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***Candida glabrata* encodes a longer variant of the mating type (*MAT*) alpha2 gene in the mating type-like *MTL3* locus, which can form homodimers.**

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Abstract

The fungal pathogen *Candida glabrata* is a haploid asexual yeast. *C. glabrata* contains orthologues of the genes that control mating and cell type identity in other fungi, which encode putative transcription factors localized in the *MAT* locus in *Saccharomyces cerevisiae* or *MTL* in other fungi. *C. glabrata* contains three copies of the *CgMTL* locus but only *CgMTL1* correctly expresses the information encoded in it. *CgMTL1* can encode the *Cga1* gene (**a** information), or the *Cgalpha1* and *Cgalpha2* genes (alpha information). *CgMTL2* contains an identical copy of the *Cga1* gene. *CgMTL3* contains an identical copy of the *Cgalpha1* gene but a longer variant of the *Cgalpha2* gene we termed *Cgalpha3*. In *S. cerevisiae* diploid cells that express *Sca* and *Scalpa* information, *Sca1* and *Scalpa2* proteins form a heterodimer, which represses genes expressed only in haploid cells and some genes involved in stress response. We constructed *C. glabrata* strains that simultaneously express *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* genes. We did not find any phenotype in these strains when grown under a large variety of stress and nutritional conditions. However, we detected an interaction between *Cga1* and *Cgalpha2* but not between *Cga1* and *Cgalpha3* by Bimolecular Fluorescence Complementation and CoIP assays.

Introduction

Candida glabrata is a fungal pathogen of humans that can cause severe systemic infections in immunocompromised patients and has not been observed to undergo sexual reproduction. However, it contains the vast majority of genes known to be required for mating (Fabre, et al. 2005: 856-73, Wong, et al. 2003: R10). In many fungi cell type determination, which is necessary for sexual reproduction, is controlled by two different alleles encoded in the mating type locus, called *MAT* in *Saccharomyces cerevisiae* or *MTL* in other fungi like *C. glabrata*, reviewed in (Butler 2010: 140-59).

C. glabrata, like *Saccharomyces cerevisiae* and the rest the *Saccharomycetaceae* family, contains in its genome three copies of the *MTL* loci, called *CgMTL1*, *CgMTL2* and *CgMTL3* (Hanson, et al. 2014: E4851-8). *CgMTL1* and *CgMTL2* are transcriptionally active while *CgMTL3* is subject to subtelomeric silencing (Muller, et al. 2008: 848-58, Ramirez-Zavaleta, et al. 2010: 1602-11, Srikantha, et al. 2003: 328-40, Yanez-Carrillo, et al. 2014: 30-4).

In *C. glabrata*, *CgMTL1*, can contain **a** information which consists of the *Cga1* gene (**a** cells), or alpha information containing the *Cgalpha1* and *Cgalpha2* genes (alpha cells). *CgMTL2* contains **a** information and *CgMTL3* alpha information. The *Cga1* gene contains two introns, but the transcript is completely processed only when the transcription originates in *CgMTL1a* but not from *CgMTL2a*, and since *CgMTL3* is silenced, only the genes contained in *CgMTL1* are correctly and efficiently expressed (Muller, et al. 2008: 848-58, Ramirez-Zavaleta, et al. 2010: 1602-11, Srikantha, et al. 2003: 328-40, Yanez-Carrillo, et al. 2014: 30-4).

The distantly related opportunistic human fungal pathogen *Candida albicans*, is a diploid yeast which has a para-sexual cycle and a single *MTL* locus (*CaMTL*), that controls cell type and sexual

reproduction. In fungi of the *Saccharomyces* lineage, like *S. cerevisiae*, and in fungi of the *Candida* clade like *C. albicans*, the *Caa1*, *Caalpha1* and *Caalpha2* genes encode transcription factors. *Sca1* and *Scalpha2* are homeodomain-containing proteins while *Scalpha1* is an *Scalpha* domain-containing DNA binding protein. Different combinations of these transcription factors control the expression of a set of cell type-specific genes (**a** or alpha specific genes), as well as a group of genes expressed only in cells that have not mated (haploid specific genes) (Baker, et al. 2011: 7493-8, Smith and Johnson 1992: 133-42); reviewed in (Butler 2010: 140-59, Haber 2012: 33-64, Johnson 1995: 552-8). Many of the haploid specific genes confer to each cell type the ability to mate with the opposite cell type, and others are involved in response to stress. In contrast to **a** or alpha haploid cells, mating of **a** and alpha cells, results in diploid cells that express simultaneously **a** and alpha information, which results in the formation of a heterodimer composed by the proteins *Sca1* and *Scalpha2* (*Sca1*-*Scalpha2* heterodimer) that represses transcription of the haploid specific genes and some other genes involved in response to stress (Galgoczy, et al. 2004: 18069-74).

Even though the general structure of the *CgMTL* loci is conserved with *S. cerevisiae*, there are important differences in the regulation and function of the genes encoded in these loci between these two closely related yeasts. For example, we have found that in *C. glabrata* there is no cell type-specific regulation of expression of genes regulated by the mating-related genes (*Cga1*, *Cgalpha1* and *Cgalpha2*), and no sexual reproduction has been detected (Butler 2010: 140-59, Ramirez-Zavaleta, et al. 2010: 1602-11). These differences between *C. glabrata* and *S. cerevisiae* despite the conservation of the mating-related genes, have led us to ask whether the *C. glabrata* *MTL*-encoded genes have a different function from that of the genes encoded at the *ScMAT* locus. To start answering this question,

we have constructed strains of *C. glabrata* expressing simultaneously the alpha and **a** information to test whether these strains can form a heterodimer between *Cga1* and *Cgalpha2*, and whether they display measurable phenotypes under a large variety of growth conditions. In the process of constructing these strains, we discovered that the alpha information encoded in *CgMTL3* is not identical to the alpha information encoded in *CgMTL1* in the *C. glabrata* sequenced strain CBS138, which contains alpha information at *CgMTL1* (Table S1).

In this paper we show that *C. glabrata* contains a longer variant of the *Cgalpha2* gene that we have called *Cgalpha3*, which is present only in *CgMTL3* (and not in *CgMTL1*alpha) in all the clinical isolates we have sequenced as well as in the CBS138 reference strain. We show that simultaneous expression of *Cga1* and either *Cgalpha2* or *Cgalpha3* in *C. glabrata* cells, results in no measurable phenotype compared to the parental strain BG14 (Table S1; (Cormack and Falkow 1999: 979-87). However, we were able to detect the formation of *Cga1*-*Cgalpha2* heterodimers but not *Cga1*-*Cgalpha3* heterodimers by Bimolecular Fluorescence Complementation assay (BiFC), although we could detect coimmunoprecipitation of *Cga1* and *Cgalpha3*. We also detected efficient *Cgalpha3* homodimer formation but no *Cgalpha2* homodimers by BiFC as measured by BiFC and coimmunoprecipitation assays (Sung and Huh 2007: 767-75).

Materials and Methods

Strains, Plasmids and Primers. All yeast and *E. coli* strains used in this work are listed in Table S1 in supporting information. Plasmids and oligonucleotides used are listed in Tables S2 and S3 respectively (supporting information).

Culture Media. Yeast cells were grown in standard yeast media as described previously (Sherman 1986), and 2% agar was added for plates. We used a variation of synthetic complete (SC), which contains 1.7 g/L yeast nutrient base (without NH_2SO_4 or aminoacids), 5 g/L NH_2SO_4 and supplemented with 0.6% of casamino acids and 2% glucose. When needed, SC was supplemented with 25 mg of uracil/L. Minimal media (YNB) contains 1.7 g/L yeast nutrient base (without NH_2SO_4 or aminoacids), 5 g/L NH_2SO_4 and 2% glucose. To select for nourseothricin resistance (Nat^{R}) in minimal medium, we used SED media, which contains 1.7 g/liter yeast nutrient base (without NH_2SO_4 or aminoacids), 1 g/L sodium glutamate and 2% glucose. Nat (GoldBio) was added to a concentration of 50 $\mu\text{g}/\text{mL}$. Rich media, YPD medium, which contains 10 g/L yeast extract, 20 g/L peptone and supplemented with 2% glucose and uracil 25 mg/mL. When required, YPD plates were supplemented with hygromycin (A.G. Scientific) at 440 $\mu\text{g}/\text{mL}$ or Nat 100 $\mu\text{g}/\text{mL}$. For media with pH adjusted to 3.5 and 6.0, 5N HCl was used. The media containing different stress producing agents, or with varying nutritional conditions (such as carbon and nitrogen sources), were prepared according to (Homann, et al. 2009: e1000783) with some modifications. The following compounds were added to rich media at the concentrations indicated in the text: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, LiCl, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, CdSO_4 , Fluconazole, SDS, calcofluor white, D-Sorbitol, NaCl, H_2O_2 , menadione, hydroxyurea (HU) and $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]$ (nitroprusside or NPS).

