$ –104, $\Delta 136$ –157) had hardly any effect on SDH4 mRNA levels (over 45% of decay). Analysis of the steady-state level of CCP1 mRNA, another Cth2 target, confirmed the results obtained with the SDH4 mRNA (data not shown). We conclude that, in addition to the TZF, the N-terminal region encompassing CR1 is essential for mRNA destabilization by Cth2.

The Cth2 N-terminal region is not required for SDH4 mRNA recognition

Deletions overlapping CR1 could impair the Cth2 destabilization activity by preventing ARE recognition, as observed for the TZF deletion, or by other means, for example, by affecting interaction with mRNA decay factors. To test whether the Cth2 $\Delta 1$ –86 protein is still able to recognize the SDH4 mRNA, we used a recently developed targeting assay where fusion of the Pop2 deadenylase subunit to a RNA-binding domain was shown to be sufficient to destabilize a reporter mRNA target containing the cognate-binding site (Finoux and Seraphin, 2006). Thus, we constructed plasmids encoding fusions of TAP–Pop2 with Cth2 $\Delta 1$ –86, or as a positive control full-length Cth2, under the control of the CTH2 promoter. A plasmid encoding TAP–Pop2 was also prepared as a negative control. These constructions were introduced into $\Delta cth1\Delta cth2$ cells that were grown in iron-rich or iron-deprivation conditions. Western blot analysis showed that the three tagged proteins, induced in low iron conditions, migrated as their sizes predicted (data not shown). RNA analysis revealed that TAP–Pop2 expression did not destabilize the SDH4 mRNA

in low iron conditions (Figure 2C, compare with empty vector). In contrast, expression of the TAP–Pop2–Cth2 fusion protein reduced the SDH4 mRNA level (59.8% decay), indicating that the fusion does not alter RNA recognition by Cth2. Interestingly, the TAP–Pop2–Cth2 $\Delta 1$ –86 fusion induced an efficient decrease of the SDH4 mRNA level (Figure 2C). As neither TAP–Pop2 nor Cth2 $\Delta 1$ –86 alone is able to induce SDH4 mRNA downregulation (see also Figure 2B), this result demonstrates that TAP–Pop2–Cth2 $\Delta 1$ –86 is still able to recognize the SDH4 mRNA ARE, stimulating the degradation of this transcript through the fused Pop2 deadenylase moiety. We conclude that Cth2 $\Delta 1$ –86 is still endowed with specific RNA recognition capacity but has lost the ability to activate SDH4 mRNA decay.

A new SDH4-derived RNA species accumulates in cth2 mutants lacking CR1

Careful examination of the northern blots revealed the presence of a new extended RNA species derived from the SDH4 locus in most *cth2* mutants that were either partially or totally unable to activate mRNA decay (see faint bands indicated by black arrowheads in Figure 2B in strains expressing Cth2 $\Delta 1$ –86, $\Delta 1$ –157, $\Delta 1$ –57 and $\Delta 36$ –57). We estimated the size of this new species to be about 1200 nucleotide (nt) long, whereas the SDH4 mRNA is roughly 850 nt long. The signal arising from this new species was weak. To check whether it was correlated with the expression of mutant proteins lacking CR1, we compared by northern blotting RNA extracted from cells expressing Cth2 $\Delta 1$ –86, Cth2 or no Cth protein at various time points after transfer to low iron conditions (Figure 3A). The extended SDH4 transcript started to be detectable 2 h after iron deprivation, its level increasing with time. Presence of this new RNA species was associated with Cth2 $\Delta 1$ –86 as it was not detected in cells expressing either no or full-length Cth2. As the extended species was best detected after

Figure 3 Mutants lacking CR1 accumulate an extended SDH4 transcript in a TZF-dependent manner. (A) Extended SDH4 transcripts accumulate during Cth2 $\Delta 1$ –86 induction. Time course analysis of normal SDH4 mRNA and extended transcript levels in cells expressing no Cth2 (–), wild-type Cth2 or Cth2 $\Delta 1$ –86 after iron removal. Northern blot analysis was performed as described for Figure 1. Methylene blue staining of ribosomal RNAs is shown as a loading control. Cth2 protein expression was monitored in parallel by western blotting as described in Figure 2B. (B) A functional TZF is required for extended SDH4 transcript production. Northern blot analysis of SDH4 mRNA and SCR1 (loading control) from cells expressing Cth2 or Cth2 $\Delta 1$ –86 and harbouring either wild-type ((ACCH)₂) or mutated ((ACCH)₂) zinc-fingers was performed as described for Figure 1. Western blot analysis of Cth2, and the Stm1 loading control, was used to control for protein expression.

