

Control of *Saccharomyces cerevisiae* pre-tRNA processing by environmental conditions

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ABSTRACT

tRNA is essential for translation and decoding of the proteome. The yeast proteome responds to stress and tRNA biosynthesis contributes in this response by repression of tRNA transcription and alterations of tRNA modification. Here we report that the stress response also involves processing of pre-tRNA 3' termini. By a combination of Northern analyses and RNA sequencing we show that upon shift to elevated temperatures and/or to glycerol-containing medium, aberrant pre-tRNAs accumulate in yeast cells. For pre-tRNA_{UAU}^{Ile} and pre-tRNA_{UUU}^{Lys}, these aberrant forms are unprocessed at the 5' ends, but they possess extended 3' termini. Sequencing analyses showed that partial 3' processing precedes 5' processing for pre-tRNA_{UAU}^{Ile}. An aberrant tRNA^{Tyr} that accumulates also possesses extended 3' termini, but it is processed at the 5' terminus. Similar forms of these aberrant pre-tRNAs are detected in the *rex1Δ* strain that is defective in 3' exonucleolytic trimming of pre-tRNAs. We further show direct correlation between the inhibition of 3' end processing rate and the stringency of growth conditions. Moreover, under stress conditions Rex1 nuclease seems to be limiting for 3' end processing of pre-tRNA^{Tyr}. Thus, our data document complex 3' processing that is inhibited by stress in a tRNA-type and condition-specific manner. This stress-responsive tRNA 3' end maturation process presumably contributes to fine-tune the levels of functional tRNA in budding yeast in response to environmental conditions.

INTRODUCTION

The unicellular organism, budding yeast, is able to adapt to sudden changes in environmental conditions by multiple levels of regulation of cell organization and gene expression. Most of these changes are caused by altered transcription efficiency, but RNA processing and decay are also affected. tRNA, the focus of this work, is transcribed by RNA polymerase III (Pol III). Control of tRNA transcription is mediated by a general negative

regulator, Maf1 (Pluta et al. 2001). Under unfavorable growth conditions such as nonfermentable carbon sources, high temperature, nitrogen starvation or stationary phase, Maf1 is dephosphorylated and binds to Pol III, leading to repression of transcription (Boguta 2013). Nutrient limitation and stress also down-regulate tRNA transcription by phosphorylation of Pol III subunit C53 (Lee et al. 2012).

Transcription by Pol III, followed by maturation of initial transcripts, generates a variety of stable and unstable tRNA species. Generally the first step in tRNA maturation is 5' leader cleavage by RNase P, a multisubunit complex consisting in yeast of nine proteins and a single RNA encoded by the *RPR1* gene (Xiao et al. 2001). The second step is 3' end processing which is more complex and involves multiple enzymes. In *S. cerevisiae* it requires Trz1, an essential endonuclease also known as RNase Z (Skowronek et al. 2014), and Rex1, a 3'-5' exonuclease (van Hoof et al. 2000; Ozanick et al. 2009). Under some conditions Rrp6, a 3'-5' exonuclease that is a component of the nuclear exosome, performs tRNA 3' end processing. It is suspected that other exonucleases may also contribute to tRNA maturation (Yoo and Wolin 1997; Copela et al. 2008). tRNA 3' end-processing also involves Lhp1, a homologue of human La protein. Lhp1 stabilizes pre-tRNAs by direct binding to the oligo U 3' termini, generated upon Pol III termination (Lin-Marq and Clarkson 1995; Stefano 1984). Binding of Lhp1 maintains the proper structure of tRNA precursors, protects them from exonucleolytic digestion, and stimulates endonucleolytic maturation (Van Horn et al. 1997; Yoo and Wolin 1997; Chakshumathi et al. 2003; Copela et al. 2008). Finally, a complex of Lsm proteins is also involved in tRNA end processing, by interaction with Lhp1. Inactivation of a single Lsm protein from this complex resulted in the accumulation of stable 5' and 3' end-extended intron-containing and 5' end-matured 3' end-extended tRNA precursors (Kufel et al. 2002). Following removal of the 3' trailer from the original transcript, the ubiquitous C, C, A nucleotides are added to the 3' terminus by nucleotidyltransferase Cca1 (Aebi et al. 1990).

After end-maturation and nuclear export, splicing of intron-containing precursors occurs in the cytoplasm. Throughout all the maturation steps modifications are added; the modified nucleotides serve numerous functions including an important role in maintaining proper tRNA structure and stability (Phizicky and Hopper 2010).

Turnover of tRNA maturation intermediates is controlled by the nuclear exosome. An indication that pre-tRNAs are normally degraded by the nuclear exosome came from the observation that their levels are increased in mutants lacking the nuclear exosome subunit Rrp6, and cofactor, Trf4 (Kadaba et al. 2004; Copela et al. 2008). Moreover, 3' turnover by the nuclear exosome appears to compete with 3' processing (Maraia and Lamichhane 2011). The inefficient pre-tRNA degradation in the exosome mutant facilitates pre-tRNA maturation; conversely, inefficient 3' end maturation directs pre-tRNAs to the exosome for degradation (Gudipati et al. 2012).

It has recently become appreciated that tRNA modifications are affected by stress (Chan et al. 2010, 2012; Preston et al. 2013; Damon et al. 2015; Alings et al. 2015; Han et al. 2015) but there are no reports regarding whether there is regulation of the activities responsible of pre-tRNA end-maturation. Here we investigated early steps of tRNA maturation for yeast grown at different temperatures (23-39°C) and in media with different carbon sources (glucose and glycerol). We show that the pattern of tRNA precursors is differently affected depending on tRNA isotype. tRNA specific down-regulation of 3' end processing by respiratory growth and elevated temperature was assessed by Northern analyses of pre-tRNA^{Ile}_{UAU}, pre-tRNA^{Tyr} and pre-tRNA^{Lys}_{UUU}. Alteration of pre-tRNA 3' end processing by stress was further confirmed by sequencing of 3' trailers of pre-tRNA^{Ile}_{UAU}. Moreover, our data suggest that the magnitude of inhibition of 3' end processing correlates with the severity of the stress. The molecular mechanism responsible for control of pre-tRNA 3' processing by

environmental conditions was investigated and a model presenting the effect of stress conditions on end maturation of pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr} is proposed.

