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ORIGINAL PAPER

Transcriptional repression by *Kluyveromyces lactis* Tup1 in *Saccharomyces cerevisiae*

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Abstract The general repression complex, constituted by the yeast Tup1 and Ssn6 factors, is a conserved global regulator of transcription in eukaryotes. In the yeast *Saccharomyces cerevisiae*, it is an important repressor of hypoxic genes, such as *ANB1*, under aerobic conditions and deletion of the *TUP1* gene causes a flocculation phenotype. The *KlTUP1* gene from the yeast *Kluyveromyces lactis* encodes for a protein with 83% similarity to Tup1 in *S. cerevisiae*. Despite the general domain conservation, the database searches showed the absence of a characteristic Tup1 glutamine-rich domain (Q1 at positions 96–116). Instead, there was a non-conserved sequence lacking the α-helix structure in this region. The ability to act as a transcriptional repressor was tested by expressing the *KlTUP1* gene, in both high- and low-copy vectors, in an *S. cerevisiae tup1* mutant strain. Repression effects were studied using the aerobic repressible reporter *ANB1–lacZ* and the effect on flocculation. In both regulatory systems, low levels of KlTup1 caused moderate (~30%) repression, but when the number of KlTup1 copies was increased, only the *ANB1* reporter raised the repression levels of *S. cerevisiae* Tup1. These results show the capability of KlTup1 to act as a repressor in *S. cerevisiae*. The lower repression reached in *S. cerevisiae* is discussed in terms of structural differences.

Keywords Yeast · Tup1 · Transcriptional regulation · Repression complex · *Kluyveromyces lactis*

Introduction

Saccharomyces cerevisiae and *Kluyveromyces lactis* are two related yeast models widely used for biotechnological purposes, with interesting metabolic differences with respect to their growth ability in different carbon sources and oxygen regulation [7]. Regarding the carbon source in *S. cerevisiae*, there is strong transcriptional regulation by the repression of genes involved in respiratory-related functions when cells are grown in glucose.

The global transcriptional repressor Tup1 forms a complex with Ssn6 (also known as Cyc8). In vivo, the repressor complex requires four monomers of Tup1 and one of Ssn6. Tup1–Ssn6 regulates a wide variety of gene families or regulons: genes regulated by glucose repression, cell type, oxygen availability, DNA damage, and other signals [16]. In *S. cerevisiae*, this complex represses the transcription of over 300 genes under standard growth conditions [8]. The Tup1–Ssn6 complex does not bind DNA directly but is recruited to the target gene promoters through interaction with a variety of sequence-specific DNA-binding proteins, such as Mig1 and Nrg1 for glucose repression or Rox1 for oxygen repression, among others [18].

In *S. cerevisiae*, the aerobic repression of hypoxic genes, such as *ANB1*, is heme-dependent and mediated through the heme-activated repressor Rox1, but also requires the general repressors Tup1 and Ssn6 [1, 17]. Full *FLO1* derepression in the absence of Tup1–Ssn6 produces the establishment of alternative nucleosome positions at this promoter and drives to a flocculation effect [14]. This is

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also an important feature of brewing yeast strains, where flocculation is a complex process dependent on the expression of specific flocculation genes such as *FLO1*.

The complex Tup1–Ssn6 represses downstream genes by two proposed mechanisms: (i) repression by altering the local chromatin structure in which the Tup1–Ssn6 complex binds to and recruits histone deacetylase complexes to specific promoters, reducing the acetylation of histones in neighboring nucleosomes, and converts the active chromatin into a repressive structure; (ii) interaction with the general transcription machinery through a specific domain in Tup1 which interacts with the subunits of the mediator polymerase complex Hrs1–Med3, Srb7, and the Srb10–Srb11 complex [8, 9].

This Tup1–Ssn6 complex is one of the largest and most important gene-regulatory circuits in budding yeast and is conserved in flies (such as Groucho in *Drosophila*), worms, and mammals (Tle1 and Tle2). In other organisms, repression by Tup1–Ssn6 homologs is essential for cellular differentiation, specifically neurogenesis, hematopoiesis, and embryonic development [12].

