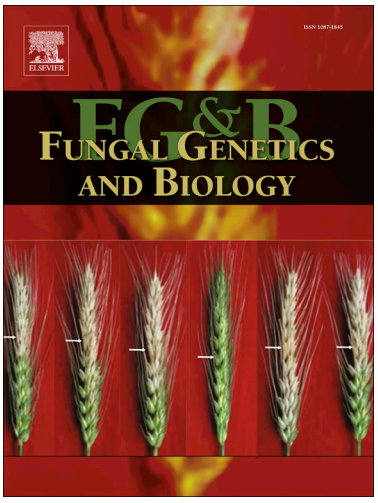

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Molecular characterization of the Silencing complex SIR in *Candida glabrata* hyperadherent clinical isolates

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Abstract

An important virulence factor for the fungal pathogen *Candida glabrata* is the ability to adhere to the host cells, which is mediated by the expression of adhesins. Epa1 is responsible for ~95% of the *in vitro* adherence to epithelial cells and is the founding member of the Epa family of adhesins. The majority of *EPA* genes are localized close to different telomeres, which causes transcriptional repression due to subtelomeric silencing. In *C. glabrata* there are three Sir proteins (Sir2, Sir3 and Sir4) that are essential for subtelomeric silencing. Among a collection of 79 clinical isolates, some display a hyperadherent phenotype to epithelial cells compared to our standard laboratory strain, BG14. These isolates also express several subtelomeric *EPA* genes simultaneously. We cloned the *SIR2*, *SIR3* and *SIR4* genes from the hyperadherent isolates and from the BG14 and the sequenced strain CBS138 in a replicative vector to complement null mutants in each of these genes in the BG14 background. All the *SIR2* and *SIR4* alleles tested from selected hyper-adherent isolates were functional and efficient to silence a *URA3* reporter gene inserted in a subtelomeric region. The *SIR3* alleles from these isolates were also functional, except the allele from isolate MC2 (*sir3*-MC2), which was not functional to silence the reporter and did not complement the hyperadherent phenotype of the BG14 *sir3*Δ. Consistently, *sir3*-MC2 allele is recessive to the *SIR3* allele from BG14. Sir3 and Sir4 alleles from the hyperadherent isolates contain several polymorphisms and two of them are present in all the hyperadherent isolates analyzed. Instead, the Sir3 and Sir4 alleles from the BG14 and another non-adherent isolate do not display these polymorphisms and are identical to each other. The particular combination of polymorphisms in *sir3*-MC2 and in *SIR4*-MC2 could explain in part the hyperadherent phenotype displayed by this isolate.

Keywords: *Candida glabrata*, hyperadherence, clinical isolates, SIR complex, polymorphisms, *SIR3*, *SIR4*

1. INTRODUCTION

In the last decades *Candida glabrata* has become a frequently isolated human fungal pathogen from disseminated infections (Diekema et al., 2012; Pfaller et al., 2014). In part, the success of *C. glabrata* as a pathogen is due to the presence of several virulence factors such as high resistance to oxidative stress (Cuellar-Cruz et al., 2008; Gutierrez-Escobedo et al., 2013), innate low susceptibility to azole antifungal drugs, rapid emergence of multiple antifungal resistant isolates after clinical exposure to these drugs (Castanheira et al., 2016; Kolaczkowska and Kolaczkowski, 2016; Pfaller et al., 2014; Pfaller et al., 2009; Salazar et al., 2018) and adherence to medical devices and host epithelial and endothelial cells (Castano et al., 2005; Desai et al., 2011; Martinez-Jimenez et al., 2013). Furthermore, an interesting correlation between the emergence of antifungal drug resistance and increased virulence and adherence to epithelial cells in *C. glabrata* has recently been described (Ni et al., 2018; Vale-Silva et al., 2017; Vale-Silva et al., 2013; Vale-Silva et al., 2016).

Adherence to biotic or abiotic surfaces in *C. glabrata* is mediated by cell wall proteins called adhesins, which are synthesized and incorporated to the cell wall in a process that depends on particular growth conditions and the genetic background of the strain (de Groot et al., 2008; Kraneveld et al., 2011). Different clinical isolates display various levels of adherence and expression of different kinds of adhesins depending on the particular clinical strain (de Groot et al., 2008; Desai et al., 2011; Gomez-Molero et al., 2015). Adherence to epithelial cells *in vitro* is mediated mostly by the Epa1 adhesin in *C. glabrata*, which is encoded by the *EPA1* gene. *EPA1* deletion in the laboratory reference strain BG14 (Cormack and Falkow, 1999), causes almost complete loss of this adherence (Cormack et al., 1999). Epa1 belongs to a family of related adhesins in *C. glabrata* encoded by the *EPA* genes, almost all of which are located near telomeres (Castano et al., 2005; De Las Penas et al., 2003). In strain BG14, as a consequence of the subtelomeric localization, the majority of the *EPA* genes are transcriptionally repressed through the mechanism of subtelomeric silencing, which depends on the Sir2, Sir3 and Sir4 proteins (referred to as the SIR complex: Silent Information Regulator), Rap1, yKu70, yKu80 and Rif1 (Castano et al., 2005; De Las Penas et al., 2003). This process in *C. glabrata* is similar to the telomere position effect (TPE) that has been previously described in *Saccharomyces cerevisiae*

(Buck and Shore, 1995; Gottschling et al., 1990; Gravel et al., 1998; Moazed et al., 1997; Rusche et al., 2003).