RESULTS

Aberrant tRNA precursors accumulate under stress conditions

Previous studies show that Maf1 regulates tRNA transcription in yeast upon changes in carbon sources (Ciesla et al. 2007). Here, we explore the effects of carbon source and temperature shifts upon tRNA end processing.

We first assessed intron-containing precursors of tRNAs by Northern analysis. RNA was isolated from wild type cells grown to exponential phase in rich glucose medium (YPD) and either shifted for two hours to various temperatures (23°C, 30°C, 37°C and 39°C) and/or to media with glycerol as the carbon source (YPGly). Each probe detected a characteristic set of processing intermediates for a given tRNA isotype (Fig. 1). For cells grown in YPD conditions, two major bands of tRNA precursors are usually detected which correspond, respectively, to the form containing the intron and extended 5' and 3' ends, generally assumed to be the primary transcript, and the end-matured, intron-containing form. Following the shift of cells to non-standard conditions, the relative ratio of these two forms changes and unexpected aberrant forms become apparent. In cells exposed to elevated temperature, the amount of the end-matured, intron-containing form of pre-tRNA^{Ile}_{UAU} decreased and a novel slower-migrating intron-containing, end-extended form was detected (marked with the solid arrow, Fig. 1A). The relative abundance of this aberrant slowest migrating species increased when the stresses of high temperature and YPGly media were combined (Fig.1A and A').

Other tRNAs were also affected by temperature and carbon source stresses. For pre-tRNA^{Tyr}, no additional slow-migrating, end-extended, intron-containing form was detected. However, an aberrant form of pre-tRNA^{Tyr} that migrated faster than the initial transcript but

slower than end-processed, intron-containing intermediate accumulated upon high temperature and carbon source stress (Fig. 1C and 1C'). For pre-tRNA_{UUU}^{Lys}, the precursors pattern resembled that observed for tRNA_{UAU}^{Ile}; that is, at elevated temperatures and/or in glycerol-containing medium, a novel form of tRNA_{UUU}^{Lys} that migrated slower than the longest end-extended, intron-containing form detected in rich media at normal temperatures was identified (Fig 1B, solid arrow). Moreover, as for tRNA^{Tyr} under stress conditions, additional forms of pre-tRNA_{UUU}^{Lys}, that migrate between the primary transcript and the end-matured intron-containing forms, were detected (Fig. 1B, dashed arrow). However, when relative ratio between primary transcript and end-matured-intron containing forms was calculated the only significant difference was observed in YPGly at 39°C (Fig. 1B'). Finally, subtle differences were visible for pre-tRNA^{Trp} and pre-tRNA^{Phe} patterns upon the shift to glycerol containing medium (Fig. S1). In sum, the results show that tRNA precursors forms are affected by environmental conditions, and the pattern of tRNA precursors changes in a tRNA-isotype and condition-specific manner.

Although the level of initial tRNA transcripts decreased upon stress treatments, the levels of mature tRNAs for given tRNAs, relative to 5S and 5.8S rRNA, as assessed by ethidium bromide staining, did not (Fig 1, bottom panels). This is probably due to the fact that tRNAs are very stable and are not degraded during the 2 hour exposure to stress and because rRNA transcription is likely coordinately decreased upon the stress conditions.

Alterations of pre-tRNA 3' termini upon temperature and carbon source stress

As the aberrant form of pre-tRNA_{UAU}^{Ile} migrated slower than the accepted primary transcript, we explored the possibility that the slow form is the real primary transcript but that under favorable growth conditions it is rapidly partially processed. We compared the pre-tRNA species that accumulate in isogenic strains with mutations in genes that encode

proteins that are involved in early steps of tRNA maturation. First, we examined the *rex1Δ* strain which lacks the 3'-5' exoribonuclease that functions in maturation of the 3' ends of 5S rRNA and some tRNAs (van Hoof et al. 2000; Copela et al. 2008; Ozanick et al. 2009; Skowronek et al. 2014). Second, we examined the *lhp1Δ* mutant lacking the Lhp1 protein that binds to the oligo U track of nascent pre-tRNAs. Lhp1 inhibits 3' exonucleolytic processing of particular pre-tRNAs and thereby assists in endonucleolytic cleavage of their trailers (Yoo and Wolin 1997; Copela et al. 2008). As a control, we also employed the *los1Δ* mutant that has a defect in tRNA nuclear export resulting in accumulation of unspliced but end-matured pre-tRNAs (Hopper et al. 1980).

Analyses of pre-tRNA^{Ile}_{UAU} in *rex1Δ* cells revealed presence of an additional, slower migrating form even when grown in standard conditions (Fig. 2A and 2D), demonstrating, as anticipated, a defect in the 3' end processing. This form of pre-tRNA^{Ile}_{UAU} was similar in migration to the form that accumulated in wild type cells subjected to carbon source and temperature stress and was the only form visible for *rex1Δ* in glycerol containing media. The data indicate that the stress conditions might affect processing of pre-tRNA^{Ile}_{UAU} 3' trailers in a condition-specific manner. Also, the partially processed intron-containing form of pre-tRNA^{Tyr} accumulated in *rex1Δ* cells (Fig. 2C and 2D). Moreover, both aberrant processing intermediates were observed for pre-tRNA^{Lys}_{UUU} in *rex1Δ* cells, even when grown in standard conditions (Fig. 2B and 2D). In contrast, aberrantly migrating forms of pre-tRNA^{Ile}_{UAU}, pre-tRNA^{Lys}_{UUU}, and pre-tRNA^{Tyr} were not detected in the *lhp1Δ* mutant (Fig. 2A-C). The observations suggest that the slowest migrating forms of pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Lys}_{UUU} detected in wild type cells under stress conditions have extended 3' ends which are protected by Lhp1, thus in *lhp1Δ* mutant these 3' extensions are sensitive to exonucleases. As expected, the control *los1Δ* strain accumulated end-mature, intron-containing precursors for all tested

tRNAs, but also the aberrant pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr} were present under stress conditions (Fig 2A and 2C, solid arrows). further confirming that they are formed due to improper early maturation in the nucleus.