Regarding the Tup1 structure previously described [18], four functionally important domains are shown in Fig. 1a: (i) *N*-terminal region of 72 amino acids which includes a domain implicated in the formation of Tup1–Ssn6 complex and in Tup1 oligomerization; (ii) intermediate region (residues 73–385) with different elements: two glutamine regions (Q1 and Q2), one ST-rich domain, and two repression domains (R1 and R2), implicated in multiple functions, such as interactions with other factors; and (iii) *C*-terminal region which contains seven WD repeats for protein–protein interactions, where the first one overlaps with the principal repression domain (R2).

To better understand the regulation of the processes where this complex is involved in yeasts, we characterized Tup1 from *K. lactis* (KlTup1). A comparative structural study and a functional analysis referring to the complementation of the phenotypes associated with the protein complex formed by Tup1–Ssn6 are presented. The repression of a hypoxic *ANB1* reporter gene and the flocculence properties in an *S. cerevisiae tup1* mutant strain are shown.

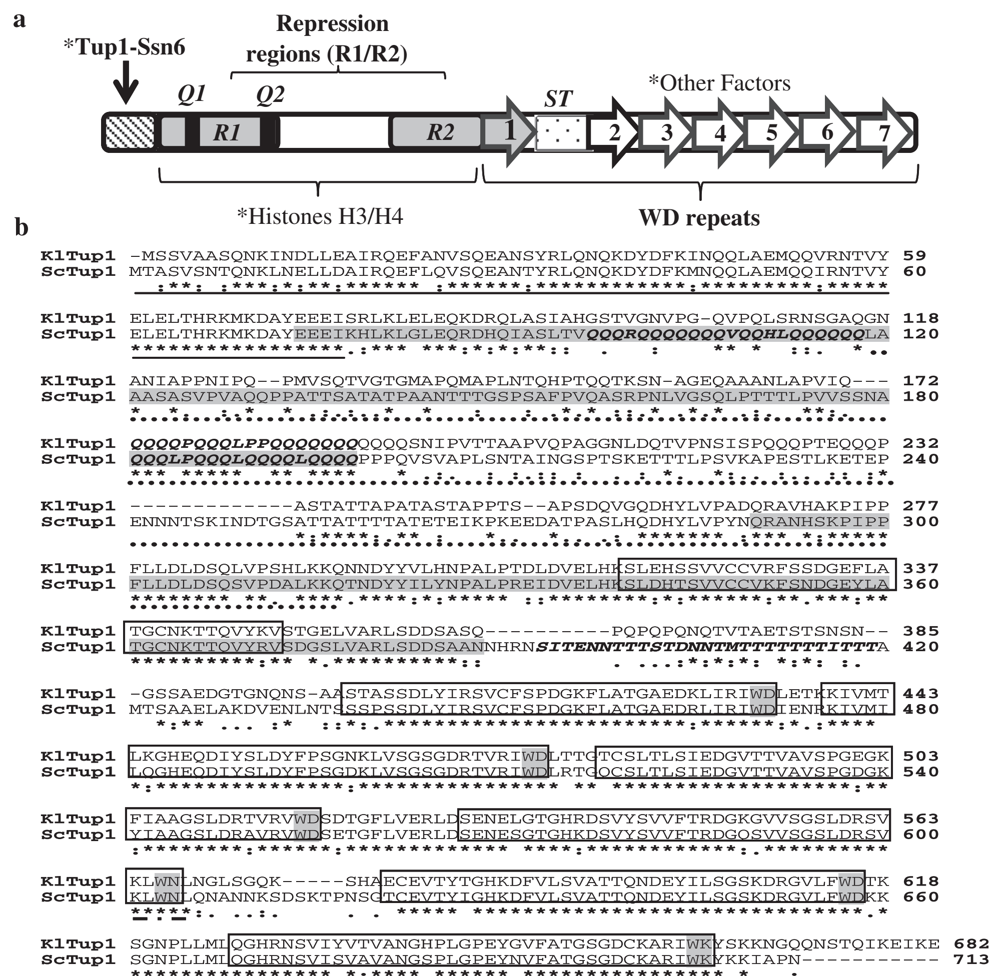

Fig. 1a, b Structural analysis of the KlTup1 protein. **a** Diagram showing the characterized functional domains of Tup1 (*asterisk* indicates interaction). **b** ClustalW alignment of Tup1 amino acid sequences from *Kluyveromyces lactis* (KlTup1) and *Saccharomyces cerevisiae* (ScTup1) with the following domains: *N*-terminal (residues 1–72) is *underlined*; *C*-terminal (residues 317–682) showing seven WD40 repeats are included in *open boxes* and the conserved WD residues are in *gray*; intermediate region shows: two repression domains (R1: residues 73–172 and R2: residues 267–365) in *gray*; two glutamine-rich regions (***Q1***: residues 96–116 and ***Q2***: residues 173–194) are in *bold* and *italics*; and the ST domain (serine-threonine-rich element, residues 392–419) is in *bold* and *italics*