In *S. cerevisiae* Rap1 binds to the telomere repeats and recruits Sir3 and Sir4 which in turn recruit Sir2, resulting in the formation of the SIR complex (Cockell et al., 1995; Hoppe et al., 2002; Longtine et al., 1989; Rine and Herskowitz, 1987; Wang and Zakian, 1990; Wright and Zakian, 1995). Sir4 is a non-globular protein which can interact with itself to form homodimers (Chang et al., 2003) and with other proteins to ensure efficient repression (Kueng et al., 2013). Sir4 binds the NAD-dependent histone deacetylase Sir2, forming a stable heterodimer and stimulating the catalytic activity of Sir2 (Hsu et al., 2013), leading to the deacetylation the N-terminal ends of H3 and H4. Sir3 contains three structural domains: the bromo-adjacent homology domain (BAH) located in the N-terminal portion of the protein, the ATPase-like domain (AAA^+) and the winged helix domain (wH) at C-terminal region. The BAH and the AAA^+ domains mediate the interaction of Sir3 with nucleosomes (Armache et al., 2011; Carmen et al., 2002) and the AAA^+ domain is also the region where Sir3 interacts with Sir4 during SIR complex establishment (Ehrentraut et al., 2011). Sir3 wH domain is necessary for the dimerization of the protein and this contributes to the stabilization of the Sir complex in nucleosomes (Oppikofer et al., 2013).

The subtelomeric silencing in *C. glabrata* differs from telomere to telomere in terms of the genetic requirements (Rosas-Hernandez et al., 2008), but mutations in any of the Sir proteins in *C. glabrata* completely lose silencing of a *URA3* reporter inserted close to several telomeres (Castano et al., 2005; De Las Penas et al., 2003). Interestingly, null mutations in any of the silencing genes in the BG14 strain results also in a hyperadherence phenotype as a result of increased simultaneous expression of several subtelomeric adhesins (Castano et al., 2005).

We have previously shown (Martinez-Jimenez et al., 2013) that 11 out of 79 *C. glabrata* clinical isolates are hyperadherent to epithelial cells *in vitro*, in a condition where *EPA1* is not expressed in our reference strain. Some of the hyperadherent isolates displayed simultaneous expression of some subtelomeric *EPA* genes that are not expressed in BG14 under these *in vitro* conditions (Martinez-Jimenez et al., 2013). Interestingly, we found several polymorphisms in the Sir3 alleles from these isolates when compared to the BG14

allele. These data suggest that the silencing machinery may be less efficient in some of these hyper-adherent isolates so that there is increased expression of subtelomeric adhesins.

Here we determined whether the Sir proteins from several hyperadherent isolates are functional in terms of their ability to silence a reporter gene inserted near three different telomeres in *C. glabrata*, as well as measuring the ability of these alleles to complement the hyperadherent phenotype of null mutants in each *SIR* gene in the BG14 background. Our results indicate that only the *SIR3* allele from MC2 clinical isolate (*sir3*-MC2), is not functional to silence a reporter gene inserted in three different subtelomeric regions. Furthermore, this allele does not complement the hyperadherent phenotype of a *sir3*Δ mutant in the BG14 background. Taken together these data suggest that natural clinical isolates of *C. glabrata* differ in their ability to establish subtelomeric silencing and that this depends on the particular alleles of Sir3 and Sir4 they possess.

2. MATERIALS AND METHODS

2.1 Strains and plasmids. Table S1 in Supplementary Material describes all the *Escherichia coli* and *C. glabrata* strains used in this study, while Table S2 lists the plasmids employed.

2.2 Primers. Table S3 describes primers used in the present work.

2.3 Media. *Candida glabrata* yeast cells were grown in standard rich yeast media (YPD) as described previously by (Sherman, 1986), adding 2% agar for solid media. When required, YPD plates were supplemented with nourseothricin (GoldBio) at 100 µg/mL.

Bacteria cells were grown in Luria-Bertani medium as described previously by (Ausubel, 2001). Strain DH10 was used for electroporation.

2.4 Transformation. All the yeast transformations with linear or supercoiled plasmids were made following the protocol described by (Castano et al., 2003). Briefly, cells were grown in YPD until the culture reached OD_{600} 1 at which point cells were collected by centrifugation and washed two times with sterile water and resuspended in 1/100 volume of 0.1M LiAc. In a separate tube a transformation cocktail was prepared containing per transformation reaction: 240 µL of 50% PEG, 36 µL of 1M LiAc, 25 µL of heat denatured

salmon sperm DNA (2mg/mL). 50 µL of the cell suspension was added to each transformation tube plus 100 ng of each plasmid DNA to transform (in a volume of 50 µL) and 301 µL of the transformation cocktail. A negative control is always added where 50 µL of water were added instead of plasmid DNA. The cell mix was incubated at 30°C for 45 min in a roller and afterwards, 43 µL of DMSO were added to each tube mixing gently. The cells were then incubated 15 min at 42°C, immediately afterwards, the tubes were centrifuged to discard the supernatant. If the selection after the transformation was for resistance to an antifungal agent, the cells were resuspended in 900 µL of YPD and incubated in a roller for 4 h at 30°C to allow for expression of the resistance cassette. Cells were then plated on hygromycin or nourseothricin containing YPD plates. If selection was for Ura^+ phenotype, the cells were resuspended in 900 µL of sterile water after the heat shock and immediately plated in selection media (SC – ura). Plates were incubated at 30°C for two days.

