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PII: S0378-1119(13)00552-0
DOI: doi: 10.1016/j.gene.2013.04.055
Reference: GENE 38583

To appear in: *Gene*

Accepted date: 15 April 2013

Please cite this article as: Morawiec, Ewa, Wichtowska, Dominika, Graczyk, Damian, Conesa, Christine, Lefebvre, Olivier, Boguta, Magdalena, Maf1, repressor of tRNA transcription, is involved in the control of gluconeogenetic genes in *Saccharomyces cerevisiae*, *Gene* (2013), doi: 10.1016/j.gene.2013.04.055

Maf1, repressor of tRNA transcription, is involved in the control of gluconeogenetic genes in *Saccharomyces cerevisiae*

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Abstract

Maf1 is a negative regulator of RNA polymerase III (Pol III) in yeast. Maf1-depleted cells manifest elevated tRNA transcription and inability to grow on non-fermentable carbon source, such as glycerol. Using genomic microarray approach, we examined the effect of Maf1 deletion on expression of Pol II-transcribed genes in yeast grown in medium containing glycerol. We found that transcription of *FBP1* and *PCK1*, two major genes controlling gluconeogenesis, was decreased in *maf1*Δ cells.

FBP1 is located on chromosome XII in close proximity of a tRNA-Lys gene. Accordingly we hypothesized that decreased *FBP1* mRNA level could be due to the effect of Maf1 on tgm silencing (tRNA gene mediated silencing). Two approaches were used to verify this hypothesis. First, we inactivated tRNA-Lys gene on chromosome XII by inserting a deletion cassette in a control wild type strain and in *maf1Δ* mutant. Second, we introduced a point mutation in the promoter of the tRNA-Lys gene cloned with the adjacent *FBP1* in a plasmid and expressed in *fbp1Δ* or *fbp1Δ maf1Δ* cells. The levels of *FBP1* mRNA were determined by RT-qPCR in each strain. Although the inactivation of the chromosomal tRNA-Lys gene increased expression of the neighbouring *FBP1*, the mutation preventing transcription of the plasmid-born tRNA-Lys gene had no significant effect on *FBP1* transcription. Taken together, those results do not support the concept of tgm silencing of *FBP1*. Other possible mechanisms are discussed.

1. Introduction

Maf1 is the only global negative regulator of RNA polymerase III (Pol III) known so far in yeast. Maf1 is regulated by its phosphorylation-dependent cellular localization (Boisnard et al., 2009; Graczyk et al., 2011; Huber et al., 2009; Lee et al., 2009; Moir et al., 2006;

Oficjalska-Pham et al., 2006; Roberts et al., 2006; Wei et al., 2009). Under repressive conditions Maf1 is imported to the nucleus and its dephosphorylated form interacts directly with Pol III complex (Oficjalska-Pham et al., 2006; Oler and Cairns, 2012; Roberts et al., 2006; Vannini et al., 2010). The association of Maf1 with Pol III under repressive conditions is correlated with dissociation of Pol III from tRNA genes and their decreased transcription (Graczyk et al., 2011; Oficjalska-Pham et al., 2006; Roberts et al., 2006; Upadhya et al., 2002). In favourable growth conditions, Maf1 is phosphorylated and located predominantly in the cytoplasm (Moir et al., 2006; Oficjalska-Pham et al., 2006; Towpik et al., 2008). The Maf1's cytoplasmic location is mediated by two mechanisms: a phosphorylation-dependent inactivation of the Maf1 nuclear location signals (NLSs) (Moir et al., 2006) and nuclear export of phosphorylated Maf1 by the exportin Msn5 (Towpik et al., 2008).

Because of the critical importance of Maf1 in Pol III transcription regulation in yeast and human (Boguta 2013; Acker et al., 2013; Johnson et al., 2007), secondary effects of Maf1 inactivation are anticipated. One reliable suggestion of cellular processes apart from Pol III transcription that are affected by Maf1 comes from genetic analysis of *maf1* mutants. *maf1* alleles which inactivate the Maf1 function in Pol III repression, including *maf1Δ* and the originally selected *maf1-1*, are temperature-sensitive when grown on non-fermentable carbon sources such as glycerol. This puzzling phenotype indicates a defect in mitochondrial function. Which mitochondrial function is compromised in Maf1-deficient cells remains to be established. In *Saccharomyces cerevisiae* two Pol III-synthesized tRNAs have been reported as mitochondrially targeted, namely tRNA-Lys and tRNA-Gln. The mitochondrial functions of these tRNAs are not completely clear although there is indirect evidence for their role in mitochondrial translation (Kolesnikova et al., 2000).

In the absence of Maf1, when the Pol III transcription is incorrectly regulated and more intense than in wild type cells, increased amounts of some cytosolic tRNAs entering

mitochondria could disturb mitochondrial translation. Defects in mitochondrial translation are the most common cause of degradation of mtDNA resulting in the formation of *petite* colonies known as [*rho*$^-$]. However, no increased [*rho*$^-$] accumulation has been observed in *maf1Δ* strains, arguing against this assumption (reviewed in Boguta, 2012; Cieśla et al., 2007).