Processing of 3' trailers consists of several steps, whereas the processing of 5' leader in pre-tRNA is a single step catalyzed by the endoribonuclease, RNase P. We investigated whether novel slow migrating form of pre-tRNA^{Ile}_{UAU} observed under stress conditions contains a 5' leader. Employing a probe specific to the 5' leader of pre-tRNA^{Ile}_{UAU}, both the conventional and the novel slow migrating forms were detected (Fig. 3A). Thus, the 5' leader sequence is present in both forms of slow migrating pre-tRNA^{Ile}_{UAU}. Therefore, the two forms of pre-tRNA^{Ile}_{UAU} likely differ only at their 3' termini and the faster migrating of the two intron-containing forms represents pre-tRNA^{Ile}_{UAU} partially processed at the 3' end. This partial 3' end trimming occurring before 5' processing is rapid under standard conditions and slowed-down under stress conditions.

We extended the analyses to detection of 5' end of the aberrant form of pre-tRNA^{Tyr}. We utilized short (probe of 17 nucleotides specific to the 5' termini of pre-tRNA^{Tyr}) which required optimization of conditions for specific detection of precursors containing 5' leader sequence. Only primary transcript and no other processing intermediates that accumulated under stress were observed (Fig. 3B). Therefore it appears that the aberrant form of pre-tRNA^{Tyr} that accumulated under stress has been processed at the 5' end and retains an unprocessed extended 3' end. In support of this, we noted that a similarly migrating intermediate form of pre-tRNA^{Tyr} accumulated under standard conditions in the *rex1Δ* mutant but was not detected in *lhp1Δ* cells (Fig. 2B), Altogether, the results of the Northern analysis indicated that the intermediates of tRNA processing which accumulate under stress

differ at their 3' ends and support the model that 3' end processing of both, pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr}, is inhibited by stress.

We assessed whether the 3' end-extended tRNA precursors observed under stress conditions could be further processed upon return to favorable growth conditions or instead are designated for degradation. Nonfunctional pre-tRNAs can be degraded by a nuclear TRAMP/exosome surveillance system (Gudipati et al. 2012; Schneider et al. 2012), therefore, we employed *rrp6Δ* mutant which impairs this quality control pathway. The fate of 3'-extended forms which accumulate in stress conditions (YPGly at 39°C) was monitored by Northern blotting at defined time points after transfer of cells back to standard conditions (YPD at 30°C) (Fig. S2). The 3' end-extended forms of tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr} progressively decrease during incubation in YPD and the end-matured intron-containing form is generated. Moreover, the levels of aberrant forms are not increased in *rrp6Δ* mutant. The results suggest that the aberrant precursors that accumulate under stress are maturation intermediates that are rather not subjected to degradation but could be further processed when cells are shifted back to permissive conditions. However, due to continued transcription after the return to favorable growth conditions we cannot exclude contribution of newly synthesized transcripts to observed pre-tRNA pattern.

Defects of 3' end-processing of pre-tRNA^{Ile}_{UAU} observed under stress conditions were further characterized by sequencing. RNA was isolated from wild type cells grown under standard conditions or shifted to YPGly and incubated for 2h at 37°C or 39°C. An oligonucleotide adapter was ligated to the 3' ends of RNAs. The RNAs with ligated oligonucleotide were reverse transcribed using a primer specific to the adapter followed by PCR with primers specific to the intron of tRNA^{Ile}_{UAU} and the adapter (Fig. 4A and 4B). Pre-tRNA^{Ile}_{UAU} was chosen for analysis due to its relatively long 60 nucleotide (nt) intron, facilitating separation of PCR amplified products by gel electrophoresis. The products were

then ligated into pGEM vector followed by transformation of *E.coli*. For each growth condition, we sequenced over 80 PCR fragments amplified from the resulting plasmids. All of the sequences were derived from pre-tRNAs prior to intron removal. As anticipated from the Northern analysis, the PCR fragments contained part of intron and 3' exon but were heterogeneous at the 3' end. End-matured pre-tRNAs became substrate of CCA nucleotidyltransferase and ended with C, CC or CCA. Other sequences corresponded to unprocessed or partially processed 3' ends (Fig. 4C, Table S1).

For cells grown in standard conditions the majority of the clones (52%) represented 3' end matured pre-tRNAs; 29% of these had single C, 12% had CC and 11% were fully modified to CCA (Fig. 4C, Table S1). Under stress conditions tRNA precursors had longer 3' extensions which are not completely cleaved (Fig. 1A) and hence, fewer clones analyzed by sequencing contained the C, CC or CCA (Fig 4C).

The remaining 48% pre-tRNA^{Ile}_{UAU} clones derived from cells grown in standard conditions possessed relatively short 3' extensions of 3-8 nucleotides (Fig. 4C, Table S1). For pre-tRNA^{Ile}_{UAU} isolated from cells shifted to stress conditions, longer 3' termini containing 9-14 nucleotides were detected (Fig. 4C). Moreover, the increase in number of clones with longer trailers correlated with temperatures elevated from 30°C to 37°C and 39°C. The results show that the rate of 3' processing of primary transcript is both temperature and carbon source dependent. The results are consistent with the hypothesis that 3' processing of primary transcripts is very fast under standard conditions, but slows down under stress, resulting in the increase of longer forms of pre-tRNA^{Ile}_{UAU}.

Additionally, mostly under stress conditions, some shorter pre-tRNA^{Ile}_{UAU} have one or two extra adenines at their 3' ends and oligoadenylation of longer form was detected only once among hundred sequenced clones (Fig. 4C, Table S1). Low frequency of oligoadenylation

supports our previous conclusion that the aberrant long pre-tRNA^{Ile}_{UAU} form which accumulates under stress conditions is not subjected to degradation (Fig. S2).

Rex1 is limiting for 3' end trimming of particular pre-tRNAs under stress conditions

Decreased efficiency of tRNA 3' end-processing under stress could be caused by down-regulation of processing enzymes. To address this possibility, we assessed whether the levels of tRNA processing proteins respond to stress. Commercially available strains encoding tagged versions of Lhp1 and Trz1, grown at conventional or stress conditions, were examined by western blotting. No effects on the levels of Lhp1 and Trz1 were observed (Fig. S3). To explore whether *REX1* expression is altered by stress, we created an HA-tagged Rex1 strain. However Rex1-HA did not have normal catalytic activity as assessed by Northern analysis of tRNAs and 5S rRNA (Fig S4, forms marked with arrows).