2.5 Construction of plasmids. *SIR* genes from the selected hyperadherent clinical isolates were cloned in the *C. glabrata* replicative plasmid pMJ22 (Yanez-Carrillo et al., 2014), GenBank Accession Number: KP238569). All the *SIR* genes were amplified with the high-fidelity enzyme Iproof (Bio-Rad) using the appropriate primers with the corresponding restriction sites (Table S3), so that the corresponding ORF plus the 5' and 3' flanking regions were amplified. The PCR products were digested with the appropriate enzymes. When necessary, PCR products were cloned in the cloning vector pMB11 (Gallegos-Garcia et al., 2012) to ensure correct digestion. To clone the *SIR2* alleles we used primers 523 Fw and 524 Rv to amplify a 3.5 kb PCR product. This PCR product was digested with *Mfe*I and *Kpn*I, which cut within the 5' and 3' flanking regions of *SIR2* respectively and filled in with Klenow enzyme to produce blunt ends. This digestion leaves 680 bp in the 5' intergenic region and 824 bp of the 3' UTR of *SIR2*. The blunt ended fragments carrying the *SIR2* alleles were cloned into *Eco*RV digested vector pMJ22 (Nat^R). To clone *SIR4* alleles we amplified a 6.3 kb fragment using primers 2191 Fw and 2192 Rv and the PCR product was cloned into the cloning vector pMB11. This intermediate plasmid was digested with *Sna*BI and *Xba*I, which cuts 500 bp upstream of the start codon of *SIR4* and 787 bp downstream of the stop codon, resulting in a 5.6 kb fragment. The *Sna*BI-*Xba*I *SIR4*

digested fragments were cloned into the replicative vector pMJ22 digested with *Eco*RV and *Xba*I.

SIR3 alleles from clinical isolates were cloned in two steps since plasmids containing *SIR3* from *C. glabrata* seem to be toxic in *E. coli*. Two PCR products were generated for each allele using primers 2178 Fw and 2122 Rv for the first 2850 bp fragment (A) containing the 5' half of the gene, and primers 2124 Fw and 2123 Rv for the second 2426 bp fragment (B) for the 3' half of the gene. Each PCR fragment was cloned separately in pMB11. We obtained the *SIR3*-A fragment from pMB11 by digesting with *Kpn*I (which cuts 944 pb upstream of *SIR3*) and with *Spe*I (which digests within *SIR3* 1888 bp from the start codon). This fragment was cloned in pMJ22 previously digested with the same enzymes. Fragment *SIR3*-B was obtained from pMB11 by digesting with *Spe*I (within *SIR3*) and *Sac*I. This fragment containing the last 1379 pb of the *SIR3* ORF and 1036 pb of the 3' UTR, was cloned in the plasmid that already contained *SIR3*-A (previously digested with *Spe*I and *Sac*I) in order to reconstitute the complete *SIR3* allele of each clinical isolate.

Clones were screened first by colony PCR and the positive clones were then verified by restriction analysis and subsequently by sequencing at the Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental (LANBAMA), IPICYT.

Sequencing of these plasmids made us aware of two mistakes in the originally published Sir3 sequencing (Martinez-Jimenez et al., 2013). We present in this work a simplified version of the published Figure with permission (Springer License No: 4224310996324), corresponding to Fig. S3.

2.6 Sequencing of *SIR* genes and plasmids. DNA from the cloned *SIR* genes from the clinical isolates was prepared using Qiagen minipreps following manufacturer's instructions and sent for sequencing to (LANBAMA-IPICYT) with specific primers. The sequences of the Sir alleles have been deposited in GenBank with the following accession numbers: *SIR2*-BG14: MG288686; *SIR2*-MC2: MG288687; *SIR2*-MC68: MG288688; *SIR4*-BG14: MG288689; *SIR4*-MC2: MG288690; *SIR4*-MC25: MG288691; *SIR4*-MC29: MG288692; *SIR4*-MC39: MG288693; *SIR4*-MC65: MG288694; *SIR4*-MC68: MG288695.

2.7 Construction of chimeras. We constructed two chimeras of the *SIR3* gene in the replicative vector pMJ22. In these chimeras, fragments of the *SIR3* allele of BG14 standard strain were exchanged for the corresponding fragments of the *sir3*-MC2 gene from the MC2 clinical isolate. The chimeras were cloned in the replicative recipient plasmid pLP189 that contains *SIR3*-BG14 and exchanging the corresponding fragments with those from *sir3*-MC2. Briefly, to obtain Chimera 1, we exchanged a 1354 bp *Spe*I/*BamH*I fragment from the *SIR3*-BG14 containing plasmid, for the corresponding fragment obtained from *sir3*-MC2 clinical isolate. Chimera 2 was obtained by replacing the 1234 bp *Xho*I/*Spe*I fragment of the *SIR3*-BG14 allele for the corresponding fragment from *sir3*-MC2. We performed complementation tests using Chimeras 1 and 2 to determine the level of silencing of the *URA3* reporter gene and adherence to HeLa in a *sir3*Δ strain.

2.8 Complementation assays. We carried out two different complementation assays to assess the function of the different alleles of the *SIR* genes from the clinical isolates. First we determined functionality by complementing the corresponding *sir*Δ strains in the BG14 background with the replicative plasmids containing the cloned *SIR* alleles from the hyperadherent isolates, and determining the level of silencing of a *URA3* reporter gene inserted at different positions in three telomeres in these strains. The level of silencing was determined using the 5-FOA sensitivity assay as described previously (Castano et al., 2005). Briefly, strains were grown to stationary phase in YPD-NAT (to maintain the plasmids), the OD_{600} of every culture was adjusted to 1 and serial dilutions were made in sterile water. 5µL (equal number of cells) of each dilution were placed with a replicator on the plates with the indicated media and incubated for 48 h at 30 °C and photographed. Strains that express the *URA3* gene cannot grow on the plates with 5-FOA due to the formation of a toxic compound. Growth in plates with 5-FOA is correlated directly with the level of silencing of the reporter gene.

