

Revisiting the role of yeast Sfp1 in ribosome biogenesis and cell size control: a chemostat study

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Saccharomyces cerevisiae *SFP1* promotes transcription of a large cluster of genes involved in ribosome biogenesis. During growth in shake flasks, a mutant deleted for *SFP1* shows a small size phenotype and a reduced growth rate. We characterized the behaviour of an *sfp1Δ* mutant compared to an isogenic reference strain growing in chemostat cultures at the same specific growth rate. By studying glucose (anaerobic)- and ethanol (aerobic)-limited cultures we focused specifically on nutrient-dependent effects. Major differences in the genome-wide transcriptional profiles were observed during glucose-limited growth. In particular, Sfp1 appeared to be involved in the control of ribosome biogenesis but not of ribosomal protein gene expression. Flow cytometric analyses revealed size defects for the mutant under both growth conditions. Our results suggest that Sfp1 plays a role in transcriptional and cell size control, operating at two different levels of the regulatory network linking growth, metabolism and cell size.

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INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, as in higher eukaryotes, homeostatic mechanisms are active to coordinate growth, metabolism and cell cycle progression (Polymenis & Schmidt, 1999; Schneider *et al.*, 2004; Sudbery, 2002). In *S. cerevisiae*, external and intracellular signals are integrated at Start during the G1- to S-phase transition, when cells become committed to a new division cycle (Futcher, 1996; Li & Johnston, 1997; Mendenhall & Hodge, 1998). Passing Start requires the attainment of a critical cell size threshold that is modulated according to the genetic background, the genealogical age and the

metabolic status of the cell (Alberghina & Porro, 1993; Jorgensen & Tyers, 2004).

The *SFP1* gene has been identified in a screen for small size (*whi*) mutants as one of the genes whose deletion caused the strongest *whi* phenotype (Jorgensen *et al.*, 2002). *SFP1* has been proposed as a regulator of cell size at Start and as a key element in the connection between growth and cell cycle progression. It encodes a zinc-finger protein promoting the transcription of a large cluster of genes involved in ribosome biogenesis (Blumberg & Silver, 1991; Fingerman *et al.*, 2003; Jorgensen *et al.*, 2002, 2004). The synthesis of ribosomes is one of the most energy demanding processes for a cell and it is highly controlled according to nutrient availability (Warner, 1999). A network of some 200 proteins is involved in rRNA transcription and processing, ribosomal protein (RP) synthesis, and assembly and transport of the mature ribosomal subunits to the cytoplasm (Grummt, 2003; Planta, 1997). The TOR and PKA signalling pathways regulate ribosome biogenesis at the transcriptional and post-transcriptional level (Li *et al.*, 2006; Powers & Walter, 1999; Zurita-Martinez & Cardenas, 2005). In addition, they are involved in controlling cell size and cell cycle progression (Schmelzle & Hall, 2000; Thevelein & de Winde, 1999). Under optimal conditions, these pathways promote cell growth, whereas under starvation they repress

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Abbreviations: BI, budding index; FC, fold change; FDR, false discovery rate; FSC, forward scattering; PI, propidium iodide; PNM, pyrimidine nucleotide metabolism; RIBi, ribosome biogenesis; RNA Pol I, RNA polymerase I; RP, ribosomal protein; TIF, translation initiation factor.

The Gene Expression Omnibus accession number for the data reported in this paper is GSE5238.

Three tables listing genes showing significantly changed expression in the *sfp1* null mutant compared to the reference strain during ethanol-limited and glucose-limited growth, and expression levels of the ribosomal protein genes, are available with the online version of this paper.

growth processes (Klein & Struhl, 1994; Zaragoza *et al.*, 1998). Recent evidence indicates that the TOR/PKA pathways regulate Sfp1 localization as well, further suggesting its involvement in this regulatory network. In addition, Sfp1 localization has been shown to be modulated by the available carbon source and the environmental conditions (Jorgensen *et al.*, 2004; Marion *et al.*, 2004). It has been proposed that Sfp1 may have a role in cell-size modulation during growth on fermentable carbon sources (e.g. glucose) while being localized in the nucleus. During growth on non-fermentable carbon sources (e.g. ethanol or glycerol) localization of Sfp1 is mostly cytoplasmic and its role in cell size regulation is less apparent (Cipollina *et al.*, 2005; Jorgensen *et al.*, 2004; Marion *et al.*, 2004). These findings are in line with ribosome biogenesis being a central element in the nutrient-dependent control of cell cycle progression and size setting. In this scenario, Sfp1 appears to be one of the key players (Jorgensen & Tyers, 2004).

Current evidence on the possible function of *SFP1* was derived from the comparison of *sfp1Δ* with the isogenic reference strain growing in shake flask batch cultures (Cipollina *et al.*, 2005; Fingerman *et al.*, 2003; Jorgensen *et al.*, 2002, 2004). However, because deletion of *SFP1* generates a slow-growing phenotype, which is particularly evident on fermentable carbon sources (Cipollina *et al.*, 2005), the growth conditions used thus far caused a significant difference in growth rate between the reference and the *sfp1Δ* mutant. The transcriptional activity of a cell, ribosome biogenesis, cell size control and cell cycle progression are dependent on the specific growth rate (Castrillo *et al.*, 2007; Regenber *et al.*, 2006). Since a regulatory role for Sfp1 has been suggested for all these processes (Cipollina *et al.*, 2005; Fingerman *et al.*, 2003; Jorgensen *et al.*, 2002, 2004) the study of a slow-growing *sfp1Δ* mutant could lead to misleading conclusions.