prolonged incubation in low iron condition, we tested whether it was detectable with all mutants lacking CR1 by analysing its presence after 5 h of iron deprivation. In these conditions, stronger signals were detected confirming earlier results and showing that the extended species was now also detectable in cells expressing the TZF alone ($\Delta 1-157/\Delta 251-285$; Supplementary Figure S2), but this was not simply explained by protein levels, (Supplementary Figure S3). Because the extended SDH4 RNA species was not detected in cells lacking Cth2 or expressing the wild-type protein, but only in cells expressing Cth2 mutants harbouring the TZF but unable to activate mRNA downregulation, these results suggested that its appearance was dependent on ARE binding by the mutated protein. To test this hypothesis, we substituted the first cysteines of the two CCCH motifs by alanines (denoted (ACCH)₂), as these mutations will breakdown the interaction network involved in zinc-finger formation and interaction with RNA (Hudson *et al*, 2004). This mutation was introduced either in the context of the full-length protein (Cth2(ACCH)₂) or combined with a CR1 deletion (Cth2 $\Delta 1-86$ /(ACCH)₂). Northern blot analysis of RNA extracted from cells expressing Cth2(ACCH)₂ demonstrated that the SDH4 mRNA was not downregulated in this context (Figure 3B). This result confirms that the (ACCH)₂ prevents RNA recognition precluding SDH4 mRNA destabilization. Interestingly, Cth2 $\Delta 1-86$ /(ACCH)₂ was unable to induce the formation of the extended RNA (Figure 3B). As all Cth2 variants were correctly expressed (Figure 3B), we conclude that formation of the extended SDH4 mRNA requires the presence of Cth2 proteins able to bind RNA through the TZF domain but unable to activate mRNA downregulation as a result of CR1 deletion.

The extended SDH4 RNA species results from alternative 3' end processing

We characterized the extended SDH4 RNA species accumulating in *cth2* mutants lacking CR1 by northern blotting using short single-stranded probes spanning the SDH4 ORF. The new RNA species overlaps the SDH4 coding region and is encoded by the same strand as the normal mRNA, but contains <350 additional nucleotides (data not shown). To further characterize this species, we cloned PCR fragments generated by circularization RT-PCR (cRT-PCR; Couttet *et al*, 1997) performed with total RNA extracted from cells expressing either full-length Cth2 or Cth2 Δ 1-86. Sequencing of resulting clones allowed us to simultaneously determine the transcription start site, poly(A) site and poly(A) tail sizes of capped SDH4 transcripts. This revealed that most SDH4-derived transcripts (22 out of 26) originate from the same transcription start site located 102 nt upstream from SDH4 initiation codon. The remaining RNAs originated from sites located a few nucleotides apart (Figure 4). There was no significant difference in the sites used in cells expressing Cth2 or Cth2 Δ 1-86, indicating that extended transcripts did not result from alternative transcription initiation.

Poly(A) sites of all SDH4 transcripts cloned from cells expressing Cth2 were mapped 147–175 nt downstream of the SDH4 stop codon, a region overlapping with SDH4 ARE sequences (Figure 4). In contrast, the poly(A) sites of the SDH4-derived transcripts isolated from cells expressing Cth2Δ1–86 were distributed throughout a larger region. In 7 of the 16 cases, they were located in the intergenic region

Figure 4 Cth2Δ1–86p induces the formation of 3' extended SDH4 transcripts. The transcriptional start sites, poly(A) sites and poly(A) tail sizes of SDH4 transcripts were determined by RT-PCR performed on total RNA cells expressing Cth2 or Cth2Δ1–86 grown for 5 h in the presence of 100 μM BPS. Transcriptional start sites and poly(A) sites are indicated by black or grey arrows, respectively, for Cth2- or Cth2Δ1–86-expressing cells, with the number of corresponding clones indicated. Average sizes of poly(A) tail lengths with standard deviation values are indicated. AU-rich elements targeted by Cth2 (Puig *et al.*, 2005) are highlighted. The positions of poly(A) sites, ARE and CSN9 ORF are also reported below on the sequence of this region.

downstream of the SDH4 coding sequence, similar to those described above (even though they were on average shifted slightly away from the SDH4 stop codon). Interestingly, in nine cases, we observed novel poly(A) sites located 443–515 nt downstream from the SDH4 stop codon (Figure 4). In six of the nine cases, the same site located 513 nt downstream from the SDH4 stop codon was used, indicating that this is an efficient poly(A) site. The average poly(A) tail sizes of these three groups of transcripts were 28 ± 15 , 32 ± 14 and 39 ± 11 nt long, respectively, consistent with their synthesis being in all cases mediated by the general 3' end processing machinery followed by partial deadenylation. These results indicated that the new SDH4-derived RNA species corresponds to 3' extended transcripts most likely generated by alternative 3' end processing. The calculated sizes of the two main species (848 and 1192 nt for normal and 3' extended SDH4 transcripts, respectively) are in perfect agreement with the sizes estimated by northern blot analysis.