2.9 HeLa cells growth and fixation. HeLa cell line is derived from human cervix carcinoma and it grows in monolayers as an epithelial cell line. Cells were grown in culture dishes (Cell Treat, Scientific Products) according to supplier´s instructions in DMEM (Minimum Essential Medium with Earle's salts) [Corning] supplemented with 10% FBS (Fetal bovine serum) (Gold-Bio) at 37°C and 5% CO_2 atmosphere in a CO_2 Incubator

(Thermo Scientific). The culture media was replaced every 2-3 days until the monolayer was confluent. Media was discarded in sterile conditions and HeLa cells were fixed with 3.7% para-formaldehyde in PBS (Phosphate Buffer Saline) for 30 minutes at 4°C. After the incubation each well was washed three times with PBS. One milliliter of PBS with penicillin 100 U/mL (Gold-Bio) and streptomycin 100 µg/mL (Sigma-Aldrich) was added to each well for conservation at 4°C.

2.10 Adherence assays.

Adherence assays were performed as described previously by (Martinez-Jimenez et al., 2013). Briefly, yeast cells carrying plasmids with the *SIR3* alleles were grown to stationary phase (48h) in YPD supplemented with nourseothricin (100µg/mL) (Sigma-Aldrich). After the first 24h of growth, the media was supplemented a second time with nourseothricin (100µg/mL) to prevent plasmid loss. OD_{600} of each culture was determined and adjusted to OD_{600} 1 with Hanks Balanced Salt Solution (HBSS: 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.6 $MgSO_4$, 137 mM NaCl, pH7.4) and $CaCl_2$ was added , to a final concentration of 5 mM. One mL of this cell suspension was added to a 24 well-plate containing the fixed HeLa cells with 500 µL HBSS with $CaCl_2$. The plate was centrifuged at 1000 rpm for 1 min and incubated one hour at room temperature. To eliminate the yeast that did not adhere to HeLa cells the wells were washed four times with HBSS with $CaCl_2$. Yeast adhered to HeLa monolayer after the washings were recovered adding 0.5 mL of 0.1 % triton, 0.5 % SDS, 10 mM EDTA in PBS to each well. Yeast cells were scraped off the well, recovered in an eppendorf tube and 10-fold serial dilutions were made in distilled, sterile water. 200 µL of each dilution were plated on YPD plates and incubated for 48 hrs at 30°C and CFUs of adherent and input cells were counted. Percentage of adherence was calculated using the formula:

% Adherence = [CFU (adherent cells) / CFU (Input cells)] * 100

Each experiment was done in biological triplicate and three technical replicates. The results shown are the mean of these measurements.

Hyperadherent clinical isolates were classified according to the level of adherence displayed to HeLa cells in vitro, under conditions where *EPA1* is not expressed in BG14

(stationary phase cells) as described by (Martinez-Jimenez et al., 2013). Briefly, isolates with adherence level between 15 to 25% were considered slightly hyper-adherent; isolates with 25-45% adherence were considered moderately hyper-adherent and higher than 45% were classified as highly hyper-adherent. By comparison strain BG14 shows ~3-5% adherence to HeLa cells under this condition, and CBS138 ~31%.

2.11 Western blot.

We constructed epitope-tagged versions of each Sir3 allele (*SIR3*-BG14, *SIR3*-CBS138 and *sir3*-MC2) at the C-terminal end with the Flag epitope and recombined each construct in its native locus in the corresponding strain backgrounds. The strains were grown in YPD and harvested in stationary phase. The protein extraction and western blot assay were done as described with minor modifications (Orta-Zavalza et al., 2013; Robledo-Marquez et al., 2016). Briefly, cells were resuspended in lysis buffer (45 mM HEPES, 400 mM Potassium acetate, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1X Complete protease inhibitors cocktail ROCHE®), 100 µL of zirconia beads were added and cells were broken using a FastPrep®-24 (MP Biomedicals) equipment, with three times for 60 s at 6 m/s. The cells were centrifuged at 15000 rpm for 40 min at 4°C, the supernatant was recovered and the protein content was determined by Bradford assay. 50 µg of total protein mixed with 2X SDS loading buffer were preheated (95°C for 8 min) and then loaded in a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted onto PVDF membranes (BIO-RAD®) and probed with anti-Flag (Sigma®) at final concentration of 3 µg/mL overnight. After washing, the membrane was probed with a goat-mouse horseradish peroxidase-conjugated secondary antibody (MERCK®). Signal was detected by ECL chemiluminiscence reagents (Pierce®) and recorded using a BioRad ChemiDoc MP System equipped with chemiluminescence.

2.12 Statistical analysis.

Statistical analysis was performed by ANOVA and Dunnett´s multiple comparison test. A P value ≤ 0.05 was considered statistically significant.

3 Results

3.1 Some clinical isolates are hyperadherent to epithelial cells under conditions where *EPA1* is not expressed in the standard strain BG14

We have previously identified eleven clinical isolates from a collection of 79, that are hyperahderent in stationary phase cultures, which is a condition in which the reference strain BG14 is non-adherent (Gallegos-Garcia et al., 2012; Martinez-Jimenez et al., 2013). Also, in contrast to BG14, some of these hyperadherent isolates (MC2, MC29, MC65 and MC68), express several *EPA* adhesins simultaneously in stationary phase (Martinez-Jimenez et al., 2013). We have focused on four hyperadherent isolates for further study (MC2, MC29, MC65 and MC68), and we included the sequenced reference strain CBS138, because it too is hyperadherent and overexpresses several *EPA* genes simultaneously in stationary phase cells (Martinez-Jimenez et al., 2013). Fig. 1 shows the adherence level of some of the chosen hyperadherent isolates in stationary phase cultures compared to the standard BG14 strain and the sequenced CBS138. The four chosen isolates represent the three classes identified previously: slightly hyper-adherent with ~15-25% adherence to HeLa cells *in vitro* (MC65); moderately hyperadherent with 25-45% (MC29 and MC68) and highly hyperadherent with >45% (MC2) (Martinez-Jimenez et al., 2013). We have also shown that the *sir2*Δ, *sir3*Δ and *sir4*Δ mutants in the BG14 background, all show hyperadherence to HeLa cells (Fig. S1) and overexpression of several *EPA* adhesins in stationary phase (Castano et al., 2005; De Las Penas et al., 2003; Rosas-Hernandez et al., 2008).