Materials and methods

Strains and media

The strains used in this work were *K. lactis* YRRL-Y1140 (CBS2359) (*MATa wt*) and *S. cerevisiae* MZ12-17Δtup1 (*MATα trp1 leu2 ura3::AZ4 tup1::TRP1*), where the *URA3* gene is disrupted with *ANB1–lacZ* (provided by Zitomer, R.S.), referred to as the *tup1* mutant strain. The media were prepared according to standard methods and yeast cells were transformed as described previously [11]. For β-galactosidase assays, cells were cultured in complete media without uracil (CM-Ura) until they reached an OD = 0.8 [11].

Cloning and sequence homology analysis

Using the information from the *K. lactis* genome available in the Génolevures database (http://www.genolevures.org/), an open reading frame (ORF) with high homology to the *S. cerevisiae TUP1* gene (KLLA0F10263g) was used to design two specific primers, ART44: 5′CCTGGATC CATGAATGAGCAGTGTTGC3′ and ART45: 5′CGTCT CGAGTTCTTTAATCTCTTT3′, and clone the *KlTUP1* gene by polymerase chain reaction (PCR) using genomic DNA from *K. lactis* YRLL Y1140 cells. The resulting fragment was digested with *BamHI* and *XhoI* restriction enzymes and cloned in specific vectors YCplac33 [*ori amp^r^ lacZ ARS1 CEN4 URA3*] and YEplac195 [*ori amp^r^ lacZ* 2*μmori URA3*] (low- and high-copy vector, respectively) [6] for complementation analysis. Other plasmids such as YCp33Sc*TUP1* and YEp195Sc*TUP1* were used [3]. The sequence homology analysis of the KlTup1 protein was carried out using different programs: multiple alignment by ClustalW from EBI (http://www.ebi.ac.uk/clustalw/) and secondary structure prediction service with the program Jpred 3 (http://www.compbio.dundee.ac.uk/www-jpred/) [4].

β-galactosidase activity and flocculence rate

For phenotypic complementation studies, β-galactosidase assays were performed as described previously [11], with extracts from aerobically grown MZ12-17Δtup1 cells transformed with the plasmids described above and indicated in Table 1. All enzyme assays were performed at least three times with two independent transformants, and the activity was normalized to the amount of protein assayed. The clearing rate (flocculence) was determined by the equation: $[A_{600}$ at $t_0 - A_{600}$ at $t_{5\ min}]/[A_{600}$ at $t_0 \times 5$ min$]$ = % culture settling out per minute [3]. Percent repression was calculated with respect to the *TUP1* gene from *S. cerevisiae* in the same vector used.

Results

Cloning and structural analysis of the KlTup1 protein

The *KlTUP1* gene was cloned as described in the Materials and methods section. Previous to the functional analysis, we characterized the structure of different domains in KlTup1 by comparing its protein sequence with the previous characterized Tup1 from *S. cerevisiae*.

ClustalW alignment of Tup1 proteins from *K. lactis* and *S. cerevisiae* is shown (Fig. 1b) and 83% similarity is observed between them. Both proteins present two highly homologous domains: the *N*-terminal Ssn6 binding domain responsible for the oligomerization of KlTup1 (residues 1–72) and the *C*-terminal domain with seven WD repeats responsible for protein–protein interactions (residues 317–682). Figure 1 also shows the intermediate region with variable homology, including two repression domains (R1: residues 73–172 and R2: 267–365), where the homology of R1 in KlTup1 is lower; two glutamine-rich regions (Q1: residues 96–116 and Q2: 173–194); and an ST domain (serine-threonine-rich element). However, even though the Q1 element is not rich in glutamine, the ST region in KlTup1p has low homology compared with Tup1 from *S. cerevisiae*.