These results demonstrate that Cth2Δ1–86 induces the use of an alternative 3' end maturation site for SDH4 transcripts. This new site is located 330 nt downstream of the normal site and thus overlaps, in the antisense direction, the CSN9

coding sequence. The extended transcripts, resulting from transcriptional read-through of the upstream poly(A) site, appear to be normally polyadenylated.

Cth2 is a nuclear factor

The effect of Cth2 on the choice of the SDH4 poly(A) site is most easily explained by a direct interaction of this factor with its target mRNA in the nucleus. Therefore, we determined the cellular localization of Cth2 by indirect immunofluorescence in cells expressing TAP-tagged Cth2 derivatives. We used two functional TAP-tagged versions of Cth2: a protein expressed under the control of the natural CTH2 promoter, and thus induced in low iron conditions (Cth2-TAP), or a protein constitutively overexpressed using the strong TPI1 promoter (TPI-Cth2-TAP) (Figure 5). Cells expressing TAP-tagged Tma46 and Fal1 were used as cytosolic and nuclear localization markers, respectively, whereas cells expressing no TAP-tagged protein were used as a negative control. For each construct, we analysed cells grown in iron-rich (+Fe) or iron deprivation (−Fe) conditions. Tma46 and Fal1 displayed cytosolic and nuclear localization, respectively, independently of the iron level in the media (Figure 5). Cth2-TAP was detectable only after growth in low iron conditions and appeared to be mostly nuclear. The protein produced by the TPI-Cth2-TAP construct was loca-

lized in the nucleus in all conditions examined, indicating that Cth2 nuclear localization is not controlled by iron concentration. We conclude that Cth2 appears to be essentially localized in the nucleus, and that its localization is not regulated by iron availability.

Mutants affecting the positioning of the 3' end processing machinery also induce the formation of extended SDH4 transcripts

The results presented above suggest that *cth2* mutants lacking the CR1 region promote the formation of extended SDH4 transcripts by interfering with polyadenylation at the normal site. This suggests that mutants weakening the 3' end processing machinery, and particularly factors affecting its positioning, should also induce the appearance of extended SDH4 RNA. We tested this possibility by comparing northern blots of SDH4 mRNAs from cells expressing Cth2Δ1–86 grown in low iron conditions with those of conditional mutants of the polyadenylation factors Rna14, Rna15 or Nab4/Hrp1 (Minvielle-Sebastia *et al*, 1998) grown in non-permissive condition (37°C). Extended SDH4 transcripts were clearly detected in *nab4* mutants (*nab4-4* and *nab4-7*) (Figure 6A, note that those are stable as iron is present and thus Cth2 is repressed). In *rna14*, *rna15* or *rna14/rna15* mutants, only very low levels of SDH4 mRNA were detectable and those

Figure 5 Cth2 nuclear localization. Cellular location of TAP-tagged proteins expressed from genomic loci was assessed by indirect immunofluorescence. C-terminally tagged Cth2 was expressed under the control of its own promoter (Cth2-TAP) or with the strong constitutive TPI promoter (TPI-Cth2-TAP). Tma46 and Fal1 proteins were used as cytosolic and nuclear markers, respectively. Cells expressing no TAP-tagged protein were used as a negative control. Cells were grown for 4 h in the presence of 300 μ M Fe (+Fe) or 100 μ M BPS (−Fe). DNA was stained with DAPI, and cells were visualized by differential interference contrast (DIC). The granular materials seen in +Fe condition are iron deposits.

Figure 6 3' extended SDH4 transcripts form in mutants of 3' end processing machinery and wild-type cells. (A) Nab4 mutants mimic the 3' end maturation effect of Cth2Δ1-86. A northern blot analysis of SDH4 transcripts and SCR1 (loading control) from cells not expressing Cth2 or expressing Cth2Δ1-86, and from cells mutated for the Nab4, Rna14 and Rna15 3' processing factors is presented. For the two left lanes, total RNAs were prepared from Δcth1Δcth2 cells transformed with the empty vector or a plasmid expressing Cth2Δ1-86, grown for 5 h at 30°C in the presence of 100 μM BPS. The 3' processing mutants were grown for 5 h at 37°C in media containing normal level of iron, that is, sufficient to repress Cth2. Positions of extended and normal SDH4 transcripts are indicated. The asterisk indicates longer read-through transcripts. (B) SDH4 3' extended transcripts are also produced in wild-type cells. SDH4 transcripts were analysed by quantitative RT-PCR using pairs of primers located upstream (PCR5') and downstream (PCR3') of the normal poly(A) site, respectively, as depicted. Cells expressing no Cth2, wild-type Cth2 or Cth2Δ1-86 were grown for 5 h in the presence 100 μM BPS. Cells carrying the thermo-sensitive nab4-7 mutation were grown for 5 h at 37°C. Percentage of 3' extended SDH4 transcripts, that is, the ratio of cDNA quantity measured by PCR3' to that of PCR5', was calculated (with standard deviations) from three independent experiments.

corresponded to species heterogeneous in size, larger than the one described above (Figure 6A). Consistent with the function of Nab4 in poly(A) site choice, these results indicate that its inactivation leads to extended SDH4 transcript formation, thus mimicking the effect of Cth2Δ1-86. In contrast, inactivating the polyadenylation machinery in the rna14 and/or rna15 mutants blocked most processing at both upstream and downstream sites.