Another possibility involving the mitochondrial scenario is a function of cytoplasmic Maf1 in postranscriptional tRNA control. In *S. cerevisiae*, unlike in vertebrates, intron-containing tRNA precursors are exported from the nucleus and pre-tRNA splicing occurs in the cytoplasm at the outer mitochondrial membrane (Yoshihisa et al., 2003). Significantly, strains lacking Maf1 do accumulate intron-containing tRNA precursors due to saturation of pre-tRNA nuclear export machinery (Karkusiewicz et al., 2011).

Lastly, Maf1 could also exert a specific effect on gene(s) required for growth on non-fermentable carbon sources and located close to tRNA gene(s) in the genome. Pol III transcription is implicated in several indirect extra-transcriptional functions, some of them that have been affecting the expression of Pol II-transcribed genes in the vicinity of a Pol III-transcribed gene (reviewed in Donze, 2012).

The purpose of the present study was to investigate in a comprehensive manner why the growth of *maf1Δ* cells is compromised on non-fermentable carbon source at elevated temperature. To address this issue, we performed a global transcriptome analysis in which *maf1Δ* mutant and its isogenic parental strain were grown in glucose-based medium, then shifted to glycerol-based medium and incubated at an elevated temperature. Simultaneous profiling of all Pol II transcripts allowed the identification of genes responding differently to those conditions in the mutant and the wild type strain. Among them two genes encoding major regulatory enzymes controlling gluconeogenesis, *FBP1* and *PCK1*, were down-regulated in *maf1Δ*. Since *FBP1* is located in close proximity of a tRNA-Lys gene on

chromosome XII, we investigated how the *FBP1* mRNA level was affected by transcription of the tRNA-Lys gene.

2. Materials and Methods

2. 1. Yeast strains and culture conditions

Yeast strain YPH500 (*MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ*) and its isogenic derivatives carrying *maf1::KanMX4* or *maf1::URA3* (*maf1Δ* hereafter) were described previously (Boguta et al., 1997; Graczyk et al., 2011).

YPH500 *fbp1*Δ and YPH500 *fbp1*Δ *maf1*Δ were constructed by replacement of *FBP1* gene by *KanMX6* cassette (Longtine et al., 1998) amplified with primers 5'AAGAATAACAGTGCGAACATATAAGAAACATCCCTCATACTACCACACATCGG ATCCCCGGGTTAATTAA3' and

5'CGTACTAAAGTACAGAACAAAGAAAATAAGAAAAGAAGGCGATCATTGAAGA ATTCGAGCTCGTTTAAAC3'. To verify correct integration of the cassette the following primers were used: 5'CGTGATTGCGCCTGAGCGAG3' (complementary to *KanMX6* cassette) and 5'TCAACGCCTTCTTACACCTG3' (binding outside of the deleted gene).

The *tK(UUU)L* gene encoding tRNA-Lys located on chromosome XII (positions 875376 to 875471) was inactivated in YPH500 and YPH500 *maf1*Δ strains by replacement of an internal fragment (position 875407 to 875465) by *TRP1* cassette (Longtine et al., 1998). For this purpose, *TRP1* sequence was PCR- amplified with hybrid primers 5'TGGAAAATTCGAAGAACTCATCCTTGTTAGCTCAGTTGGTAGAGCGTTCGGCTT ATTGACCACACCTCTAC3' and

5'TAATAGGCGTGAAGCAAAGGAAGTTGATAAAAAAAAAGTAAAGAACTCCTCGA ATTCGAGCTCGTTTAAAC3' which contained sequences homologous to 5' and 3' regions of *tK(UUU)L*. PCR product was than introduced to YPH500 and YPH500 *maf1*Δ cells following selection of transformants on minimal glucose medium lacking tryptophan. Correctness of manipulations was verified by PCR using two additional primers, 5'AAGTTAGCCCGGGACCATGC3' and 5'TCAACGCCTTCTTACACCTG3' both binding outside the gene in question (data not shown).

Rich medium (YP, 1% peptone, 1% yeast extract) plus 2% glucose (YPD) or 2% glycerol (YPGly) or minimal glucose medium supplemented with appropriate amino acids were used for growth as required (Sherman, 2002). For transcriptome analysis or Northern hybridization an overnight culture grown in YPD was inoculated into 100 ml of fresh pre-warmed YPGly ($A_{600}\approx$ 0.1) and grown at 30^{o}C to OD_{600}=0.8-1.0, transferred for 2 h to 37^{o}C and then harvested for RNA isolation. For RT-qPCR analysis of strains carrying plasmids yeast pre-cultures were grown at 30^{o}C in minimal glucose medium lacking leucine, then transferred to glycerol rich medium for 5 h, shifted to 37^{o}C for 2 h and harvested.

2.2. RNA isolation

RNA was obtained as described by Schmitt et al (1990), with centrifugation and incubation in 65°C carried out for twice as long as in the original procedure (Schmitt et al., 1990). Quantity and quality of RNA was analysed by NanoDrop 1000 (Thermo ND-1000) and verified by visualization on ethidium bromide-stained formaldehyde-agarose gels.

2.3. Microarray analyses

For microarray analyses *maf1Δ* and its wild-type parent strain were grown and RNA was prepared in parallel to minimize experimental inconsistency. Additionally, RNA quality was

analysed on RNA Nano Chip (Agilent) with the use of a bioanalyzer (Agilent Bioanalyzer 2100).