3.2 *SIR2* alleles from the hyperadherent clinical isolates are functional for silencing a subtelomeric *URA3* reporter

The simultaneous expression of several subtelomeric adhesins in the hyperadherent isolates, suggested that the subtelomeric silencing machinery might work inefficiently in these isolates resulting in expression of several *EPA* genes simultaneously.

We decided to clone the *SIR* genes (*SIR2*, *SIR3* and *SIR4*) of some hyperadherent isolates, to determine whether the *SIR* alleles from the isolates are functional using two assays: a)

the ability to silence a reporter gene (*URA3*) inserted close to the right telomere of chromosome E (telomere E_{-R}), and b) the ability to adhere to HeLa cells *in vitro*.

We cloned the *SIR2* alleles with their own promoter and 3' UTR region, from isolates MC2, MC29, MC65, MC68, the non adherent isolate MC39 (as a control), our reference strain BG14 and the sequenced strain CBS138 in the replicative vector pMJ22 (Materials and Methods). Each plasmid was transformed into the *sir2*Δ strain in the BG14 background to test whether these *SIR2* alleles are functional. The first assay consists in measuring the ability to repress the *URA3* reporter inserted 1.3 kb from the telomere E_{-R}, between *EPA3* and the telomere (Fig. 2a), where *EPA3* forms a cluster with *EPA1* and *EPA2*. The ability to repress the reporter was evaluated by a growth assay on plates containing 5-FOA, where expression of *URA3* results in the production of a toxic compound and cell death. The results are shown in Fig. 2b. The *sir2*Δ strain transformed with the empty vector, cannot grow on 5-FOA since there is no subtelomeric silencing and *URA3* is expressed. However, all the *SIR2* alleles from each hyperadherent isolate as well as that from the BG14, CBS138 or the non-adherent isolate MC39, were able to complement the absence of the endogenous *SIR2* gene, allowing silencing of the reporter as judged by the strong growth of each strain on 5-FOA plates of every strain.

We sequenced the *SIR2* genes from isolates MC2 and MC68 and from strain BG14, and there is only one non-synonymous change that leads to a change in the amino acid sequence of the protein (which we refer here simply as polymorphisms) with respect to *SIR2*-BG14. This polymorphism is the same for both clinical isolates (change P304S, data not shown, Genbank accession numbers: MG288687 and MG288688 respectively).

3.3 *SIR4* alleles from the hyperadherent clinical isolates are functional for silencing a subtelomeric *URA3* reporter

We next tested whether the *SIR4* genes from the selected hyperadherent isolates (MC2, MC29 and MC65) and control strains cloned in the replicative vector pMJ22, could complement a *sir4*Δ strain containing the *URA3* reporter inserted at -1.3 kb from telomere E_{-R} in the BG14 background. The results indicate that, similar to the case for the *SIR2* alleles from these isolates, all the *SIR4* alleles were able to complement the *sir4*Δ strain for

silencing of the subtelomeric *URA3* reporter (Fig. 2d). The *SIR4* genes from these isolates were sequenced and, unlike *SIR2* genes, *SIR4* alleles displayed multiple non-synonymoous changes compared to *SIR4*-BG14 (Fig. S2).

3.4 *sir3* from MC2 hyperadherent isolate is not functional

We next cloned the *SIR3* genes from isolates MC2, MC29, MC65, MC68, BG14 and CBS138 in the replicative vector and transformed them individually into a *sir3*Δ mutant in the BG14 background containing the *URA3* reporter inserted between *EPA3* and the telomere E_{-R}. While almost all the *SIR3* genes from the hyperadherent isolates were functional as judged by the ability to repress the *URA3* reporter, the *sir3* allele from isolate MC2 (*sir3*-MC2) was unable to complement the *sir3*Δ mutation for silencing of the reporter as indicated by the lack of growth of this strain in plates containing 5-FOA (Fig. 2c). The sequences of these alleles have been published (Martinez-Jimenez et al., 2013) and a simplified version of the data is presented in Fig. S3.

3.5 *sir3* allele from MC2 isolate is not functional for silencing the *URA3* reporter at other telomeres in the *sir3*Δ strain

Silencing at different telomeres in *C. glabrata* is differentially regulated (Rosas-Hernandez et al., 2008), so we next asked if the *sir3*-MC2, was also not able to silence a *URA3* reporter inserted near other telomeres. To do this, we transformed the plasmids containing the *sir3*-MC2 allele into two different *sir3*Δ strains: the first one contains the *URA3* reporter inserted 2.23 kb from the right telomere of chromosome C (C_{-R}) and the second strain the reporter is inserted 23.69 kb from the right telomere of chromosome I (I_{-R}). As shown in Fig. 3a and 3b, the plasmid containing *sir3*-MC2 is unable to complement the *sir3*Δ, since the strains containing this plasmid fully express the *URA3* reporter at the other two telomeres tested.

3.6 *sir3* allele from MC2 does not decrease the hyper-adherence to HeLa cells of the BG14 *sir3*Δ strain

We then wanted to know whether the *sir3*-MC2 allele was also non-functional for silencing several natural subtelomeric genes, which in *C. glabrata* are mostly genes from the *EPA*

family. As a measure of the ability of *sir3*-MC2 to silence the native *EPA* genes, we determined the adherence level of the *sir3*Δ strain (in the BG14 background) complemented with the replicative plasmids containing either the *sir3*-MC2 allele or the *SIR3*-BG14 or *SIR3*-CBS138 alleles.

