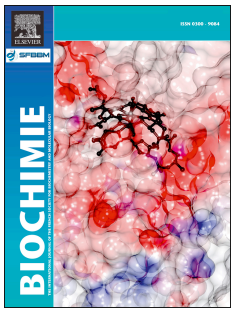

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Sfl1 is required for *Candida albicans* biofilm formation under acidic conditions

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Abstract

Candida albicans is a common *Candida* species, responsible for infections in various anatomical sites under different environmental conditions, aggravated in the presence of its biofilms. As such, this study aimed to reveal the regulation of *C. albicans* biofilms under acidic conditions by the transcription factor Sfl1, whose role on biofilm formation is unclear. For that, microbiologic and transcriptomic analyses were performed with the knock-out mutant *C. albicans sfl1*Δ/*sfl1*Δ and its parental strain SN76, grown in planktonic and biofilm lifestyles at pH 4 (vaginal pH). The results revealed that despite being a filamentation repressor Sf1 is required for maximal biofilm formation under acidic conditions. Additionally, Sfl1 was found to induce 275 and 126 genes in biofilm and planktonic cells, respectively, with an overlap of 19 genes. The functional distribution of Sfl1 targets was similar in planktonic and biofilm modes but an enrichment of carbohydrate metabolism function was found in biofilm cells, including some genes encoding proteins involved in the biofilm matrix production. Furthermore, this study shows that the regulatory network of Sfl1 in acidic biofilms is complex and include the positive and negative regulation of transcription factors involved in adhesion and biofilm formation, such as *AHR1*, *BRG1*, *TYE7*, *TEC1*, *WOR1*, and various of their targets. Overall, this study shows that Sfl1 is a relevant regulator of *C. albicans* biofilm formation in acidic environments and contributes to a better understanding of *C. albicans* virulence under acidic conditions.

Keywords: *Candida* biofilm, filamentation, acidic environment, Sfl1 targets, regulation network

1. Introduction

Candida albicans is the most frequent causative agent of various fungal infections, from mild to life-threatening severity [1]. Its success as a major fungal pathogen relies on various pathogenic traits, among which the ability to produce filamentous forms and to develop high recalcitrant biofilms [2]. These features are modulated by the environmental conditions to which *C. albicans* cells are exposed within the human host, including various pH levels. Indeed, *C. albicans* is capable of colonize and/or infect several anatomical sites including the mouth, vagina, blood and gut [3,4]. We have recently demonstrated that pH 4 (vaginal environment) impairs the filamentation ability of *C. albicans* cells either in planktonic and biofilm modes [5]. Nevertheless *C. albicans* was capable to produce highly adhered biofilms under acidic conditions, although with lower total biomass than those developed under neutral conditions. We have also revealed that the acidic conditions trigger the regulation of several genes involved in adhesion and biofilm maturation. Of note, besides targets of known biofilm regulators we have found that acidic conditions also trigger the regulation of several documented targets of Sfl1 [6], which role in biofilm formation is unknown.

The transcription factor Sfl1 has been reported as a repressor of filamentation, flocculation and microcolony formation in *C. albicans* [7–9]. In contrast we found a defective biofilm formation in a *SFL1* deletion mutant grown under acidic conditions [6]. This was an interesting finding since Sfl1 is a hyphal repressor, although being required for full virulence and pathogenesis [10]. More important, acidic conditions repress or retard filamentation via Sfl1, which act as both activator and repressor, of morphological repressors and inducers, respectively [8,10]. Sfl1 represses and is repressed by Sfl2, a morphological inducer, depending on the conditions. Low pH or low temperature were shown to induce Sfl1 expression, which block Sfl2 expression, preventing or retarding

filament development. In contrast high temperature or pH will induce Sfl2, reducing Sfl1 expression levels [8,10]. The acidic-specific regulation of Sfl1 and the phenotype of the *SFL1* deletion mutant biofilm under acidic conditions suggest that Sfl1 may act as an acidic biofilm regulator, however the genomic mechanisms underlying this regulation are unknown. As such, this study aimed to reveal Sfl1 target genes in *C. albicans* acidic biofilms in order to clarify Sfl1 function in fungal development and its biofilm regulatory network.

2. Methods

2.1 Strains and initial growth conditions

In this study the knock-out mutant *C. albicans sfl1*Δ/*sfl1*Δ was used along with its parental strain *C. albicans* SN76 [10]. The strains were subcultured for 48 h on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) at 37 °C. Then, some colonies were used to inoculate 40 ml of Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany). The inoculum was incubated for 18 h at 37 °C under 120 rev/min. Then, the cell suspension was centrifuged at 5000 g for 10 min at 4 °C, the supernatant was discarded and the cells were washed twice Phosphate Buffered Saline 1x (PBS; pH 7). The pellet was resuspended in Roswell Park Memorial Institute medium (RPMI; Sigma-Aldrich, St. Louis, Missouri), buffered with 3-(N-Morpholino) propane sulfonic acid (MOPS; Sigma-Aldrich, St. Louis, Missouri) and adjusted to pH 4.0 with lactic acid. Three independent inoculums were prepared for each experiment.