OFF media for *MET3* promoter: the media used was YNB supplemented with a mixture of almost all amino acids as described by (Zordan, et al. 2013: 1675-86) except that L-Tyrosine, PABA and Myo-Inositol were not added to the mix. ON media for *MET3* promoter: media was the same as the OFF conditions but omitting also methionine and cysteine.

Bacteria strains were cultured in Luria-Bertani (LB) as described (Ausubel 2001). LB medium contains 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl. All plasmids were introduced into *Escherichia coli* strain DH10 by electroporation, and 50 µg/mL carbenicillin (Invitrogen) was added to select for plasmids. For LB solid media, 1.5% agar was used.

Yeast transformation. For transformation of *C. glabrata* with either supercoiled or linearized plasmids, we followed the LiOAc method previously described (Castano, et al. 2003: 905-15).

Construction of strains expressing simultaneously *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* from *CgMTL1*

We used strain CGM531 (*mtl1,2,3*) Δ (Table S1, supporting information) as recipient strain for the construction of *Cga1* and *Cgalpha2* or *Cgalpha3* expressing strains from *CgMTL1* locus. The strains were constructed by the two-step replacement method. For the *CgMTL1a1*-*alpha2* strain, plasmid pRM4 (Table S2) containing *Cga1* and *Cgalpha2* genes with their respective promoters and the flanking upstream and downstream regions from *CgMTL1*, was digested with *MfeI* and integrated at the *CgMTL1* locus by homologous recombination after transformation into strain CGM531 [(*mtl1,2,3*) Δ , Table S1]. The correct integration at *CgMTL1* was confirmed by PCR with the appropriate oligos (329 and 291 for the 5' side and 14 and 161 for the 3' side, Table S3). Cells of the integrant strain were grown in non-selective rich media to allow for homologous recombination and

excision of the vector. The correct segregant in which the *mtl1Δ* locus had been replaced by the construct expressing *Cga1-Cgalpha2* was identified by PCR (oligos 329 and 161 to generate a PCR fragment across the construct). To generate the strain containing *CgMTL1a1-alpha3*, we used the same strategy as above, except pRM50 digested with *MfeI* was used to integrate at *CgMTL1* by homologous recombination. Oligos for diagnostic PCR of the integrant were 772 and 329 for the 5' side and 14 and 149 for the 3' side. Oligos to identify the correct segregant were 771 and 330 for the 5' end and 334 and 161 for the 3' end. Even though we don't have antibodies to detect the native *Cgalpha2* and *Cgalpha3* proteins in these strains, we have generated N-terminal fusions of these proteins with mCherry and expressed them under the copper inducible P_{MTI} promoter. The cells grown under inducing conditions (1 mM CuSO_4), clearly show red fluorescence mostly colocalizing with the nucleus and also in the rest of the cell in the case of *Cgalpha3*. In the case of *Cga1* we tagged this protein at the N-terminus with the Flag epitope expressed under the P_{MTI} promoter and we could easily detect the fusion protein by Western blot (data not shown).

Construction of fusion plasmids for Bimolecular fluorescence complementation assay (BiFC) for *C. glabrata*.

We generated a set of plasmids (Table S2, supporting information) derived from pCU and pCN (Zordan, et al. 2013: 1675-86), which contain the inducible promoter of the gene *MET3* followed by a multiple cloning site. One set of plasmids contains the N-terminal fragment of the optimized version of YFP called Venus (VN) after the multiple cloning site, a second set of plasmids contains the C-terminal fragment of Venus YFP (VC). After the VN or VC fragments, we cloned in frame the sequence for 5 repetitions of amino acids G and A which function as a linker between the Venus

fragment and the corresponding *MTL* gene (Fig. 4A). VC and VN with the linker sequence were synthesized as PCR fragments using oligos 1687, 1685, 1694 and 1692 in which the reverse primer contains the linker sequence (Supplementary Table S3). The PCR reaction was performed with high fidelity enzyme. The PCR product was digested with the appropriate enzymes and cloned into pCU or pCN digested with *XbaI* and *SpeI* to generate plasmids pRM90, pRM101 y pRM103. In these recipient vectors we cloned in different combinations *Cga1* cDNA, *Cgalpha2* and *Cgalpha3* generated by PCR using the primers designed with the appropriate restriction enzyme sites and the high fidelity enzyme iProof (BioRad). Recipient plasmids were sequenced. The genes that were cloned into the recipient vectors were also sequenced and the junction of the fusion was verified to be correct. All sequencing reactions were done in LANBAMA-IPICYT. Details of the recipient vectors will be described elsewhere (manuscript in preparation).

Bimolecular fluorescence complementation assay (BiFC). Strains containing the appropriate combinations of two plasmids were grown overnight in OFF media (see Culture Media). After 12 h of growth, cells were washed twice with sterile water and resuspended in the ON media, which lacks Met and Cys. The OD₆₀₀ was adjusted to 0.2 and cells were incubated at 30°C for 2.75 h after which aliquots were taken and fluorescence was measured in a FACScalibur flow cytometer (BD Biosciences). Baseline levels of the cytometer were adjusted using a strain containing two vectors, one with the VN-YFP fragment and the other with VC-YFP fragment but without gene fusions (non fluorescent). The levels of this strain were adjusted to a value of 10 units of fluorescence. This strain culture was gated using forward scatter and ten thousand events were collected and measured per sample. As positive control, we used N-terminal fusions of *Scalpha2* with either the VC or the VN YFP

fragments expressed simultaneously in the (*mtl1,2,3*) Δ strain of *C. glabrata*. The negative control was coexpression in the (*mtl1,2,3*) Δ strain of *C. glabrata* of *Scalpa2-VC* and *Scalpa2-VC*. Experiments were performed 3 times independently. The results shown were calculated as follows: we obtained the average of the geometric mean (arbitrary units of fluorescence) for each strain in the ON media and the mean value of the geometric mean of the negative control was subtracted to each strain. The differences that were significant are indicated by an asterisk

Sensitivity assay to chronic stress on solid media. Cell cultures of each strain were grown in YPD to stationary phase (48 h) at 30°C with shaking. Each culture was diluted to an OD₆₀₀ of 0.5 in sterile water. This cell suspension was serially diluted in 96-well plates to 10⁻⁵ and equal volumes of each dilution were spotted onto YPD plates and YPD containing different concentrations of each compound tested. Plates were incubated at 30°C for 7 days and photographed every day after day 2. Each experiment was repeated at least 3 times.

Growth curves in liquid media and calculation of duplication time. To quantitate duplication times of each strain in different stress conditions we used the method previously described with some modifications (Gutierrez-Escobedo, et al. 2013). Cells were grown in YPD liquid media to stationary phase (48 h). The OD₆₀₀ of each culture was adjusted to 0.01 in each media to be tested (YPD at pH 3.5, pH 6.0 and pH 7; or YPD with NaCl at 0.5M, 1.0M and 1.5M). 300 μ L of each cell suspension were transferred to 100-well plates (Honeycomb plates, Growth Curves, USA) and incubated at 30°C with continuous shaking in a Bioscreen C equipment (Growth Curves, USA). OD₆₀₀ was continuously monitored every 15 min for 24 to 48 h. To calculate duplication times of each strain, only the data

corresponding to the logarithmic phase of growth of each curve were used for each medium tested, as described in (Murakami, et al. 2008: 113-21).

Fluconazole and hydrogen peroxide (H₂O₂) sensitivity assays and calculation of MIC₅₀. Stationary phase cultures (grown in YPD at 30°C for 48 h) of each strain were diluted in fresh YPD to obtain cell suspensions at a concentration of 10⁴ cells/mL. 150µL of each cell suspension was transferred to 100-well Honeycomb plates that already contained 150µL of YPD with different concentrations of fluconazole (from 0.0078 to 256µg/mL). Plates were incubated in a Bioscreen C machine at 35°C with shaking and OD₆₀₀ was recorded every 15 min for 24 h. MIC₅₀ values for each strain were calculated as previously described based on the dose-response curve (Orta-Zavalza, et al. 2013: 1135-48) and using the GraphPad PRISM software (San Diego, CA). Hydroxyurea and sodium nitroprusside (NPS) sensitivity determination was made in the same way except that the incubation temperature was 30°C and the concentrations used are indicated in Table 2.