Samples for microarray hybridization were prepared with an Affymetrix GeneChip 3' IVT Express Kit and hybridized to an Affymetrics GeneChip Yeast Genome 2.0 Array. The microarray was scanned with an Affymetrix GeneChip Scanner 3000. Data analyses were performed with Affymetrix scanner software and Partek® Genomics Suite™ 6.4 (Partek Incorporated). Probe set values in the mutant were divided by probe set values in the wild-type. Differentially regulated transcripts were selected from an initial pool of 5 744 probe sets for 5 841 of the 5 845 genes present in *S. cerevisiae.* After Principal Component Analysis (PCA), gene expression change was analysed by the ANOVA method. A list of genes with significantly different expression between the two strains was created with the FDR (false discovery rate) set at 0.05, p-value = 0.05, and the minimum fold change of 2.5. The microarray analysis was performed at Laboratory of Microarray Analyses, IBB PAS and the Faculty of Biology University of Warsaw.

2.4. Northern blotting

Total RNA (20 μg) was resolved on 1% agarose gel with 0.92% formaldehyde in 1xNBC buffer (0,5 M boric acid, 10 mM sodium citrate, 50 mM NaOH) for 1,5 h at 100 V and transferred o/n by capillary blotting with 20x SSC (3 M NaCl, 0,3 M sodium citrate) onto nitrocellulose membrane. RNA was cross-linked to the membrane by UV irradiation and drying for 10 min at 65°C. The membrane was prehybridized for 3 h in 65°C in buffer (7% SDS, 0,5 M Na_2HPO_4, 1 mM EDTA, 1% BSA) and hybridized in the same solution with an oligonucleotide probe labelled with [α-^{32}P]ATP. Probes were prepared by PCR amplification using the following primers: FBP1F 5'GCCAACTCTAGTAAATGGAC 3', FBP1R 5'GTAGAGGATCCGTACATGGC 3', PCK1F 5' TGGCTCTTAGTGACGAAGTC 3',

PCK1R 5’ AACTGACCAGCGTTCCAGAC 3’, ACT1F 5’ TTCCCATCTATCGTCGGTAG 3’, ACT1 R 5’ GTGGTGGAGAAAGAGTAACC 3’ followed by purification and labelling with HexaLabel DNA labelling Kit (Fermentas). After hybridization the blots were washed 3x 15 min at 37°C with 2xSSC and 0,1% SDS and exposed to a phosphorimaging screen (Fujifilm). Signal was quantified using an FLA-7000 PhosphorImager (Fujifilm). Band intensities were quantified using Multi Gauge v 3.0 software.

Table 1. Oligonucleotides used for qPCR.

Gene name	Primers sequences
FBP1	5' ACAAACACGGGCTCATACGC 3'
	5' TACATGGCATAGCAAGCGGC 3'
ACT1	5' CATGTTCCCAGGTATTGCCGA 3'
	5' GTCAAAGAAGCCAAGATAGAA 3'
$tDNA_i^{Met}$	5' GCGCCGTGGCGCAGTGGAAGCGCG 3'
	5' GCGCCGCTCGGTTTCGATCCGAGGAC 3'
$tDNA^{Leu}$	5' GGTTGTTTGGCCGAGCG 3'
	5' TGGTTGCTAAGAGATTCGAACTC 3'

2.5. Reverse transcription-qPCR (RT-qPCR)

cDNA for each sample was synthesized by reverse transcription of total RNA using Superscript II reverse transcriptase (Invitrogen) and used for quantitative PCR (primer sequences: Table1), performed according to the manufacturer’s instructions using SYBR Green PCR Master Mix (PE Applied Biosystems) and an Applied Biosystem 7000 or 7300

unit. The specificity of individual real-time PCR products was determined by performing melting curve analysis. Melting temperatures were determined with the Dissociation Curve software (PE Applied Biosystems).

2.6. Plasmid construction and mutagenesis

For plasmid construction, a 2090-bp DNA fragment containing *FBP1* and *tK(UUU)L* genes was PCR-amplified with 5'CCATTCCATTCGCTACTTCC3' and 5'TCAACGCCTTCTTACACCTG3' primers, cloned in pDrive plasmid (Qiagen PCR cloning Kit) and subcloned as a PstI -XbaI fragment in pRS315. To inactivate intragenic B-box promoter of tRNA-Lys, a single base substitution of C 56 to G was introduced by PCR using 5'TCAGGGGTT**G**GAGCCCCCTA 3'and 5' TAGGGGGCTC**C**AACCCCTGA 3' primers and appropriate procedure (Newman et al., 1983).

3. Results

3.1. Identification of Pol II genes differentially expressed in S. cerevisiae **cells** *lacking Maf1*

We performed a microarray analysis to investigate whether Maf1, the negative regulator of Pol III-directed transcription, plays any role in the regulation of Pol II-transcribed genes. Using an Affymetrix GeneChiP array, the transcriptome profile was determined for wild type and *maf1Δ* cells grown in a glycerol medium at 30^{o}C to OD = 0.8 and shifted to 37^{o}C for two hours. The whole experiment was performed in triplicate and Principal Component Analysis (PCA) indicated a good correlation between the replicates.

Surprisingly, few genes were differentially expressed in *maf1*Δ relative to the wild type suggesting a very specific pattern of deregulation of Pol II-transcribed genes in this mutant

(Table 2). We hypothesized that those transcriptional effects exerted by Maf1 on Pol II-transcribed genes were indirect because no DNA-binding motif has been reported for Maf1 and we could not identify any common DNA sequence in the upstream regions of the affected genes.

