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A Versatile Overexpression Strategy in the Pathogenic Yeast Candida albicans: Identification of Regulators of Morphogenesis and Fitness

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Abstract

Candida albicans is the most frequently encountered human fungal pathogen, causing both superficial infections and life-threatening systemic diseases. Functional genomic studies performed in this organism have mainly used knock-out mutants and extensive collections of overexpression mutants are still lacking. Here, we report the development of a first generation C. albicans ORFeome, the improvement of overexpression systems and the construction of two new libraries of C. albicans strains overexpressing genes for components of signaling networks, in particular protein kinases, protein phosphatases and transcription factors. As a proof of concept, we screened these collections for genes whose overexpression impacts morphogenesis or growth rates in C. albicans. Our screens identified genes previously described for their role in these biological processes, demonstrating the functionality of our strategy, as well as genes that have not been previously associated to these processes. This article emphasizes the potential of systematic overexpression strategies to improve our knowledge of regulatory networks in C. albicans. The C. albicans plasmid and strain collections described here are available at the Fungal Genetics Stock Center. Their extension to a genome-wide scale will represent important resources for the C. albicans community.

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Introduction

Candida albicans is a normal member of human natural cavities, especially of the gastrointestinal and urogenital tracts [1,2]. In addition to its commensal activity and under specific conditions, this yeast becomes one of the major invasive fungal pathogen of humans and can cause both mucosal and life-threatening disseminated infections [3,4]. The significant mortality rate associated with candidiasis in immunocompromised patients drives the research efforts to improve our knowledge of C. albicans biology and pathogenesis [5].

During the last decade, progresses in gene inactivation methodologies have been the driving force to characterize C. albicans molecular processes [6,7]. Several collections of heterozygous and homozygous knock-out (KO) mutants have been generated. These resources are now invaluable to study C. albicans regulatory networks, virulence, hyphal morphogenesis, biofilm formation, identify drug targets and evaluate the mode-of-action of antifungal compounds [8–20]. However, the use of KO mutants shows some limitations. First, since C. albicans is an obligate diploid organism with no known meiotic cycle, two rounds of gene disruption are required to produce each deletion mutant. The
pioneering works for the construction of homozygous KO mutants in the C. albicans genome mentioned above were indeed tedious and time-consuming. Second, gene deletion approaches are not optimal in the case of functional redundancy or essential genes. To date, less than 25% of C. albicans genes have been functionally characterized, indicating that new approaches must be developed for the study of this pathogen.

The use of both systematic KO and overexpression (OE) approaches to investigate cellular processes has proven highly successful in the model yeast Saccharomyces cerevisiae. Genome-wide collections of S. cerevisiae OE strains have been assembled and used to perform large-scale functional analyses, leading to the identification of new signalling pathways, new targets and functions for transcription factors or protein kinases and, more largely, to improve our image of the functional landscape of the cell [21–27]. In contrast, OE strategies have not been exploited extensively in C. albicans. Fu et al. [28] established a collection of 26 heterozygous OE strains for genes encoding glyco phosphatidylinositol-anchored (GPI) proteins. This study demonstrated the role in adherence for the product of the IFF4 gene, one of 11 members of the IFF genes family. More recently, Salini et al. [29] constructed an OE library of 103 transcription factors which has been used in two independent screens, demonstrating a role for the Tec1 transcription factor in the response of white cells to pheromones [29] and identifying a critical function for the Brg1 transcription factor in C. albicans biofilm formation, filamentous growth and virulence [30]. Other studies have used OE of selected genes in order to test the relevance of regulatory networks and functional pathways inferred from gene expression studies [31–34]. Nevertheless, the collections of OE strains yet available are focused and despite the encouraging results obtained in the studies mentioned above, a flexible collection of OE plasmids encompassing the 6200 C. albicans genes and the corresponding C. albicans OE strains are still lacking.

Here, we constructed two collections of C. albicans OE strains, enriched for genes encoding protein kinases, protein phosphatases, transcription factors and other signalling proteins. These new resources took advantage of the highly efficient Gateway® technology to provide versatility to our system, leading to a first generation C. albicans ORFome. To test the functionality and applications of our strategy, we performed screens for regulators of morphogenesis and growth rate in C. albicans, two research areas of crucial interest for the development of new antifungal strategies. Our results highlight the value of using gene OE as a complement to gene inactivation to both uncover gene function and reveal new regulators in C. albicans. They also pave the way for the development of genome-wide OE approaches for this major pathogen.

**Results and Discussion**

**Development of Gateway Vectors for Overexpression in Candida albicans**

In order to develop a collection of C. albicans OE strains, we have taken advantage of the Gateway® methodology that enables recombination-mediated cloning of PCR-amplified ORFs into a donor vector and their subsequent recombination-mediated transfer into a variety of customized destination vectors [35]. We developed two conditional OE destination vectors named Clp10-P<sub>PCK1</sub>-GTW-TAPtag and Clp10-P<sub>P1 TET</sub>-GTW (Fig. 1A and 1B respectively), both being derivatives of the C. albicans Clp10 integrative vector [36]. Clp10-P<sub>PCK1</sub>-GTW-TAPtag carries a Gateway® cassette flanked by the gluconeogenesis-induced C. albicans PCK1 promoter (P<sub>PCK1</sub>; [37]) and an in-frame sequence encoding a tag for tandem-affinity purification (TAPtag; Fig. 1B; [38]). Expression from P<sub>PCK1</sub> is achieved in the presence of casamino acids and repressed in the presence of glucose. Clp10-P<sub>P1 TET</sub>-GTW contains the TET promoter (P<sub>P1 TET</sub>; [39]) that is activated in the presence of tetracycline derivatives. In contrast to Clp10-P<sub>PCK1</sub>-GTW-TAPtag, Clp10-P<sub>P1 TET</sub>-GTW is not equipped with a TAPtag but with a unique barcode system (Fig. 1B and Materials and Methods for the barcoding procedure).