To assess whether Rex1 level or function is reduced under stress condition by another means, we monitored the ability of overexpressed Rex1 to suppress tRNA processing defects. We transformed yeast with a multicopy plasmid encoding the native Rex1 and analyzed tRNAs by Northern blotting (Fig. 5). As anticipated, this plasmid was functional because it complemented the *rex1*Δ defects of 5S rRNA and pre-tRNA processing (Fig. S5, forms marked with arrows). When the blot was hybridized with the probe specific to pre-tRNA^{Ile}_{UAU}, only a subtle effect of *REX1* overexpression upon pre-tRNA^{Ile}_{UAU} processing was observed (Fig. 5A) and differences were not statistically significant (Fig. 5B). However, the levels of aberrant form of pre-tRNA^{Tyr} (intron-containing with unprocessed 3' trailer) which accumulated under stress conditions were significantly decreased when Rex1 was overproduced (Fig. 5A). At the same time the relative levels of end-mature intron-containing pre-tRNA^{Tyr} increased upon *REX1* overexpression (Fig. 5B).

Additionally we found that the examined defects of 3' processing of pre-tRNAs were not suppressed neither by overproduction of Lhp1 nor Trz1 (Fig. S6). Northern analysis suggests further inhibition of 3' processing by the excess of Lhp1 which probably limits the access of Rex1 and other nucleases to the 3' end (Fig. 6A).

These cumulative data provide compelling evidence in support of our conclusion that the pre-tRNA 3' processing activity of Rex1 is limited under stress conditions.

DISCUSSION

The effect of repression of tRNA transcription under various types of stress in Maf1-dependent fashion has been thoroughly studied and well defined (Boguta 2013). The condition employed in this study - a nonfermentable carbon source together with an elevated temperature are known to lower tRNA transcription (Ciesla et al. 2007). Also, the general level of tRNA modification was reported to change upon oxidative stress, DNA damage or hydroxyl peroxide and temperature (Chan et al. 2010, 2012; Alings et al. 2015). Furthermore, hypomodified tRNAs or tRNAs containing mutations that destabilize their structure were shown to be degraded at elevated temperatures via the RTD pathway (Chernyakov et al. 2008; Whipple et al. 2011). In addition, heat shock as well as methionine starvation, or oxidative stress, caused tRNA cleavage in the anticodon loop by Rny1, creating tRNA halves (Thompson et al. 2008; Thompson and Parker 2009). All those processes, show different levels of response of cells to changes of environmental conditions.

However, there has not been a previous report that systematically exams the effects of growth conditions upon tRNA maturation, although differences in pre-tRNAs intermediates at elevated temperature were reported previously for some intron-containing yeast tRNA families (O'Connor and Peebles 1991; Kufel et al. 2002; Kufel and Tollervey 2003), Here,

we employed Northern analyses and RNA sequencing to examine the effects of alterations of temperature and carbon source upon precursors processing of several tRNAs.

Defects in tRNA maturation were detected by the accumulation of aberrant tRNA processing intermediates upon shift of cells to stress conditions - high temperature and/or medium with a nonfermentable carbon source. These intermediates can be categorized into two types. The first is represented by the slowest aberrantly migrating form which contains the 5' leader, intron, and extended 3' trailer. This form was most thoroughly examined for pre-tRNA^{Ile}_{UAU} and its abundance was found to correlate with the severity of the stress. The second type of aberrant tRNA form observed for pre-tRNA^{Tyr} at higher temperatures contained extended 3' trailers and introns but processed 5' termini. Both types of intermediates were detected for pre-tRNA^{Lys}_{UUU} when the stresses of high temperature and YPGly media were combined (Fig. 1B); however, these effects were subtle.

Similar aberrant precursor forms of pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr} were reported resulting from defects in 3' end processing of pre-tRNA due to the *rex1Δ* mutation or to the combination of *rex1Δ* with partial inactivation of Trz1 endonuclease (Skowronek et al. 2014). Moreover, an earlier report detected slow migrating tRNA^{Ile}_{UAU} and tRNA^{Lys}_{UUU} forms dependent on the presence of Lhp1 (Yoo and Wolin 1997). When the isogenic *rex1Δ* and *lhp1Δ* mutants were used as controls, we established that both types of the stress-related processing intermediates co-migrated with the precursors which are normally trimmed at the 3' ends by Rex1. We thus propose that Rex1 activity is limiting under stress conditions. This idea is supported by partial suppression of the defect in tRNA^{Tyr} maturation upon *REX1* overexpression (Fig. 5A). Additionally, since accumulation of stress-related forms of pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr} was not detected in *lhp1Δ* mutant, we infer that Lhp1 is also involved in control of tRNA maturation by stress (Fig. 2). Together, the results allow us to

form a model in which defects of pre-tRNA processing observed under stress conditions are interconnected to inefficient processing of 3' trailers (Fig 6).

Formation of the 3' end begins with termination of tRNA gene transcription in which intrinsic 3' exonuclease activity is mediated by the C11 subunit of Pol III. Mutations in C11 that decrease cleavage activity resulted in increased 3' oligo(U) length, but only by one or two nucleotides (Huang et al. 2005). Although it was not experimentally addressed, it is conceivable that stress conditions decrease the activity of endogenous C11. Thus, in addition to impaired 3' processing, inefficient cleavage might have an impact upon the length of U tracts detected in 3' trailers of tRNA^{Ile}_{UAU} under stress conditions.

The C11 subunit forms a subcomplex C11/C53/C37 and altered activity of this complex results in read-through of termination signals (Landrieux et al. 2006; Huang et al. 2005; Rijal and Maraia 2013). The two tRNA^{Ile}_{UAU}-encoding genes in the yeast genome possess the CTTTCTC sequence at their 3' ends, followed by a tract of 9 or 10 T(s), respectively. Such long tracts of T(s) should function as strong transcription termination signals since 4-6 T(s) are sufficient for termination in eukaryotic cells (Hamada 2000; Braglia 2005). Theoretically, another T tract that could potentially serve for transcription termination is located about 50 nucleotides further downstream. Transcription terminated at this site would generate much longer transcripts than what was detected. Thus, transcription termination readthrough appears not to cause the accumulation of slow migrating form of tRNA^{Ile}_{UAU} under stress conditions.