Consistent with the requirement of Maf1 for growth in media with a non-fermentable carbon source, two genes essential for gluconeogenesis, *FBP1* and *PCK1*, were down-regulated in the *maf1*Δ mutant. According to microarray analysis, level of *FBP1* mRNA (encoding fructose-1,6-bisphosphatase) was decreased over 6.5 fold and level of *PCK1* mRNA (coding for phosphoenolpyruvate carboxykinase) was decreased 2.5 fold in *maf1Δ*.

Table 2. List of genes with known function whose expression was 2.5 fold or more differentially regulated in *maf1Δ* mutant compared to those of the wild-type parent strain

Gene Symbol	Gene Product	p-value	Fold-Change (*maf1Δ* vs wt)
FBP1	Fructose-1,6-bisphosphatase, key regulatory enzyme in the gluconeogenesis pathway, required for glucose metabolism	0.00487	-6.5441
LEU2	Beta-isopropylmalate dehydrogenase, catalyzes the third step in the leucine biosynthesis pathway	0.00002	-4.3543
COS8	Nuclear membrane protein, member of the DUP380 subfamily of conserved, often subtelomerically-encoded proteins	0.00071	-4.1701
MF(ALPHA)1	Mating pheromone alpha-factor, made by alpha cells; interacts with mating type a cells to induce cell cycle arrest and other responses leading to mating	0.0024	-2.9476
PCK1	Phosphoenolpyruvate carboxykinase, key enzyme in gluconeogenesis,	0.02965	-2.5025

3.2. Inactivation of gene encoding Maf1 prevents induction of FBP1 and PCK1 mRNAs during growth on non-fermentable carbon source.

Both, *FBP1* and *PCK1*, are among multiple genes in *S. cerevisiae* known to be regulated in a carbon source-dependent manner (Roberts and Hudson, 2006; Schüller, 2003; Turcotte et al., 2010). Consistently with published data (Mercado et al., 1994), in a wild-type yeast strain expression of *FBP1* and *PCK1* was completely repressed when cells were grown in glucose-rich medium but was significantly higher when the cells were grown in a medium with glycerol as shown by Northern blot analysis (Fig. 1). Under the growth conditions used for the microarray analysis, i.e. growth in a glycerol medium with a subsequent shift to elevated temperature, the level of *FBP1* and *PCK1* mRNAs was even higher (Fig. 1).

The *maf1Δ* cells behaved differently: in a glucose medium the expression of both genes was as low as in the wild type, but upon transfer to a glycerol medium followed by the temperature shift a less-spectacular increase of expression was observed and the steady-state levels of *FBP1* and *PCK1* mRNA were respectively, about four fold and three fold lower than in wild type *MAF1* strain. Thus, the Northern blotting result confirmed the earlier microarray data. Altogether, these results clearly indicate a role of Maf1 in the control of expression of two crucial regulatory enzymes of gluconeogenesis with a particularly strong effect on *FBP1* transcription.

3.3. Investigation of the role of adjacent tRNA-Lys gene in the control of FBP1 expression

The strong effect of Maf1 deficiency on the induction of *FBP1* raised the question regarding the mode of action of Maf1.

We speculated that the inefficient induction of *FBP1* on a non-fermentable carbon source could somehow result from the highly active Pol III transcription in *maf1*Δ cells.

The *FBP1* gene is located on chromosome XII adjacent to the *tK(UUU)L* gene that encodes tRNA-Lys (Fig. 2A). According to our earlier data (Cieśla et al., 2007) the tRNA-Lys level is elevated nearly seven-fold in *maf1*Δ cells grown in glycerol medium at 37°C. That actively transcribed tRNA-Lys gene could down-regulate transcription of the *FBP1* gene located in its proximity by a mechanism called tRNA-gene-mediated silencing (tgm), where tRNA genes negatively regulate transcription of adjacent Pol II transcribed genes (Hull et al., 1994; Kendall et al., 2000). Consistent with the above assumption, an effect of *maf1*Δ on tgm silencing has been reported (Moir et al., 2006).

To test whether *FBP1* transcription is indeed affected by tgm silencing, we inactivated the *tK(UUU)L* gene on chromosome XII in the control and *maf1*Δ strains by insertion of a *TRP1* cassette (see Materials and Methods). Next, by using RT-qPCR, we investigated whether the prevention of *tK(UUU)L* transcription exerts any effect on the level of *FBP1* mRNA (Fig. 2B). We also verified the Maf1-mediated control of tRNA transcription (Fig. 2C) and growth phenotypes of examined strains (Fig. S1).

As expected, under the applied growth condition (glycerol medium with a shift to elevated temperature) transcription of tRNA-Met and tRNA-Leu was low in the wild type strain but was markedly higher in the *maf1*Δ mutant (Fig. 2C). In agreement with the results of the Northern blot assay, *FBP1* transcription was high in wild type and low in *maf1*Δ (Fig, 2B). Inactivation of the *tK(UUU)L* gene enhanced the *FBP1* gene expression in the wild type strain and, to a lesser extent, also in the *maf1*Δ mutant (Fig. 2B). These results suggest that the intensity of transcription of the *FBP1* gene may be dependent on the expression of the adjacent tRNA-Lys gene. This conclusion was confirmed when the same experiment has been repeated applying minor change in growth protocol (Fig. S2).