**Optimisation of Tetracycline-dependent Overexpression in Candida albicans**

Expression from the P<sub>P1 TET</sub> promoter requires a C. albicans-adapted reverse Tet-dependent transactivator (cartTA) that binds the tetO sequences in the P<sub>P1 TET</sub> promoter in a tetracycline-dependent manner and drives transcription through the activation domain of the Gal4 protein of S. cerevisiae [39]. Different plasmids allowing expression of cartTA in C. albicans are available among which pNIM1 whereby cartTA is expressed from the promoter of the ADH1 gene and that harbours a P<sub>TET</sub>-GFP fusion (Fig. 1C–a; [39]). We reasoned that tetracycline-dependent OE of genes cloned downstream of P<sub>TET</sub> on Clp10-P<sub>TET</sub>-GTW plasmids might be enhanced by removing the P<sub>TET</sub>-GFP fusion from pNIM1 and expressing cartTA from a stronger promoter than that of the ADH1 gene. Therefore we produced two derivatives of the pNIM1 plasmid: pNIM1ΔP<sub>TET</sub>-GFP (Fig. 1C–b) lacks the P<sub>TET</sub>-GFP fusion; pNIMX (Fig. 1C–c) lacks this fusion and carries the cartTA coding region placed under the control of the strong and constitutive C. albicans TDH3 promoter (P<sub>TDH3</sub>; [40]). In order to test the relative efficiency of the pNIM1, pNIM1ΔP<sub>TET</sub>-GFP and pNIMX plasmids plasmids driving OE from the P<sub>P1 TET</sub> promoter, these plasmids were introduced in a C. albicans strain that harboured a fusion between P<sub>P1 TET</sub> and the gLUC59 luciferase reporter gene [41]. Results presented in Fig. 1D showed that luciferase levels achieved from the strains harbouring pNIM1ΔP<sub>TET</sub>-GFP or pNIMX were respectively 3 or 5 times higher than those obtained in a C. albicans strain harbouring pNIM1. Noticeably, luciferase levels achieved from the strain transformed with pNIMX and the P<sub>P1 TET</sub>-LUC59 fusion were above those observed in a C. albicans strain harbouring a P<sub>FACT1</sub>-LUC59 fusion (Fig. 1D). Thus, C. albicans strains harbouring pNIMX were subsequently used to drive expression from the P<sub>P1 TET</sub> promoter.

**Validation of the OE-Gateway Vectors Developed for Candida albicans and Quantification of the OE Level**

We verified that Gateway®-cloning of ORFs into both plasmids allowed efficient OE of proteins by transferring the GFP [42] and UME6 ORFs into these vectors. UME6 was selected as its OE has been shown to trigger hyphal formation [43,44]. As shown in Fig. 2A, OE of UME6 resulted in the formation of hypha in conditions that do not normally trigger C. albicans morphogenesis. Production of TAP-tagged GFP and Ume6 proteins was also observed in strains harbouring derivatives of Clp10-P<sub>PCK1</sub>-GTW-TAPtag and grown under gluconeogenic conditions (Fig. 2B).

We also compared the strengths and expression kinetics of the P<sub>PCK1</sub> and P<sub>P1 TET</sub> promoters. Strains harbouring a P<sub>PCK1</sub>-gLUC59 fusion or a P<sub>P1 TET</sub>-gLUC59 fusion and pNIMX were shifted to gluconeogenic conditions or grown in the presence of 3 μg mL<sup>−1</sup> anhydrotetracycline (ATC), respectively, and luciferase activity was recorded at different time points following the shift. Results in Fig. 2C showed that an increase in luciferase activity was detectable after 1 h when using P<sub>P1 TET</sub> while 4–8 h were needed to see such an increase when using P<sub>PCK1</sub>. Moreover, expression levels obtained from P<sub>P1 TET</sub> were ca. 30 times those achieved from
Figure 1. Gateway-adapted OE systems for C. albicans. Schematic maps of the Clp10-P<sub>PCK1</sub>-GTW-TAPtag (A) and Clp10-P<sub>TET</sub>-GTW (B) vectors. The presence of attR recombination sites allows Gateway<sup>®</sup>-mediated cloning of ORFs in place of the GTW-B/CmR cassette. ORFs are expressed from the PCK1 promoter (P<sub>PCK1</sub>-A) or the TET promoter (P<sub>TET</sub>-B) that are induced in gluconeogenic growth conditions or in the presence of tetO. The figure shows the expression levels of various constructs at different time points, with error bars indicating standard deviation.
of tetracycline derivatives (doxycycline, anhydrotetracycline), respectively. In the first case, ORFs are fused to a TAPtag coding region, thus allowing production of proteins TAPtagged at their C-terminus. In the second case, each ORF is associated to a unique barcode (BC). Derivatives of Clp10-PCCK1-GTW-TAPtag and Clp10-PTET-GTW plasmids can be targeted to the C. albicans RPS1 locus when linearized with SstI or SceI and C. albicans transformants are selected for uridine prototrophy conferred by the URA3 gene. C. Schematic maps of the different transactivation cassettes used to promote expression from the TET promoter. The pNIMX cassette (c) is a derivative of pNIM1 (a) [39]. pNIMX was generated by deleting the PTET-GFP fusion in pNIM1, yielding pNIM1APter-GFP (b), and subsequently exchanging the pDONR207 promoter upstream of the cartTA region by the TDH3 promoter (pTDH3). Relevant restriction sites are shown: A: AclI, B: BamHI, Bg: BglII, K: KpnI, N: NcoI, S: SacI, X: XbaI. D. The pNIMX transactivator cassette provides enhanced PTET-driven OE. C. albicans strains harbouring the Clp10-PTET-GLTUC95 plasmid, with the gltUC95 luciferase reporter gene under the control of PTET, and either pNIM1, pNIM1APter-GFP or pNIMX (CEC1909, CEC2249 or CEC3083 respectively) were grown in YPD liquid medium supplemented with 50 µg/mL Dox. A C. albicans strain harbouring Clp10-PTET-GLTUC95 plasmid (CEC3088) and expressing the gltUC95 reporter gene constitutively was used as a control and grown in the same conditions. Data represent luciferase specific activity detected from the corresponding PCR products from the start codon to the phenotypes associated with the OE of those 257 genes for both sets of strains (Fig. 3A; Table S1). In summary, our procedure testing the effect of gene OE on a given phenotype.