2.2 Planktonic growth and biofilm formation

The inoculums prepared were further used to grow cells in planktonic and biofilm modes. For that, the cell density was adjusted to $1x10^5$ cells/ml, using a Neubauer

haemocytometer (Marienfeld, Lauda-Königshofen, Germany). For the planktonic growth, the cell suspensions were placed in 25 ml Erlenmeyer flasks and incubated for 24 h at 37 °C under agitation (120 rev/min). In order to form biofilms the cell suspensions were placed into wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) (200 µl per well) and incubated under the same conditions than planktonic cells [5]. After incubation, the medium was removed and biofilms were washed with PBS 1x. Planktonic and biofilm cells were analyzed as described in the next sections (all analyses were performed in triplicate).

2.3 Evaluation of filamentation ability

In order to evaluate the impact of *SFL1* deletion in filamentation ability of *C. albicans* the strains were grown in the planktonic and biofilm lifestyles as described above, but both in the absence and presence 10 % Fetal Bovine Serum (FBS; Sigma-Aldrich, St. Louis, Missouri). For the analysis of filamentous forms of biofilm cells, the biofilms were scraped from the microtiter plates' wells with PBS 1x and the filamentous forms of biofilm cells were counted in an optical microscope using a Neubauer chamber (results were presented as percentage of filamentous forms). For the analysis of planktonic cells' filaments, aliquots of the cell suspensions were analyzed microscopically as described for biofilm cells [5].

2.4 Determination of cell cultivability

In order to determine the impact of *SFL1* deletion on cell cultivability of planktonic and biofilm cells the strains were grown as described above and analyzed through the colony forming units (CFUs) counting methodology [11]. Briefly, biofilms were scraped from the microtiter plates with PBS 1x and the suspensions were serially diluted in PBS 1x and

plated on SDA. The plates were incubated for 24 h at 37 °C and the number of CFUs on the plates was counted. The results were presented as Log (CFU/ml). The same procedure was performed with the suspensions of planktonic cells.

2.5 Transcriptomic analysis

The analysis of genes regulated by Sfl1 in planktonic and biofilm cells was performed using species-specific DNA microarrays [6]. For this, the transcriptome of *C. albicans* *sfl1*Δ/*sfl1*Δ cells was compared with the transcriptome of *C. albicans* SN76 cultivated under the same conditions. This dual-channel analysis was performed with cells grown in planktonic and biofilm lifestyles. The experimental setups used to cultivate the cells were the same as described above but using 24-well polystyrene microtiter plates for the development of biofilms (1 ml per well). Biofilms were scraped from the microplates with PBS 1x and the suspensions were sonicated (Ultrasonic Processor, Cole-Parmer, IL, USA) for 30 s at 30 W, in order to separate the cells from the biofilm matrix [12]. Then, the cell suspensions (planktonic and biofilm cells) were centrifuged at 3000 g for 10 min at 4°C and the pellets were used for RNA extraction.

2.5.1 RNA extraction

The extraction of RNA from the cells was performed using the RiboPure – Yeast Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The concentration and purity of the extracted RNA were determined by spectrophotometry and its integrity was confirmed in an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) > 7 were used for the microarrays analysis.

148 #### 2.5.2 Microarrays

149 cDNA synthesis, hybridization, and scanning were performed using the Agilent protocol for
150 two-color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling
151 v6.9 (Agilent Technologies), according to the manufacturer's instructions. One hundred
152 nanograms of total RNA of each isolate were used to synthesize labeled cDNA (with
153 Cyanine 3-CTP and cyanine 5-CTP), using Agilent T7 Promoter Primer and T7 RNA
154 polymerase Blend (Agilent Technologies, Cat.5190–2305). Six hundred nanograms of
155 labelled cDNA were hybridized in the microarray. Hybridizations were carried out using
156 Agilent gasket slides in a rotating oven for 17 h at 65°C. Slides were then washed
157 following manufacturer's instructions and scanned in an Agilent G2565AA microarrays
158 scanner. Probes signal values were extracted using Agilent Feature Extraction Software.
159 Three biological replicates and three technical replicates were performed. Data were
160 normalized using median centering of signal distribution with Biometric Research Branch
161 BRB-Array tools v3.4.o software 36. Final statistical analysis was carried out using LIMMA
162 package in MeV software (MultiExperiment Viewer 4.8.0) 36 with a cut-off p-value of 0.01.
163

164 ### 2.6 Measurement of gene transcription by quantitative real-time PCR
165 ### (qRT-PCR)