Domain characterization of the Tup1 *N*-terminus

Considering the complex and multiple interactions described for this factor, a further characterization of the most conserved domains was performed. The *N*-terminal region of Tup1 encompassing residues 1–72 is required for both the oligomerization of Tup1 and the association with Ssn6 [15]. Prediction of the secondary structure using the Jpred 3 program [4] of the *N*-terminal regions of both proteins from *K. lactis* and *S. cerevisiae* was made. As shown in Fig. 2, the secondary structure at positions 1–72 was conserved in KlTup1 where the line HELIX shows residues likely to adopt α-helical structures, indicated with an H. Two glutamine-rich regions have been described in Tup1 from *S. cerevisiae* (Fig. 1a, denoted by Q1 and Q2). The first Q1 element (residues 173–194), whose secondary structure predicts another α-helix in Tup1, is not present in KlTup1, as shown in Fig. 2, while the second Q2 region (residues 173–194) is conserved in both proteins. Therefore, the region 1–72 of KlTup1 conserved both the sequence and the secondary structure. However, the sequence encompassing positions 96–116 lacks a Q1-rich element and the α-helical structure. This structural difference has not been previously reported and is included in a repression-associated region.

```
KlTup1
AA       1  MSSVAASQNKINDLLEAIRQEFANVSQEANSYRLQNQKDYDFKINQQLAEMQQVRNTVYELELTHRKMKDAY 72
HELIX       ---------HHHHHHHHHHHHHHHHHHHHHHHHH------HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH

ScTup1
AA       1  MTASVSNTQNKLNELLDAIRQEFLQVSQEANTYRLQNQKDYDFKMNQQLAEMQQIRNTVYELELTHRKMKDA 72
HELIX       ---------HHHHHHHHHHHHHHHHHHHHHHHHHH------HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH

KlTup1
AA      73  EEEISRLKLELEQKDRQLASIAHGSTVGNVPGQVPQLSRNSGAQGNANIAPPNIPQ 128
HELIX       HHHHHHHHHHHHH-------------------------------------------

ScTup1
AA      73  YEEEIKHLKLGLEQRDHQIASLTVQQQRQQQQQQVQQHLQQQQQLAAASASVPV 128
HELIX       HHHHHHHHHHHHHH---------HHHHHH---HHHHHHHHHHHHHHHH--------
```

Fig. 2 Predicted secondary structure of the *N*-terminus sequence from *K. lactis* and *S. cerevisiae* Tup1 protein sequences (residues 1–72 and 73–128). The *line* HELIX shows residues likely to adopt α-helical structures, indicated with an *H* (Jpred 3 program) [4]. The Q1 region in ScTup1 is in *gray*

```
KlTup1  320  -HSSV--VCCVRFSSDGE------FLATGC-NKTTQVYKVSTGE-------------LVARLSDDSASQ   365
ScTup1  343  -HTSV--VCCVKFSNDGE------YLATGC-NKTTQVYRVSDGS-------------LVARLSDDSAAN   388

KlTup1  404  SSDLY--IRSVCFSPDGK------FLATGAEDKLIRIWDLETK-------------KIVMTLK        445
ScTup1  441  SSDLY--IRSVCFSPDGK------FLATGAEDRLIRIWDIENR-------------KIVMILQ        482

KlTup1  446  GHEQD--IYSLDYFPSGN------KLVSGSGDRTVRIWDLTTG-------------TCSLTLS        487
ScTup1  483  GHEQD--IYSLDYFPSGD------KLVSGSGDRTVRIWDLRTG-------------QCSLTLS        524

KlTup1  488  -IEDG--VTTVAVSPGEGK-----FIAAGSLDRTVRVWDSDTGF-------------LVERLDSENELGT 536
ScTup1  525  -IEDG--VTTVAVSPGDGK-----YIAAGSLDRAVRVWDSETGF-------------LVERLDSENESGT 573

KlTup1  537  GHRDS--VYSVVFTRDGK------GVVSGSLDRSVKLWNLNGLSGQK-----SHAECEVTYT         585
ScTup1  574  GHKDS--VYSVVFTRDGQ------SVVSGSLDRSVKLWNLQNANNKSDSKTPNSGTCEVTYI         627

KlTup1  586  GHKDF--VLSVATTQNDE------YILSGSKDRGVLFWDTKSGN-------------PLLMLQ        627
ScTup1  628  GHKDF--VLSVATTQNDE------YILSGSKDRGVLFWDKKSGN-------------PLLMLQ        669

KlTup1  628  GHRNS--VIYVTVANGHPLGPEYGVFATGSGDCKARIWKYSKKNGQQNSTQIKEIKE              682
ScTup1  670  GHRNS--VISVAVANGSSLGPEYNVFATGSGDCKARIWKYKKIAPN                         713
```