Establishment of a Collection of Candida albicans OE Strains

Based on these results, we generated two new collections of C. albicans OE strains. We focused our study on 384 C. albicans ORFs encoding 76 protein kinases (PKs), 36 protein phosphatases (PPs), 179 transcription factors (TFs) and 93 other proteins related to signalling. Corresponding PCR products from the start codon to the penultimate codon were cloned into the pDONR207 donor vector. Following Sanger and Illumina/Solexa sequence validation, a total of 338 (93.1%) derivatives of pDONR207 were obtained (Fig. 3A and Table S1).

ORFs cloned into pDONR207 were subsequently transferred into the Clp10-PCCK1-GTW-TAPtag and uniquely barcoded Clp10-PTET-GTW-TAPtag plasmids. A total of 315 Clp10-PCCK1-GTW-TAPtag derivatives and 337 Clp10-PTET-GTW derivatives were obtained (Fig. 3A; Table S1). These plasmids were subsequently introduced at the RPS1 locus in C. albicans wild-type strains CEC161 or CEC2907, respectively. Of the resulting 298 strains that harboured a Clp10-PCCK1-GTW-TAPtag derivative, 277 produced a TAP-tagged protein when grown in gluconeogenic conditions with approx. 15% showing relatively low levels of protein production (Fig. 3 and data not shown). Eventually, 277 C. albicans PCCK1-driven OE strains and 302 C. albicans PTET-driven OE strains were obtained, with 257 genes being represented in both sets of strains (Fig. 3A; Table S1). In summary, our procedure had more than 70% success rate in both cases, reflecting a near 90% success rate at each step. Results presented below focus on the phenotypes associated with the OE of those 257 genes for which PCCK1-driven and PTET-driven OE strains were available. These genes encode 48 PKs (44% of all annotated C. albicans PKs), 27 PPs (66% of all annotated C. albicans PPs), 123 TFs (51% of all annotated C. albicans TFs) and 59 other proteins related to signalling (Fig. 3B).

Screening for Genes Affecting Morphogenesis upon PCCK1-driven OE

The ability of C. albicans to switch between yeast and hyphal forms is considered a major requirement for virulence and biofilm formation [11,45–48]. Thus, we performed a screen to identify C. albicans genes whose PCCK1-driven OE triggers pseudohyphal or hyphal growth under conditions that normally promote yeast growth. As shown in Fig. 2A and 4, gluconeogenic conditions required for expression from PCCK1 are associated with growth in the yeast form only. Hence, the 257 C. albicans PCCK1-OE strains described above were grown individually in YNB 2% casamino acids at 30°C for 18 h and the cultures were observed microscopically. Eleven strains displayed pseudohyphal or hyphal growth in inducing conditions as shown in Fig. 4. The corresponding genes are listed in Table S2 and included 9 TFs, 1 PP and 1 PK subunit.

Seven of these genes have been previously associated with morphogenesis including those encoding the Ccn1 G1 cyclin and the Cas5, Fkh2, Rfg1, Sfl1, Sfl2 and Brg1 transcription factors (Table S2). Indeed, inactivation of CCN1, CAS5, FKH2, SFL2 and BRG1 results in defects in filamentation [13,17,30,34,49–54]. On the other hand, Sfl1 and Rfg1 have been described as repressors of filamentation in C. albicans [10,13,35–38]. However, the role of RFG1 is not restricted to this function since its OE triggers pseudohyphal growth [59]. Noticeably, OE of SFL2 and BRG1 has been previously shown to trigger hyphal growth [30,54,60,61]. In contrast, several genes identified in this screen were not known for their role in morphogenesis including those encoding the Sa6 phosphatase and the Suc1, Grf10, and Orf19.217 putative transcription factors (Table S2). Interestingly, we have observed that inactivation of GRF10 and ORF19.217 did not impair morphogenesis on a variety of hypha-inducing media despite the effect of their OE on morphogenesis (data not shown). These observations are concordant with results published by Homann et al. [13]. Thus, these results confirmed previous published data obtained with either KO or OE strategies and indicated that our OE approach could reveal genes with novel roles in C. albicans pseudohyphal or hyphal differentiation.

Screening for Genes Affecting Morphogenesis in Liquid Medium upon PTET-driven OE

Next, we performed a similar screen with the PTET-driven OE collection. Indeed, gene OE from PTET is highly advantageous since it can be used in any medium supplemented with a tetracycline derivative, while PCCK1-driven OE is strictly dependent upon gluconeogenic growth conditions. Moreover, we have observed that the level of OE is considerably higher with the PTET system (Fig. 2C). Thus, the 257 C. albicans PTET-driven OE strains were grown individually in liquid YPD supplemented with 3 µg.mL⁻¹ ATc at 30°C for 18 h and the cultures were observed microscopically. In these conditions, we observed that PTET-driven OE of 21 genes induced filamentation or pseudofilamentation (Fig. 5A and Table S2), among which 6 exhibited a weak phenotype (Fig. S2). This gene set included BRG1, SFL2, SFL1, RFG1, CAS5, FKH2 and ORF19.217 already identified in our screen of PCCK1-driven OE strains. In contrast, we did not observe filamentation upon PTET-driven OE of CAS5, GRF10, SALS and CCN1. Finally, PTET-driven OE of 14 additional genes triggered pseudofilamentation and/or filamentation (Fig. 5A, Fig. S2 and Table S2). These included TEC1, EFH1, CPH1, PCL1, RAD53, SKN7 and STE11 whose role in morphogenesis was previously uncovered using KO [17,18,30,62–72] and OE mutants
Figure 2. Functionality of the Gateway® OE systems. A. $P_{PKC1}$-driven and $P_{TET}$-driven OE of $UME6$ but not GFP triggers morphogenesis. C. albicans strains with integrated Clp10-P$_{PKC1}$-GTW-TAPtag or Clp10-P$_{TET}$-GTW derivatives harbouring the GFP (CEC2407 or CEC2992, respectively) or $UME6$ (CEC1097 or CEC2994, respectively) ORFs were observed microscopically upon growth in gluconeogenic conditions.
or YPD supplemented with 50 µg.mL⁻¹. Dax at 30°C for 18 h. Scale bar = 5 µm. B. Production of TAPTagged proteins. C. albicans strains with integrated Clp10-PCK1-GTW-TAPTag derivatives harbouring the GFP (CEC2407) or UME6 (CEC1097) ORFs were grown in SD (−) or YNB 2% casamino acids (+) for 6 h. Whole cell extracts were separated by SDS-PAGE and probed with a peroxidase-coupled antibody allowing the detection of TAPtagged proteins in gluconogenic conditions. Proteins of interest are indicated by an arrow along with their deduced size. C. Kinetics of expression from the PCK1 or TET promoters. C. albicans strains with integrated Clp10-PCK1-GTW-TAPTag or Clp10-PCL1-GTW derivatives harbouring the GFP (CEC2407 or CEC2992, respectively) or gLuc59 (CEC1906 or CEC3083, respectively) ORFs were grown in YNB 2% casamino acids or YPD supplemented with 3 µg.mL⁻¹ ATc for 18 h at 30°C. Data represent luciferase specific activity detected from the different strains at the indicated time points of growth under inducing conditions. Assays were performed in duplicate and means and SD are shown.