166 In order to validate the results obtained in the microarray analysis, the level of mRNA
167 transcripts of a set of specific genes (*TEC1*, *WOR1* and *AHR1*) was assessed using qRT-
168 PCR. The experimental conditions used to grow planktonic and biofilm cells and to extract
169 the RNA were as described above for the microarray analysis. After RNA extraction, the
170 complementary DNA (cDNA) was obtained with the iScript cDNA Synthesis Kit (Bio-Rad,
171 Hercules, CA, USA), according to the manufacturer's instructions, using 0.5 µg of total
172 RNA. Then, qRT-PCR was performed using mixtures consisted of 125 ng of the
173 synthesized cDNA, SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), dH_2O

(Cleaver Scientific Ltd, Warwickshire, UK) and the primer pair respective to the target gene (50 µM). Negative controls (dH_2O) and non-reverse transcriptase controls were also included in each run**.** The primers were designed using Primer3 web software using the gene sequences available in *Candida* Genome Database [13]. The specificity of each primer pair for its target gene was confirmed applying a PCR to genomic DNA extracted from *C. albicans* cells. qRT-PCR (CF X96 Real-Time PCR System; Bio-Rad, Hercules, CA, USA) was performed at 98 °C for 2 min (initial denaturation), followed by 98 °C for 5 s (denaturation) and 57 °C for 5 s (primer annealing), for 40 cycles. In each cycle it was generated a melting curve running a dissociation stage at 60 °C, allowing the verification of the amplification product specificity. In order to ensure that multiple plates could be compared control samples were included on each plate. Furthermore, the transcript level of *ACT1* mRNA was used as an internal control. The relative gene expression levels were calculated using the ΔCt method [14], using the Ct value determined for each sample, normalized with the Ct of the internal control gene (Ct = 22.15 ± 1.11). Each reaction was performed in triplicate.

2.7 Bioinformatic analyses

The functional description of Sfl1 targets was obtained with FungiFun 2.2.8 tool [15] using the classification ontology provided by the Functional Catalogue (FunCat) database [16]. A functional enrichment analysis was also conducted on FungiFun, using the whole *C. albicans* genome as background. Additionally, *Candida* Genome Database (CGD) [17] was used to search for Sfl1 targets described as biofilm-induced and -repressed in previous studies. CGD was also used to search for Sfl1 targets annotated as required for adhesion and biofilm formation (verified and computationally predicted phenotypes). Furthermore targets displaying a relevant role in biofilm formation, according to CGD, were

199 analyzed in the PathoYeastract database [18] in order to determine their regulatory
200 associations. PathoYeastract was also used to search for Sfl1 targets already identified.
201

202 ### 2.8 Statistical analyses

203 In the functional enrichment analysis of Sfl1 targets performed with FungiFun tool
204 the statistical Fisher-exact test with Benjamini-Hochberg correction [15,19],
205 available in the tool, was used. Additionally, the results obtained for *C. albicans*
206 *sfl1*Δ/*sfl1*Δ and SN76 strains regarding filamentation, cell cultivability and gene
207 expression (qRT-PCR analyses), were compared using the t test implemented in
208 GraphPad Prism 6 software. All tests were performed with a confidence level of
209 95%.
210

211 ## 3. Results

212 ### 3.1 Role of Sfl1 in filamentation and cell cultivability

213 Cells of *C. albicans sfl1*Δ/*sfl1*Δ and of its parental strain SN76 grown for 24 h in planktonic
214 and biofilm modes, under acidic conditions (RPMI at pH 4) supplemented or not with FBS,
215 were analyzed in terms of percentage of filamentous forms. In the absence of FBS, a
216 statistically significative difference was found between the two strains. Planktonic and
217 biofilm cells of *C. albicans* SN76 displayed around 17% (P-value ≤0.01) and 10% (P-value
218 ≤0.05) more filaments than those of *sfl1*Δ/*sfl1*Δ, respectively (Figure 1A). In contrast, in the
219 presence of FBS, there was not a statistical difference in the percentage of filaments
220 between the strains, either in planktonic or biofilm modes. Additionally, *C. albicans* SN76
221 cells displayed higher percentage of filaments in presence of FBS than in its absence, but
222 *sfl1*Δ/*sfl1*Δ cells presents similar percentage in both conditions and lifestyles (Figure 1A).

Cells of *C. albicans sfl1*Δ/*sfl1*Δ and its parental strain SN76 were also analyzed in terms of cell cultivability. The results revealed that *sfl1*Δ/*sfl1*Δ cells display statistically (P-value ≤0.05) lower cell cultivability than those of SN76 strain, in either planktonic or biofilm modes. The difference between the strains was of 1 order of magnitude (Log (CFUs/ml)) in planktonic cells and 2 orders of magnitude in biofilm cells (Figure 1B).