As shown in Fig. 4a, the *sir3*-MC2 allele did not significantly decrease the level of adherence to HeLa cells of the *sir3*Δ strain in the BG14 background, displaying a similar level of adherence to the *sir3*Δ strain complemented with the empty vector. The allele from the hyperadherent sequenced strain CBS138 (*SIR3*-CBS138), was also not able to complement the *sir3*Δ mutant (Fig. 4a). Instead, the strain containing the native *SIR3*-BG14 allele displayed a decrease in the level of adherence compared to the vector control that was statistically significant.

To determine whether the levels of the Sir3-MC2 protein are comparable to the Sir3-BG14, we constructed C-terminal epitope tagged versions of Sir3 from BG14, CBS138 and MC2 strains to generate Sir3-Flag-BG14, Sir3-Flag-CBS138 and Sir3-Flag-MC2 C-terminal, epitope tagged translational fusions. Protein extracts from the strains carrying these constructs recombined in the corresponding *SIR3* locus were used to detect the protein levels by western blot. The results show that all three tagged proteins from each allele can be detected and are synthesized at equivalent levels in all three strains (Supplementary Fig. S4). These data indicate that lack of complementation of the Sir3-MC2 allele is not due to lack of synthesis or instability of the protein.

3.7 *SIR3* allele from BG14 is dominant over the *SIR3* allele from MC2 clinical isolate

Taking into account the fact that *sir3*-MC2 does not complement the *sir3*Δ strain, we considered that the *sir3*-MC2 allele would be recessive in the presence of the native *SIR3* in the BG14 strain. We first transformed the BG14 strain with the *sir3*-MC2 allele and measured adherence to HeLa cells. As expected for a recessive allele, the BG14 strain transformed with the *sir3*-MC2 allele displayed almost the same level of adherence as the strain transformed with the empty vector (Fig. 4b). In fact, there were no significant differences in the adherence of the BG14 strain transformed with any of the *SIR3* alleles tested, suggesting that the *sir3*-MC2 allele is recessive to the wild-type (Fig. 4b). The low

level of adherence in this experiment is due to the fact that under stationary phase growth conditions, the BG14 strain is non-adherent because the adhesins are not expressed.

We performed the converse experiment in which the MC2 strain was transformed with the *SIR3* alleles from MC2, BG14 and CBS138. As shown in Fig. 4c the MC2 isolate transformed with the *SIR3*-BG14 allele, had a significantly lower adherence to HeLa cells than the strain transformed with the empty vector or the untransformed original isolate. Indeed, the MC2 isolate transformed with the *SIR3*-CBS138 allele or with an extra copy of its own *sir3* allele also displayed a decreased level of adherence compared to the empty vector control, although not as low as with *SIR3*-BG14 allele.

3.8 A combination of several changes in the amino acid sequence of Sir3 from MC2 isolate are required to abolish the silencing function.

We had previously sequenced the *SIR3* gene from the hyperadherent isolates and found that Sir3 from MC2 (Sir3-MC2) contains five changes in the amino acid sequence or polymorphisms with respect to the Sir3 protein from BG14. We decided to determine whether all the polymorphisms are responsible for the loss of function of the Sir3-MC2 protein, since the other *SIR3* alleles from other isolates also contain polymorphisms and some are shared with isolate MC2 (Martinez-Jimenez et al., 2013) and Fig. S3.

We constructed two chimeras of Sir3 between the *sir3*-MC2 and *SIR3*-BG14 allele to split the polymorphisms into two groups as shown in Fig. 5a. We then transformed the two chimeras into the *sir3*Δ strain containing the *URA3* reporter between *EPA3* and E_R telomere and measured the level of silencing of the reporter. The results show that both chimeras were functional for silencing the reporter: chimera 1 (carrying the last two polymorphisms of the Sir3-MC2) and chimera 2 (carrying the first three polymorphisms of the Sir3-MC2, which are the same three polymorphisms that Sir3-CBS138 contains) (Fig. 5b).

Furthermore, we measured the adherence levels to HeLa cells of the BG14 *sir3*Δ strain complemented with each of the two chimeras, and interestingly, we found that only chimera 1 complements the hyperadherent phenotype of the *sir3*Δ strain to a similar level to the Sir3-BG14 allele (Fig. 5c). Instead, chimera 2, which is identical in protein sequence as Sir3-CBS138, does not complement the hyper adherent phenotype of the *sir3*Δ strain. This

is the same adherence level displayed by the *sir3*Δ strain complemented with p*SIR3*-CBS138 (Fig. 5c).

4 Discussion

Adhesion is an important step in the establishment of infection by *Candida glabrata*. Different adhesins such as the Epa family or other cell wall proteins (Awp, Pwp, Aed proteins) play important roles mediating tissue adhesion in this yeast (Cormack et al., 1999; de Groot et al., 2008). Expression of the *EPA* genes can be regulated by different signals like growth phase (de Groot et al., 2008; Gallegos-Garcia et al., 2012), oxidative stress (Juarez-Cepeda et al., 2015), environmental stimuli (Domergue et al., 2005), or subtelomeric silencing (Castano et al., 2005; De Las Penas et al., 2003; Juarez-Reyes, 2012; Rosas-Hernandez et al., 2008). In this work we characterized the Sir proteins in *C. glabrata* hyper adherent clinical isolates since this complex is crucial in heterochromatin formation during the establishment and/or maintenance of silencing.