We have studied the role of *SFP1* by growing an *sfp1Δ* mutant and the isogenic reference strain in continuous cultures where the specific growth rate is equal to the dilution rate (D , expressed as h^{-1}), which can be easily controlled (Hoskisson & Hobbs, 2005). Thus, chemostat cultivation allowed for a comparison of both strains at the same specific growth rate. Consequently, nutrient-dependent parameters could be evaluated separately from growth-rate-dependent ones. To assess the effects of metabolism and carbon source on Sfp1 activity we analysed two different cultivation conditions, aerobic ethanol-limited cultures and anaerobic glucose-limited cultures. We compared the global transcriptional profile of the *sfp1Δ* mutant with that of the reference strain under both conditions. In addition, cell cycle parameters and cell size distributions of the populations at the single cell level were analysed by flow cytometry. Our results provide new insights concerning the role of Sfp1 as a transcriptional activator. Moreover, we propose that Sfp1 is involved in modulating size setting independent of growth rate and carbon source. This function appears to be partially uncoupled from the role of Sfp1 in transcriptional control.

METHODS

Strains and growth conditions. The prototrophic reference *S. cerevisiae* strain CEN.PK 113-7D (*MATa MAL2-8c SUC2*) and the isogenic strain CEN.PK 111-32D *sfp1Δ* (*MATa MAL2-8c SUC2 leu2-3,112 sfp1Δ::KILEU2*) were used in this study. Cells were grown at 30 °C in 2 l chemostats (Applikon), with a working volume of 1.0 l. Cultures were fed with a defined mineral medium that limited growth by glucose or ethanol. The medium composition was based on that used by Verduyn *et al.* (1992). The concentration of substrate carbon in the reservoir medium was 250 mmol C l^{-1} for ethanol-limited cultures and 830 mmol C l^{-1} for glucose (anaerobic)-limited cultures (residual glucose concentration <0.3 mM). Glucose-limited cultures were grown at a dilution rate (D) of 0.05 h^{-1} under anaerobic conditions due to the wash-out of glucose (anaerobic)-limited *sfp1Δ* cultures at $D=0.10 h^{-1}$. Anaerobiosis was maintained by sparging the medium reservoir and the fermenter with pure nitrogen gas at 0.5 $l min^{-1}$. Furthermore, Norprene tubing and butyl septa were used to minimize oxygen diffusion into the anaerobic cultures. Ethanol-limited cultures were grown at $D=0.10 h^{-1}$ under aerobic conditions with the airflow set at 0.5 $l min^{-1}$. For both culture conditions the stirrer speed was 800 r.p.m. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH using an Applikon ADI 1030 Biocontroller. The working volume of the cultures was kept constant by means of an electrical level sensor. The off-gas was cooled by a condenser connected to a cryostat set at 2 °C, and analysed as previously described (van Maris *et al.*, 2003). Chemostat cultures were assumed to be in steady-state when, after at least five volume changes, the culture dry weight, specific carbon dioxide production rate and oxygen consumption rate (for aerobic cultures), changed by less than 2% during 24 h. Steady-state samples were taken from cultures grown for less than 18 generations in the chemostat. Dry weight, extracellular metabolites, dissolved oxygen and gas profiles had to be constant over at least three volume changes prior to sampling for RNA extraction and cytofluorimetric analysis.

Analytical methods. Culture supernatants were obtained after centrifugation of samples from the chemostats. For glucose and ethanol determination and carbon recovery calculation, culture supernatants and media were analysed by HPLC on an Aminex HPX-87H ion-exchange column using 5 mM H_2SO_4 as the mobile phase. Culture dry weights were determined via filtration as previously described (Postma *et al.*, 1989).

Microarray analysis. Sampling of cells from chemostats and total RNA extraction were performed as previously described (Piper *et al.*, 2002). Probe preparation and hybridization to Affymetrix Genechip microarrays were performed according to the Affymetrix instructions. The one-cycle eukaryotic target labelling assay was used, starting with 15 μg total RNA. The quality of total RNA, cDNA, rRNA and fragmented cRNA was checked using the Agilent Bioanalyser 2100 (Agilent Technologies). Results for each growth condition were derived from three independent cultures.

Data acquisition and analysis. Acquisition and quantification of array images and data filtering were performed using Affymetrix GeneChip Operating Software (GCOS) version 1.2. Before comparison, all arrays were globally scaled to a target value of 150, using the average signal from all gene features, with GCOS (version 1.2). To eliminate insignificant variations, genes with expression values below 12 were set to 12 and genes for which maximum expression was 12 over the 12 arrays were discarded, as described previously (Piper *et al.*, 2002). From the 9335 transcript features on the YG-S98 arrays, a filter was applied to extract 6383 yeast ORFs, representing 6084 different genes. This discrepancy was due to several genes being represented more than once. To represent the variation in triplicate measurements, the coefficient of variation was calculated as the standard

deviation divided by the mean as previously described (Boer *et al.*, 2003).

For additional statistical analyses, Microsoft Excel running the Significance Analysis of Microarrays (SAM, version 2.21) add-in was used for pair-wise comparisons between the reference strain and the *sfp1Δ* mutant (Tusher *et al.*, 2001). To determine the genes with significantly changed expression according to SAM an expected false discovery rate (FDR) of 1 % and a fold change (FC) of 2 were used as input values. Enrichment for specific functional categories among the differentially expressed genes compared to their genome-wide occurrence was computed using FunSpec (Robinson *et al.*, 2002). The *P*-value was computed by considering a hypergeometric distribution.