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[30,45,65–67]. TEC1 and CPH1 are well-characterized regulators of morphogenesis. TEC1 encodes a TEA/ATTS transcription factor regulating hypha-specific genes as well as biofilm formation and pheromone signalling [11,29,64]. CPH1 encodes a transcription factor required for mating and hyphal growth on solid media and lies in the same Cek1-MAPK pathway than the Ste11 protein [62,73,74]. PCL1 encodes a cyclin homolog whose expression is induced upon filamentous growth [56]. It was also recently shown to be required for agar invasion at elevated temperature [72]. The Rad53 protein kinase is involved in DNA-replication and DNA-damage checkpoint pathways and its deletion is known to nearly eliminate cells affected in both pathways activation and can be used to identify targets of genes whose OE is toxic [23,27]. Hence, we assessed to what extent our P TET-driven OE system could trigger changes in C. albicans growth rate.

The 257 P TET-driven OE strains were surveyed during a growth kinetic in 96-well plates in the presence or absence of ATc. For each strain, a ratio equalling to the doubling time monitored in non-inducing conditions (YPD) divided by the doubling time observed under inducing conditions (YPD supplemented with 3 µg.mL⁻¹ ATc) was calculated. This identified 17 genes whose OE decreased C. albicans growth rate (≥2 fold) and 2 genes whose OE increased C. albicans growth rate (≥2 fold) (Fig. 6A). The latter two genes encode the bZIP domain-containing protein of the ATP/CREB family Rca1 and a putative TF of unknown function, Orf19.4125 (Fig. 6A). We did not observe other phenotypes associated with the OE of these genes in a wild-type strain (data not shown). The ral1Δ mutant is viable but slow-growing and displays increased invasive growth [13,17], consistent with our observations. This gene clearly plays important roles in C. albicans biology since it controls both the susceptibility to different antifungals [20] and carbonic anhydrase expression via the cAMP/PKA/Elg1 signalling pathway [78].

A majority of the genes whose OE resulted in decreased growth rate (13/17 genes or 76.4%) were among those whose OE triggered filamentation (Fig. 5A, 5B and 6A). Indeed, filamentation results in optical density readings that are not correlated with the actual growth rates. Nevertheless, we observed cell death when the BEM1, TCK2 or EFH1 genes were overexpressed (Fig. 6B). Decreased growth was also observed when strains overexpressing TCK2 or EFH1 were grown on solid medium (Fig. 6C). BEM1 has previously been shown to be essential in C. albicans [79,80]. Despite the requirement of this gene for pseudohyphal and hyphal growth in S. cerevisiae and Yarrowia lipolytica, respectively [81,82], the role of Bem1 in C. albicans morphogenesis remains unclear [79,80]. None of the two other genes (TCK2 and EFH1) were previously associated with cell growth. In particular, TCK2 encodes a plasma membrane protein similar to the highly conserved serine/threonine casein kinase 1 (CK1) of S. cerevisiae and plays role in damaging oral epithelial cells and hyphal branching [83]. Thus, our results revealed genes involved in C. albicans fitness, including genes not previously described for such a role. However, if one excludes genes whose OE triggers morphogenesis, these genes represent a minor fraction of those we have tested. This is despite the fact that many of them have regulatory functions and suggests that C. albicans might be more robust than S. cerevisiae to the

Impact of P TET-driven OE on C. albicans Growth Rate

In the yeast S. cerevisiae, OE of up to 15% of the gene repertoire results in growth defects at the colony level. This is often due to pathway activation and can be used to identify targets of genes whose OE is toxic [23,27]. Hence, we assessed to what extent our P TET-driven OE system could trigger changes in C. albicans growth rate.

Screening for Genes Affecting Morphogenesis in Solid Medium upon P TET-driven OE

We additionally performed a screen of our P TET-driven OE strain collection on YPD solid medium supplemented with 3 µg.mL⁻¹ ATc and identified 17 genes whose OE triggered filamentation (Fig. 5B). Noticeably, three of these genes had not been identified in the screen performed in liquid conditions, namely SFU1, ORF10 and ORF19.7227. Moreover, the phenotype associated to the OE of CSRI and KNS1 was relatively weak (Fig. 5B). CSRI (or ZAP1) encodes a zinc-finger TF involved in zinc homeostasis and in regulation of biofilm matrix production [33,75]. It has been shown that deletion of CSRI/ZAP1 affects filamentous growth [10,13,17,33,76]. Similarly, a homoygous transposon insertion in ORF19.7227 that encodes a putative protein phosphatase inhibitor (PPI) decreases colony wrinkling but does not block true hyphal growth in liquid media [16], consistent with our observations. In contrast, the putative Ser/Thr PK KNS1 and the TF SF1 had not been previously associated to a function in filamentous growth. Indeed, KNS1 remains uncharacterized and SFU1 encodes a transcriptional repressor of iron-responsive genes [77].