4.1 *SIR3* allele from MC2 clinical isolate (*sir3*-MC2) is not functional

The hyper adherent phenotype and the over expression of several *EPA* genes simultaneously in clinical isolate MC2, led us to propose that subtelomeric silencing in this strain is not efficient when compared to BG14 (Martinez-Jimenez et al., 2013). In this work we found that only the *sir3*-MC2 allele was not able to silence the reporter gene inserted at telomere E-$_R$ or at two other telomeres (C-$_R$ and I-$_R$) (Fig. 2c and Fig. 3a-b), while the rest of the Sir3 alleles and all the Sir2 and Sir4 alleles from the studied clinical isolates were capable of silencing the reporter inserted 1.3 kb from the telomere E-$_R$. These results suggest that the *sir3*-MC2 allele could not establish and/or maintain the subtelomeric silencing to prevent expression of the reporter gene. Even though the subtelomeric silencing differs from telomere to telomere in *C. glabrata*, the Sir3 protein is absolutely required in all of them (Rosas-Hernandez et al., 2008). Under conditions where there is no silencing in the BG14 background, the genes normally present in subtelomeric regions can be expressed, and as a result, strains become hyper-adherent. The *sir3*-MC2 allele was not able to decrease the hyper-adherent phenotype of the BG14 *sir3*Δ strain, this suggests that the Sir3-MC2 protein is not functional to establish subtelomeric silencing in this genetic background (Fig.4a). Together these data suggest that Sir3-MC2 protein is not functional.

We have determined that an epitope tagged version of the Sir3-MC2 protein is expressed at similar levels to the Sir3-BG14 or Sir3-CBS138, indicating that the lack of complementation is not due to instability of the protein or lack of expression (Fig. S4)

4.2 *SIR3* allele from BG14 is dominant over the *SIR3* allele from MC2 clinical isolate

Saccharomyces cerevisiae Sir3 (*Sc*Sir3) is a histone and nucleosome binding protein that can bind nucleosomes mainly via its conserved BAH domain located at the N-terminal region, and its AAA^+ domain in the central region plays also a minor role in this association (Buchberger et al., 2008; Carmen et al., 2002). It has been shown using *in vitro* assays that *Sc*Sir3 protein has preference for residue K16 in histone H4 when it is hypoacetylated (Hecht et al., 1995; Liou et al., 2005; Wang et al., 2013). *Sc*Sir3 can form homodimers through its winged helix domain at the C-terminal end of the protein and also interacts with *Sc*Sir4 protein through its C-terminal domain (Behrouzi et al., 2016; Moretti et al., 1994; Oppikofer et al., 2013).

Our results show that *SIR3*-BG14 allele is dominant over the *sir3*-MC2, since the introduction of the *SIR3*-BG14 allele in the MC2 clinical isolate partially complements the hyperadherence phenotype (Fig. 4c). Interestingly, the MC2 strain transformed with *SIR3*-CBS138, displayed decreased adherence when compared to this isolate transformed with the empty vector. This is reminiscent of the data reported by (Halliwell, 2012), in which an extra copy of *SIR3*-CBS138 in the CBS138 strain, increased the subtelomeric silencing. This led to the proposal that *SIR3*-CBS138 allele is inefficient and silencing in this strain is *SIR3* dosage dependent. However, an extra copy of its own *sir3* (*sir3*-MC2) in MC2 strain did not significantly decrease adherence to epithelial cells compared to the vector transformed control. This suggests that the *sir3*-MC2 allele is even weaker in function than the *SIR3*-CBS138 allele. It should be noted that the conditions used in our experiments (stationary phase cultures) are thought to be similar to conditions found *in vivo* where silencing in some strains is very efficient. So differences in the ability to silence subtelomeric adhesin-encoding genes might have a large impact on the ability of *C. glabrata* to adhere to different surfaces.

4.3 The combination of multiple amino acid polymorphisms in Sir3-MC2 is required to abolish the functionality of the protein

We have previously reported the presence of multiple amino acid changes in Sir3-MC2, two of them are located in the C-terminal half of the protein, one within the AAA^+ and the other in the winged helix domain respectively (Martinez-Jimenez et al., 2013) (Fig. S3). Mutational analysis in the BAH and AAA^+ domains of *Sc*Sir3 has shown that silencing can be partially or totally abolished by certain mutations (Ehrentraut et al., 2011; Sampath et al., 2009). Sir3-MC2 allele contains the changes I739N and D998G relative to BG14 in the AAA^+ and winged helix domains respectively. The other three polymorphisms present in Sir3-MC2 allele (S236T; N268H; I503L), do not fall within any of the described domains and are shared with the sequenced strain CBS138, which is a functional allele for silencing of the subtelomeric *URA3* reporter, but is also a hyperadherent strain (Martinez-Jimenez et al., 2013). The unique combination of five polymorphisms in the Sir3-MC2, results in a very high level of adherence and lack of silencing of a subtelomeric reporter gene, suggesting that this particular combination of polymorphisms is required to render this protein non-functional, while all the other combinations of polymorphisms in Sir3 of the studied clinical isolates were at least partially functional. For example there is only one difference between Sir3-MC65 sequence (slightly adherent) and Sir3-MC2 (highly adherent), which is the polymorphism I739N in the AAA^+ domain in Sir3-MC2 but absent in Sir3-MC65, yet Sir3-MC65 can silence the reporter, suggesting that the five polymorphisms in Sir3-MC2 are required to completely abolish Sir3 function. In fact, the experiment using the two chimeras separating the polymorphisms in the *sir3*-MC2 ORF (chimera 1 contains the last two amino acid changes and chimera 2 the first three, like CBS138) supports this idea, since only chimera 1 is functional for both phenotypes assayed here: silencing of the reporter (Fig. 5 b) and complementation of the hyperadherent phenotype of the BG14 *sir3*Δ strain (Fig. 5 c). Interestingly, chimera 2, which has the same amino acid sequence as Sir3-CBS138, displays the same phenotype as Sir3-CBS138 (functional for silencing of the reporter and hypeadherent). One possibility is that this combination of polymorphisms disrupts the interaction of Sir3-MC2 homodimers and/or the interaction with Sir4-BG14.