Originally tgm silencing was identified as a negative effect of transcription of a plasmid-encoded tRNA gene on the expression of a neighbouring Pol II gene (Hull et al., 1994;

Kendall et al., 2000). To apply a similar assay, we cloned a chromosomal fragment containing both *tK(UUU)L* and *FBP1* in a centromeric plasmid. Additionally, a point mutation $C_{56}G$ was introduced in the B-box of the promoter of the tRNA-Lys gene to prevent its transcription (Newman et al., 1983). Plasmids containing *FBP1* adjacent to either the wild type or the mutated silent allele of the tRNA-Lys gene were introduced to *fbp1*Δ and *fbp1*Δ *maf1*Δ yeast mutants and *FBP1* expression was quantified by RT-qPCR. In agreement with the results for the chromosomal *FBP1* gene, transcription of the plasmid-borne *FBP1* was high in the presence of Maf1 and low when Maf1 was depleted (Fig. 3A). As expected, the levels of tRNA-Met and tRNA-Leu were higher in *maf1*Δ (Fig. 3B). However only little (or no) effect of point mutation in B-box of *tRNA-Lys* promoter on the level of *FBP1* mRNA was observed in *maf1*Δ cells (Fig, 3A, compare *fbp1*Δ*maf1*Δ[FBP1-tDNA] and *fbp1*Δ*maf1*Δ[FBP1-tDNA*] bars).

4. Discussion

In this work we present evidence that Maf1, a general negative regulator of Pol III transcription, is involved in the expression of several genes transcribed by Pol II. Microarray analysis identified some Pol II genes repressed in cells lacking Maf1 and grown under restrictive conditions (glycerol medium and a shift to elevated temperature). Exploring the mechanism of the Maf1 effect on transcription of Pol II genes we focused here on *FBP1* which encodes fructose-1,6-bisphosphatase, an enzyme required in gluconeogenesis, the metabolic pathway generating glucose from non-carbohydrate substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids. The rationales for this choice were: (i) this enzyme catalyzes a nonreversible step of gluconeogenesis; (ii) the gene is localized in close proximity of a tRNA gene; (iii) *maf1*Δ cells cannot grow on non-fermentable carbon sources (such as glycerol).

As mentioned above, the *FBP1* gene is located on chromosome XII adjacent to the *tK(UUU)L* gene (encoding tRNA-Lys) that is transcribed by Pol III. These genes are in opposite orientation and close to each other (the 5'ends of *FBP1* coding sequence and the *tK(UUU)L* transcription unit are 584 bp apart). We speculated that active transcription of the tRNA-Lys gene could negatively regulate the expression of the adjacent Pol II-transcribed gene, *FBP1*.

To test this hypothesis, we deleted *tK(UUU)L* locus by its replacement with a *TRP1* cassette. Than we investigated the expression of *FBP1* in wild type, *maf1Δ* and *tK(UUU)L*-deleted strains. *FBP1* gene expression was decreased when *MAF1* gene was deleted and increased when tRNA-Lys gene was deleted as compared with a wild type strain. Strikingly, the level of *FBP1* expression in strain deprived of both Maf1 protein and the tRNA-Lys gene was higher than in strain lacking Maf1 alone. This result indicated that disruption of neighboring tRNA gene leads to increased transcription of *FBP1*.

We then introduced a point mutation in the internal promoter of the tRNA-Lys gene to prevent its transcription. In this case, *FBP1* expression was estimated in strains with the chromosomal *FBP1* gene deleted and transformed with a centromeric plasmid harboring the *FBP1-tK(UUU)L* locus. RT-qPCR results showed decreased levels of *FBP1* mRNA in the absence of Maf1, but the point mutation of the tRNA-Lys promoter only slightly induced expression of *FBP1* mRNA as compared to the effect of the disruption of the tRNA-Lys gene in the chromosomal locus. Possibly inactivation of chromosomal *tK(UUU)L* locus by insertion of *TRP1* cassette potentially changes local chromatin structure upstream of *FBP1*. Chromatin remodeling is described as basic mechanism controlling *FBP1* promoter; SWI/SNF functions as a major chromatin-remodeling complex, whereas SAGA (Spt-Ada-Gcn5-acetyltransferase complex) is required for stable recruitment of Cat8 co-activator (Biddick, Law, Chin, et al., 2008; Biddick, Law, and Young, 2008).

Significantly, Cat8 binding site located in *FBP1* promoter almost overlaps 5' end of *K(UUU)L* locus and inserted *TRP1* gene, which is also actively transcribed, may favor with Cat8 recruitment. In contrast these effects are not expected for point mutation in tRNA-Lys gene promoter.

Since the point mutation in the tRNA-Lys promoter had only little effect on *FBP1* expression when both genes were harbored in a plasmid, the active transcription status of the tRNA-Lys gene is not critical for regulation of *FBP1* expression.