3.2 Transcriptomic targets of Sfl1

A transcriptomic analysis, using microarrays technology, was carried out with planktonic and biofilm cells of *C. albicans sfl1*Δ/*sfl1*Δ and SN76 strains grown in planktonic and biofilm lifestyles, under acidic conditions. In order to identify Sfl1 targets, the levels of gene expression displayed by the parental strain were compared with those of the mutant strain, in each growth mode. For this analysis, only genes showing a difference of expression between the strains of at least 1.5-fold were selected as (i) Sfl1-induced targets – higher expression in the parental than in the mutant strain – or (ii) repressed targets - lower expression in the parental than in the mutant strain. The results revealed that Sfl1 induces the expression of 275 genes in biofilms and 126 genes in planktonic cells, with an overlap between the two growth modes of 19 genes (Figure 2 and Supplementary Table S1). Among biofilm Sfl1-induced targets 66 presented a strong regulation (more than 2-fold alteration in the expression) and among planktonic targets 18 were strongly induced (Supplementary Table S2). Additionally, Sfl1 was found to repress the expression of 295 genes in biofilm cells (101 strongly repressed) and of 350 genes in planktonic cells (135 with strong repression), (Supplementary Table S2) with an overlap between the lifestyles of 56 genes (Figure 2 and Supplementary Table S1).

All Sfl1 targets found in this study were searched in PathoYeastract in order to infer about their previous annotation as Sfl1-regulated. This analysis revealed that 16 and 17 Sfl1-induced targets found in planktonic and biofilm cells, respectively, were previously

reported as Sfl1-induced. Also 69 and 46 repressed targets found in planktonic and biofilm cells, respectively, were previously reported as Sfl1-repressed, according to PathoYeastract (Supplementary Table S2). In addition, all biofilm Sfl1 targets were searched in Candida Genome Database in order to infer about their annotation as biofilm-regulated. This analysis revealed that 96 and 38 Sfl1-induced and -repressed targets, respectively, were annotated as biofilm-induced and -repressed (Supplementary Table S2).

3.3 Functional distribution of Sfl1 targets

The functional distribution of Sfl1 targets identified in planktonic and biofilm cells was analyzed in using FungiFun tool. This analysis revealed an enrichment of genes with functions related to “metabolism”, “transport” and “binding”, both in planktonic and biofilm cells, and a similar relative percentage of unclassified targets (Figure 3). The most enriched class in both growth modes was “metabolism”, represented by ~37% and ~25% of Sfl1-induced and -repressed targets, respectively (Figure 3). Among Sfl1-induced targets, 6 and 7 subclasses of the “metabolism” class were found to be statistically enriched in planktonic and biofilm cells, respectively. The subclasses with the most statistically significant result were the “secondary metabolism” (P-value=0.0001) in planktonic cells and “carbohydrate metabolism” (P-value=0.003) and “aminoacidic pathway” (P-value=0.002) in biofilm cells. Regarding the “transport” main class, the most statistically significant subclass was “cellular import” (P-value≤ 0.05) for both planktonic and biofilm lifestyles (Supplementary Table S3).

3.4 Biofilm regulatory network of Sfl1

The Sfl1 targets found in biofilm mode were searched in *Candida* Genome Database in order to identify genes annotated as required for *C. albicans* adhesion and biofilm

development. This analysis revealed that 7 and 16 Sfl1-induced genes are required for adhesion and biofilm formation, respectively (Supplementary Table S2). Then, the regulatory associations between biofilm-related targets and Sfl1 were analyzed using PathoYeastract. This analysis revealed that Sfl1 induces the expression of three transcription factors (Tye7, Ahr1 and Brg1), which are positive regulators of other Sfl1-induced targets also required for biofilm formation. Additionally, Sfl1 was found to repress the expression of two transcription factors (Tec1 and Wor1) with a relevant role in the biofilm formation, which also repress other Sfl1 targets required for biofilm formation. The regulation of Ahr1, Tec1 and Wor1 by Sfl1 in *C. albicans* biofilm cells was further confirmed by qRT-PCR (supplementary Figure S1). Considering these results, the proposed regulatory network of Sfl1 in *C. albicans* acidic biofilms is presented in Figure 4A. Among the 20 Sfl1-induced targets presented in Figure 4A, the heat shock protein Hsp21 showed the highest induction of expression (fold change of 2.7). In addition, Vps15 and Psa2 also presented a strong induction (more than two-fold change) and the remaining targets had a moderate regulation (more than 1.5-fold change) (Figure 4B).