Promoter analysis was performed using the web-based software Regulatory Sequence Analysis Tools (RSA-Tools) (van Helden *et al.*, 2000). A 'pattern discovery' search was performed for the promoters (from position -800 to -1) of the genes that were differentially expressed between the *sfp1Δ* and the reference strain under both the analysed growth conditions. Two rounds of analyses were performed by setting the oligonucleotide size at 6 and 8, respectively. Elements significantly over-represented were found only in the promoters of the downregulated genes of glucose-limited cultures. In particular, only two elements were found that coincided with RRPE and PAC elements as described in Hughes *et al.* (2000). The gene annotation was made according to the MIPS Comprehensive Yeast Genome Database (available at <http://mips.gsf.de/genre/proj/yeast>) and the Saccharomyces Genome Database (available at <http://www.yeastgenome.org>).

All microarray data are available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE5238.

Flow cytometric analysis. FITC and propidium iodide (PI), both supplied by Fluka, were used to stain total proteins and DNA, respectively, on fixed cells. The flow cytometric analyses were performed as already described (Cipollina *et al.*, 2005; Vanoni *et al.*, 1983). The coefficient of variation (cv) was used as a quantitative measure of the size heterogeneity of the population. It was calculated from the histograms of protein distributions as the standard deviation divided by the mean.

Determination of the percentage of cells with a DNA content of 1C (G1 cells) was performed on cells stained with PI. PI fluorescence and forward scattering (FSC) values for each cell were presented in a dot plot. Because FSC values are representative of the cell dimensions and G1 cells are characterized by small size (Porro *et al.*, 1997), in each dot plot two regions were detectable: one with events characterized by lower mean FSC (i.e. smaller cells) and 1C DNA content (G1 cells), and the other with events having higher mean FSC and DNA content >1C. By manually selecting the region with smaller cells it is possible to determine the fraction of the population with a DNA content of 1C. Cytofluorimetric analyses were always performed in duplicate, with very good reproducibility. The data shown always refer to one representative experiment.

RESULTS

Physiology of the *sfp1Δ* and reference strains in aerobic ethanol- and anaerobic glucose-limited chemostat cultures

While *S. cerevisiae* CEN.PK 113-7D has a maximum specific growth rate (μ_{\max}) of 0.40 h⁻¹ when aerobically growing in shake flasks on minimal medium with glucose as carbon source (Flikweert *et al.*, 1999), its *sfp1Δ* derivative

has a much slower μ_{\max} of 0.16 h⁻¹. To prevent growth-rate-related variations obscuring data interpretation when comparing the physiology of the reference and the *sfp1Δ* mutant, both strains were grown at an identical growth rate in chemostat cultures. The reference strain CEN.PK 113-7D and the isogenic *sfp1Δ* mutant were therefore grown in chemostat cultures under ethanol- and glucose (anaerobic)-limitation.

Under this cultivation set-up, fresh medium continuously flows into the fermenter, while the same amount of culture broth flushes out so that the operative volume is kept constant. Therefore, the culture is continuously diluted by a factor called the dilution rate (D , h⁻¹). The steady state is reached when the measured growth parameters (in this specific case metabolite consumption/production rate, biomass concentration and budding index) become constant. At steady state, the population is in exponential balanced growth, and the specific growth rate for each growing cell in the population [$\mu = \ln(2)$ (duplication time)⁻¹] equals the dilution rate (Alberghina *et al.*, 1998; Monod, 1950; Novick & Szilard, 1950).

A major advantage of chemostat cultures compared to shake flasks is that the nutritional/environmental conditions (pH, aeration, specific growth rate and agitation) can be set and tightly controlled by the user. In addition, very slow growth rates can be reached while maintaining continuous exponential growth and an optimal physiology (Regenberg *et al.*, 2006; Saldanha *et al.*, 2004). This approach allowed us to grow the reference strain and the *sfp1Δ* mutant at the same specific growth rate. This was obtained by always setting an identical dilution rate for both the reference and the mutant strain. Similarly, all the other cultivation parameters were always identical for the two strains (pH, medium composition, temperature and aeration).

By changing the nutrient supply and the aeration setting, two metabolic conditions were analysed, i.e. ethanol (aerobic)- and glucose (anaerobic)-limitation. These two different set-ups were chosen as paradigms for two very different metabolic states, fully respiratory (with no glucose present) and fully fermentative, respectively (Cortassa *et al.*, 1995; Daran-Lapujade *et al.*, 2004; Kappeli, 1986). It would have been of interest to study the condition of respiratory metabolism during aerobic glucose-limitation. However, the mutant strain exhibited sustained metabolic oscillations under this growth condition, making it impossible to reach the required steady state (data not shown).

For each growth condition the dilution rate (D), which is equal to the specific growth rate (μ), was chosen to be close to the maximum growth rate of the *sfp1Δ* mutant for that specific condition. Ethanol-limited cultures were grown at $D=0.10$ h⁻¹ while glucose (anaerobic)-limited cultures were grown at $D=0.05$ h⁻¹, corresponding to a duplication time of 6.9 and 13.8 h, respectively.

As shown in Table 1, the two strains showed a very similar behaviour. As expected, during growth on ethanol both

Table 1. Physiological parameters of the CEN.PK 113-7D reference strain and the *sfp1Δ* mutant during ethanol- and glucose (anaerobic)-limited growth

Ethanol-limited cultures were grown at $D=0.10\text{ h}^{-1}$, in aerobiosis. Glucose-limited cultures were grown at $D=0.05\text{ h}^{-1}$, in anaerobiosis. Data represent the mean \pm SD of three independent chemostat cultivations. RQ, respiratory coefficient ($q_{\text{CO}_2}/q_{\text{O}_2}$); Y_{SX} , yield of biomass; C rec, carbon recovery; Ref., reference strain; NA, not applicable.