Viability quantification after acute exposure to H₂O₂ and menadione. Stationary phase cultures (grown in YPD at 30°C for 48 h) of each strain were diluted in fresh YPD and incubated at 30°C with shaking. The dilutions were made so that after 8 duplications each culture would have reached an OD₆₀₀ 0.5. At this point, cultures were divided and treated with different concentrations of H₂O₂ or menadione for 2 h. Cells were then washed with sterile water and resuspended in sterile water. The OD was determined and the cell suspensions were adjusted to OD₆₀₀ 0.01 in fresh YPD and placed in 100-well Honeycomb plates to start outgrowth curves at 30°C in a Bioscreen C machine. Viability was calculated as previously described in (Gutierrez-Escobedo, et al. 2013). Experiments were performed at least 4 times.

Western Blot and Co-immunoprecipitation experiments. We constructed a set of plasmids containing N-terminal fusions of either Flag or cMyc with *Cgalpha2* and *Cgalpha3* separated by the G-A linker and under the inducible promoter P_{MTI} . We generated another compatible plasmid containing the **a1** gene fused at the N-terminus with the Flag epitope (separated by the G-A linker) under the P_{MTI} promoter (Supplementary Table S2). The appropriate combinations of plasmids were transformed in the null (*mtl1,2,3*) Δ strain. Cells were grown in SC liquid media for 12 h, and cultures were separated into two tubes. To one tube we added 2mM $CuSO_4$ to induce P_{MTI} and the equivalent volume of sterile water was added to the control tube. Both tubes were incubated for 2 h at 30°C with shaking. After the induction cells were collected by centrifugation. Protein extracts, CoIP and Western blots were performed as previously described (Orta-Zavalza, et al. 2013: 1135-48). Briefly, 1mg of total protein from each strain extract was used to immunoprecipitate Flag or cMyc tagged proteins using 50 μ L of anti-c-Myc or anti-Flag agarose beads (Sigma) and incubated for 1.5 hrs at 4°C with gentle shaking. The tubes were centrifuged and the IPs washed three times with lysis buffer after which the beads were resuspended in 40 μ L of SDS-loading buffer. These IPs were loaded onto two 12% SDS-polyacrylamide gels. After electrophoresis, the gels were blotted onto PVDF membranes (BioRad) and probed with either mouse anti-Flag (Sigma) or mouse anti-cMyc (Millipore) primary antibodies. The membranes were washed three times and then probed with goat anti-mouse HRP-conjugated secondary antibody (Amersham). The membranes were detected by using ECL chemiluminescence reagents and recorded using a BioRad ChemiDocTM MP System equipped with chemiluminescence.

Murine infection studies. Yeast cultures used to inoculate mice were grown to stationary phase (48 h). Cells were washed with sterile PBS and resuspended in 1/10 of the volume of PBS. The OD₆₀₀ was measured and the cell suspensions were adjusted to have 4x10⁸ cells/mL in PBS and cell counts were verified by OD₆₀₀, by counting in a hemocytometer and by plating serial dilutions for c.f.u. Seven to eight-week old BALB/C female mice were inoculated with a total of 4x10⁷ cells (in a volume of 100μL of each cell suspension) through tail vein injection. Each yeast strain was inoculated into 6 mice and each group was kept in different cages until day 7 post-infection when they were euthanized according to the guidelines of the Bioethics Committee for Animal Care and Treatment of our institution. The kidneys, liver and spleen from the animals were retrieved and homogenized. Different dilutions of these homogenates were plated on YPD-penicillin-streptomycin plates and incubated at 30°C for 48 h. Colony forming units (c.f.u.) were counted. Data from one representative experiment is reported in Fig. 3C using GraphPad PRISM software (San Diego, CA), but three independent experiments were made with very similar results.

RT-PCR. RNA was extracted from stationary phase cells grown in YPD using TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. RNAs were treated with DNaseI (Sigma-Aldrich, St. Louis, MO). cDNA synthesis and PCR reactions were made using AccessQuick RT-PCR kit (Promega, Madison, WI). Oligonucleotides used are listed in Supplementary Table S3 (oligos 330 and 469 for *Cga1* gene; oligos 290 and 291 for *Cgalpha2* and *Cgalpha3* genes and oligos 219 and 220 for the *ACT1* gene). cDNA synthesis was performed at 45°C for 45 min and the PCR reaction at 60°C for **a1**, 55°C for *Cgalpha2* and *Cgalpha3* and 57°C for *ACT1* gene.

Results

***Candida glabrata* encodes different versions of the *Cgalpha2* gene in the *CgMTL1*alpha and *CgMTL3*alpha loci.**

C. glabrata contains three mating type-like loci called *CgMTL1*, *CgMTL2* and *CgMTL3*, which are arranged in a similar disposition to the three mating loci of *S. cerevisiae* (Ramirez-Zavaleta, et al. 2010: 1602-11, Srikantha, et al. 2003: 328-40, Yanez-Carrillo, et al. 2014: 30-4). The vast majority of clinical isolates studied, contain **a**-type information at *CgMTL2* and alpha-type at *CgMTL3*, while *CgMTL1* can contain either **a**, or alpha information (Fig. 1A). The **a**-type information consists of the **a1** gene, and in clinical isolates with type **a** information in *CgMTL1*, both copies of the **a1** gene (at *CgMTL1* and *CgMTL2*) are identical (data not shown). The alpha information consists of the *Cgalpha1* and *Cgalpha2* genes. We initially cloned and sequenced the *Cgalpha1* and *Cgalpha2* genes from *CgMTL1* from the sequenced strain CBS138 (http://www.candidagenome.org/cgi-bin/gbrowse2/gbrowse/cglab_cbs138) and compared it with the alpha information from *CgMTL3* of our reference strain BG2 (Cormack and Falkow 1999: 979-87). Surprisingly, we found that even though the *Cgalpha1* genes present at *CgMTL1* in strain CBS138 and *CgMTL3* (in strains CBS138 and BG14) are identical, the *Cgalpha2* gene present at *CgMTL1* (CAGL0B01265g) is different from the one present at *CgMTL3* (CAGL0B00264). We have designated this gene at *CgMTL3*, *Cgalpha3* (Fig. 1B). Next we decided to determine whether *Cgalpha3* is conserved in *CgMTL3* in other *C. glabrata* clinical isolates. We cloned and sequenced *CgMTL1* and *CgMTL3* loci from 3 additional clinical isolates that contain alpha information both at *CgMTL1* and *CgMTL3*, and we found that all the isolates with alpha information at *CgMTL1* contain the *Cgalpha2* gene at this locus, while all of the isolates contain the longer version we

have designated *Cgalpha3* at *CgMTL3* (Fig. 1B). The predicted protein sequences of *Cgalpha2* and *Cgalpha3* are identical up to the first 172 amino acids, and then *Cgalpha2* has 14 amino acids more for a total of 186 amino acids, while *Cgalpha3* has 38 more amino acids for a total of 210 amino acids (Fig. 1B and Fig. S1, supporting information).

Construction of strains expressing simultaneously the *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* genes simultaneously.

In both *S. cerevisiae* and *C. albicans* simultaneous expression of **a** and alpha information results in the formation of a heterodimer between the **a1** and alpha2 proteins (**a1**-alpha2), which controls a set of genes that are important to maintain cell-type identity as well as other genes, some involved in response to stress (for example *HOG1* in *S. cerevisiae*) or virulence-related (for example *ECE1* and some others in *C. albicans*) (Miller and Johnson 2002: 293-302, Tsong, et al. 2003: 389-99).