4. Discussion

The formation of *Candida* biofilms in the vaginal environment (on vaginal walls or intrauterine devices) is correlated with complicated and recurrent vulvovaginal candidiasis episodes, which may have serious consequences [20]. Despite increasing studies focusing on *C. albicans* biofilms, their transcription regulation under acidic conditions, such as those found in the vaginal environment (pH around 4), is poorly known. This study revealed that Sfl1 is a relevant transcription regulator of *C. albicans* biofilm formation under acidic conditions and its regulatory network was characterized.

The function of Sfl1 as repressor of the development of filamentous forms of *C. albicans* under neutral conditions has been described by some studies [7,8,10]. In this

study a similar effect under acidic conditions was found (Figure 1A), in line with the previous suggestion that acidic conditions may repress the filamentation via *SFL1*. Of note, the effect of *SFL1* deletion on the filamentation ability, despite being statistically significant under acidic conditions (Figure 1A), did not have the exacerbated impact previously demonstrated under neutral conditions [7,8,10]. Curiously, the presence of FBS, an inducer of filamentation, used in previous studies to potentiate the effect of *SFL1* deletion [10], did not have a significant effect in the knock-out mutant strain under acidic conditions, in either planktonic or biofilm modes (Figure 1A). Furthermore, the FBS led to an increase of filamentation in the parental strain similar to that caused by the deletion of the *SFL1* gene (maximum of 30%) (Figure 1A). These findings suggest that under acidic conditions the promotion of filamentation, either through an external stimulus or genetic alteration, is highly impaired.

Despite the repressor effect of Sfl1 on the filamentation we have previously shown that *C. albicans sfl1*Δ/*sfl1*Δ strain form defective biofilm under acidic conditions [6]. In this study, the microbiologic analyses with this strain corroborate the previously observed phenotype, showing a statistically lower cell cultivability in mutant biofilms than in those of its parental strain (Figure 1B). Importantly, a lower cultivability was also found in planktonic cells of the knock-out mutant strain (Figure 1B). This result suggests that part of the defective biofilm formation in the absence of *SFL1* gene may arose from the defective cell growth observed in planktonic cells. However, the impact of *SFL1* deletion in the cultivability of biofilm cells was twice than that detected in planktonic cells suggesting that, other factors besides the defective cell growth may have affected the biofilm formation of the mutant strain. In fact, this is a curious result, because repressors of filamentation usually promote the formation of biofilms when absent, as the filaments are known to contribute to the accumulation of cells within the biofilms. Nevertheless, some filamentation repressors have been identified as required for maximal biofilm formation,

such as *ACE2*. These results have been explained by the regulation of genes with significant impact on adhesion and biofilm maturation [21,22]. This prompted us to analyze the target genes of Sfl1 in planktonic and biofilm modes, under acidic conditions.

By microarrays' analysis 476 and 570 Sfl1 target genes were identified in *C. albicans* biofilm and planktonic cells, respectively (Figure 2). Of note, only a small number of Sfl1 targets found in this study (17 targets), are previous documented targets of Sfl1, according to PathoYeastract (Supplementary Table S2), probably reflecting the acidic conditions used in this study. Furthermore, only ~10% of Sfl1 targets found in biofilm cells were also identified as Sfl1 targets in planktonic cells (Figure 2 and Supplementary Table S1), showing that Sfl1 displays a biofilm-specific regulatory action. Additionally, several Sfl1-induced targets found in biofilm cells (96 genes) are annotated as biofilm-induced in Candida Genome Database, corroborating the potential role of Sfl1 as a transcriptomic regulator of acidic biofilms.

Despite the difference in the number of genes regulated by Sfl1 in planktonic and biofilm modes (Figure 2 and Supplementary Table S1), in general, their functional distribution is similar, with an enrichment of the "metabolism", "binding function" and "transport" classes (Figure 3). Nevertheless, among "metabolism" functional class, the "carbohydrate metabolism" was the most statistically enriched subclass found among biofilm Sfl1-induced targets but the "secondary metabolism" was the most statistically enriched subclass among planktonic cells. The higher abundance of Sfl1-induced targets related to carbohydrate metabolism in biofilms may not only contribute to nutrient utilization in the hostile environment as may also be related to the carbohydrate content of the biofilm matrix. Indeed, some biofilm Sfl1-induced targets, including *XOG1* and *GCA1*, encode proteins previously suggested to display a relevant role in the production and delivery of biofilm matrix carbohydrates [23,24]. Being that the biofilm matrix is considered an important feature of biofilms these results suggest a potential role of Sfl1 in the defense

of *C. albicans* biofilms. Accordingly, a higher abundance of Sfl1-induced genes related to defense and virulence function was found among biofilm than planktonic cells (Figure 3).