Low levels of extended SDH4 transcript are detectable in cells expressing wild-type Cth2.

The observation of extended SDH4 mRNAs in cells expressing Cth2 proteins lacking CR1, which are generally unable to destabilize the SDH4 transcript, suggests that the extended species may be formed in wild-type cells but very rapidly degraded. To test for the presence of traces of extended

transcripts in wild-type cells, we used a sensitive quantitative RT-PCR assay (qRT-PCR). Briefly, RNAs, extracted from cells grown in low iron conditions expressing Cth2, Cth2Δ1-86 or no Cth2 protein and from nab4-7 cells grown at 37°C, were reverse transcribed with gene-specific primers. Quantification was performed by qPCR using two sets of primers amplifying regions located upstream or downstream from the normal SDH4 poly(A) site, respectively. Comparing the ratio of these two values (from three independent biological replicates) reported the fraction of extended SDH4 transcript present in these cells. Consistent with the northern blot analysis, a large fraction of the SDH4 transcript was extended in cells expressing Cth2Δ1-86 ($18.1 \pm 6.6\%$; Figure 6B). A similar situation was also confirmed for the nab4 mutant ($4.88 \pm 1.71\%$). Interestingly, the fraction of extended SDH4 transcript detected in CTH2 wild-type cells was significantly higher than in cells expressing no Cth factors ($0.61 \pm 0.25\%$ versus $0.11 \pm 0.07\%$). We conclude that low but significant levels of extended SDH4 transcripts are also detectable when the wild-type Cth2 protein is expressed. Cth2 may, similar to Cth2Δ1-86, induce the production of extended SDH4 transcripts but those could be unstable, explaining their low abundance.

Wild-type Cth2 destabilizes extended SDH4 transcripts

The observation that wild-type Cth2 induces the formation of extended SDH4 transcript, together with the fact that it induces degradation of target mRNAs, suggests that Cth2 may destabilize the extended transcripts. This would explain why those accumulate only to low levels in cells expressing Cth2, when compared with cells expressing Cth2Δ1-86, the latter being unable to stimulate their degradation.

To test this possibility, we measured the half-lives of normal and extended SDH4 transcripts in cells expressing Cth2Δ1-86 or co-expressing Cth2Δ1-86 and Cth2. We first constructed a reporter plasmid where the transcription of the SDH4 gene (including the downstream poly(A) site and an oligo(G) tract inserted in the 3'UTR) was driven by the GAL1 promoter, allowing its use for transcriptional chase experiments. This reporter was introduced together with a plasmid encoding Cth2Δ1-86 in Δcth1Δcth2 and Δcth1CTH2 yeast strains and transcriptional chase experiment was performed (Figure 7). The extended SDH4 transcript produced by the reporter plasmid was readily detectable, demonstrating that its production is independent of the promoter driving SDH4 expression. Importantly, the extended transcript was detectable both in cells expressing Cth2Δ1-86 alone, and in those expressing both Cth2 and Cth2Δ1-86. As extended SDH4 transcripts were undetectable by northern blotting in wild-type cells, this demonstrates that Cth2Δ1-86 is able to induce its formation, even in the presence of fully functional Cth2. However, the amounts of both extended and normal SDH4 mRNAs were dramatically reduced in cells co-expressing the wild-type Cth2 protein. Plotting the amount of RNA remaining as a function of time indicated that the extended SDH4 transcript is as stable as the SDH4 reporter mRNA in cells expressing only Cth2Δ1-86 (half-lives of 9 and 10 min, respectively; Figure 7). Co-expression of Cth2 with Cth2Δ1-86 destabilized both the SDH4 mRNA and extended SDH4 transcript, even though the effect was significantly stronger for the latter one (half-lives of 5 and 3 min, respectively). These observations suggest that extended SDH4 transcripts

Figure 7 Wild-type Cth2 destabilizes extended SDH4 transcripts. The structure of the SDH4_{G18L} reporter is indicated. The SDH4 transcript is under the control of the galactose-inducible and glucose-repressible GAL1 promoter and contains a stretch of 18 G inserted just after the stop codon. The plasmid contains 640 nt downstream of the SDH4 ORF encompassing the poly(A) site of the extended SDH4 transcript. Cells expressing wild-type Cth2 and Cth2Δ1-86, or only Cth2Δ1-86, were transformed with a plasmid encoding the SDH4_{G18L} reporter and grown for 5 h in the presence of galactose to express the SDH4 reporter and of 100 μM BPS to express Cth2 proteins. Glucose was added to stop transcription of the reporter and samples were collected at the indicated time points. Total RNAs were analysed by northern blotting using 5' end-labelled oligonucleotide complementary to the G₁₈ sequence, or random-labelled probe against SCR1 (loading control). Positions of normal and extended transcripts and their cognate decay intermediates are indicated.

synthesized in cells expressing wild-type Cth2 are rapidly targeted to degradation.