Carbon source	Strain	Specific consumption or production rate [mmol (g dry biomass) ⁻¹ h ⁻¹]					RQ	Y_{SX} [g (mol C) ⁻¹]	C rec (%)
		q_{SX}^*	$q_{\text{CO}_2}^\dagger$	$q_{\text{O}_2}^*$	q_{EtOH}^\dagger	q_{Gly}^\dagger			
Ethanol	Ref.	3.8 \pm 0.04	3.3 \pm 0.09	6.9 \pm 0.19	NA	NA	0.47 \pm 0.00	13.3 \pm 0.3	96 \pm 1
	<i>sfp1Δ</i>	3.9 \pm 0.20	3.5 \pm 0.21	7.4 \pm 0.47	NA	NA	0.48 \pm 0.00	13.7 \pm 0.5	100 \pm 0
Glucose	Ref.	3.2 \pm 0.14	5.6 \pm 0.16	NA	4.4 \pm 0.07	0.4 \pm 0.03	NA	2.6 \pm 0.1	96 \pm 1
	<i>sfp1Δ</i>	3.2 \pm 0.05	5.6 \pm 0.01	NA	4.9 \pm 0.02	0.5 \pm 0.03	NA	2.7 \pm 0.1	99 \pm 2

*Millimoles of substrate or oxygen consumed per gram of biomass per hour.

†Millimoles of CO₂ or product [ethanol (EtOH) or glycerol (Gly)] formed per gram of biomass per hour.

strains displayed a fully respiratory metabolism with a respiratory coefficient (RQ) of ~ 0.47 and a yield of ~ 13 g biomass (carbon moles)⁻¹. No trace secondary metabolites were found in the medium. These data were consistent with previously published findings for the reference strain and indicated that ethanol was completely metabolized (Daran-Lapujade *et al.*, 2004). During anaerobic glucose-limited growth we observed, as expected, significant production of glycerol and ethanol. Ethanol was generated through alcoholic fermentation as the sole mode of glucose dissimilation. Production of glycerol was required for the non-respiratory oxidation of NADH. As expected, the biomass yield on glucose under this growth condition was very low due to the low ATP yield from alcoholic fermentation. Under both steady-state conditions, the *sfp1Δ* mutant and the reference strain exhibited very similar phenotypes. The specific substrate and oxygen consumption rates (q_{SX} and q_{O_2}) as well as the specific production rate of carbon dioxide (q_{CO_2}) and secondary metabolites showed very similar values for both strains. Similarly, the yield of biomass on carbon was identical, indicating a similar efficiency of substrate utilization and a comparable flux distribution.

Deletion of *SFP1* causes major transcriptional changes during growth on glucose

A genome-wide transcriptional analysis was performed for the reference and the *sfp1Δ* strains for each growth condition. All data presented in this work were derived from three independent chemostat cultures. The average CV for all genes for triplicate experiments was below 0.19 for each of the four conditions. Furthermore, the levels of *ACT1* and *PDA1*, common loading standards for conventional Northern analysis, varied by less than 9% over the different cultures. For all tested conditions 5828 transcripts ($\sim 95\%$ of annotated ORFs) were above the detection limit. Comparison of transcript levels between the *sfp1Δ* and the reference strains revealed, for each of the two conditions,

the genes whose expression significantly changed (SAM, FC threshold=2 and FDR=1% as described in Methods). In this study we will refer to the genes whose expression levels were higher and lower in the mutant strain compared to the reference strain as 'up regulated' and 'down regulated', respectively. This terminology does not imply any hypothesis regarding regulatory mechanisms.

Comparison of the *sfp1Δ* and the reference strain during ethanol-limited growth revealed 91 transcripts whose expression significantly changed (33 up- and 58 down-regulated; see Supplementary Table S1, available with the online version of this paper). No enrichment for functional categories or features (localization, complexes, etc.) could be identified among genes either up- or downregulated under this condition. Very similar results were obtained after lowering the FC threshold to 1.5 when performing SAM analysis.

During anaerobic glucose-limited growth 217 genes displayed significantly changed expression (see Supplementary Table S2, available with the online version of this paper), 62 being upregulated and 155 down-regulated (Fig. 1a). Again, no enrichment for functional categories was found among the genes that were upregulated. However, close to 50% of the downregulated genes (72 genes) belonged to only four different functional categories that were significantly enriched compared to their corresponding genome-wide distribution (see table in Fig. 1a). The ribosome biogenesis cluster ('RiBi regulon') was the largest, containing genes involved in processing of rRNA and assembly and transport of mature ribosomal subunits (Jorgensen *et al.*, 2004). Genes encoding translation initiation factors (TIF) and RNA polymerase I (RNA Pol I) were also significantly represented, as was the pyrimidine nucleotide metabolism (PNM) cluster. The remaining 83 downregulated genes represented miscellaneous features. Surprisingly, no ribosomal protein (RP) genes were present among the downregulated sequences under any of the analysed conditions.