Several transcription factors were found among Sfl1 targets, suggesting that the regulatory network of this transcription factor is very complex. Among the transcription factors regulated by Sfl1 in biofilm cells, five possess a known role in the biofilm development, with three of them being induced by Sfl1 (Ahr1, Brg1 and Tye7) and two being repressed (Tec1 and Wor1). In addition, 13 Sfl1-induced targets also displaying relevant roles in the biofilm formation are documented targets of one or more of the five transcription factors targeted by Sfl1, according to PathoYeastract (Figure 4). Furthermore, four genes required for the initial adhesion of biofilm cells were also identified among Sfl1-induced targets. Considering these results, we propose the biofilm regulatory network of Sfl1 presented in Figure 4. It may be suggested that the defective phenotype of *C. albicans* sfl1 mutant biofilms developed under acidic conditions may, at least in part, be explained by lower expression of several genes required for biofilm development and higher expression of others that repress their expression. As such, Sfl1 seems to be a relevant transcriptomic regulator of *C. albicans* acidic biofilms. Nevertheless, it should be noted that further studies are required to confirm Sfl1 role under acidic conditions in other culture media than RPMI.

5. Conclusions

This study suggests that, despite its role as repressor of filamentation, Sfl1 is required for maximal *C. albicans* biofilm formation under acidic conditions, probably due to its regulation of genes with relevant roles in adhesion and biofilm development. Additionally, the results also suggest a potential role of Sfl1 in the defense mechanisms of biofilms. This study shows that Sfl1 may be an interesting target to control acidic biofilms and thus further studies, including *in vivo*, are required to ascertain the relevance of this

transcription factor. Overall, this study will contribute to a better understanding of the transcriptomic alterations underlying the development of *C. albicans* acidic biofilms and to a better management of diseases caused by them.

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Data availability statement: The microarrays data have been deposited in GEO (accession number: GSE206208).

Conflicts of interest: The authors declare no potential conflict of interests.

Authors' contributions: Bruna Gonçalves, Mariana Henriques e Sónia Silva contributed to the study conception and design. Material preparation, data collection and analysis were

performed by Bruna Gonçalves, Ana Barbosa and Ana Raquel Soares. The first draft of the manuscript was written by Bruna Gonçalves and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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Figure captions

Figure 1. Impact of *SFL1* deletion on the filamentation and cell cultivability of *Candida albicans* planktonic and biofilm cells at acidic conditions. Percentage of filamentous forms **(A)** and cell cultivability **(B)** of *C. albicans* SN76 and *sfl1Δ/sfl1Δ* cells grown for 24 h in RPMI at pH 4, in planktonic and biofilm modes. The percentage of filamentous forms was evaluated in presence of FBS. Asterisks represent statistical difference between the strains at the same conditions (***P*-value ≤0.01; **P*-value ≤0.05).

Figure 2. Venn diagram presenting the number of Sfl1 target genes in *Candida albicans* planktonic cells and biofilms grown at acidic conditions. Number of genes whose expression was found to increase or decrease (above or below 1.5-fold) in *C. albicans* SN76 cells grown at pH 4, in planktonic and biofilm modes, in comparison with the transcript levels registered in cells of the knock-out mutant strain *sfl1*Δ/*sfl1*Δ, under similar conditions (microarrays analysis). The genes found to be induced and repressed by Sfl1 exclusively in biofilms or planktonic cells and in both conditions are shown in Supplementary Table S1.

Figure 3. Functional distribution of *C. albicans Sfl1* targets at acidic conditions. Genes found to be induced and repressed by Sfl1 in *C. albicans* SN76 cells grown at pH 4, in planktonic (right graph) and biofilm (left graph) modes, were clustered according to their biological function, through FungiFun database (black and white bars correspond to Sfl1-induced- and -repressed genes, respectively). The relative percentage shown correspond to the number of target genes included in each functional class compared to the total number of Sfl1-induced or repressed genes found in the corresponding lifestyle.

Figure 4. Regulation of Sfl1 *C. albicans* biofilm targets with role in adhesion and biofilm formation. (A) Proposed regulatory cascade of genes with reported role in adhesion and biofilm formation, found to be regulated by Sfl1 in *C. albicans* biofilms developed for 24 h at pH 4. Black boxes represent transcription factors, arrows (→) represent positive regulation (dashed for potential regulation) and lines with bar (—ı) represent negative regulation. **(B)** Levels of mRNA transcripts of Sfl1-targets found in *C. albicans* SN76 biofilms in comparison to those found in *sfl1*Δ/*sfl1*Δ, as determined by microarray analysis. The results that give rise to this Figure are fully detailed in Supplementary Table S2.