Analysis of the northern blot also revealed the presence of degradation intermediates (Figure 7) resulting from the blockage of a 5'–3' exonuclease mediated by the oligo(G) sequence (Decker and Parker, 1993). Intermediates corresponding both to the SDH4 mRNA and extended transcript were detected, indicating that these two RNA species are, at least in part, degraded by a nuclear and/or cytoplasmic 5'–3' mRNA degradation pathway.

Cth2 modulates the 3' end processing of other ARE-containing mRNAs

The results presented above demonstrate that Cth2 prevents the use of the normal poly(A) site of the SDH4 mRNA contributing to its destabilization. Cth2 could also induce read-through of the normal poly(A) site of other ARE-containing mRNA targets. To test this hypothesis, we measured by qRT-PCR the levels of total and read-through transcripts originating from three other ARE containing Cth2 targets: CCP1, SDH2 and ACO1, and from the control ACT1 transcript that does not appear to be regulated by Cth2 (Puig *et al*, 2005). This assay was performed using RNA extracted from cells expressing wild-type Cth2, Cth2Δ1-86 or no protein. In addition, we analysed *nab4-7* cells defective in poly(A) site choice. For each cell type, we calculated the fraction of extended transcript for the four loci (Figure 8). The basal transcriptional read-through observed in the absence of Cth2 was quantitatively variable from one locus to the next (SDH2: $1.74 \pm 0.92\%$; ACO1: $0.020 \pm 0.010\%$; CCP1: 0.071 ± 0.019 and ACT1: $1.4 \times 10^{-4} \pm 6.5 \times 10^{-5}\%$). Remarkably, on expression of Cth2Δ1-86, a strong increase in the fraction of 3' extended transcripts was observed for the three ARE-containing mRNAs (SDH2: $15.4 \pm 8.97\%$; ACO1: $1.01 \pm 0.90\%$ and CCP1: $4.71 \pm 2.73\%$) but not for ACT1 ($2.77 \times 10^{-4} \pm 1.76 \times 10^{-4}$). Interestingly, expression of

Cth2 also specifically increased the fraction of extended ARE-containing transcripts (SDH2: $20.24 \pm 11.49\%$; ACO1: $0.054 \pm 0.014\%$ and CCP1: $0.44 \pm 0.18\%$) without affecting the level of ACT1 extended transcript ($1.86 \times 10^{-4} \pm 1.18 \times 10^{-4}\%$). Importantly, alteration of a general factor involved in poly(A) site selection (*nab4-7*) also increased the fraction of extended transcripts. However, all the loci, including ACT1, were in this case affected (SDH2: $45.13 \pm 6.14\%$; ACO1: $1.66 \pm 0.97\%$; CCP1: $7.83 \pm 2.61\%$ and ACT1: $0.04 \pm 3 \times 10^{-3}\%$). Altogether, these results show that Cth2 affects not only the poly(A) site choice of the SDH4 mRNA but also of other ARE-containing mRNA targets.

Discussion

Our data demonstrate that Cth2 has at least two molecular functions. On the one hand, as described earlier, Cth2 destabilizes ARE-containing mRNA targets (Puig *et al*, 2005). On the other hand, our results demonstrate that Cth2 also controls poly(A) site selection, inducing the read-through of the normal site and production of extended transcripts. These two functions seem related, however, as extended transcripts appear particularly unstable, at least for the SDH4 locus.

Dissection of the Cth2 protein reveals that it contains at least two functional domains: the TZF region and CR1. The TZF regions of hTTP family members have been shown earlier to be essential for RNA binding *in vitro* (Lai *et al*, 1999, 2003; Hudson *et al*, 2004). Using an *in vivo* protein-targeting assay, we show that this is also the case for the *S. cerevisiae* factor. The TZF of Cth2 is essential both to promote mRNA degradation and to stimulate the formation of extended transcripts. In addition, we identified CR1 as a functionally important region of Cth2 that, in contrast to the TZF, is only required for mRNA decay. CR1 is conserved in *Saccharomyces* species but we could not clearly identify

similar regions in more distantly related proteins, possibly because it is too small and poorly defined. Moreover, deletion of CR1 and adjacent sequences is required to completely inhibit mRNA decay. This indicates the presence of several cooperating sequence elements in this region. The detailed molecular mechanism(s) by which they contribute to mRNA