The *CgMTL3* locus in *C. glabrata* is silenced by the assembly of silent chromatin at the left telomere of chromosome B (Ramirez-Zavaleta, et al. 2010: 1602-11). However, silencing at this locus is leaky and it is possible that in a small fraction of natural *C. glabrata* isolates that contain the *Cga1* gene in *CgMTL1* (and alpha information at *CgMTL3*), the cells could express *Cga1* and *Cgalpha3* simultaneously. To investigate the consequences of efficient simultaneous expression of both types of mating information in every cell in a population of *C. glabrata* cultures, we decided to construct two different strains. In the first strain we introduced by homologous recombination in the *CgMTL1* locus, the *Cga1* gene followed by the *Cgalpha2* gene, each one with its own promoter (see Materials and Methods). In the second strain we introduced the *Cga1* and *Cgalpha3* genes in *CgMTL1* (Fig. 2A).

These strains contain deletions of both *CgMTL2* and *CgMTL3* loci so the only source of mating information comes from *CgMTL1* (strains CGM1552 and CGM1569 respectively, Table S1 supporting information). Integration of these constructs in the *CgMTL1* locus is important since the **a1** gene contains two introns, which are correctly spliced only when the transcript originates at *CgMTL1* but not at *CgMTL2* (Muller, et al. 2008: 848-58, Ramirez-Zavaleta, et al. 2010: 1602-11). We first determined that both genes integrated in the *CgMTL1* locus were expressed in each of the new strains, using RT-PCR. As shown in Fig. 2B the *Cga1* gene is correctly expressed and processed in both constructed strains, and by comparison, the *Cga1* gene transcript originating at *CgMTL2* is expressed but not processed as previously determined. We included as a positive control an over-expressing construct in which we integrated the *Cga1* gene driven by the *PGK1* promoter, integrated in *CgMTL1* (**a1-OE**) and as negative control, we used the recipient null strain that contains no mating information [*(mtl1,2,3)*Δ strain]. The *Cgalpha2* or *Cgalpha3* genes are also expressed correctly as measured by RT-PCR compared to *Cgalpha2* from *CgMTL1* from the control strain CBS138 (*CgMTL1*alpha) (Fig. 2C).

Strains expressing either *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* simultaneously display no observable phenotype under many different stress conditions.

The *Sca1*-*Scalpa2* heterodimer formed in diploid cells of *S. cerevisiae* represses a set of genes most of which are normally expressed in haploid cells; but there are other genes repressed by this heterodimer, notably the *ScHOG1* gene is repressed, which confers sensitivity to high concentrations of NaCl (Galgoczy, et al. 2004: 18069-74). We decided to first characterize the growth phenotype under

different kinds of stress of the strains simultaneously expressing *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3*, compared with the parental, strain BG14 (*CgMTL1a*), and with the (*mtl1,2,3*) Δ null mutant. The results for three of the conditions tested are shown in Figs. 3A, B, C and D and the rest of the conditions tested are summarized in Tables 1 and 2. In Fig. 3C we show the duplication times for the strains expressing *Cga1* and *Cgalpha2* or *Cgalpha3* compared to the parental BG14 or the null *mtl* mutant when grown in different concentrations of NaCl. It is clear from the data that all the strains have the same duplication time in each NaCl concentration. Fig. 3A and B presents the minimal inhibitory concentration (MIC_{50}) for each strain under oxidative stress by hydrogen peroxide (H_2O_2) and stress caused by the antifungal drug fluconazole. Again, the MIC_{50} for H_2O_2 or fluconazole is the same for every strain tested regardless of the genes expressed from *CgMTL1* or even in the absence of *MTL* information.

In Table 1 we present the data obtained from chronic exposure to compounds that generate different kinds of stress, as measured by growth on plates containing stress-generating compounds at several concentrations. We qualitatively assessed colony growth and morphology after 24, 48 and 72 hrs. It can be seen in Table 1 that we could not observe any difference in the growth or colony morphology between the strains expressing *Cga1* and either *Cgalpha2* or *Cgalpha3* and the BG14 parental strain in any kind of stress tested (growth in the presence of metals, membrane or cell wall damaging agents, osmotic or oxidative stress, low pH or anaerobiosis). Supplementary Figs. S2 and S3 show some other examples of colony growth under stress conditions for these strains. We then measured growth of these strains in either chronic or acute exposure to some compounds in liquid media. A summary of the results is shown in Table 2 and some examples of exposure to stress conditions in liquid are shown in

Supplementary Figs. S4, S5 and S6. As can be seen there is no *in vitro* condition tested where we could measure a difference between the strains expressing both types of information and the parental BG14 strain or the triple null (*mtl1,2,3*) Δ strain.

Strains simultaneously expressing α and alpha information, efficiently colonize target organs in a mouse model of systemic infection.

Since we saw no phenotype *in vitro* of the strains expressing *Cga1* and either *Cgalpha2* or *Cgalpha3*, we decided to determine their ability to colonize and persist within the host in a mouse model of systemic infection. Fig. 3D shows that there is no difference in the colonization of kidney, liver and spleen between the BG14 strain and the strains expressing both types of information or the null (*mtl1,2,3*) Δ strain, suggesting that the cells are competent to colonize efficiently the three organs tested in a murine systemic model of infection.

The *C. glabrata* proteins *Cga1* and *Cgalpha2* interact directly in vivo and *Cgalpha3* forms homodimers.

We wanted to determine whether the protein *Cga1* could interact with either *Cgalpha2* or *Cgalpha3* (or both) when simultaneously expressed in a cell. It should be noted that most of the amino acids involved in the interaction between *Cga1* and *Cgalpha2* in the heterodimer, are conserved in both of the *C. glabrata* orthologues (Supplementary Fig. S1B). For this purpose, we performed a co-immunoprecipitation assay using N-terminal Flag and cMyc tagged proteins. In these constructs the epitopes are separated from each protein by the G-A linker and are expressed from the inducible

promoter P_{MTI} . The appropriate combinations of translational fusions for heterodimer formation (Flag-*Cga1* and cMyc-*Cgalpha2* or Flag-*Cga1* and cMyc-*Cgalpha3*) and for homodimer formation (Flag-*Cgalpha2* and cMyc-*Cgalpha2*; Flag-*Cgalpha3* and cMyc-*Cgalpha3*) were transformed into the null (*mtl1, 2, 3*) Δ mutant and expression of both fusions was induced by addition of 2 mM CuSO_4 to the growth media. Immunoprecipitation was performed using anti-cMyc coupled agarose beads, and the immunoprecipitated proteins were run in SDS acrylamide gels. One set of the samples (input) were analyzed by Western blot with mouse anti-cMyc antibodies to detect the input proteins. The other set (CoIP) was analyzed with mouse-anti-Flag antibodies to detect whether the Flag-*Cga1*, Flag-*Cgalpha2* or Flag-*Cgalpha3* fusion proteins co-immunoprecipitate with cMyc tagged *Cgalpha2* or *Cgalpha3*. As shown in Fig. 4A Flag-*Cga1* coimmunoprecipitates with both cMyc-*Cgalpha2* and cMyc-*Cgalpha3* suggesting that these proteins interact (Fig. 4A lanes 10 and 12). Fig. 4A also shows that *Cgalpha3* forms homodimers efficiently (Fig. 4A lane 8) but *Cgalpha2* only forms homodimers very inefficiently (if at all), since the signal we detected is very weak (Fig. 4A lane 9). We could not detect *Cgalpha2*-*Cgalpha3* heterodimers (Fig. 4A lane 11).

To determine whether *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* directly interact, we also used Bimolecular Fluorescence Complementation assay (BiFC) (Sung and Huh 2007: 767-75). We generated compatible plasmids encoding fusion proteins of either the N-terminal or the C-terminal half of the Venus fluorescent YFP protein (VN or VC) with the NH₂-terminus of each *CgMTL*-encoded protein (*Cga1*, *Cgalpha2* and *Cgalpha3*) separated by the G-A linker (Fig. 4B). The fusion proteins are expressed under the control of the inducible promoter of the *MET3* gene (Fig. S7).

We transformed into *C. glabrata* combinations of these plasmids so as to co-express in the same cell both halves of the YFP protein fused to each of *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* proteins to test whether a functional YFP is regenerated. If *Cga1* interacts directly with *Cgalpha2* or *Cgalpha3*, both halves of YFP will be brought close together and the YFP protein will be functional and will emit fluorescence under excitation by UV light, which can be measured using a FACS machine. For the positive control for these experiments we used N-terminal fusions of *S. cerevisiae* alpha2 protein (*Scalpha2*), with either the VC or VN fragments of Venus-YFP, since it is well documented that it forms homodimers (Smith and Johnson 1992: 133-42, Tan and Richmond 1998: 660-6). We co-expressed these fusions in *C. glabrata* and measured fluorescence emission by flow cytometry. As shown in Fig. 4C, we could clearly detect fluorescence from cells co-expressing both fusions of *Scalpha2* (positive control).