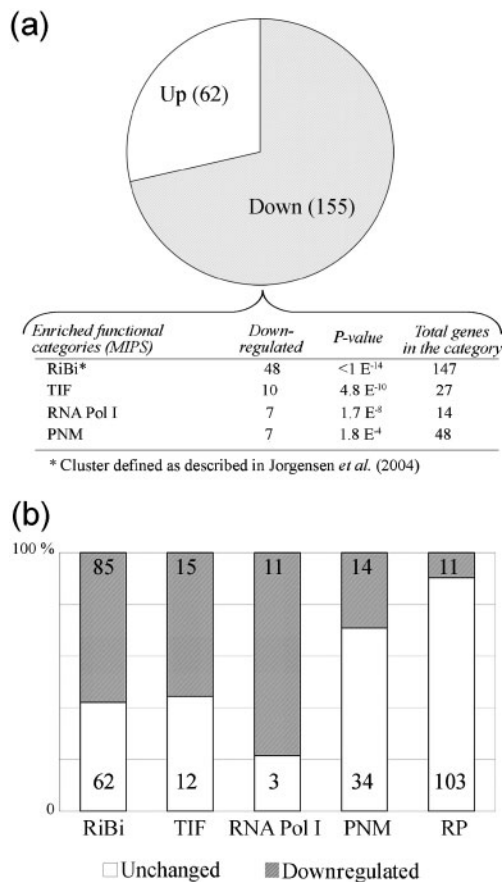


Fig. 1. Genes showing significantly changed expression during glucose (anaerobic)-limited growth. (a) 217 transcripts significantly changed their expression levels in the *sfp1Δ* mutant compared to the reference strain during glucose (anaerobic)-limited growth (SAM analysis, FDR=1% and FC=2). Enrichment for specific functional categories (calculated as described in Methods) was found only for the set of downregulated genes. The *P*-value was computed with a hypergeometric distribution. The enriched categories and their *P*-values are given in the table (RiBi, ribosome biogenesis; TIF, translation initiation factors; RNA Pol I, RNA polymerase I; PNM, pyrimidine nucleotide metabolism). (b) A *t*-test was performed ($P \leq 0.05$) among all the genes in the complete dataset that showed a FC lower than -1.5 . Thus, significant enrichment was observed for each of the four functional groups shown in the table in Fig. 1(a). Changes in the ribosomal protein gene (RP) cluster were also examined. The numbers of genes significantly downregulated or with unchanged expression are shown on the bars. On the *y*-axis the percentage, based on the total number of genes for each functional category, of downregulated and unchanged genes is indicated.

In the first round of analysis, SAM software was used to find significant changes (see Methods) because it allowed a stringent statistical control over the complete dataset. Once the over-represented functional categories were found, we focused on these categories to try and find if more genes belonging to them were downregulated, although to a

lesser extent, in the complete dataset (all the genes above the expression threshold were considered; see Methods). For this we performed a *t*-test on all those genes that showed a FC lower than -1.5 . Thus, a significant enrichment was observed for each of the four functional groups (Fig. 1b). The same analysis was performed for the RP category, but again this cluster was not significantly represented (Fig. 1b).

RiBi and RP are independent transcriptional clusters

The lack of downregulation of RP genes in the *sfp1Δ* strain during glucose-limited growth was surprising. Indeed, previous data suggested that Sfp1 was involved in both RiBi and RP transcriptional regulation (Jorgensen *et al.*, 2004; Marion *et al.*, 2004). To obtain a quantitative measure of such a different behaviour we compared our dataset of anaerobic glucose-limited growing cultures with a published dataset, referring to an *sfp1Δ* and an isogenic reference strain growing on rich medium in shake flasks (Jorgensen *et al.*, 2004). As shown in Fig. 2, in both datasets RiBi, TIF, RNA Pol I and PNM gene clusters emerged as the functional categories highly represented among the downregulated genes. However, shake flask cultures of the *sfp1Δ* mutant showed a significant downregulation of the RP cluster compared to the reference strain (123 of 129 RP genes showed a FC lower than -1.5). In our dataset, only 11 RP genes of the 114 ORFs displayed a FC below -1.5 . It is important to note that in our experimental setup, as previously described, the reference and the mutant strains were growing at the same specific growth rate. This was not the case for the dataset derived from cultures growing in shake flasks using rich medium. Under these conditions the mutant strain grew much more slowly than the reference strain (Jorgensen *et al.*, 2002). Our data suggest that the RP and RiBi genes may be regulated, at least partly, through independent mechanisms. In particular, Sfp1 appeared responsible for the transcriptional regulation of genes involved in ribosome biogenesis (RiBi, TIF, RNA Pol I and PNM) but not of RP genes.

The existence of such a differential regulation was further underlined by an *in silico* promoter analysis. The promoters of the 155 genes that were downregulated during glucose-limited growth were specially enriched for the presence of RRPE and PAC elements [RRPE=GAAAAWWTT, PAC=GMRATGARNT (Hughes *et al.*, 2000); see Methods]. It has been previously shown that these elements are typical of the gene clusters involved in ribosome biogenesis, while they are not present in RP gene promoters (Hughes *et al.*, 2000; Wade *et al.*, 2001; Wang *et al.*, 2004). We found that among the 155 analysed promoters, 106 and 55 contained RRPE and PAC, respectively, suggesting the existence of a functional link between Sfp1 activity and the genes containing these promoter elements. Finally, it should be noted that not all the genes that were downregulated and that contained