decay remains to be deciphered. Our results indicate that at least a fraction of the SDH4 mRNAs and extended transcripts targeted by Cth2 are degraded by the 5'–3' pathway, as cognate decay intermediates are detected. This is consistent with some results obtained in mammalian cells (Stoecklin *et al*, 2006). However, we cannot exclude that a 3'–5' mRNA decay pathway is also implicated in their decay, as also proposed for the mammalian system. An interesting possibility would be that post-translational modification (e.g. phosphorylation) of key residue(s) of Cth2 induces interaction with, and stimulation of, the decay machinery and that these events would be dependent on the presence of the Cth2 N-terminal 86 residues. This model would be consistent with results obtained in mammalian cells that indicate an essential function for post-translational modification of TTP and homologues for their function (Benjamin *et al*, 2006; Hitti *et al*, 2006; Cao *et al*, 2007). Moreover, this could explain the moderate sequence conservation of this region, especially if several partially redundant residues are involved. Our observation of doublet bands corresponding to Cth2 in western blot analyses support the existence of post-translational modification of this factor; however, their nature and implication (if any) in mRNA decay remain to be established. Besides CR1, comparison of Cth sequences from *Saccharomyces* species revealed the presence of two other conserved regions: CR2 and CR3. Deletion analysis failed so far to reveal a function for these regions: they could be involved in Cth2 activities not detected by assaying levels of target mRNAs.

Our results indicate that the production of extended transcripts requires mRNA binding by Cth2. Given that conserved ARE sequences overlap the poly(A) sites of the SDH4 mRNA(s), a possibility could be that Cth2 competitively inhibits pre-mRNA access to the 3' end processing machinery. Such a model is consistent with the observation that weakening the polyadenylation machinery by mutating the Nab4 factor involved in the selection of the poly(A) site also induces the formation of extended transcripts. In fact, the similarity between the ARE sequence (Puig *et al*, 2005) and proposed Nab4- or RNA15-binding sites (Gross and Moore, 2001; Perez-Canadillas, 2006) suggests that a direct competition may exist for RNA binding by these factors. However, alternative models involving a direct interaction of RNA-bound Cth2 with specific components of the polyadenylation machinery can also be proposed. In the specific case of *SDH4*, the presence of a second poly(A) site located downstream leads to the formation of defined extended transcripts when Cth2 is present. For the *ACO1*, *SDH2* and *CCP1* mRNAs, such defined transcripts were not detected possibly because of heterogeneity resulting from multiple 3' ends. The extended transcripts observed in conditions of Cth2 induction, namely

Figure 8 Cth2p alters the 3' end maturation of several ARE-containing mRNA. The presence of extended transcripts derived from the *SDH2*, *CCP1*, *ACO1* and *ACT1* loci was assessed by quantitative RT-PCR. PCR5' and PCR3' depicted as black bars on the schematic representation of genomic regions for each locus tested, corresponded to PCR obtained using two pairs of primers located upstream and downstream from the predicted main poly(A) sites, respectively. Positions of ARE are indicated by small black boxes. This analysis was performed as indicated for Figure 6, using the same total RNA samples. Percentages and standard deviations were calculated from three independent experiments.

low iron levels, differ thus from the one recently reported for the CTH2 transcripts, which were only described in iron-replete conditions (i.e. when Cth2 is repressed) and proposed to result from an unconventional mechanism of 3' end formation (Ciais *et al*, 2008).

For the SDH4, ACO1, SDH2 and CCP1 mRNAs, significant levels of extended transcripts are detected in cells expressing wild-type Cth2. This demonstrates that their production is not a peculiarity resulting from the expression of mutated Cth2 proteins lacking the CR1 region. Rather, the extremely low level of extended transcript present in wild-type cells is likely to result from their severe instability. Co-expression of Cth2 and Cth2Δ1–86 results in the production of extended, unstable transcripts. This co-dominance further supports that the truncated protein is partially functional (e.g. capable to bind ARE sequences), competes with the wild-type factor for RNA binding and polyadenylation inhibition, but is unable to induce the subsequent decay of RNA to which it is associated.

As Cth2 affects both poly(A) site choice and stability of the interacting transcript, it is difficult to quantify whether it similarly affects the stability of the normal mRNA and the extended transcript *in vivo*. Indeed, a fraction of the normal transcripts escaping Cth2 may remain relatively stable. In contrast, all extended transcripts are bound by Cth2 during their synthesis and thus likely to be destabilized. The concentration of (activated) Cth2 is thus likely to be a critical factor determining the fraction of target transcript ultimately degraded. Consistent with this notion, prolonged expression of Cth2Δ1–86 increases the level of the extended SDH4 transcript, indicating that at early time points, Cth2Δ1–86 quantities are limiting. Cth2 implication in poly(A) site choice appears to take place in the nucleus, consistently with our observation of the predominant nuclear localization of Cth2. In fact, Cth2 recruitment to ARE targets is likely to occur co-transcriptionally and thus it will be of interest to test whether it interacts with the transcriptional machinery or chromatin. An early recruitment of Cth2 to target RNAs during synthesis could ensure a rapid response of cell to change in iron concentration. However, the observation that Cth2 destabilizes the extended transcript when co-expressed with Cth2Δ1–86 suggests that it may also interact later with mature mRNAs containing ARE, in the nucleus and/or the cytoplasm, to specifically activate their decay. Cth2 is thus likely to be, as reported for mammalian TTP, a shuttling protein, acting in several cellular compartments.