Interestingly, regardless of the growth conditions, for tRNA^{Ile}_{UAU} we detect partial processing of 3' trailer before the 5' leader removal (Fig. 3A). Previous evidence has led to the generally accepted model that in yeast, pre-tRNA 5' processing occurs prior to 3' processing (O'Connor and Peebles 1991; Yoo and Wolin 1997; Lee et al. 1991). However, an exception was reported for tRNA^{Trp} for which the 3' trailer is removed before the 5' leader

(Kufel and Tollervey 2003). The data reported here provide in vivo evidence supporting the model proposed by Copela et al. (Copela et al. 2008), that partial 3' end trimming can proceed 5' processing also for tRNA_{UAU}^{Ile} (Fig. 6).

It is possible that the observed changes in processing of pre-tRNA_{UAU}^{Ile} under stress could be caused by lower availability of the enzymes involved in 3' end processing. Pre-tRNA_{UAU}^{Ile} has a relatively long trailer (up to 14 nt) and thus belongs to tRNA precursors which required both Trz1 and Rex1 for 3' end processing (Ozanick et al. 2009; Skowronek et al. 2014). However, overexpression of *REX1* is not sufficient to overcome the stress-induced defect in pre-tRNA_{UAU}^{Ile} 3' end processing (Fig. 5A). Additionally, for both Lhp1 and Trz1 we did not observe significant differences in their protein levels under stress (Fig. S3). However, we cannot exclude possibility that the activity/specificity of these enzymes is lower. Another possible explanation for stress-mediated inhibition of 3' trailer processing would be a structural change of pre-tRNA_{UAU}^{Ile} that hinders enzyme access to the 3' termini. In support of this notion, it has been reported that partial m⁵C modification at position 48 and m⁷G at position 64 can be introduced prior to 5' end processing of pre-tRNA_{UAU}^{Ile} (Ohira et al. 2009) and levels of these two modifications are increased under various stress conditions (Chan et al. 2010). It is conceivable that changes in folding/structure of pre-tRNAs could impact their 3' end processing under stress. It has recently become appreciated that tRNA modification which could alter tRNA structure are affected by growth conditions. The effect of stress was reported for tRNA thiolation (Kamenski et al. 2007; Damon et al. 2015; Han et al. 2015), m⁵C methylation (Preston et al. 2013) and other modifications (Alings et al. 2015). It is worth noting that, activities of at least two enzymes responsible for end processing, RNase P and RNase Z, depend not on substrate sequence but proper structure (Mondragón 2013; Takaku et al. 2004).

The form of pre-tRNA^{Tyr} which accumulates under stress conditions is processed at its 5' end. In this case, decreased activity of Rex1 exonuclease might account for inhibition of the processing of 3' trailer. A role of endonucleolytic cleavage seems to be less probable because, inactivation of Trz1 alone did not result in the accumulation of the tRNA^{Tyr} intermediate which contained intron and 3' trailer (Skowronek et al. 2014) and, overproduction of Trz1 had no significant effect on accumulation of aberrant pre-tRNA forms as well (Fig. S6B).

Our study provide evidence for an environmental response of tRNA processing for several tRNA species highlighting the effects of temperature and/or carbon source stress. It is quite possible that additional processing steps in these and other tRNAs might be effected by various types of stress. It is known that effects of diverse environmental stresses elicit distinct responses at the level of pre-mRNA processing (Kuhn et al. 2001; Pleiss et al. 2007; Bergkessel et al. 2011). Although the mechanism of adaptation and physiological function of the observed response require further clarification, our data document that the impact of environmental conditions also occurs for tRNA maturation.

MATERIAL AND METHODS

Strains, plasmids, and media

Yeast strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and *rex1Δ*, *lhp1Δ*, and *los1Δ* derivatives were obtained from the yeast deletion collection (Open Biosystem). The plasmid harboring *REX1* was obtained from M. Rosbach and is described in (Abruzzi et al. 2007). YEp24 plasmid was used as the control vector.

YPD or YPGly complete media contained 1% yeast extract (Fisher Scientific), 1% peptone (BD), and 2% glucose (Fisher Scientific) or 2% glycerol (Sigma), respectively. Minimal

medium (SC) contained 2% glucose and 0.67% yeast nitrogen base (BD). SC –ura and Sc-leu contained supplements required for growth (at 20 µg/ml each, except uracil or leucine, respectively).

LBA X-gal IPTG plates contained 1% bactotrypton, 0.5% yeast extract, 20 mg/ml Agar, (all from BD), 0.5% NaCl, 1 mg/ml ampicillin, 1.5% X-Gal and, 0,8 M IPTG.

Northern blot analysis

Overnight yeast cultures were resuspended in fresh YPD media to $OD_{600} = 0.15$ and grown to exponential phase ($OD_{600} = 0.5-0.8$). The cells were collected by centrifugation and washed. Then the cultures were resuspended in YPGly media and/or shifted to different temperatures in the range of 23°C –39°C. After incubation under stress conditions for 2h, the cells were harvested, washed and re-suspended in TSE buffer (0.01 M Tris, 0.01 M EDTA, 0.1 M NaCl, pH 7.5). TSE saturated phenol was added to the samples and the mixture was incubated at 37°C with shaking for 1h followed by 30 min incubation on ice, and then a second phenol extraction was performed, followed by ethanol precipitation for at least 1h at -80°C. 3-5 µg of RNA was separated by electrophoresis on a 10% polyacrylamide, 8 M urea gel, transferred onto positively charged nylon membranes, and hybridized with DIG-labeled probes, as described previously (Wu et al. 2013). For some probes, the temperature of hybridization and washing protocol were modified to increase specificity of the signal. Some blots were developed using photographic films for higher resolution. Calculation of RNA signals was conducted using Total LabQuant software (Totalab).

For RNA hybridization the following DIG-labeled oligonucleotides were used: JW0011

(tRNA^{Ile}_{UAU}) 5'-
GGCACAGAACTTCGGAAACCGAATGTTGCTATAAGCACGAAGCTCTAACCACT
GAGCTACA-3'; ND0060 (tRNA^{Lys}_{UUU},) 5'-
CTCCTCATAGGGGGCTCGAACCCCTGACATTTCCGGTATCCTTGCTTAAGCAAATG

CGCT-3'; SRIM15 (tRNA^{Tyr}) 5'-
 GCGAGTCGAACGCCCGATCTCAAGATTTACAGTCTTGCGCCTTAAACCAACTTGG
 CTACC-3'; WHIT27 (tRNA^{Ile}_{UAU} -5' end) 5'-CTACACGAGCATTTCGAAAGA-3',
 tY(GUA)D (tRNA^{Tyr} 5' end of tY(GUA)D gene) 5'-GAGAGTCGATTTCTTGC-3'.