Fig. 3 The alignment of the Tup1 WD repeats. Regions of the WD repeats of Tup1 proteins from *K. lactis* and *S. cerevisiae* are aligned to maximize homology, where the conserved GH and WD residues are in *gray*. The residue numbers refer to the first residue in each repeat, with reference to each full-length Tup1 protein

Domain characterization of the Tup1 *C*-terminus

The carboxy-terminal fragment is critical for protein function. Tup1p belongs to a family of eukaryotic proteins characterized by a highly conserved region consisting of seven repeats named WD40. This repeat is a degenerate sequence repeat that has been characterized as containing a variable region, in both length and composition, and a core region, which is more uniform in length and contains certain conserved amino acids, including GH at the *N*-terminus and WD at the *C*-terminus [5]. This motif is present in many proteins and its structure is involved in diverse cellular processes and seems to mediate protein–protein interactions [13].

The amino acid alignment of the *C*-terminal domain with the WD repeats of Tup1 proteins from *K. lactis* and *S. cerevisiae* was made (Fig. 3), showing the high homology and the conserved amino acids for protein–protein interactions and functional activity already characterized in Tup1 from *S. cerevisiae* [18].

Repression analysis of KlTup1 in an *S. cerevisiae tup1* mutation

To determine whether the *K. lactis TUP1* homolog was able to functionally substitute for the *S. cerevisiae TUP1* gene, we expressed different plasmid DNA (in low- and high-copy vectors) in an *S. cerevisiae tup1* strain. We tested two different phenotypes: (i) aerobic repression of the hypoxic *ANB1* gene, measured by β-galactosidase activity and (ii) repression of the *FLO1* gene by flocculence assay, as shown in Table 1.

The *S. cerevisiae tup1* strain exhibits phenotypes typical of a *tup1* knockout, including defective repression of an *ANB1–lacZ* reporter gene and flocculation. As shown in Table 1, the *KlTUP1* gene was able to complement an *S. cerevisiae tup1* mutation at different rates, depending on the copy number expressed in the cells.

Regarding the effect on the hypoxic *ANB1* promoter, as shown in Table 1, KlTup1 repressed it only to 37% when a low-copy vector (YCp33Kl*TUP1*) was used, but complete repression was almost achieved when a high-copy vector (YEp195Kl*TUP1*) was present in the cells, compared with values obtained for *S. cerevisiae* Tup1. With respect to the effect on the *FLO1* promoter, expression of KlTup1 in a low-copy vector allows 31% flocculence, while in a high-copy vector, it was only 68%.

We can conclude that KlTup1 acts as repressor in *S. cerevisiae* but less efficiently (only 30% of our low-copy reporter systems). Increasing the copy number enhances the repression activity on the *ANB1* promoter but not as much on the *FLO1* promoter (68%), probably reflecting a different promoter context.

Table 1 Levels of β-galactosidase activity in extracts from aerobically grown MZ12-17Δtup1 cells (*ANB1–lacZ*) transformed with plasmid DNA (vectors and *TUP1* gene from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*) were determined and expressed in Miller units. Flocculence assays were determined and clearing rate expressed as percent culture settling out per minute. Values are ± standard deviation. Percent repression is expressed according to the equation (*tup1Δ − mutant*)/(*tup1Δ −* wild type)

Plasmid DNA	Effect of *KlTUP1* gene on repression			
	ANB1–lacZ		Flocculence	
	Activity	Percent repression	Clearing rate	Percent repression
YCplac33	6.5 ± 0.7	0	0.045 ± 0.006	0
YCp33Sc*TUP1*	0.18 ± 0.03	100	0.032 ± 0.007	100
YCp33Kl*TUP1*	4.18 ± 0.54	37	0.041 ± 0.009	31
YEplac195	6.5 ± 0.61	0	0.045 ± 0.004	0
YEp195Sc*TUP1*	0.065 ± 0.009	100	0.020 ± 0.006	100
YEp195Kl*TUP1*	0.16 ± 0.05	98	0.028 ± 0.004	68