We also transformed combinations of two plasmids in *C. glabrata*, one containing the N-terminal fusions of the VC fragment of Venus YFP with *Cgalpha2* or *Cgalpha3* and the other containing the fusions with the complementing Venus fragment (VN) with *Cga1*, *Cgalpha2* or *Cgalpha3*.

As shown in Fig. 4C, we detected a weak fluorescent signal indicative of the formation of *Cga1*-*Cgalpha2* heterodimers when both fusion proteins were coexpressed in the *mtl* null strain (*Cgalpha2*-VC; *Cga1*-VN); but not *Cga1*-*Cgalpha3* heterodimers (*Cgalpha3*-VC; *Cga1*-VN). We were also able to detect the formation of *Cgalpha3* homodimers when VN-*Cgalpha3* and VC-*Cgalpha3* were coexpressed, but not when VC-*Cgalpha2* and VN-*Cgalpha2* were coexpressed, indicating that *Cgalpha2* does not form homodimers but can interact with *Cga1*.

Discussion

Even though *C. glabrata* has a relatively close phylogenetic relationship with *S. cerevisiae* there are important differences regarding maintenance of cell-type identity and sexual reproduction in these organisms. *C. glabrata* has conserved the *CgMTL1*, *CgMTL2* and *CgMTL3* loci in a similar disposition to the three mating loci of *S. cerevisiae* (*ScMAT*, *ScHMR* and *ScHML* respectively). However, the regulation of expression of the genes encoded in these loci as well as the expression of cell-type specific genes is not conserved. For example, *CgMTL2* is transcriptionally active in *C. glabrata* while it is efficiently silenced in *S. cerevisiae*, which is essential to determine cell-type identity (Muller, et al. 2008: 848-58, Ramirez-Zavaleta, et al. 2010: 1602-11). Also, *C. glabrata* does not maintain expression of cell-type specific genes; *i.e.* genes that in *S. cerevisiae* are expressed in a cell-type-specific fashion due to control by the allele present in the *MAT* locus. In *C. glabrata* these so-called cell-type-specific genes are all expressed regardless of the information present in the *MTL* loci, or even in the absence of mating information [*(mtl1,2,3)*Δ] (Ramirez-Zavaleta, et al. 2010: 1602-11).

***C. glabrata* contains a longer variant of the *Cgalpha2* in the *CgMTL3* locus called *Cgalpha3*.**

In this work we have shown first that *C. glabrata* encodes two variants of the *Cgalpha2* gene that we have designated *Cgalpha2* and *Cgalpha3*. *Cgalpha2* is encoded in the *CgMTL1* locus (in clinical isolates with alpha information in this locus), and *Cgalpha3* is present in *CgMTL3*. *CgMTL3alpha3* is conserved in *C. glabrata*, since it is present in 4 different clinical isolates that we have sequenced (data not shown). However, *CgMTL3alpha3* is silenced in *C. glabrata* and therefore only a small fraction of the population of any given culture can express *CgMTL3alpha3*.

Simultaneous expression of *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* causes no observable phenotype in *C. glabrata*.

In diploid cells of *S. cerevisiae* and *MTL* heterozygotic *C. albicans* cells, the *Cga1*-*Cgalpha2* heterodimer is formed, which is a transcriptional regulator with properties different from either of the two proteins alone (Goutte and Johnson 1993: 359-71, Johnson 1995: 552-8, Miller and Johnson 2002: 293-302). In *S. cerevisiae*, *Sca1*-*Scalpa2* represses a group of genes that are involved in a variety of processes, for example some genes unique to haploid cells, some involved in meiosis, sporulation, and response to pheromones and osmotic stress. In *C. albicans*, genes involved in a morphogenetic switch, some genes involved in mating related functions, and some involved in virulence are all repressed by the heterodimer (Bennett and Johnson 2003: 2505-15, Galgoczy, et al. 2004: 18069-74). We constructed *C. glabrata* strains expressing both *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* to determine whether these strains display a growth phenotype under stress conditions. As shown in Tables 1 and 2, we could not find any growth phenotype of these strains, even though we tested growth under many different stress or nutritional conditions.

The fact that *C. glabrata* does not seem to maintain cell type identity, that is, the so-called cell type specific genes are expressed regardless of the information present at *CgMTL1*, suggests that in *C. glabrata* these genes might have evolved to regulate a different set of genes (Ramirez-Zavaleta, et al. 2010: 1602-11). In a preliminary experiment in which we analyzed the transcriptome of cells expressing *Cga1*-*Cgalpha2* or *Cga1*-*Cgalpha3* we did not see a difference in the expression of the so-called cell type-specific genes when compared with the null mutant strain (*mtl1,2,3*) Δ (data not shown).

We still need to analyze the transcriptome of strains expressing only one of the *CgMTL* related genes and under several conditions in order to determine what genes, if any, are regulated by these putative transcription factors. We will also perform ChIP seq experiments to find if these proteins bind to DNA targets.

***Cga1* and *Cgalpha2* can interact to form a heterodimer as determined by CoIP and BiFC.**

In *C. glabrata*, both variants *Cgalpha2* and *Cgalpha3*, share homology to the *Scalpha2* gene, and have conserved many of the amino acids involved in the interaction with *Cga1* (Suppl. Fig. S1A). To determine whether these proteins interact with each other or form homodimers we performed coimmunoprecipitation and BiFC experiments. By CoIP we found that we could coimmunoprecipitate *Cga1* with both *Cgalpha2* and *Cgalpha3* (Fig. 4A lanes 10 and 12) suggesting that *Cga1* forms a complex with *Cgalpha2* and also with *Cgalpha3*. We also showed that *Cgalpha3* can efficiently form homodimers, but *Cgalpha2* does not (Fig. 4A, lanes 8, 9). To determine whether *Cga1*-*Cgalpha2* and *Cga1*-*Cgalpha3* interact directly we made the BiFC assay. We found evidence of a weak interaction between *Cga1* and *Cgalpha2* but not between *Cga1* and *Cgalpha3*, as measured by BiFC (Fig. 4B). The fact that we could not detect direct interaction between *Cga1*-*Cgalpha3* by BiFC, is probably not due to lack of synthesis of the *Cgalpha3* VC and VN-YFP fusion proteins in *C. glabrata*, since we could detect the *Cgalpha3* homodimer by this assay. Instead it is possible that *Cga1*-*Cgalpha3* forms a complex but the spatial structure of this complex does not result in a close proximity of VN and VC fragments of YFP (*Cga1*-VN and *Cgalpha3*-VC containing strain). Perhaps in these strains the interaction between molecules of *Cgalpha3* is substantially more efficient than the interaction between

Cga1 and *Cgalpha3*, possibly forming complexes mainly formed by *Cgalpha3* molecules. So even though *Cgalpha3* can interact with *Cga1* (Fig. 4A lane 12), these heterodimers are not abundant and therefore the fluorescent signal is not detected.

However, the possibility of the *Cga1-Cgalpha3* heterodimer formation is noteworthy since in natural *C. glabrata* strains, which are haploid, would not normally express both *Cga1* and *Cgalpha2* in a single cell since the *Cga1* mRNA is only processed correctly when transcription initiates in *CgMTL1* (not from *CgMTL2*) and *Cgalpha2* is only present in *CgMTL1* (not in *CgMTL3*). Therefore, the only combinations that would occur naturally would be *Cga1* and *Cgalpha3* (from *CgMTL1a* and *CgMTL3alpha* respectively), or *Cgalpha2* and *Cgalpha3* (from *CgMTL1alpha* and *CgMTL3alpha* respectively), none of which seem to form heterodimers. However, artificial expression of both *Cga1* and *Cgalpha2* (our strain construct), which forms heterodimers, resulted in no observable phenotype associated with it. Clearly if these proteins have a function in *C. glabrata*, it is not similar to the one described in *S. cerevisiae*.