It has been reported that some other tRNA genes can inhibit transcription from adjacent Pol II promoters *in vivo*. Those phenomena, named tRNA gene mediated (tgm) silencing, were primarily observed for plasmids harboring tRNA and Pol II-transcribed genes. The Sandmeyer's lab found that a single base pair mutation (C56G, the mutation also used in our work) that inactivated a *SUP2* tRNA-Tyr gene increased the level of transcript from a neighboring sigma element (an isolated long terminal repeat of retrotransposon Ty3) (Kinsey and Sandmeyer, 1991). In the Engelke's lab, different Pol II-transcribed promoters have been reported as being repressed by various tRNA genes placed upstream at varied distances and in either orientation. Moreover, mutations that decreased tRNA gene promoter strength of those tRNA genes alleviated that repression (Hull et al., 1994). It should be noted that in the original articles describing tgm silencing (Hull et al., 1994; Kinsey and Sandmeyer, 1991) the inactivation of a plasmid-born tRNA gene induces a 3 to 9 fold transcription increase of the adjacent Pol II-transcribed gene. Low effect of plasmid-born tRNA on adjacent Pol II gene described in current paper definitely is not in the same range of fold induction (Fig, 3A).

An important molecular mechanisms for tgm silencing requires targeting of the tRNA gene to the nucleolus and mutations that disrupt nucleolar structure release the reporter Pol II gene from the repressive effects (Kendall et al., 2000; Wang et al., 2005). A genome-wide localization experiment with high-resolution oligonucleotide arrays indicated that condensin

is bound to all tRNA genes in the yeast genome (D'Ambrosio et al., 2008) and that condensin mutants no longer exhibit tgm silencing and lose the nucleolar localization of tRNA gene (Haeusler et al., 2008). Strikingly, the tgm impairment and mislocalization of tRNA genes observed in the condensing mutants is not associated with transcription defect as determined for the tRNA-Leu3(CAA) gene (Haeusler et al., 2008).

Nevertheless, a genome-wide analysis has shown (i) that natural Pol II-transcribed promoters tend to be severely underrepresented upstream of tRNA genes, (ii) an over-representation of genes transcribed divergently relative to a tRNA gene, and (iii) that tRNA genes exert a modest but significant inhibitory effect (3.5 fold less than control genes) on adjacent Pol II-transcribed promoters (Bolton and Boeke, 2003). A direct analysis of *PTR3* transcription, from a promoter located very close to a tRNA gene, shows that tgm silencing can also operate in a native chromosomal setting (Bolton and Boeke, 2003). The possible interference between tRNA genes and neighboring Pol II-transcribed genes was analyzed for six natural chromosomal loci identified as having Pol II transcription modulated in Pol III transcriptional mutants. The relevant tRNA genes were replaced with *URA3* cassette (similar strategy as used in our work). Three (*ACO1*, *ARO8* and *YEL033W*) of the six genes showed a consistent but modest modification in transcript level by Northern blot analysis (Conesa et al., 2005). A similar tRNA position effect was also reported initially for the tRNA-Thr(CGU) gene upstream of *CBT1* in the native chromosomal locus (Simms et al., 2004). However, more detail subsequent analysis demonstrated that tRNA-Thr(CGU) gene in its natural context serves as an insulator between *STE6*, an upstream gene, and *CBT1*, preventing the *STE6* regulatory elements from affecting *CBT1* transcription (Simms et al., 2008).

tgm silencing was assayed with a plasmid harboring artificial loci containing the *SUP4* tRNA gene adjacent to a *GAL1* promoter-driven *HIS3* gene. A wild type *MAF1* strain presented [His^-] phenotype on galactose media (Hull et al., 1994). Strikingly, *maf1Δ* generates [His^+]

colonies (Moir et al., 2006). Thus, the absence of Maf1 protein allowed the expression of *GAL1* promoter-driven *HIS3* gene contrary to expectations because the tRNA was supposed to be better expressed in a *maf1Δ* strain in comparison with wild type one. That paradoxical effect of Maf1 was not explained (Moir et al., 2006).

Here we observed strong negative effect of *maf1Δ* on expression of *FBP1* gene which is adjacent to the tRNA-Lys(UUU). However, the point mutation in the tRNA-Lys promoter had only little effect on *FBP1* mRNA level when both genes were harbored in a plasmid. In summary we concluded that another, yet unknown mechanism accounts for decreased transcription of *FBP1* in *maf1Δ* cells. Possibly Maf1 by itself could be involved in the *FBP1* expression. Maf1 has been previously shown to modulate some Pol II genes in humans (Johnson et al., 2007). Genome-wide analysis of Maf1 occupancy in yeast identified the fragment (iYLR377C; 584 bp) between *tK(UUU)L* and *FBP1* ORF as specifically enriched by Maf1 protein (Oficjalska-Pham et al., 2006). According to the straightforward interpretation, Maf1 enrichment on this fragment is due to Pol III-transcribed *tK(UUU)L*, but we cannot exclude that Maf1 could be directly involved in control of *FBP1* transcription. It deserves further study.

Acknowledgements

We are grateful to Joël ACKER for stimulating discussions. Work in Poland was supported by Foundation for Polish Science within International PhD Project " Studies of nucleic acids and proteins - from basic to applied research", co-financed from European Union - Regional Development Fund Biology and by the National Science Centre, Poland (grant N301693740).

In France, this work was supported by grants from the French National Research Agency (2010-BLAN-1605-01), from a Partenariat Hubert Curien (PHC) Polonium (grant number 27665NJ) of the French foreign office, and from CEA's International Relations Division (DRI).