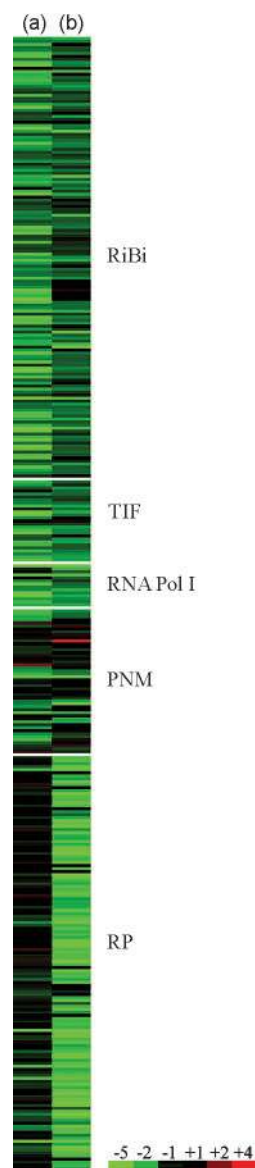


Fig. 2. Transcriptional profile of an *sfp1Δ* mutant compared to the isogenic reference strain during chemostat and shake flask growth. Transcriptional data relative to the five clusters that emerged as Sfp1 target candidates are reported. (a) Data from this study: *sfp1Δ* versus reference strain, during glucose (anaerobic)-limited growth, $D=0.05\text{ h}^{-1}$. (b) Data derived from the literature: *sfp1Δ* versus reference strain, during shake flask growth, in rich medium with glucose as the carbon source. The image was created using the visualization software TreeView (<http://rana.lbl.gov/EisenSoftware.htm>). The colours corresponding to the FC are shown in the key.

RRPE and PAC elements in their promoters were involved in ribosome biogenesis. These genes represented miscellaneous features whose phenotype, if any, did not clearly emerge from our analysis.

Analysis of cell size and cell cycle parameters

In glucose-grown batch cultures the main phenotypic trait resulting from *SFP1* deletion is a very reduced cell size (*whi* phenotype) accompanied by a slow growth rate (Cipollina *et al.*, 2005; Jorgensen *et al.*, 2002). This size phenotype is less evident during batch growth on ethanol, when the growth rate defects of the mutant are marginal (Cipollina *et al.*, 2005). We wondered whether the *whi* phenotype was maintained in continuous cultures where nutrient-dependent modulation of cell size can be evaluated separately from growth-rate-dependent variables. Therefore the average cell size for each strain and growth condition was measured by a flow cytometric approach on FITC-stained cells (see Methods). This gives quantitative information on the protein content distribution of the cells in the analysed populations. It has been reported previously that this parameter is a good estimate of the cell size (Alberghina & Porro, 1993; Vanoni *et al.*, 1983).

During ethanol-limited growth the average size was 285.0 and 174.3 for the reference and mutant strain, respectively (the cell size is expressed as channel number, relative units; Fig. 3a). During glucose-limited growth the average size of the reference and the mutant strains was 326.0 and 254.2, respectively (Fig. 3b). Surprisingly, under both growth conditions the protein distributions of the mutant strain were shifted towards lower values. Thus, the *sfp1Δ* strain was characterized by a lower general 'size setting' compared to the reference population, with smaller dimensions both at birth and at division [dividing and newborn cells are mainly represented in the right- and left-hand sides of the histograms, respectively (Porro *et al.*, 1997)]. The cell volume distributions, measured with a Coulter counter analyser, were in good agreement with the cell protein distributions (data not shown).

The bimodal distribution of the reference population growing under ethanol limitation (Fig. 3a) reflects a high heterogeneity in cell size ($cv=0.38$) and a population structure which is typical of budding yeast growing slowly in poor media with a low budding index (BI) (Alberghina *et al.*, 1991; Vanoni *et al.*, 1983). The *sfp1Δ* strain growing on ethanol showed a right-skewed size distribution reflecting a comparable degree of heterogeneity ($cv=0.43$), although a true bimodal shape was not detected.

To assess whether defects in size setting of the mutant strain correlate with alterations in cell-cycle progression we determined the percentages of cells in G1 and in S + G2 + M phases in our cultures. We used two independent approaches: manual determination of BI and flow cytometric analysis. The BI represents the percentage of budded cells in the population which are in the S + G2 + M phase, with a DNA content greater than 1C. The remaining part of the population, namely the unbudded cells, is in the G1 phase, with a DNA content of 1C. The flow cytometric analysis was performed on PI-stained cells, whose fluorescence intensity is proportional to the DNA content. The percentage of G1 cells was determined from dot plots of FSC and PI fluorescence on

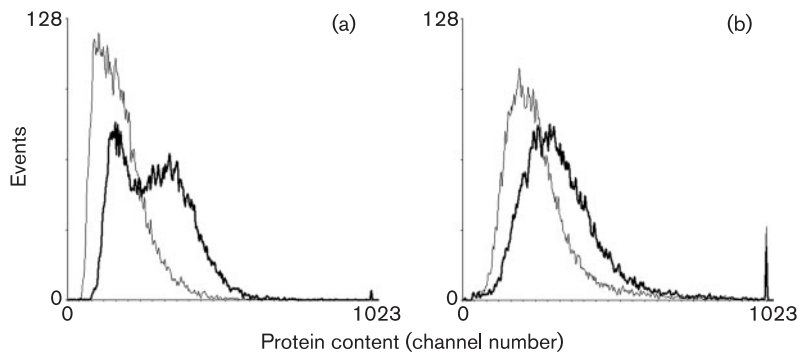


Fig. 3. Cell size distribution of the *sfp1Δ* (thin line) and the reference strain (thick line) during ethanol- (a) and glucose (anaerobic)- (b) limited growth. Samples were collected during steady state. Fixed cells were stained with FITC and analysed by flow cytometry. The fluorescence intensity [expressed as channel number, relative units] is shown on the *x*-axis and represents the size of single cells.