The strong interaction between cMyc-*Cgalpha3* and Flag-*Cgalpha3* by CoIP, indicative of the formation of a *Cgalpha3* homodimers, contrasts with the lack of homodimers between cMyc-*Cgalpha2* and Flag-*Cgalpha2* by either CoIP or BiFC (Fig. 4A, lane 9; Fig. 4B), or *Cgalpha2-Cgalpha3* heterodimer formation (Fig. 4A, lane 11; Fig. 4B). It is not known yet whether *Cgalpha3*, or *Cgalpha3* homodimers can bind to DNA and have a specific function. We are in the process of constructing strains that only express *Cgalpha3* from *CgMTL1* as well as strains carrying each one of the *MTL* genes individually in *CgMTL1*. The fact *C. glabrata* has conserved both variants and that *Cgalpha3* but not *Cgalpha2* can form homodimers, might suggest that these proteins have different functions. For

example, *Cgalpha2* could interact with *Cga1* to regulate a specific set of genes while *Cgalpha3* homodimers could regulate a different set of genes or possibly a group of common genes but in a different manner. We are in the process of expressing epitope tagged proteins, individually and in combinations to perform ChIP-seq experiments to determine whether these proteins bind to DNA and to what sequences. We are also constructing fusions of these proteins with YFP and Cherry fluorescent proteins to determine localization of each of the *MTL* related proteins. These data will help understand the role these genes play in *C. glabrata*.

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The authors declare that they have no conflict of interest.

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Table 1: Simultaneous expression of *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* does not result in an observable growth phenotype on solid media with various stress-causing agents. The same number of cells of each of the four strains tested were serially diluted and spotted onto rich solid media containing the indicated concentrations of each stress-causing agent. Strains used are: parental BG14 (*MTL1a1*), the triple null mutant (*mtl1,2,3*) Δ , strains expressing *Cga1* and *Cgalpha2* and *Cga1* and *Cgalpha3* from *CgMTL1* respectively.

Strains used:		BG14 (<i>MTL1a1</i>) (<i>mtl1,2,3</i>) Δ <i>MTL1 a1, alpha2</i> <i>MTL1 a1, alpha3</i>	
Type of Stress	Agent	Concentration	Growth Phenotype ^a
Metal	Cu	13mM, 15mM	NP
	Li	1M	NP
	Zn	1mM	NP
		5mM	
		10mM	
	Cd	0.5mM	NP
		2.5mM	
10mM			
Membrane damage	Fluconazole	100 μ M	NP
	SDS	0.04%	NP
Cell Wall damage	Calcofluor White	20 μ M	NP
Osmotic stress	Sorbitol	1.5M	NP
	NaCl	1.5M	NP
		1.8M	
Redox imbalance	pH	1.33	NP
		1.9	

		2.4	
		5.5	
		7.5	
		8.0	
		8.5	
		8.93	
Oxidative stress	H₂O₂	10mM	NP
		20mM	
		40mM	
		100mM	
	Menadione	30μM	NP
		60μM	
		80μM	
		110μM	
Anaerobiosis	No agent	No agent	NP

^a NP means that there is no observable growth phenotype (colony size or morphology) between the strains expressing *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* and the parental BG14 or the triple mutant (*mtl1,2,3*)Δ strains.

Table 2. Simultaneous expression of *Cga1* and *Cgalpha2* or *Cgalpha3* does not result in a detectable phenotype when logarithmic phase cells are exposed to different types of stress-causing compounds in liquid media. Logarithmic phase cells growing in rich media were exposed to the indicated concentrations of H₂O₂ for 2 h and % survival was estimated by performing outgrowth curves in a Bioscreen C apparatus and calculated as described in (Murakami, et al. 2008: 113-21). The rest of the experiments were chronic treatments in liquid media with menadione, nitroprussiate, fluconazole or hydroxyurea. Stationary phase cultures (48 h at 30°C in YPD) of each strain were used to inoculate fresh YPD liquid media with the indicated concentrations of each compound at an OD₆₀₀ of 0.01, placed in 100-well Honeycomb plates and incubated for 48 h at 30°C in a Bioscreen C. MIC₅₀ values were calculated as described (Orta-Zavalza, et al. 2013: 1135-48).

Strains used		BG14 (<i>MTL1a1</i>) (<i>mtl1,2,3</i>) Δ <i>MTL1 a1, alpha2</i> <i>MTL1 a1, alpha3</i>	
Condition	Agent	Concentration	Growth Phenotype ^a
DNA damage	Hydroxyurea	10mM	NP
		25mM	
		35mM	
		50mM	
Reactive Nitrogen Species	NPS	400mM	NP
Oxidative stress	H ₂ O ₂	10mM	NP
		15mM	
		20mM	

		300mM	NP
	Menadione	100µM	
		150µM	
		200µM	

^a **NP** means that there is no growth phenotype as estimated by the duplication time in each condition, between the strains expressing *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* and the parental BG14 or the triple mutant (*mtl1,2,3*)Δ strains.

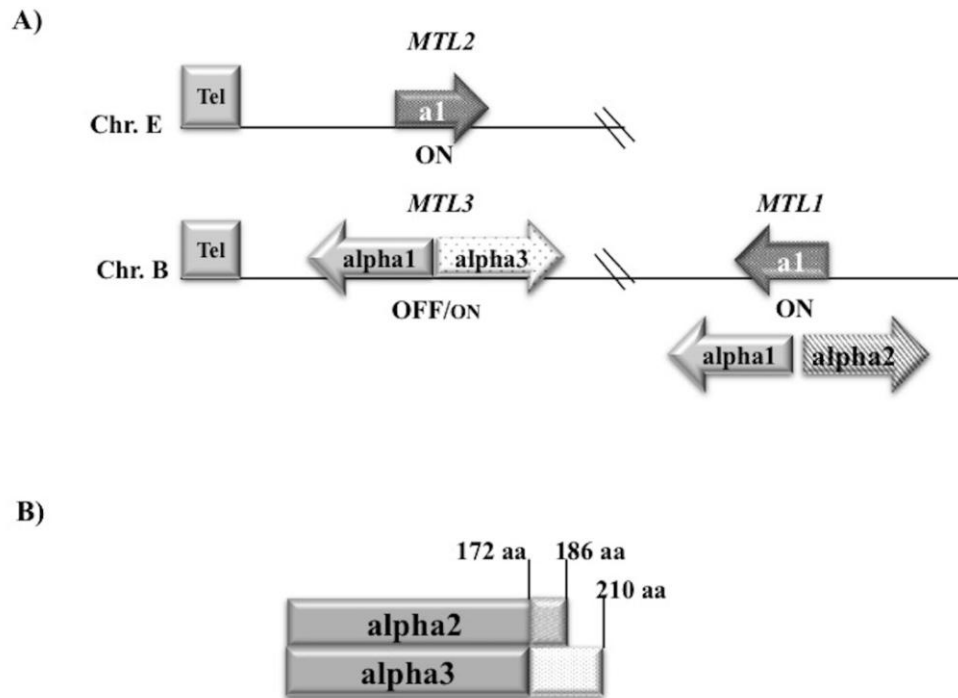


Figure 1. *Candida glabrata* contains a longer version of the *Cgalpha2* gene at the *CgMTL3* locus. (A) *C. glabrata* contains three *CgMTL* loci distributed in two chromosomes. *CgMTL1*, localized in chromosome B can contain the *Cga1* gene (**a** information) or the *Cgalpha1* and *Cgalpha2* genes (alpha information). *CgMTL2*, in chromosome E contains an identical copy of the *Cga1* gene. *CgMTL3*, which is close to the left telomere of chromosome B contains alpha information: an identical copy of the *Cgalpha1* gene, but a variant of *Cgalpha2* termed *Cgalpha3*. *CgMTL1* and *CgMTL2* are transcriptionally active (ON) while *CgMTL3* is subject to subtelomeric silencing (OFF/ON). (B)

Schematic representation of the putative proteins alpha2 and alpha3. alpha2 and alpha3 are identical up to amino acid 172 and only differ at their C-termini. From amino acid 172, alpha2 has 14 more amino acids for a total of 186, and alpha3 contains 24 more amino acids after aa 172 for a total of 210 amino acids.