Legends to Figures

Figure 1. Analysis of *FBP1* and *PCK1* transcripts confirms specific role of Maf1 in their induction during growth on non-fermentable carbon source. Total RNA was extracted from wild-type (wt) and mutant (*maf1Δ*) strains grown at 30°C to exponential phase in glucose medium or in medium with glycerol with a shift to 37°C for two hours. RNA was analysed by Northern hybridization with probes specific for *FBP1* (**A**) and *PCK1* (**B**). All hybridizations were internally controlled by co-hybridization with *ACT1* and all hybridizations with a given probe were analyzed on the same filter.

Figure 2. Disruption of chromosomal tRNA-Lys *tK(UUU)L* gene leads to enhanced expression of adjacent *FBP1* gene.

A. Schematic representation of the *FBP1-tK(UUU)L* region of *S. cerevisiae* chromosome XII. *FBP1* open reading frame and *tK(UUU)L* transcription unit are represented by arrows. Chromosome XII coordinates (in kb) are indicated by a ruler on the top. *FBP1* and *tK(UUU)L* genes are transcribed from opposite DNA strands. **B**. Part *tK(UUU)L* gene was replaced with *TRP1* cassette (*tDNA*Δ). RNA was purified from wild type (wt), *maf1*Δ, *tDNA*Δ or *maf1*Δ *tDNA*Δ cells grown in glycerol medium at 30°C to OD 0.8 and shifted to 37°C for 2 h. *FBP1* mRNA was quantified by RT-qPCR and normalized to *ACT1* mRNA. *FBP1* mRNA relative level was normalised to 1 for wt. **C.** Levels of tRNA-Met and tRNA-Leu were determined by

RT-qPCR in the same samples as in panel B. Experiments were performed in triplicate. Error bars represent the standard error.

Figure 3. Mutation in *tK(UUU)L* gene promoter has no effect on expression of adjacent *FBP1* gene.

RNA was purified from *fbp1*Δ and *fbp1*Δ *maf1*Δ cells transformed with plasmids harboring either *FBP1* and *tK(UUU)L* ([FBP1-tDNA]) genes or the *FBP1* gene and promoter inactivated *tK(UUU)L* gene ([FBP1-tDNA*]). For RNA purification yeast pre-cultures were grown at 30^{o}C in minimal glucose medium lacking leucine, then transferred to rich glycerol medium for 5 h, shifted to 37^{o}C for 2 h, and harvested. **A.** *FBP1* mRNA was quantified by RT-qPCR and normalized to *ACT1* mRNA. *FBP1* mRNA relative level was normalized to 1 for *fbp1*Δ cells transformed with plasmids harboring *FBP1* and *tK(UUU)L* ([FBP1-tDNA]). **B**. Levels of tRNA-Met and tRNA-Leu were determined by RT-qPCR in the same samples (as described for panel A). Experiments were performed in triplicate. Error bars represent the standard error.

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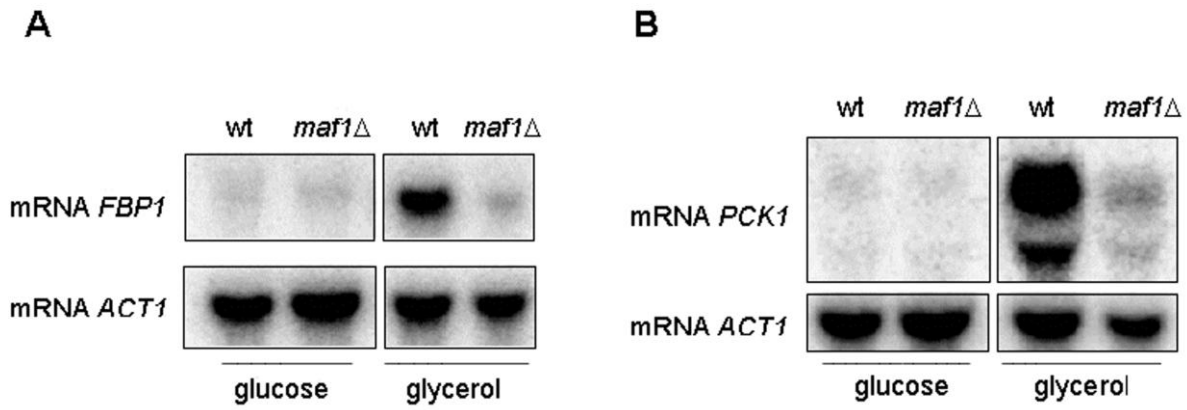