RNA 3' adapter ligation and sequencing

700 ng of RNA from each sample was treated with DNase according to manufacturer's instructions (TURBO™ DNase, Life Technologies) prior to ligation with Universal miRNA Cloning Linker (NEB). Ligation was conducted using truncated T4 RNA Ligase 2 (NEB) in the manufacturers buffer supplemented with 12% PEG MW 8000; the reaction was incubated for 2h at room temperature followed by phenol: chloroform extraction and ethanol precipitation. The entire sample was used for reverse transcription with Super Script II Reverse Transcriptase (Life Technologies), and performed according to manufacturer's protocol with 2 pmol of primer specific to the linker (5'-ATTGATGGTGCCTACAG-3') and incubation time for reverse transcription extended to 1.5h. Negative controls were prepared using RNA without linkers for RT. cDNA was used for PCR with primers specific to the linker and pre-tRNA intron (5'-CAACATTCGGTTTCCG-3'). PCR product was analyzed by electrophoresis on 2.5% agarose gel. The proper size product was cut from the gel, purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and ligated into pGEM®-T Easy vector (Promega) followed by transformation of *E. coli* which was plated on LBA- X-gal IPTG selection plates. White colonies were selected and plasmids were isolated with QIAprep Spin Miniprep Kit (Qiagen) and sequenced at the laboratory of sequencing DNA and oligonucleotide synthesis IBB PAS. Sequencing results were analyzed with Sequence Scanner 2 (Applied Biosystem) and online Multi Align tool (Corpet 1988).

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



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FIGURES LEGENDS

Figure 1. Patterns of tRNA precursors differ depending on growth conditions and tRNA isotype. (A-C) The wild type yeast strain was grown in media with glucose as the carbon source (YPD) at 30°C to exponential phase and then shifted for 2h to different temperatures (23-39°C) and/or to media containing glycerol as the carbon source (YPGly). RNAs were isolated and analyzed by Northern hybridization with probes specific to indicated tRNAs. Due to the high levels of mature tRNAs with respect to processing intermediates, different exposures were taken from blot hybridized with the same probe to assess pre-tRNAs and mature tRNAs in this and following figures. The major and minor forms of pre-tRNA precursors which accumulate under stress conditions are indicated by solid and dashed arrows, respectively. The following graphic representations for tRNA species were used in this and all subsequent figures:  tRNA precursor containing introns and unprocessed 5' and 3' ends,  tRNA precursor with intron and unprocessed 3' end,  end-matured intron-containing pre-tRNA,  mature tRNA. Positions of binding of probes to each form are marked with a black line above tRNA graphic representations. This and all succeeding Northern blots are representative of at least three independent biological replicates of experiments in which ethidium bromide stained 5.8S and 5S rRNA serve as the loading controls. (A'-C') Calculation of pre-tRNA ratios from at least 3 biological replicates. The bars represent the ratios between the levels of following forms; for tRNA^{Ile}_{UAU} the ratios between the slower migrating (aberrant) and faster migrating (standard), forms containing 5' and 3' termini and intron; for tRNA^{Lys}_{UUU} the ratio between end-extended intron-containing forms (primary transcript) and end-matured intron-containing form and for tRNA^{Tyr} the

ratios of the aberrant 5' processed 3' end-containing (aberrant) form with respect to the end-matured intron-containing tRNA (standard). Standard deviation and p-value calculated as a two-tailed t-test are presented on the graphs. Stars above the bars designate respectively * p-value <0.05, ** p-value < 0.005, *** p-value < 0.0005.

Figure 2. Both exonucleolytic 3' end maturation pathway and chaperone function of Lhp1 influence the precursor pattern of tested tRNAs. (A-C) Wild type and indicated mutant strains were grown at 30°C to exponential phase and then shifted for 2h to 30°C or 37°C and/or YPGly. RNA was isolated and analyzed by Northern hybridization with probes specific to tRNA_{UAU}^{Ile}, tRNA_{UUU}^{Lys} and tRNA^{Tyr}. Ethidium bromide stained 5.8S rRNA was used as a loading control. The major and minor forms of pre-tRNA precursors which accumulate under stress conditions are indicated by solid and dashed arrows, respectively. (D) Calculation of ratios between various forms of pre-tRNA (specified in the legend to Fig. 1) from at least 3 biological replicates.

Figure 3. Specie- specific tRNA processing intermediates under stress conditions: tRNA_{UAU}^{Ile} contains the 5' leader sequence whereas the aberrant tRNA^{Tyr} species is processed at the 5' end. Wild type and *rex1Δ* mutant strains were grown in YPD at 30°C and then shifted for 2h to 37°C and/or YPGly. RNA was isolated and analyzed by hybridization with probes specific to 5' leaders of both tRNA_{UAU}^{Ile} genes and tRNA^{Tyr} tY(GUA)D. Forms of tRNA precursors which accumulate under stress conditions are indicated by solid arrows. Hybridization with other specific probes recognizing mature and pre-tRNAs were employed as controls. The bottom panels present ethidium bromide stained 5.8S and 5S rRNA used as the loading controls.

Figure 4. Sequencing of pre-tRNA^{Ile}_{UAU} forms document 3' end processing defects under stress conditions. (A) Outline of the strategy for linker ligation and sequencing of pre-tRNA 3' ends. (B) Section of pre-tRNA^{Ile}_{UAU} subjected to sequencing. (C) Summary of results obtained for pre-tRNA^{Ile}_{UAU} sequencing of RNAs isolated from cells grown in YPD 30°C or shifted for 2h to YPGly at 37°C or 39°C. Percent of pre-tRNA^{Ile}_{UAU} molecules with 3' processed or 3' extended ends were calculated from total number of sequenced clones which corresponded to genomic sequence and/or additional modification (A or CCA).

Figure 5. Effect of *REXI* overexpression on 3' end processing of pre-tRNA^{Ile}_{UAU} and pre-tRNA^{Tyr} under stress. (A) Wild type strain transformed either with the multicopy vector or the vector containing *REXI* was grown in minimal media to exponential phase and then shifted to YPD or YPGly at the indicated temperatures for 2h. Isolated RNA was analyzed by Northern blotting with probes specific for tRNA^{Ile}_{UAU} and tRNA^{Tyr}. The bottom panels present ethidium bromide stained 5.8S and 5S rRNA used as the loading controls. Forms of tRNA precursors which accumulate under stress conditions are indicated by solid arrows. (B) Calculation of RNA ratios from at least 3 biological replicates was performed as described in legend to Fig. 1. Standard deviation and p-value calculated as a two-tailed t-test are presented on the graphs, * above the bars represent p-value =0.05.