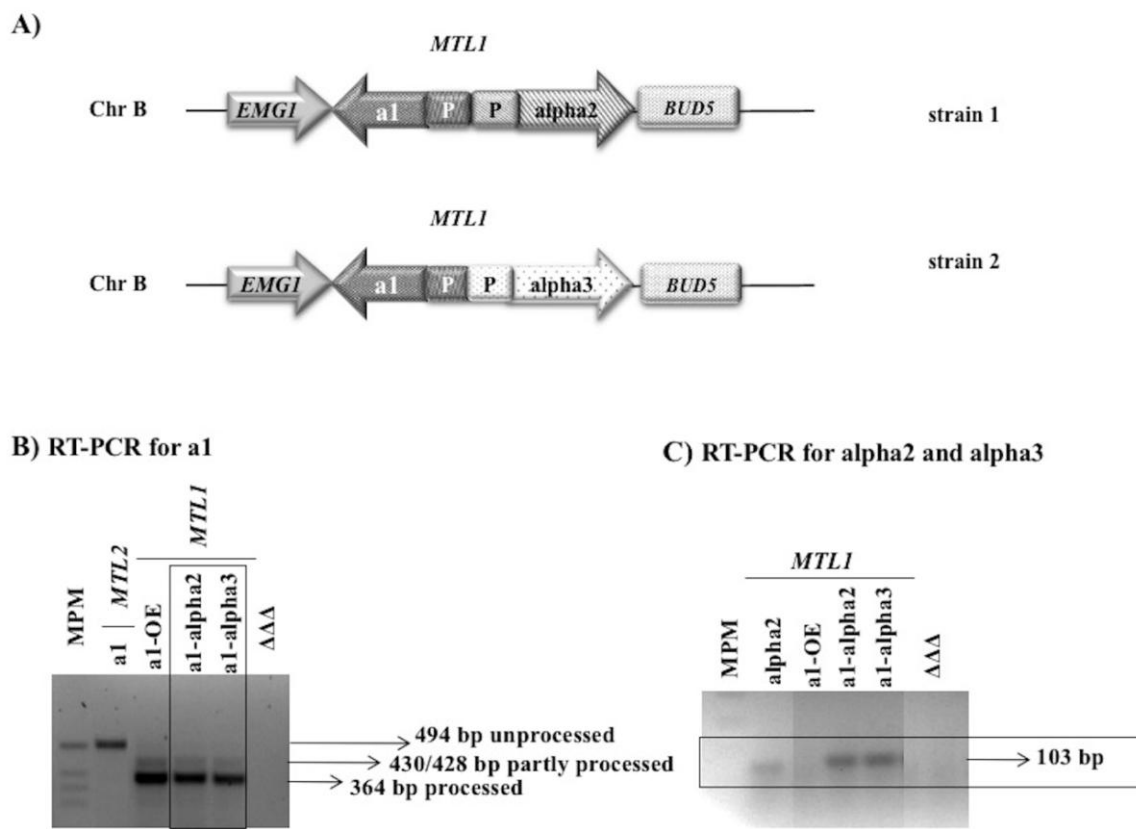


Figure 2. Structure of the *CgMTL1* locus of strains that simultaneously express *Cga1* and *Cgalpha2* genes or *Cga1* and *Cgalpha3* genes from this locus. (A) We constructed two *C. glabrata* strains using as parental strain the null *mtl* mutant (*mtl1,2,3*) Δ . In the first strain we introduced by homologous recombination at *CgMTL1* the *Cga1* gene driven by its own promoter and immediately adjacent to it, the *Cgalpha2* driven by its own promoter. The second strain contains *Cga1* and *Cgalpha3* each one also driven by their own promoters. (B) RT-PCR to determine transcription and processing of the *Cga1* gene in the constructed strains. RNA was extracted from stationary phase cultures of the indicated

strains. The *Cga1* gene contains two introns, the unprocessed mRNA is 494 bp long, the partially processed RNAs are 428 and 430 bp respectively and the completely processed is 364 bp long. In the strains constructed, *CgMTL1a1*-alpha2 and *CgMTL1a1*-alpha3 (**a1**-alpha2 and **a1**-alpha3 | *MTL1*), the main RNA detected corresponds to the completely processed RNA (364 bp long), although the partially processed product can also be detected. As controls we included strain CBS138 (**a1** | *MTL2*), which contains the *Cga1* gene only in *CgMTL2* and it is not processed as previously described. We also included the triple null (*mtl1,2,3*) Δ mutant ($\Delta\Delta\Delta$), in which we cannot detect any signal and a strain overexpressing the *Cga1* gene from *CgMTL1* (**a1**-OE). (C) RT-PCR to determine transcription of the *Cgalpha2* and *Cgalpha3*. We used oligos that anneal to the region where *Cgalpha2* and *Cgalpha3* are identical. The same strains as in panel (B) were used. Both strains expressing *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* show a transcript of the expected size (103 bp). The control is CBS138, which contains alpha information at *MTL1*.

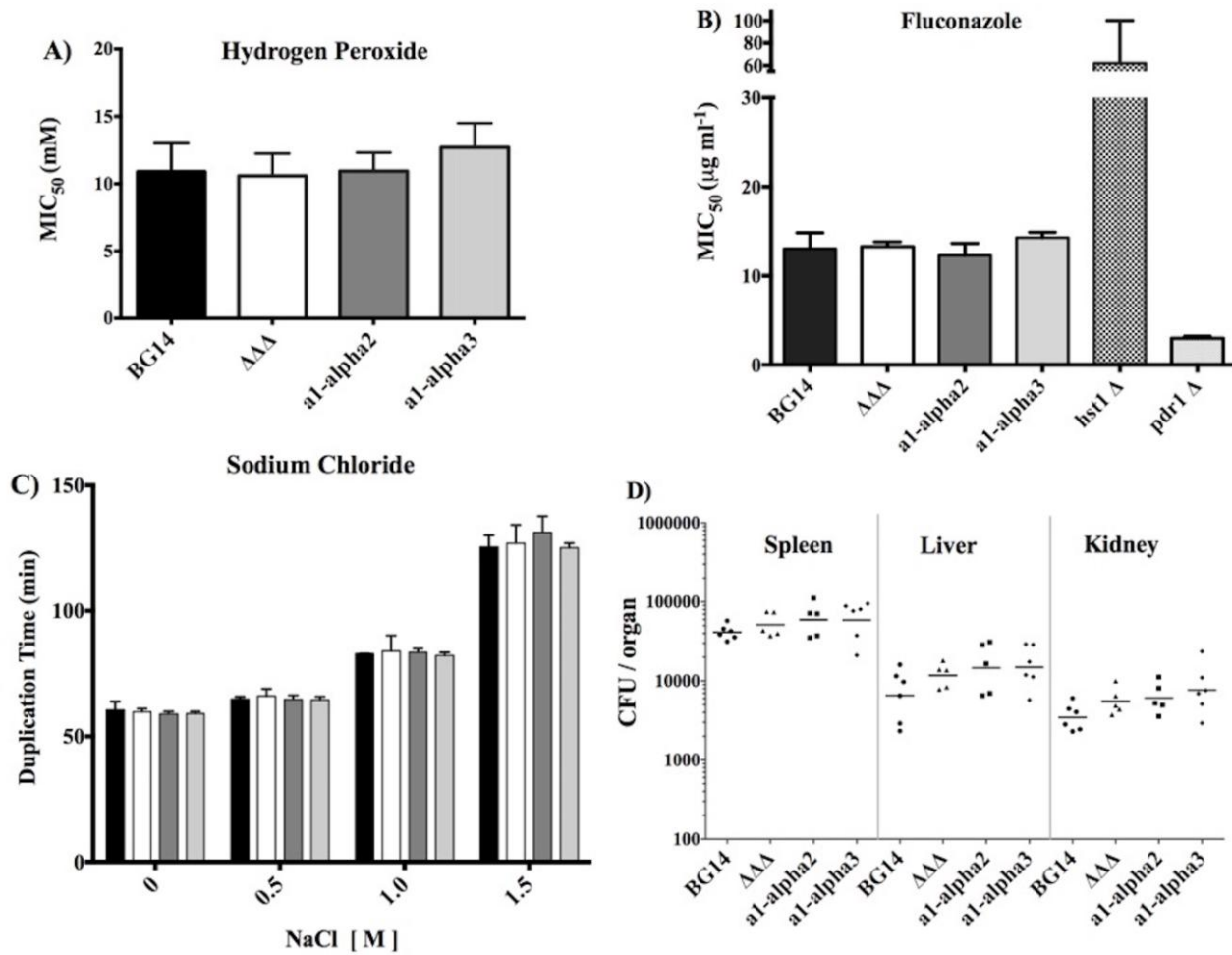


Figure 3. Strains expressing simultaneously *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* display no observable phenotype under many stress and nutritional conditions. (A) Stationary phase cells of the indicated strains were diluted in fresh YPD media so that an OD₆₀₀ of 0.5 was reached after 8 duplications. Cells were then diluted in fresh rich media at an OD₆₀₀ of 0.01 with different concentrations of H₂O₂ ranging from (0.3125 to 50 mM), and placed in 100-well Honeycomb plates and incubated at 30°C in a Bioscreen C machine where OD₆₀₀ was monitored every 15 min for 24 h. MIC₅₀ was determined based on the dose response curve as described (Orta-Zavalza, et al. 2013: 1135-