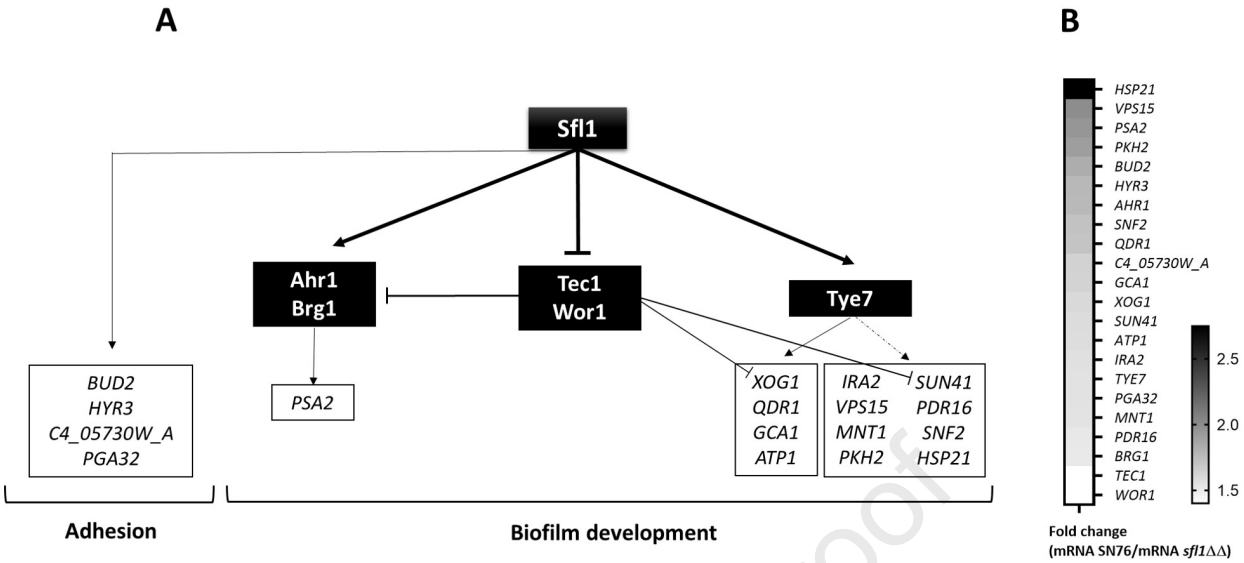
Supplementary Material

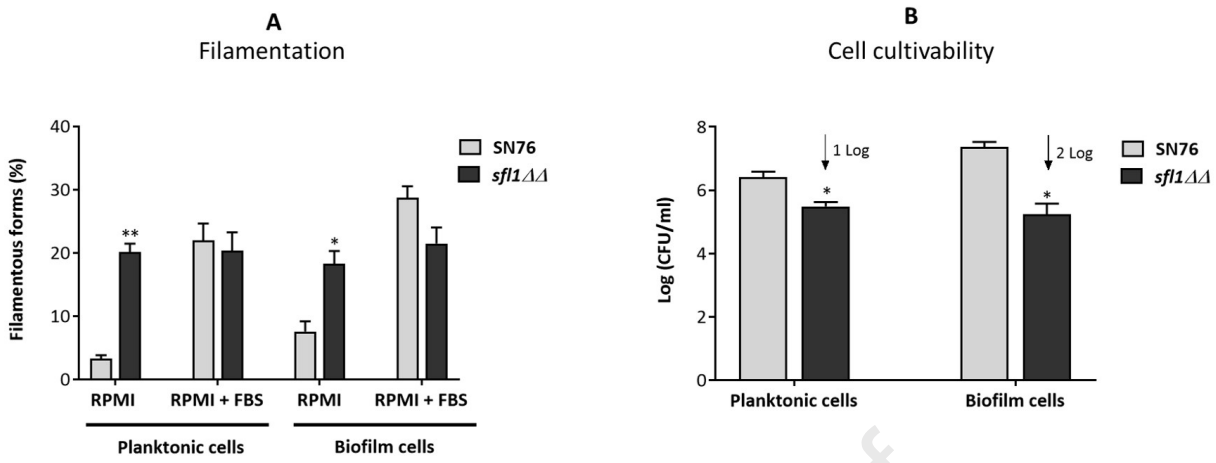
Figure S1. Transcript levels, estimated by qRT-PCR, of *C. albicans TEC1*, *WOR1* and *AHR1* genes. Are presented the levels produced in *C. albicans sfl1*Δ/*sfl1*Δ and SN76 biofilms cells cultivated 24 h in RPMI at pH 4. The values of the transcript levels were normalized using as internal control the levels of *ACT1* mRNA. Error bars represent standard deviation. Asterisks represent statistical difference between the conditions (* p-value <0.05; **p-value <0.001).