Figure 6. Model presenting the effect of stress conditions on end-maturation of pre-tRNA^{Tyr} and pre-tRNA^{Ile}_{UAU}. (A) Under standard growth conditions tRNA^{Tyr} is first processed at the 5' terminus by RNase P, then the 3' end is matured by an exo- and/or endonucleolytic pathway. The step of 3' removal is very fast under standard conditions (indicated by a green arrow), but under stress it is inhibited (marked with a red dashed arrow). Thus, 5'-end-processed 3'-end-extended precursors of tRNA^{Tyr} can be detected under standard conditions and they accumulate to higher levels under stress. (B) tRNA^{Ile}_{UAU} has a longer

primary transcript than previously assumed (3' extension marked with yellow) and it is removed in two steps. The first step is very fast under standard conditions (indicated by a green arrow) and the 3' end is partially trimmed before the 5' end is removed. Inhibition of this step by stress (marked with a red dashed arrow) results in accumulation of pre- tRNA^{Ile}_{UAU} unprocessed at both 3' and 5' termini and part of these accumulated pre-tRNAs have longer 3' extensions. The second step of 3' end removal is done in concert with 5' end cleavage, thus precursors processed at one end- are not detected.

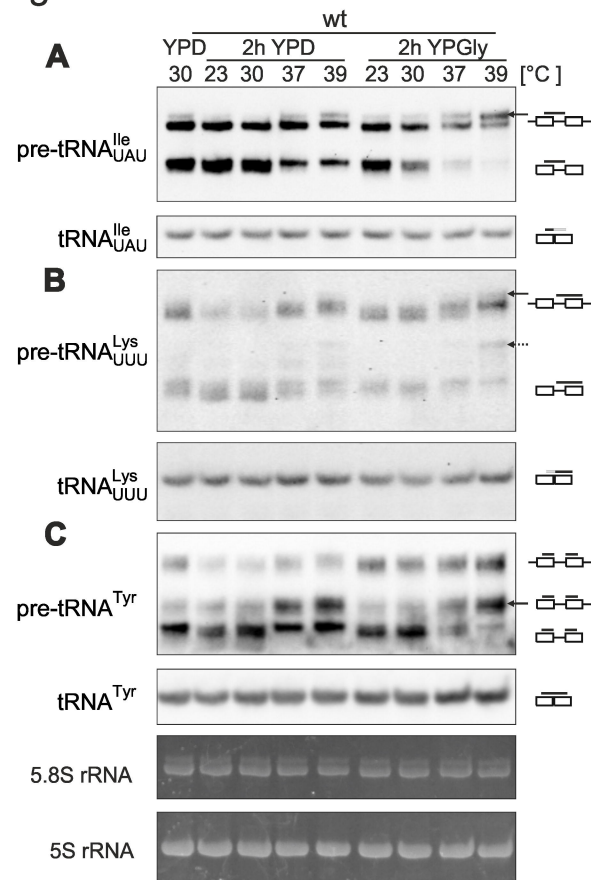
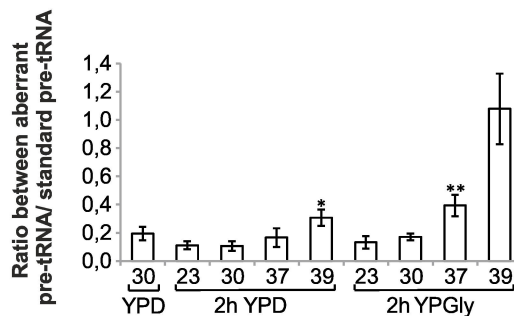
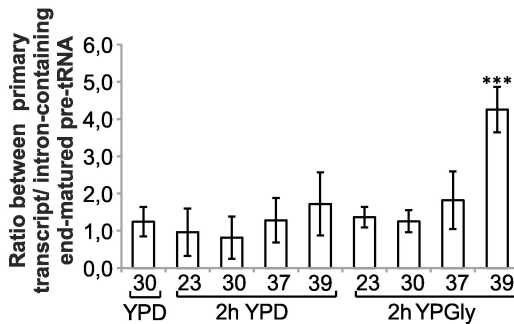
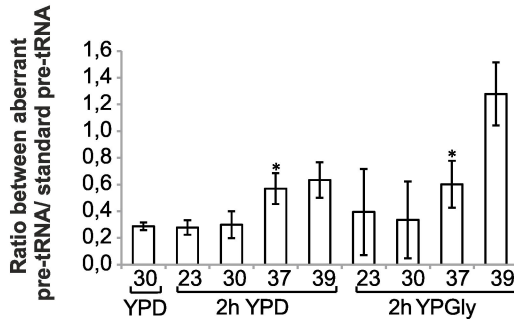
**A'****B'****C'**