We also noted that five of the 18 genes identified in the screen in liquid medium were not recovered in the screen on solid medium [PCL1, RFG1, CAS5, RIM11, ORF19.4125; Fig. 5A and B].

Taken together, the three screens performed using our P TET-driven and P PCK1-driven OE strain collections showed overlaps but specificities in the sets of genes that were identified based on the
Figure 3. Establishment of two collections of \textit{C. albicans} OE strains. A. Schematic of the OE strain construction pipeline. \textit{C. albicans} ORFs were amplified from their start codon to their penultimate codon and cloned into the pDONR207 vector using Gateway®-mediated recombination. The resulting plasmids were analyzed individually by Sanger sequencing of the ORF-5' and 3' ends and in pools by Illumina/Solexa sequencing. Validated ORFs were transferred into Clp10-\textit{P}_{\text{PCK1}}-GTW-TAPtag or Clp10-\textit{P}_{\text{TET}}-GTW and the resulting plasmids were introduced at the \textit{RPS1} locus in \textit{C. albicans} strain CEC161 or CEC2907, respectively. Production of a TAPtagged protein of the appropriate size was subsequently tested by Western-blot analysis of protein extracts of the \textit{C. albicans} OE strains grown in YNB +2% casamino acids medium for 18 h in the case of the \textit{P}_{\text{PCK1}}-driven OE strains. For each step the success rate is indicated along with the number of validated plasmids or strains that have been obtained. B. Distribution of the 257 overexpressed ORFs overlapping both collections across functional categories.

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Overexpression Strategy in *C. albicans*

- **GFP**
  - ~62kDa

- **UME6**
  - ~180

- **SUC1**
  - ~90

- **SFL2**
  - ~110

- **GRF10**
  - ~110

- **BRG1**
  - ~85

- **SFL1**
  - ~130
  - ~260

- **CAS5**
  - ~105

- **FKH2**
  - ~100

- **SAL6**
  - ~110

- **ORF19.217**
  - ~80

- **CCN1**
  - ~100
deleterious effects of gene OE and/or that our OE system is not sufficient to reveal such phenotypes.

Conclusions

In this study, we have reported the development of two collections of OE strains and the potential of OE screens in uncovering novel components of regulatory pathways in C. albicans. To date, OE screens have been rarely used for the identification of genes conferring specific phenotypes in C. albicans. Fu et al. [28] have established a collection of 26 C. albicans OE strains whereby genes encoding GPI-anchored proteins are overexpressed from a tetracycline-repressible promoter [84]. Sahni et al. [29] have constructed a collection of 107 C. albicans OE strains whereby genes encoding transcription factors are overexpressed from a tetracycline-inducible promoter [39]. These collections were developed using promoter replacement at the targeted gene through a split-marker strategy [28] or allelic exchange between an ADH1 allele and an OE plasmid obtained by restriction enzyme mediated cloning [39]. Therefore, these resources lack some of the versatility and reusability that is associated with the partial C. albicans ORFeome and collections of OE plasmids developed here. Indeed, our strategy was based on the highly efficient Gateway® recombinatorial cloning methodology [35,85] that provided the possibility to shuttle ORFs between plasmids allowing gluconeogenesis- or tetracycline-inducible expression and production of tagged or untagged proteins. Moreover, because our OE plasmids used an integrative vector that is targeted highly efficiently to the C. albicans RPSI locus, development of collections of OE strains in various genetic backgrounds is rather straightforward (AN, SBB and CE, unpublished data). Hence, our work has laid the ground for the establishment of a C. albicans ORFeome and a genome-wide collection of C. albicans OE strains, a collaborative project that is ongoing in our laboratory and that of C. Munro (University of Aberdeen; [86]).

Several of the genes that we have identified in our morphogenesis alteration screen were already known for their role in filamentation such as BRG1, CPH1, SFL2 and TEC1, thus validating our screen (Table S2). However, by comparison with previous studies, we noticed that OE of three genes present in our previous studies, we noticed that OE of three genes present in our

Materials and Methods

Strains and Media

All C. albicans strains used in this study are listed in Table 1. Strains were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SD minimal medium [0.67% yeast nitrogen base (YNB, Difco) with 0.4 or 2% glucose] supplemented if necessary with arginine, histidine and uridine, at 20 mg L−1 and 2% agar for solid media. OE from PCK1 was triggered in YNB plus 2% casamino acids liquid cultures at 30°C whereas OE from P_TET was induced by the addition of 50 μg ml−1 doxycycline (Dox - Fluka) or 3 μg ml−1 anhydrotetracycline (ATc - Fisher Bioblock Scientific) in YPD at 30°C. ATc was preferred over Dox as this semi-synthetic tetracycline derivative has been described for its lower toxicity and its higher efficiency in the binding of the TetR repressor protein [92]. Furthermore, we have observed that 2 μg ml−1 ATc reproduced the effect of 50 μg ml−1 Dox, either on solid or in liquid medium and this concentration was not deleterious for growth or morphogenesis of C. albicans (Fig. S1 and data not shown). Dox- and ATc-containing cultures were maintained in the dark as these compounds are light sensitive.

Plasmids harbouring a Gateway® cassette were propagated in Escherichia coli strain TOP10 ccdB (Invitrogen). Other plasmids were propagated in E. coli strain DH5α [93]. E. coli strains were grown in LB medium. Antibiotics were used at the following
Figure 5. P_{TET}-driven OE screens confirm results obtained with the P_{PKI} promoter and reveal the role of other *C. albicans* genes in morphogenesis. A. P_{TET}-driven OE of 21 genes promotes pseudo-filamentation or filamentation in liquid media. *C. albicans* strains with integrated Clp10-P_{TET}-GTW derivatives harbouring ORFs for the indicated genes were grown in YPD or YPD supplemented with 3 μg mL⁻¹ ATc.
for 18 h. Both cultures were observed microscopically and revealed OE-associated pseudofilamentation or filamentation. Genes whose $P_{PCK1}$-driven OE results in pseudo-hyphal or hyphal growth are shown on the left panel whereas others genes are placed on the right panel. OE of 15 genes showing the strongest phenotypes are represented, the other 6 are shown in Fig. S2. Scale bar = 5 µm. B. $P_{PCK1}$-driven OE of 17 genes promotes filamentation on solid media. Cultures of $C. albicans$ wild-type strains with integrated Clp10-P$_{PCK1}$-GTW derivatives harbouring ORFs for the indicated genes were spotted on YPD or YPD supplemented with 3 µg·mL$^{-1}$ Aτc and were observed after 5 days of growth at 30 °C. C. Overlap between the three morphogenesis screens. This venn diagram was obtained with the online software Gliffy (http://www.gliffy.com) and summarises results obtained in the screens performed with the $P_{PCK1}$ and $P_{TET}$ promoters. Circle size is proportional to the number of genes identified.