the *x* and *y* axes respectively. As shown in Table 2, the two approaches gave very similar results. The BI was ~28 % for ethanol-limited cultures and ~50 % for glucose (anaerobic)-limited cultures. This latter value was unexpectedly high; however, high BI values appear to be typical of populations growing under anaerobiosis (L. Brambilla, personal communication). From the BI it was also possible to calculate the duration of the budded and unbudded phases (Vanoni *et al.*, 1983) (Table 2). Intriguingly, all the data clearly indicated that while cell cycle parameters changed according to the two conditions, the *sfp1Δ* mutant did not exhibit any significant difference in the distribution throughout the cell cycle phases compared to the reference strain, in particular in the G1 phase where the main control over cell size takes place. Therefore, these findings indicate that the size defects and the alterations in cell cycle progression can be ‘uncoupled’ and are fully consistent with previous publications showing that an extended G1 phase does not always result in entering Start at a smaller cell size (Jorgensen & Tyers, 2004; Popolo *et al.*, 1982).

DISCUSSION

Continuous cultures: subsiding growth rate effects

The advantages of using chemostat cultures for physiological and metabolic studies have been widely recognized

(Brauer *et al.*, 2005; Hoskisson & Hobbs, 2005; Porro *et al.*, 2003; Wu *et al.*, 2006). Nevertheless, they have not yet found wide application in molecular and cell biology.

One of the advantages of this approach is that cells are continuously growing exponentially, even at very slow growth rates such as those used in this study. No evidence of starvation characterizes these slow-growing cultures and the transcription of ribosomal protein genes, an indicator of cellular fitness, is fully active (see Supplementary Table S3, available with the online version of this paper) (Regenberg *et al.*, 2006; Saldanha *et al.*, 2004).

The use of chemostat cultures was the only strategy that allowed us to grow the reference and the *sfp1* null mutant strains at the same, albeit slow, specific growth rate, with any desired carbon source, while maintaining an optimal cell physiology.

Data reported here, while confirming some previous findings on the role of Sfp1 in yeast physiology, revealed new features which otherwise would have remained masked by growth-rate-dependent effects. In fact, the slow-growing phenotype of a *sfp1Δ* mutant during growth in shake flasks ‘per se’ generates a number of effects that are difficult to uncouple from those directly generated by the absence of *SFP1*. Our findings indicate that the mutant and reference strains share a similar physiology when growing at the same specific growth rate. Consistently, the absence of *SFP1* did not affect the efficiency of carbon utilization,

Table 2. Cell cycle parameters for the growth conditions used in this study

Cells collected at steady state were fixed, stained with PI and analysed by flow cytometry. Budding index (BI) was manually determined by counting, using an optical microscope, the number of budded cells in a total of 500 cells. Data represent the mean \pm mean deviation of two independent experiments. Ref., Reference strain, (CEN.PK 113-7D). The doubling time (T_d) was calculated as $\ln 2/D$, where D is the dilution rate. The duration of the budded phase (T_b) was calculated as described by Vanoni *et al.* (1983): $T_b = [\ln(1 + BI/100)] \times T_d / \ln 2$. The duration of the G1 phase (T_{G1}) is equal to $T_d - T_b$.

Growth condition	Strain	% IC*	BI (%)	T_d (h)	T_b (h)	T_{G1} (h)
Ethanol-limited, aerobic, $D=0.10 \text{ h}^{-1}$	Ref.	72.8	27.8 ± 0.5	6.9	2.4	4.5
	<i>sfp1Δ</i>	73.5	30.6 ± 4.5	6.9	2.7	4.2
Glucose-limited, anaerobic, $D=0.05 \text{ h}^{-1}$	Ref.	51.5	48.8 ± 3.8	13.8	7.9	5.9
	<i>sfp1Δ</i>	44.5	50.7 ± 6.7	13.8	8.2	5.6

the activity of the fermentative and respiratory pathways or the global flux distributions. Therefore, the chosen cultivation approach was revealed to be a good starting point for further studies concerning the activity of Sfp1 as a transcriptional regulator and modulator of size setting in response to different nutrients.

Sfp1 is a positive regulator of ribosome biogenesis during growth on glucose

Previous studies have suggested that Sfp1 is a transcriptional activator of the RiBi gene family and of RP genes (Jorgensen & Tyers, 2004; Marion *et al.*, 2004; Wang *et al.*, 2004). We observed the downregulation of the RiBi cluster, but not of RP genes, in glucose-limited *sfp1Δ* cultures, indicating the involvement of Sfp1 in the regulation of RiBi but not RP genes (Fig. 2).

This finding was recently confirmed by additional experiments in which glucose pulses were added to carbon-limited cultures of both the *sfp1* null mutant and the reference strain. The dynamics of the transcriptional response induced by such perturbation were analysed over time. Preliminary results indicated that the primary transcriptional target of Sfp1 is indeed the RiBi cluster and not the RP genes. Furthermore, these data strongly indicated that the lack of involvement of *SFP1* in RP gene transcriptional regulation was not due to the slow growth rate applied in the present work. On the contrary, this phenomenon was evident after the glucose pulse when the growth conditions were comparable to growth in shake flasks (preliminary data, not shown).

At first glance these data appeared to be in contrast with previously reported results for an *sfp1Δ* strain. However, they were consistent with the observation that over-expression of *SFP1* results in a delay between the induction of the RiBi and the RP genes, which gave the first indication of the Sfp1-dependent transcription of the RiBi cluster (Jorgensen *et al.*, 2002).