Discussion

Most of the functional domains defined in Tup1 from *S. cerevisiae* are highly conserved in KlTup1 (Fig. 1), where they share the same general domain structures, with an *N*-terminal domain required for tetramerization, a poorly conserved middle region, and a *C*-terminal domain of seven WD repeats, as shown in other Tup1-like eukaryotic proteins [18].

The 72 *N*-terminal residues of Tup1 are necessary and sufficient for the formation of the Tup1–Ssn6 complex. It has been suggested that Ssn6 may serve as an adaptor between DNA-bound proteins and Tup1 [13]. The formation of two α-helical segments in the first 72 amino acids of KlTup1 is confirmed (Fig. 2) and, therefore, the Ssn6 interaction domain is conserved. Regarding the two glutamine-rich elements (Q1 and Q2), Q2 (residues 173–194) is highly conserved (Figs. 1 and 2) but the Q1 region (residues 96–116) is not present, and the α-helix predicted in this segment is not formed in the KlTup1 sequence (Fig. 2). This difference could have several consequences on the structure and function for KlTup1, since the amino acid sequence (residues 96–116): i) could have other functions; or ii) is a structural modification with different repression response; or iii) simply has no influence on protein function.

The crystal structure of the Tup1 WD domain has been determined and reveals that the seven repeated units form a circular, propeller-like structure with seven blades, each made up of four β strands [5, 13]. The *C*-terminus of KlTup1 shows that the seven WD repeats (Fig. 3) are well conserved. This domain is responsible for protein–protein interactions and transcriptional repression.

In the middle region, KlTup1 shows an ST region (residues 365–385) with a lower percentage of serine-threonine amino acids compared to Tup1. Deletion of this ST region between WD1 and WD2 in Tup1 from *S. cerevisiae* has not shown loss of repression, suggesting that this region, which is unique to yeasts Tup1 and KlTup1 [18], is not present in other higher eukaryotic homologs, and is not important for repression. Therefore, the lower repression of KlTup1 found in this work is probably not due to this structural divergence.

The results obtained from measuring using two different repression systems, β-galactosidase activity and the clearing rate (for flocculence determination) (Table 1), show the function of KlTup1 as a repressor. When KlTup1 was expressed in a low-copy vector, only 30–31% repression was observed in both regulatory systems. This is probably due to the lower homology in the middle region (including the Q1 and ST elements) between the two factors. However, we found that repression, by a high-copy KlTup1, is complete at the hypoxic *ANB1* promoter where the aerobic DNA-binding repressor Rox1p recruits the Tup1–Ssn6 complex. Moreover, these results suggest the ability to form a heterologous complex between KlTup1 and ScSsn6 in vivo.

The flocculence rate is partially restored in low-copy KlTup1 at similar levels as in *ANB1–lacZ*; however, high-copy KlTup1 is not able to compensate the repression defects, as in the reporter, with only 68% repression in the FLO regulation system responsible for flocculation when a high-copy vector was used. This difference between the two repression systems analyzed may reflect the different promoter features, such as different chromatin context or other promoter-specific factors recruiting the Tup1–Ssn6 complex to the promoter. As described previously [14], in the absence of Tup1–Ssn6, *FLO1* derepression is complete because of the establishment of an alternative nucleosome pattern at this promoter. Therefore, it is possible that, when expressing KlTup1, even in a high-copy vector, some interactions necessary for chromatin remodeling at *FLO1* promoter have changed.

We have reported the characterization of KlTup1 as a yeast transcriptional repressor, evidenced by its ability to

complement repression phenotypes in an *S. cerevisiae tup1* mutant strain. Its repression capability is diminished and some structural differences may account for this finding. The new information is especially relevant for further repression studies in *K. lactis*, where many of the transcriptional regulators of respiro-fermentative metabolism, such as the KlMig1 or the KlHap2/3/4 complex, do not regulate transcription as their *S. cerevisiae* homologs [2, 10].

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