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Construction of a $C. albicans$ Partial ORFeome

The detailed method for the cloning of $C. albicans$ ORFs in the pDONR207 vector has been described [94]. Briefly, for each of the selected ORF, a forward primer including the attB1 site and the first 10 codons of the ORF and a reverse primer including the attB2 site and the last ten codons of the ORF were designed and synthesized at Pasteur-Genopole-Ile-de-France oligonucleotide synthesis platform (Table S1). ORFs were amplified from genomic DNA of $C. albicans$ strain SC5314 [95] using Eppendorf Triple Master Taq polymerase and 30 cycles of amplification with elongation time varying from 1 to 3 min. according to the ORF size. The resulting PCR products were checked by agarose gel electrophoresis, ethanol precipitated and, following resuspension in Tris-EDTA (TE), mixed with the donor plasmid pDONR207 (Invitrogen), and subjected to a recombination reaction with Invitrogen Gateway® BP Clonase$^\text{TM}$. The recombination mixtures were transformed into E. coli strain DH5α and one transformant per ORF was selected for further study. Plasmids were prepared using the Millipore$^\text{TM}$ MultiScreen$^\text{TM}$ 96-well Filtration System and Millipore$^\text{TM}$ MultiScreen$^\text{TM}$ PLASMID. The cloned ORFs were sequenced from the 5′- and 3′- ends using Sanger sequencing. Moreover, a pool of the 347 plasmids was subjected to Illumina/Solexa sequencing in order to obtain full length sequencing of the ORFs. Sequencing reads were aligned to the ORF sequences available from the Candida Genome Database [96] using CLC Genomics Workbench version 4. Polymorphisms were compared to a database of SNPs obtained following Illumina/Solexa sequencing of $C. albicans$ strain SC5314 (Diogo et al., manuscript in preparation). All plasmids with mutations causing a non-sense mutation or a frame-shift were excluded. Among the remaining 312 plasmids, we detected 494 synonymous and 405 non-synonymous mismatches. Among these, 362 synonymous and 312 non-synonymous mismatches had also been identified by Solexa/Illumina sequencing of strain SC5314 suggesting that they correspond to genuine SNPs. This indicated that our cloning procedure was responsible for 132 synonymous and 196 non-synonymous mutations in 422 kb insert sequences corresponding to one mutation at every 1286 bp. Information on these mutations is available in Table S1.

Construction of Gateway®-compatible $C. albicans$ OE Vectors

The sequences of oligonucleotides used for cloning purposes are listed in Table S3. Two Gateway®-compatible vectors for conditional OE in $C. albicans$ were constructed. First, oligonucleotides Nco-3′Sce and Nco-3′Sce were annealed and inserted into the NcoI site of the $C. albicans$ Clp10 integrative vector [36]. This vector designated Clp10S is bearing the 18 bp I-SceI site that is not found in the $C. albicans$ genome. Then, the $C. albicans$ PCK1 promoter region ($P_{PCK1}$) was amplified from $C. albicans$ strain SC5314 genomic DNA using primers PRPKC1PR and TAPFUR. The TAPtag coding region was amplified using oligonucleotides TERPVUII and TAPFUF and plasmid pFA-TAP-URA3, a derivative of pFA-GFP-URA3 [97] where the PsA/A6r fragment harbouring the GFP coding region has been replaced by a PsA/Asr fragment carrying the TAPtag coding region amplified from plasmid pBS1479 [38] using oligonucleotides Tap1-PstI and Tap2-AsrI that allow the addition of a (Gly-Ala)$_3$ coding linker 5′ of the TAPtag coding sequence. Both PCR products were mixed and a fusion product was amplified using primers TERPVUII and PRPKC1PR and cloned into the TOPO-TA cloning vector (Invitrogen). The $KpnI$/PstI $P_{PCK1}$-TAPtag cassette was excised from the resulting plasmid and cloned into $KpnI$/EcoRV-digested Clp10S, yielding Clp10S-$P_{PCK1}$-TAPtag. The Gateway® Rb cassette was excised from pBS-RB using EcoRV and cloned into EcoRV-digested Clp10S-$P_{PCK1}$-TAPtag, yielding Clp10S-$P_{PCK1}$-GTW-TAPtag. In order to construct the Clp10S-$P_{TET}$-GTW plasmid, a tetracycline-inducible promoter ($P_{TET}$) was amplified from plasmid pTET25 [39] using oligonucleotides TETKpn and TetATGE5, and cloned into $KpnI$/EcoRV-digested Clp10S-$P_{PCK1}$-GTW-TAPtag, yielding Clp10S-$P_{TET}$-TAPtag. This vector was amplified using Vect32 and Vect33 and the PCR product was digested with EcoRV and self-ligated yielding plasmid Clp10S-$P_{TET}$ that has three stop codons downstream of the EcoRV site. The EcoRV-digested Gateway® Rb cassette was cloned into EcoRV-digested Clp10S-$P_{TET}$, yielding Clp10S-$P_{TET}$-GTW. Subsequently, a derivative of Clp10S-$P_{TET}$-GTW was constructed by $Sau$ digestion and ligation of the annealed Vect30 and Vect31 oligonucleotides. This vector was designated Clp10S-$P_{TET}$-GTW. In this vector, the I-SceI site is closer to the $RPS1$ sequences that are used for integration at the $C. albicans$ $RPS1$ locus than in the Clp10P-$P_{PCK1}$-GTW-TAPtag. Hence transformation efficiency and integration at the $RPS1$ locus are higher when using I-SceI-digested Clp10P-$P_{PCK1}$-GTW derivatives as compared to I-SceI-digested Clp10P-$P_{PCK1}$-GTW-TAPtag derivatives (data not shown). Yet, the use of $Sau$ digestion to target derivatives of these plasmids at the $RPS1$ locus is still preferred.