Overall, our analysis demonstrate that Cth2 is a multi-functional protein not only involved in mRNA decay but also, surprisingly, able to modulate polyadenylation. Together with Nab2 and Npl3, Cth2 is thus one of the first factors regulating the choice of the poly(A) site of target mRNAs described in yeast. Furthermore, we present here the first example of a direct implication of a hTTP family member in 3' end processing. These factors had only been shown so far to control gene expression in various organisms through other post-transcriptional processes. Because Cth2 inhibits the

normal polyadenylation of several target mRNAs where ARE sequences are located near the poly(A) site, we surmise that this property, even though not essential, facilitates the degradation of these transcripts. This results possibly from the presence of a long 3'UTR that could favour the action of the mRNA decay machinery. Further analysis of Cth2 will be required to understand mechanistically how it stimulates RNA degradation. Conversely, it will be of interest to test whether other TTP family members, including Cth1 in yeast and mammalian factors, also control poly(A) site choice.

Materials and methods

Strains and media

S. cerevisiae strains used in this study are listed in Supplementary Table S1. Unless otherwise stated, cells were grown at 30°C in selective synthetic media with 2% glucose, supplemented with 300 μM of Fe(NH₄)₂(SO₄)₂ or 100 μM of BPS when necessary. For transcriptional chase, 4% glucose (final concentration) was added to cells growing in 2% galactose. Plasmids and oligonucleotides used in this study are listed in Supplementary Tables S2–S4.

RNA analysis

Total RNA prepared by hot phenol extraction was analysed by northern blotting as described earlier (Daugeron *et al*, 2001b). The plasmidic SDH4 reporter RNA was detected with an oligonucleotide probe and other RNAs were detected using random-primed PCR probes. Signals were detected with a Phosphorimager (GE Healthcare).

Mapping of 5' and 3' ends of the SDH4 transcripts, and determination of poly(A) tail size, was carried out by cRT-PCR (Couttet *et al*, 1997) as detailed in Supplementary data.

qRT-PCR with primer sets located upstream and downstream of the normal poly(A) site was performed in biological triplicates using a LightCycler480 (Roche) for the SDH4, SDH2, ACO1, CCP1 and ACT1 loci (see Supplementary Table S5). The ratio of the downstream to upstream RNA quantities was expressed as a percentage of 3' extended molecules.

Protein analysis

TAP-tagged proteins were detected as described earlier (Rigaut *et al*, 1999), whereas the Stm1 protein was detected using rabbit polyclonal antibodies and goat anti-rabbit-HRP IgG secondary antibody (Pierce). ECL signals were detected with a Las3000 device (Fuji). Indirect immunofluorescence was as described earlier (Daugeron *et al*, 2001a) except that the TAP-tagged proteins were detected using rabbit anti-protA antibody (Sigma) (dilution 1/1000) and secondary goat anti-rabbit Cy3-conjugated antibodies (1/200). Cells were visualized using a Zeiss Axioplan 2 microscope linked to a cool Snap camera (Princeton Instruments).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank W Keller and L Minvielle-Sebastia for providing material, our group members, especially E Simon, for support and C Barrandon, S Camier, H Le Hir, A Lebreton and J Saulière for useful comments. MP was supported by the Research Ministry and ARC. This study was supported by La Ligue contre le Cancer (Equipe labellisée 2008), the CNRS, Agence Nationale de la Recherche (ANR-07-BLAN-0093-01) and the ESF Eurocore project Euxosome.

References

Bakheet T, Williams BR, Khabar KS (2006) ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic Acids Res* **34**: D111–D114

Barreau C, Paillard L, Osborne HB (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res* **33**: 7138–7150