Fig.2

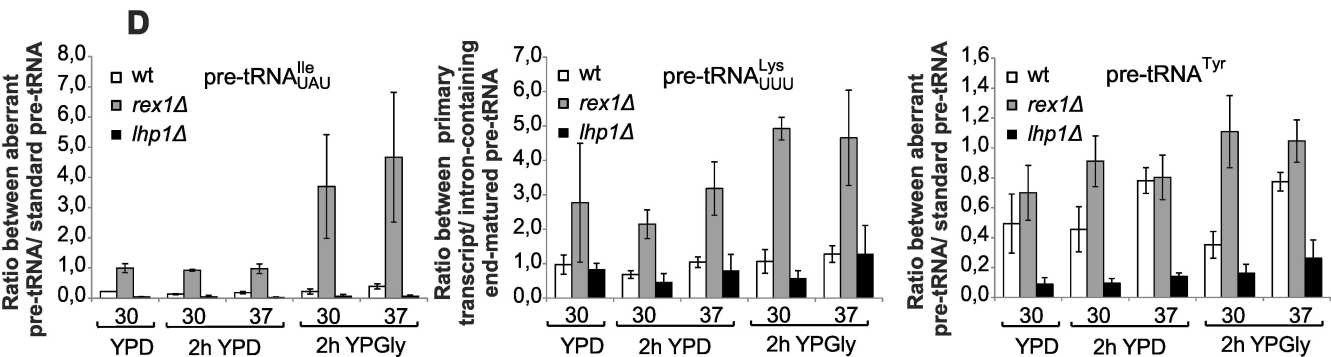
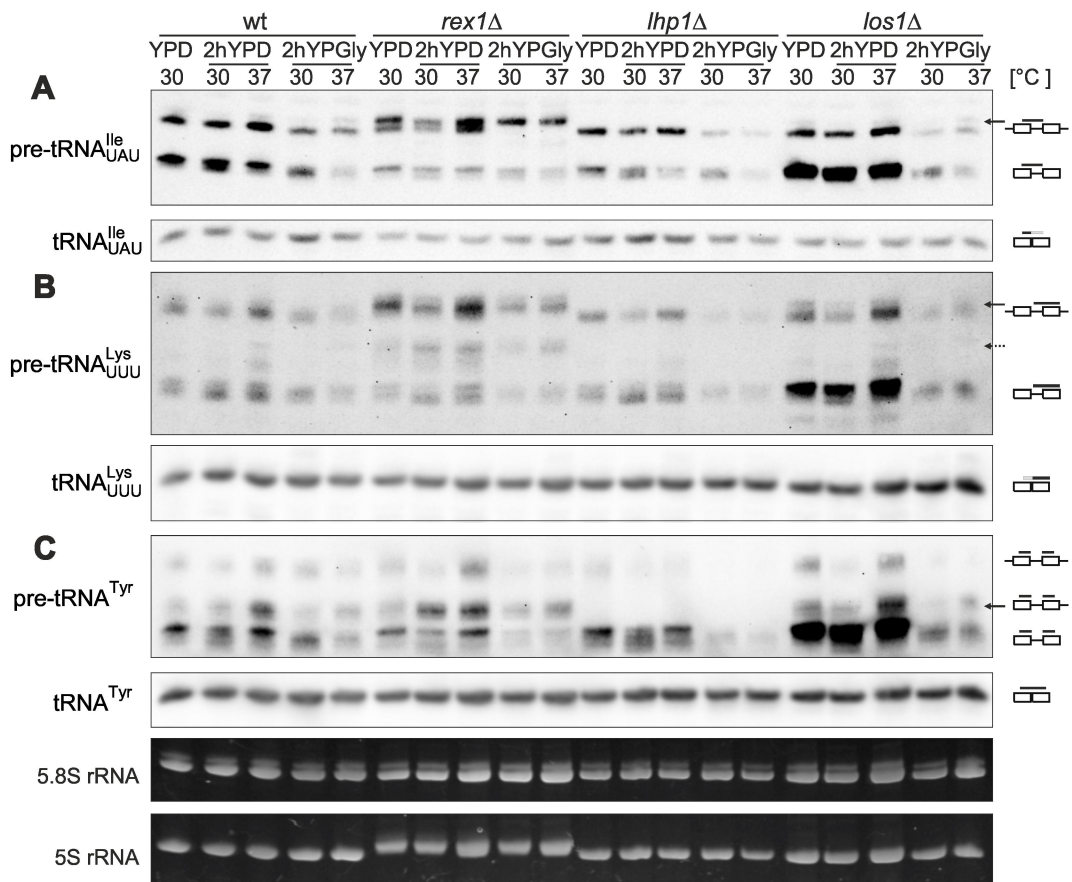
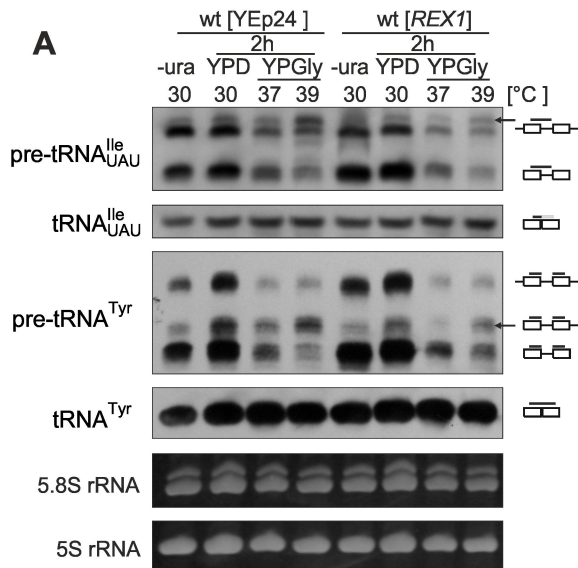


Fig. 5



B

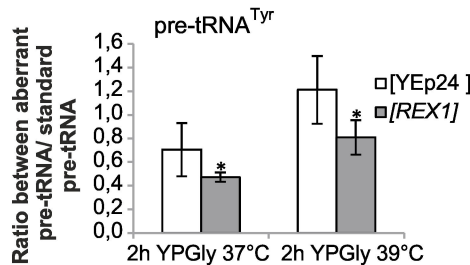
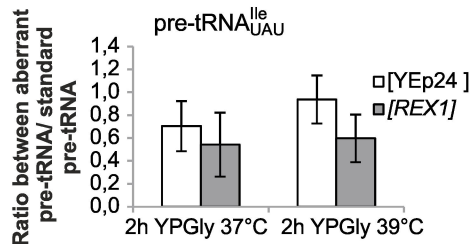


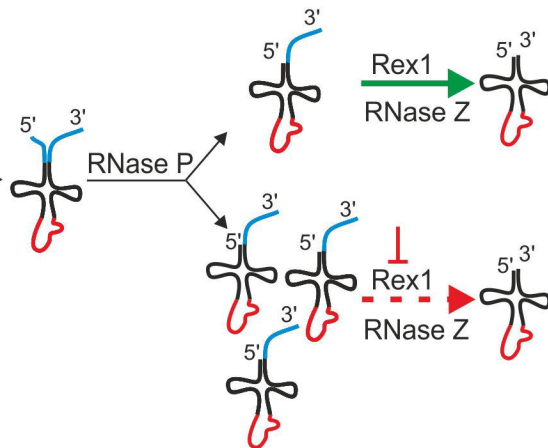
Fig. 6

A

standard growth conditions

tRNA^{Tyr}
primary transcript

stress growth conditions



B

standard growth conditions

tRNA^{Ile}_{UAU}
primary transcript

stress growth conditions