A collection of Clp10P-$P_{TET}$-GTW derivatives was generated by the incorporation of specific molecular barcodes. We used the set of molecular barcodes previously designed for the construction of the S. cerevisiae deletion collections [98]. Briefly, these barcodes consist of a specific 20 bp sequence flanked by universal primer sequences (U1 and U2 or D1 and D2). These barcodes were amplified by PCR using genomic DNA prepared from a pool of the S. cerevisiae heterozygous deletion collection and primers Sac-U1 and Sac-U2 or Sac-D1 and Sac-D2. The resulting PCR products were digested with SacI and ligated into SacI-digested and dephosphorylated Clp10P-$P_{TET}$-GTW. Individual clones were recovered after E. coli TOP10 cells$^R$ transformation and the cloned barcodes were sequenced. Only plasmids with a tag showing a unique ID in the TAG4 yeast barcode array and without mismatch in the common primers U1-U2 or D1-D2 were kept. In total, 936 barcoded derivatives of Clp10P-$P_{TET}$-GTW were obtained.
Figure 6. $P_{\text{TET}}$-driven OE of 20 genes results in decreased or increased growth rate. A. Impact of OE on the growth rates of 257 OE strains. Genes whose OE decreases or increases growth rate (>2 fold and >2 fold, respectively) upon growth in liquid medium are listed in red or green boxes respectively. Genes are classified based on their OE phenotype from the more affected to the less affected. Genes in orange correspond
Construction of C. albicans OE Strains

Detailed methods for the transfer of C. albicans ORFs from pDONR207 into the Clp10-P<sub>TDH3</sub>-GTW-TAPtag or barcoded Clp10-P<sub>TDH3</sub>-GTW plasmids as well as the integration of the resulting expression plasmids at the RPS1 locus have been described [94]. Brieﬂy, an aliquot of each derivative of pDONR207 was mixed with 50 ng of one of the destination plasmids and subjected to a recombination reaction with Invitrogen Gateway® LR Clonase™. The recombination mixtures were transformed into E. coli strain DH5α and one transformant was used for plasmid preparation as described above. EcoRV digestion was used to verify the cloning of the appropriate ORF. The expression plasmids bearing P<sub>Bgl</sub> were digested by Stul (or E-Sce I if necessary) and transformed into C. albicans strain CEC161 according to Walther and Wendland [99]. Transformants were selected for prototrophy and veriﬁed by PCR using primers ClpUL and ClpUR that yield a 1 kb product if integration of the OE plasmid has occurred at the RPS1 locus alternatively. The expression plasmids bearing P<sub>Bgl</sub> were transformed into C. albicans strain CEC2907 following Stul or EcoRI linearization. CEC2907 is a derivative strain of CEC161 transformed with pNMX (Fig. 1B). pNMX is a derivative of pNIM1 [39] that was modiﬁed in two steps. First pNIM1 (Fig. 1B.a) was digested by Nol I and BgIII, treated to create blunt ends and self-ligated to reconstitute an Nol I site, yielding pNIM1<sub>a</sub>-<sub>TET</sub>-GFP (Fig. 1B.b). Next, the ADH1 promoter (P<sub>ADH1</sub>) was replaced by the TDH3 promoter (P<sub>TDH3</sub>) as follows: a region of <i>car</i><i>TA</i> (containing the start codon) was excised from pNIM1<sub>a</sub>-<sub>TET</sub>-GFP with <i>Bgl</i>II and subcloned into G<sub>a</sub>-digested BLUESCRIPT-SK(-)-P<sub>TDH3</sub>. The resulting plasmid was linearized with XbaI, treated to create blunt ends and digested by XbaI. The XbaI(blunt)-XbaI fragment containing the P<sub>TDH3</sub>-<i>cartTA</i> fusion was then subcloned in pNIM1<sub>a</sub>-<sub>TET</sub>-GFP linearized with BstBI, treated to create blunt ends and digested by <i>Bgl</i>II, yielding pNIM2 (Fig. 1B.c), in which P<sub>TDH3</sub> is inserted upstream of <i>car</i><i>TA</i>. Integration of pNIMX digested with <i>Bgl</i>II and <i>Stu</i>II at the <i>ADH1</i> locus in strains CEC2907 was veriﬁed by PCR using primers NIM1<sub>verif</sub> and ADH1<sub>verif</sub>. Analysis of TAPtagged Proteins by Western Blotting

A 20 μL culture in SD or YNB 2% casamino acids was inoculated at OD<sub>600</sub> = 0.05 with a freshly grown colony. 10 ODs of exponentially growing cells were collected by centrifugation after 4–6 h of growth at 30°C, resuspended in lysis buffer (0.1 M NaOH, 0.5M EDTA, 2% SDS, 2% β-mercaptoethanol) and incubated 10 min at 90°C [100]. The lysate was neutralized with 5 μL 4 M acetic acid, incubated 10 min at 90°C and 50 μL loading buffer (0.25 M Tris-HCl pH 6.8, 50% glycerol, 0.05% bromophenol blue) were added. Proteins were separated on an Invitrogen 10% NuPage gel, transferred onto nitrocellulose and TAPtagged proteins were detected using peroxidase-coupled anti-peroxidase antibodies (Sigma) and an ECL kit (GE Healthcare).