- Benjamin D, Schmidlin M, Min L, Gross B, Moroni C (2006) BRF1 protein turnover and mRNA decay activity are regulated by protein kinase B at the same phosphorylation sites. *Mol Cell Biol* **26**: 9497–9507
- Bucheli ME, He X, Kaplan CD, Moore CL, Buratowski S (2007) Polyadenylation site choice in yeast is affected by competition between Npl3 and polyadenylation factor CFI. *RNA* **13**: 1756–1764
- Cao H, Deterding LJ, Blackshear PJ (2007) Phosphorylation site analysis of the anti-inflammatory and mRNA-destabilizing protein tristetraprolin. *Expert Rev Proteomics* **4**: 711–726
- Carrick DM, Lai WS, Blackshear PJ (2004) The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis. *Arthritis Res Ther* **6**: 248–264
- Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**: 451–464
- Ciais D, Bohnsack MT, Tollervey D (2008) The mRNA encoding the yeast ARE-binding protein Cth2 is generated by a novel 3' processing pathway. *Nucleic Acids Res* **36**: 3075–3084
- Couttet P, Fromont-Racine M, Steel D, Pictet R, Grange T (1997) Messenger RNA deadenylation precedes decapping in mammalian cells. *Proc Natl Acad Sci USA* **94**: 5628–5633
- Daugeron MC, Kressler D, Linder P (2001a) Dbp9p, a putative ATP-dependent RNA helicase involved in 60S-ribosomal-subunit biogenesis, functionally interacts with Dbp6p. *RNA* **7**: 1317–1334
- Daugeron MC, Mauxion F, Seraphin B (2001b) The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation. *Nucleic Acids Res* **29**: 2448–2455
- Decker CJ, Parker R (1993) A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. *Genes Dev* **7**: 1632–1643
- Fenger-Gron M, Fillman C, Norrild B, Lykke-Andersen J (2005) Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Mol Cell* **20**: 905–915
- Finoux AL, Seraphin B (2006) *In vivo* targeting of the yeast Pop2 deadenylase subunit to reporter transcripts induces their rapid degradation and generates new decay intermediates. *J Biol Chem* **281**: 25940–25947
- Foury F, Talibi D (2001) Mitochondrial control of iron homeostasis. A genome wide analysis of gene expression in a yeast frataxin-deficient strain. *J Biol Chem* **276**: 7762–7768
- Gross S, Moore CL (2001) Rna15 interaction with the A-rich yeast polyadenylation signal is an essential step in mRNA 3'-end formation. *Mol Cell Biol* **21**: 8045–8055
- Hau HH, Walsh RJ, Ogilvie RL, Williams DA, Reilly CS, Bohjanen PR (2007) Tristetraprolin recruits functional mRNA decay complexes to ARE sequences. *J Cell Biochem* **100**: 1477–1492
- Hitti E, Iakovleva T, Brook M, Deppenmeier S, Gruber AD, Radzioch D, Clark AR, Blackshear PJ, Kotlyarov A, Gaestel M (2006) Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. *Mol Cell Biol* **26**: 2399–2407
- Hudson BP, Martinez-Yamout MA, Dyson HJ, Wright PE (2004) Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d. *Nat Struct Mol Biol* **11**: 257–264
- Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H, Han J (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* **120**: 623–634
- Lai WS, Blackshear PJ (2001) Interactions of CCCH zinc finger proteins with mRNA: tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. *J Biol Chem* **276**: 23144–23154
- Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, Blackshear PJ (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. *Mol Cell Biol* **19**: 4311–4323
- Lai WS, Kennington EA, Blackshear PJ (2003) Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. *Mol Cell Biol* **23**: 3798–3812
- Lund MK, Kress TL, Guthrie C (2008) Autoregulation of Npl3, a yeast SR protein, requires a novel downstream region and serine phosphorylation. *Mol Cell Biol* **28**: 3873–3881
- Minvielle-Sebastia L, Beyer K, Krecic AM, Hector RE, Swanson MS, Keller W (1998) Control of cleavage site selection during mRNA 3' end formation by a yeast hnRNP. *EMBO J* **17**: 7454–7468
- Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS, Wilusz J (2002) The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J* **21**: 165–174
- Perez-Canadillas JM (2006) Grabbing the message: structural basis of mRNA 3'UTR recognition by Hrp1. *EMBO J* **25**: 3167–3178
- Proudfoot N, O'Sullivan J (2002) Polyadenylation: a tail of two complexes. *Curr Biol* **12**: R855–R857
- Puig S, Askeland E, Thiele DJ (2005) Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. *Cell* **120**: 99–110
- Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* **17**: 1030–1032
- Roth KM, Wolf MK, Rossi M, Butler JS (2005) The nuclear exosome contributes to autogenous control of NAB2 mRNA levels. *Mol Cell Biol* **25**: 1577–1585
- Stoecklin G, Colombi M, Raineri I, Leuenberger S, Mallaun M, Schmidlin M, Gross B, Lu M, Kitamura T, Moroni C (2002) Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. *EMBO J* **21**: 4709–4718
- Stoecklin G, Mayo T, Anderson P (2006) ARE-mRNA degradation requires the 5'-3' decay pathway. *EMBO Rep* **7**: 72–77
- Thompson MJ, Lai WS, Taylor GA, Blackshear PJ (1996) Cloning and characterization of two yeast genes encoding members of the CCCH class of zinc finger proteins: zinc finger-mediated impairment of cell growth. *Gene* **174**: 225–233