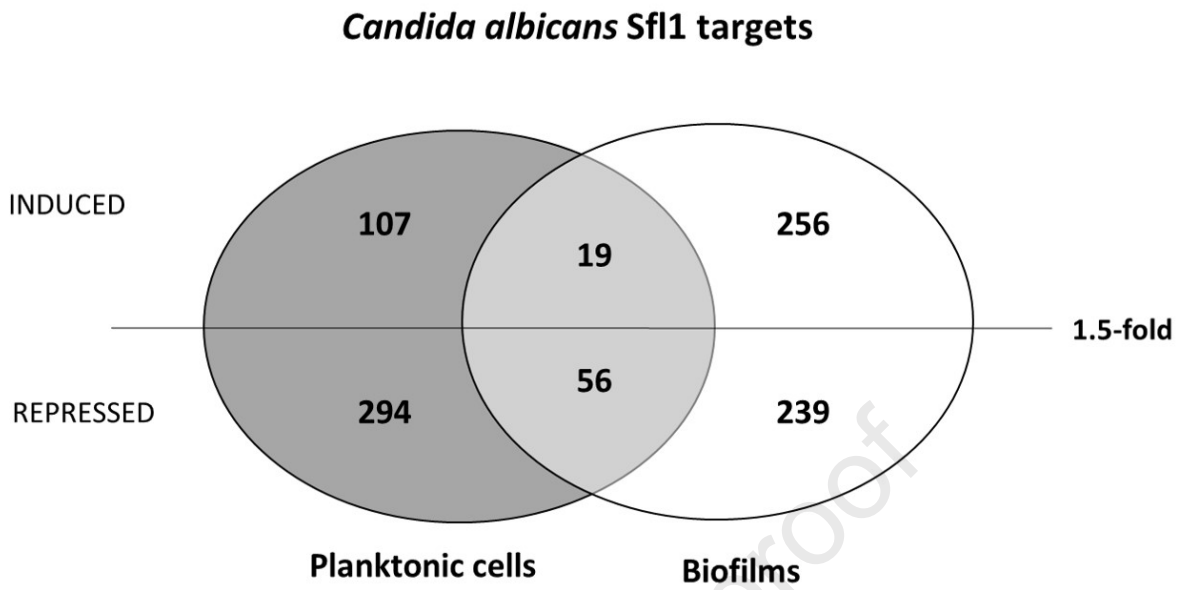
Table S1. List of *Candida albicans* Sfl1-induced and -repressed targets exclusively found in biofilms or planktonic cells and in both conditions

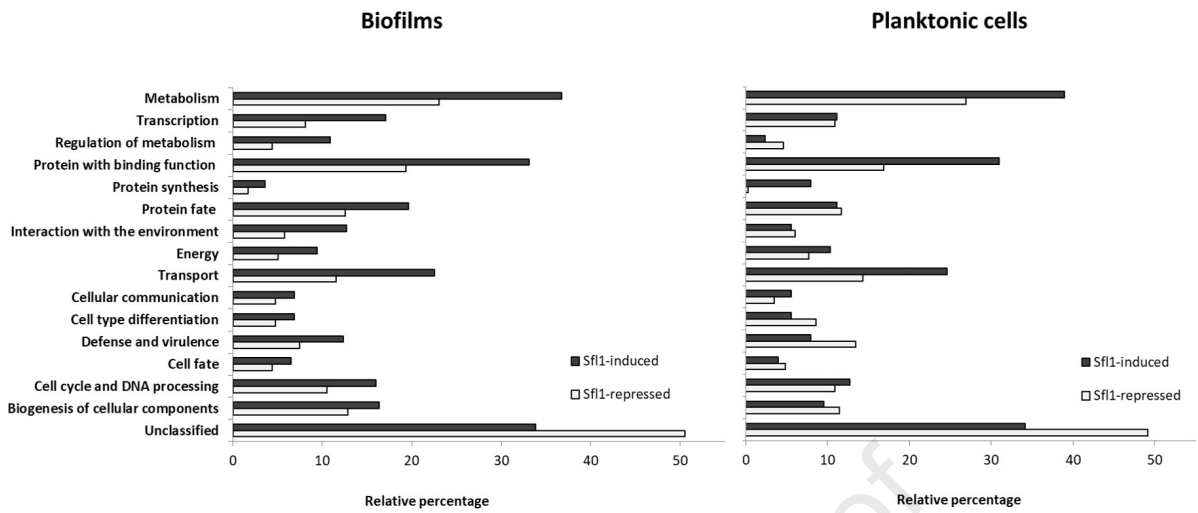
Table S2. List of Sfl1-induced and -repressed targets found in planktonic and biofilm cells of *Candida albicans*, respective fold-change and results of bioinformatic analyses performed in PathoYeastract and *Candida* Genome Database

Table S3. Functional distribution of Sfl1-induced and -repressed targets found in planktonic and biofilm cells of *C. albicans*









Highlights

- Sfl1 is required for maximal *C. albicans* biofilm formation under acidic conditions
- Sfl1 regulates the expression of genes with roles in adhesion and biofilm formation
- Sfl1 has a potential role of in the defense mechanisms of *C. albicans* biofilms
- Sfl1 may be an interesting target to control acidic biofilms