For the RP genes, Rap1 together with the Fhl1–Ifh1 complex is the main transcriptional regulator (Klein & Struhl, 1994; Martin *et al.*, 2004; Rudra *et al.*, 2005). The activity of all these factors is regulated via partially overlapping pathways (involving TOR and PKA), allowing a coordinate expression of all the components required for ribosome biosynthesis. This explains the similar behaviour that the RiBi and RP gene clusters usually show. However, the use of independent but parallel and partially redundant regulatory pathways is often strategically employed by cells to fine-tune transcriptional regulation. We hypothesize that this is the case for the RiBi and RP clusters. Downregulation of the RP genes observed in *sfp1Δ* mutants during growth in shake flasks might be a secondary effect due to growth rate defects. Finally, several expression profiles published elsewhere (Saldanha *et al.*, 2004; Wade *et al.*, 2001) together with the observed differences in the promoter regions lend further support to our hypothesis on the differential regulation of RiBi and RP gene clusters.

The observed expression defects of the genes related to rRNA transcription (the RNA Pol I cluster) and processing (RiBi cluster) might well explain the phenotype of the mutant strain. This is characterized by low amounts of rRNA and a delay in rRNA processing steps (Fingerman *et al.*, 2003). Moreover, consistent with previous data on Sfp1 localization (Jorgensen *et al.*, 2004; Marion *et al.*, 2004), our findings indicated that during growth on glucose, but not on ethanol, Sfp1 acts as activator of RiBi cluster transcription, thus sustaining the growth potential of the cell.

The signal inducing the transcriptional activity of Sfp1 in response to the available carbon source remains to be elucidated. In anaerobic glucose-limited chemostats, while glucose is efficiently transported and a sustained glycolytic flux is active inside the cell, the extracellular residual glucose concentration is low (<0.3 mM) compared to the K_m of the high-affinity sensing systems (Ozcan *et al.*, 1996; Rolland *et al.*, 2000). This suggests that high levels of extracellular glucose are not required for stimulating Sfp1 activity. The positive signal could be mainly generated at the intracellular level and sustained by fermentative metabolism.

The mechanisms through which Sfp1 exerts its transcriptional control remain unknown. Attempts to prove physical interaction of Sfp1 with promoters of RiBi genes have failed (Fingerman *et al.*, 2003; Jorgensen *et al.*, 2004). So far, only weak interactions with some RP gene promoters have been identified, but this point is still controversial (Fingerman *et al.*, 2003; Jorgensen *et al.*, 2004; Marion *et al.*, 2004). It is possible that Sfp1 acts through indirect mechanisms, modulating the chromatin structure or acquiring and regulating other activators. Similar mechanisms of transcriptional control have recently been shown for other important factors involved in RP and rRNA gene regulation (Li *et al.*, 2006; Rohde & Cardenas, 2003; Tsang *et al.*, 2003). Such strategies would allow coordinated transcriptional control and be compatible with the low expression levels of Sfp1 itself (Ghaemmaghami *et al.*, 2003).

Deletion of *SFP1* causes a small size phenotype

We have shown that *SFP1* is involved in cell size control. This role seems to be independent of the metabolic status of the cell and the growth rate. In fact, the *sfp1Δ* mutant showed a *whi* phenotype during growth on both ethanol and glucose. This suggests that the control of size setting might act through a different pathway compared to the transcriptional control of the RiBi cluster that is promoted by Sfp1 mainly during glucose-limited growth. We specifically searched for common genes or genes with a shared function with expression patterns that were significantly changed under both growth conditions; no statistically valid results were obtained that could explain the observed *whi* phenotype. However, an inspiring suggestion emerging from this analysis was related to the expression of two RNA Pol I genes, *RPA49* and *RPA14*. It has been previously shown that the deletion of each of them caused a strong *whi* phenotype (Jorgensen *et al.*,

2002). We observed that *RPA14* is downregulated in *sfp1Δ* cultures during ethanol-limited growth (FC=−3.2) but not during glucose-limited growth (FC=−1.2). Vice versa, *RPA49* is downregulated during glucose- but not ethanol-limited growth (FC=−2.4 and 1.0, respectively). These two RiBi genes, encoding potential Start repressors (Jorgensen *et al.*, 2002), could be the link between the observed transcriptional alterations of the *sfp1Δ* mutant and the small size phenotype.

To see whether the size defects might be connected with alterations in cell cycle progression we performed a more detailed analysis of the cell cycle phases. Surprisingly, no alterations were found in the mutant strain, indicating that deletion of *SFP1* can affect size setting without altering the distribution throughout the cell cycle. It has been hypothesized that an intimate link exists between ribosome biogenesis, cell cycle control and size setting (Jorgensen *et al.*, 2004; Sudbery, 2002). Our results suggest that these phenotypes can be partially uncoupled if the growth rate defects of the mutant are removed. We suggest that Sfp1 may act at two regulatory levels, modulating ribosome biogenesis and size setting. This is further supported by the observation that the presence of defects in ribosome biogenesis is not enough to explain the generation of a *whi* phenotype (Rudra *et al.*, 2005).

Our results, together with previous findings, provide strong evidence that Sfp1 plays a key role in linking growth potential with cell size control. A future challenge lies in further studying the molecular mechanisms that are at the base of this control in order to understand the complex regulatory networks that, in all eukaryotes, govern growth, size setting and cell-cycle progression.

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