Figure 1

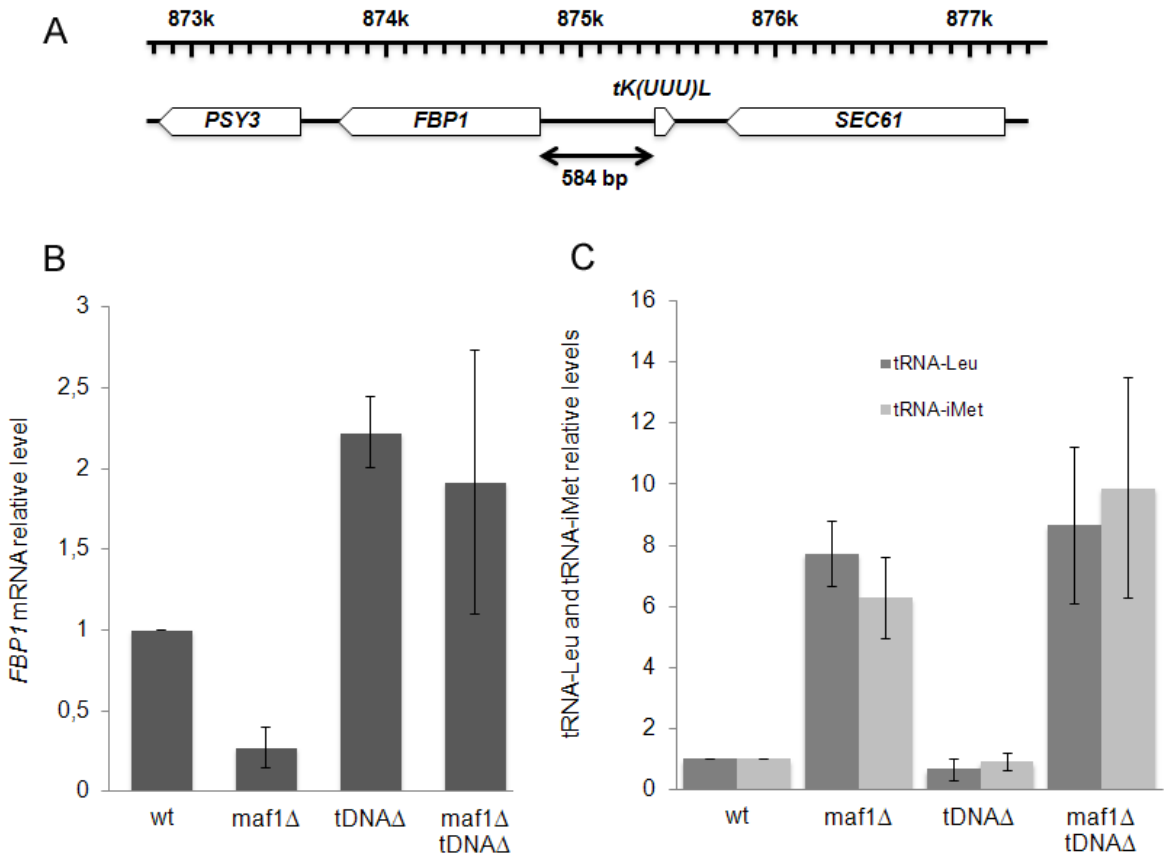

Figure 2

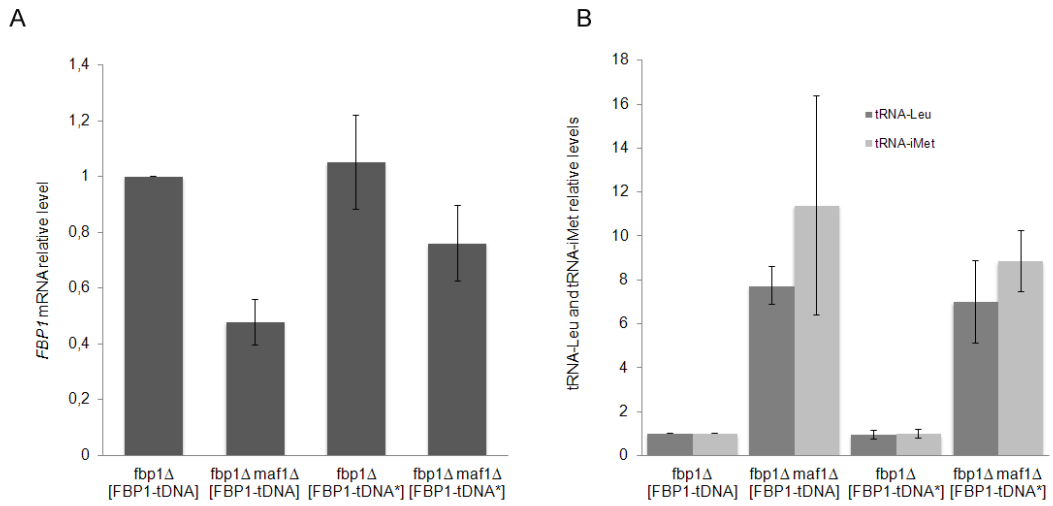

Figure 3

Highlights

- The effect of Maf1 on expression of Pol II-transcribed genes in yeast was analysed
- Transcription of *FBP1* and *PCK1* genes was decreased in the absence of Maf1
- Effect of adjacent tRNA-gene inactivation on expression of *FBP1* gene was examined
- The mechanism of Maf1-mediated effect on gluconeogenesis is discussed

Abbreviations

FBP1- yeast gene encoding fructose-1,6-bisphosphatase

PCK1- yeast gene encoding phosphoenolpyruvate carboxykinase

Pol II-RNA polymerase II

Pol III- RNA polymerase III

RT-qPCR- reverse transcription quantitative polymerase chain reaction

SDS- sodium dodecyl sulfate

EDTA- ethylenediaminetetraacetic acid

BSA- bovine serum albumin

ATP-adenosine tri-phosphate

cDNA- complementary DNA

tgm silencing- tRNA-gene mediated silenncing

ORF- open reading frame