Luciferase Assays

100 μL of YNB 2% casamino acids or YPD supplemented with 3 μg.ml<sup>-1</sup> ATC were inoculated with a freshly grown colony on SD 2% glucose resuspended in dH<sub>2</sub>O (in the case of P<sub>TDH3</sub>-driven OE strains) or an overnight culture in YPD at 30°C (in the case of P<sub>TET</sub>-driven OE strains). At each time point, a volume equivalent to 20 OD was centrifuged (2–5 min at 3500 rpm) and resuspended in 200 μL of R-luc buffer (NaCl 0.5 M, Na<sub>2</sub>HPO<sub>4</sub> 0.1 M pH 6.7,

Table 1. Strains used in this study.

| Strain name | Genotype | References |
|-------------|----------|------------|
| BWP17       | ura3Δ::imm434/ura3Δ::imm434 his1Δ::imm434/his1Δ::imm434 arg4Δ::imm434/arg4Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 | [101] |
| CEC161      | ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/HIS1 arg4Δ::hisG/ARG4 | [102] |
| CEC955      | ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/HIS1 arg4Δ::hisG/ARG4 ADH1/adh1::ADH1p-cartTA::SAT1::PTET-cartGFP | This study |
| CEC988      | ura3Δ::imm434/ura3Δ::imm434 arg4Δ::arg4Δ::hisG/HIS1 his1Δ::hisG RSPI/RPS1::Clp10-P<sub>TGDH1</sub>-glUC59 | This study |
| CEC1097     | ura3Δ::imm434/ura3Δ::imm434 arg4Δ::arg4Δ::hisG/ARG4 his1Δ::hisG/HIS1 RSPI/RPS1::Clp10-P<sub>TGADH3</sub>-UM66-TAPtag | This study |
| CEC1215     | ura3Δ::imm434/ura3Δ::imm434 arg4Δ::arg4Δ::hisG/ARG4 his1Δ::hisG/HIS1 RSPI/RPS1::Clp10-P<sub>TGADH3</sub>-glUC59 | This study |
| CEC2249     | ura3Δ::imm434/ura3Δ::imm434 arg4Δ::arg4Δ::hisG/ARG4 ADH1/adh1::ADH1p-cartTA::SAT1::PTET-cartGFP RSPI/RPS1::PTET-glUC59 | This study |
| CEC2407     | ura3Δ::imm434/ura3Δ::imm434 arg4Δ::arg4Δ::hisG/ARG4 RSPI/RPS1::Clp10-P<sub>TGADH3</sub>-GFP-TAPtag | This study |
| CEC3083     | ura3Δ::imm434/ura3Δ::imm434 arg4Δ::arg4Δ::hisG/ARG4 ADH1/adh1::PTDGADH3-cartTA::SAT1::PTET-glUC59 | This study |
EDTA 1 mM). For luciferase assays, 100 μL of cells were mixed with 20 μL of 2 μM coelenterazine before luminescence (integration time: 1000 ms) and absorbance (wavelength: 610 nm) were measured using a microplate reader (TECAN Infinite 200). The final luminescence value is obtained by the following formula: Luminescence unit/Absorbance.

Spotted Assays
Strains in the P_{TEt}‐driven OE collection were grown in 96‐well plate in YPD (30 h; 30°C) and spotted on YPD plates supplemented or not with 3 μg.mL⁻¹ ATc using the RoToR robot (Singer Instrument). Alternatively, 5‐fold serial dilutions of 3 mL overnight cultures in SD at 30°C were spotted on SD plates supplemented or not with 3 μg.mL⁻¹ ATc. In both cases, plates were grown at 30°C for 2–5 days and scanned with Epson perfection 4490. Spotting assays were performed in triplicate.

Growth Kinetics
Strains in the P_{TEt}‐driven OE collection were grown in 96‐well plates in YPD (30 h; 30°C) and inoculated at a final OD₆₀₀ = 0.1 in 100 μL YPD supplemented or not with 3 μg.mL⁻¹ ATc. Growth at 30°C was monitored every 20 minutes using a microplate reader (TECAN Sunrise). Doubling‐time (DT) was calculated by dividing the doubling time between OD₆₀₀ = 0.15 and OD₆₀₀ = 0.6. Growth curves were performed in triplicate.

Microscopy and Image Analysis
Cells were observed with a Leica DM RXA microscope (Leica Microsystems). Images were captured with a Hamamatsu ORCA II‐ER cooled CCD camera, using the Openlab software version 3.5.1 (Improvision Inc.), and then processed with Adobe Photoshop 10.0 software.

Supporting Information
Figure S1 Comparison of doxycycline (Dox) and anhydrotetracycline (ATc). A. 50 μg.mL⁻¹ Dox or 2 μg.mL⁻¹ ATc induce P_{TEt} to a similar extent. C. albicans strains with integrated Clp10‐P_{TEt}‐GTW derivatives harbouring ORFs (CEC2992 or CEC3083, respectively) were grown in YPD supplemented with 50 μg.mL⁻¹ Dox or 2 μg.mL⁻¹ ATc for 18 h at 30°C. Data represent luciferase specific activity detected from the different strains at 0 and 18 h of growth under inducing conditions. Assays were performed in duplicate and means and SD are shown. B. Effects on morphogenesis are similar between 50 μg.mL⁻¹ Dox and 2 μg.mL⁻¹ ATc. C. albicans strain SC5314 and a strain overexpressing UME6 (CEC2994) were grown in YPD medium and spotted on YPD medium supplemented or not with tetracycline analog (50 μg.mL⁻¹ Dox or 2 μg.mL⁻¹ ATc). Pictures were taken after 5 days of growth at 30°C. C. ATc shows lower inhibition of C. albicans hyphal growth than Dox. C. albicans strain SC5314 was grown in YPD liquid medium supplemented or not with different concentrations of Dox or ATc for 18 h at 30°C and observed microscopically. Scale bar = 5 μm.

Table S1 Summary of the collections provided in this study.

Table S2 Candida albicans genes whose P_{PKet}‐driven or P_{TEt}‐driven OE triggers pseudohyphal or hyphal growth.

Table S3 Oligonucleotides used in this study.

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Author Contributions
Conceived and designed the experiments: AN SG SBB AF CE. Performed the experiments: MC AN VC SZ SG SBB AF ML DD TR. Analyzed the data: AN DD TR CE. Wrote the paper: AN CE. Revised the manuscript: MC SZ SBB AF ML DD TR.

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