48). Data shows the mean of the three experiments. (B) Stationary phase cultures of the indicated strains were inoculated at an OD₆₀₀ of 0.01 in YPD with different concentrations of fluconazole (from 0.0078 to 256µg/mL). Cell suspensions were placed in 100-well Honeycomb plates, incubated at 30°C and OD₆₀₀ was monitored every 15 min for 24 h. MIC₅₀ was determined based on the dose response curve as described (Orta-Zavalza, et al. 2013: 1135-48). (C) Stationary phase cultures of the indicated strains were inoculated at an OD₆₀₀ of 0.01 in YPD with the indicated concentrations of NaCl. Cell suspensions were placed in 100-well Honeycomb plates, incubated at 30°C and OD₆₀₀ was monitored every 15 min for 24 h. Duplication times were calculated as previously described (Orta-Zavalza, et al. 2013: 1135-48). (D) Strains with *Cga1* and *Cgalpha2* or *Cga1* and *Cgalpha3* display equally efficient colonization of organs as the parental strain, in a mouse model of systemic infection. Groups of 6 mice were infected with 4×10^7 cells of the indicated strains by the tail vein. Seven days postinfection mice were euthanized and the indicated organs homogenized and plated to determine colony-forming units (c.f.u.). Data from a representative experiment is presented. Each symbol represents one mouse and the horizontal small lines represent the mean of the 6 mice used per group inoculated with each *C. glabrata* strain.

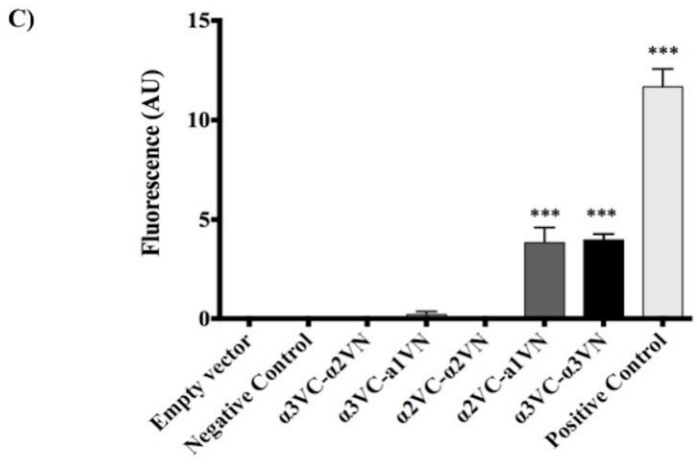
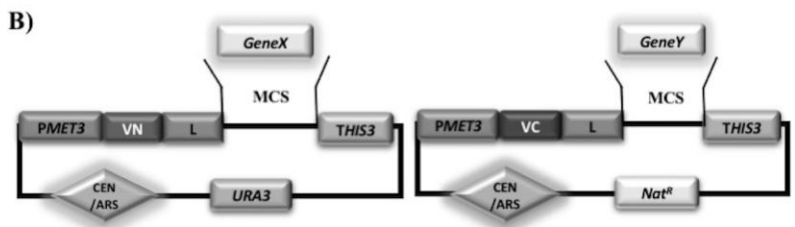
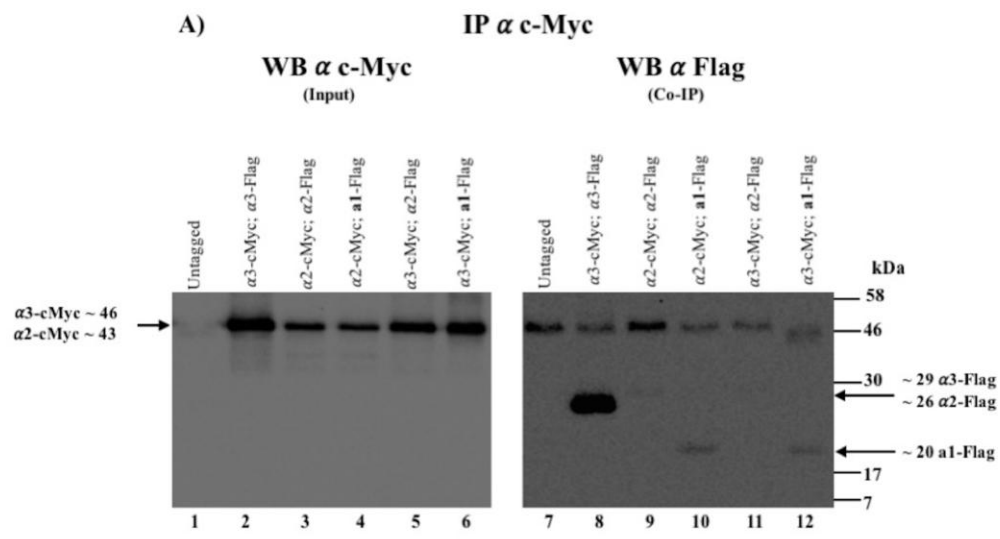


Figure 4. *Cga1* can interact directly with *Cgalpha2* in *C. glabrata*. Physical interaction between *Cga1* and *Cgalpha2* or *Cgalpha3* was determined by CoIP experiments. (A) Cells of the (*mtl1,2,3*) Δ strain

containing the indicated plasmids with the two specified epitope tagged fusions driven by the P_{MTI} were induced by addition of 2mM CuSO_4 . Cell lysates were immunoprecipitated using anti-cMyc coupled agarose beads (IP α cMyc). The immunoprecipitates were run in two SDS-acrylamide gels and transferred. One membrane was probed anti-cMyc antibodies (input) and the second set was probed with anti-Flag antibodies (CoIP). Lanes 1 and 7: untagged strain are cell lysate from the $(mtl1,2,3)\Delta$ strain with no plasmid. The rest of the lanes are strain $(mtl1,2,3)\Delta$ with plasmids containing the indicated fusions. Lanes 2 and 8: cMyc- $Cg\alpha3$ and Flag- $Cg\alpha3$; lanes 3 and 9: cMyc- $Cg\alpha2$ and Flag- $Cg\alpha2$; lanes 4 and 10: cMyc- $Cg\alpha2$ and Flag- $Cga1$; lanes 5 and 11: cMyc- $Cg\alpha3$ and Flag- $Cg\alpha2$; lanes 6 and 12: cMyc- $Cg\alpha3$ and Flag- $Cga1$.

Direct interaction between the $CgMTL$ encoded proteins was assessed by Bimolecular Fluorescence Complementation assay (BiFC). (B) Schematic representation of the plasmids used for the BiFC assay. Plasmids contain translational fusions of the VN or VC fragments of the Venus-YFP fused to the N-terminal end of $Cga1$, $Cg\alpha2$ or $Cg\alpha3$ separated by a linker of five repetitions of G and A residues (L). Two plasmids containing the appropriate combinations of the fusions were transformed into *C. glabrata* null strain $(mtl1,2,3)\Delta$. The positive control is the $Sc\alpha2$ protein, which forms homodimers (VN- $Sc\alpha2$ and VC- $Sc\alpha2$). The negative control were cells expressing two plasmids with the N-terminal fusions of the $Sc\alpha2$ with the same YFP fragment (VC- $Sc\alpha2$, VC- $Sc\alpha2$). (C) The strains containing the indicated pair of plasmids were grown overnight in OFF media. After two washes, cells were induced by switching to ON medium for 2.75 h (Materials and Methods). Fluorescence was measured by flow cytometry. Results shown were calculated as follows: The average geometric mean of fluorescence values obtained for the negative control strain were

subtracted from the average geometric mean fluorescence obtained for each strain with different combinations of plasmids. The average geometric mean obtained for the negative control was 38.8 ± 0.56 units, while the positive control average was 51.4 ± 0.95 units.

Results shown are the mean of three independent experiments. Values obtained are statistically significant, and are indicated by asterisks ($p < 0.001$).