Both Sir3 and Sir4 from several clinical isolates have many polymorphisms in certain domains of the proteins compared to other silencing proteins with additional activities such as Sir2 and yKu70 (one or no polymorphisms), and with other types of proteins such as two different enzymes involved in proline biosynthesis (no polymorphisms) (Table S4). This may indicate that Sir3 and Sir4 can accumulate more mutations to allow for different levels of silencing of target genes. This could result in more variability in the expression of subtelomeric, adhesin-encoding genes, which can increase the possibility of adhering to different tissues within the host. This is in contrast to other proteins, which have highly conserved activities and may be subject to strong selective pressure, and display much less variation (Table S4).

Here we have shown that only the *sir3*-MC2 allele was non functional, but in fact other silencing proteins could be defective in the MC2 background and/or the rest of the studied clinical isolates. Due to the requirement of the particular combination of polymorphisms in Sir3-MC2 and the presence of multiple changes in the sequence of Sir4-MC2 protein compared to Sir4-BG14 (Fig. S2), it is possible that the interactions between Sir3-MC2 and Sir4-MC2 could be affected in this background (less efficient), even though polymorphisms in Sir4-MC2 are not located in C-terminal coiled coil domain, which is possibly the main domain through which it interacts with Sir3. Another possibility, and probably one which has a stronger impact, is that the dimerization of Sir3-MC2 during the formation of the *SIR* complex could be compromised and, consequently, the *SIR* complex would be less stable.

Interestingly, both, Sir3 and Sir4 from the non-adherent isolate MC39, are identical in amino acid sequence to the corresponding BG14 proteins, supporting the idea that BG14 and MC39 alleles are the most efficient at subtelomeric silencing and that the hyper-adherent isolates have polymorphisms with respect to these efficient alleles. In fact, it is worth noting that all the hyperadherent isolates share two polymorphisms in both proteins, regardless of the level of adherence (S236T and I503L in Sir3 and K72N and L635S in Sir4) (Fig. S2 and S3).

Future experiments will be necessary to assess whether the alleles from the hyperadherent isolates form less stable SIR complexes than those from BG14 or MC39, and whether Sir3-BG14 can form dimers with Sir3-MC2 and other combinations. We are also characterizing

other silencing proteins in these hyper-adherent clinical isolates and the interactions between them.

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Conflict of interest:

The authors declare that they have no conflict of interest

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Figure Legends

Fig. 1 Adherence of *Candida glabrata* clinical isolates to HeLa cells *in vitro*. Adherence was calculated as percentage of *C. glabrata* stationary phase cells that remained adhered to the HeLa cells monolayer after extensive washing. Data represents the mean of three independent biological replicates with three technical replicates each. Statistical analysis was done using ANOVA with Dunnett´s multiple comparison test ($P<0.05$). Clinical isolates MC2; MC65, MC29, MC68 and CBS138 strain are statistically different from the BG14 strain except clinical isolate MC39 which is non-adherent like BG14. NA means Non Adherent; SA is Slightly Adherent; MA is Moderately Adherent and HA: is Highly Adherent.

Fig. 2 *SIR3* allele from the isolate MC2 is not functional, while the *SIR2* and *SIR4* alleles from all the hyperadherent clinical isolates tested are functional to silence a subtelomeric reporter. **a** Schematic representation of the *URA3* reporter gene location. **b** The *SIR2* allele from all the clinical isolates complement the *sir2*Δ in the BG14 background. The *SIR2* allele from each isolate was introduced into the *sir2*Δ and the expression of the *URA3* reporter was measured by growth on 5-FOA containing plates, which is correlated with the level of silencing of the reporter. Serial dilutions of stationary phase cultures were spotted

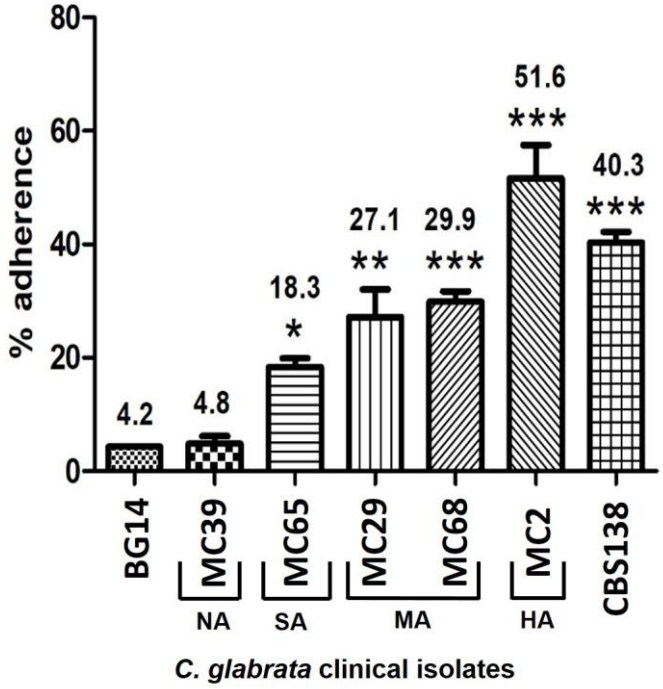
on the plates with the indicated media and incubated for 48 h at 30 °C and photographed. **c** The *sir3* allele from MC2 clinical isolate (*sir3*-MC2) does not complement the *sir3*Δ. The experiment was done as described for complementation with the *SIR2*. **d** The *SIR4* alleles from representative hyperadherent clinical isolates from each category complement the *sir4*Δ. The experiment was done as described in **2 a** and **b**. NA means Non Adherent; SA is Slightly Adherent; MA is Moderately Adherent and HA: is Highly Adherent.

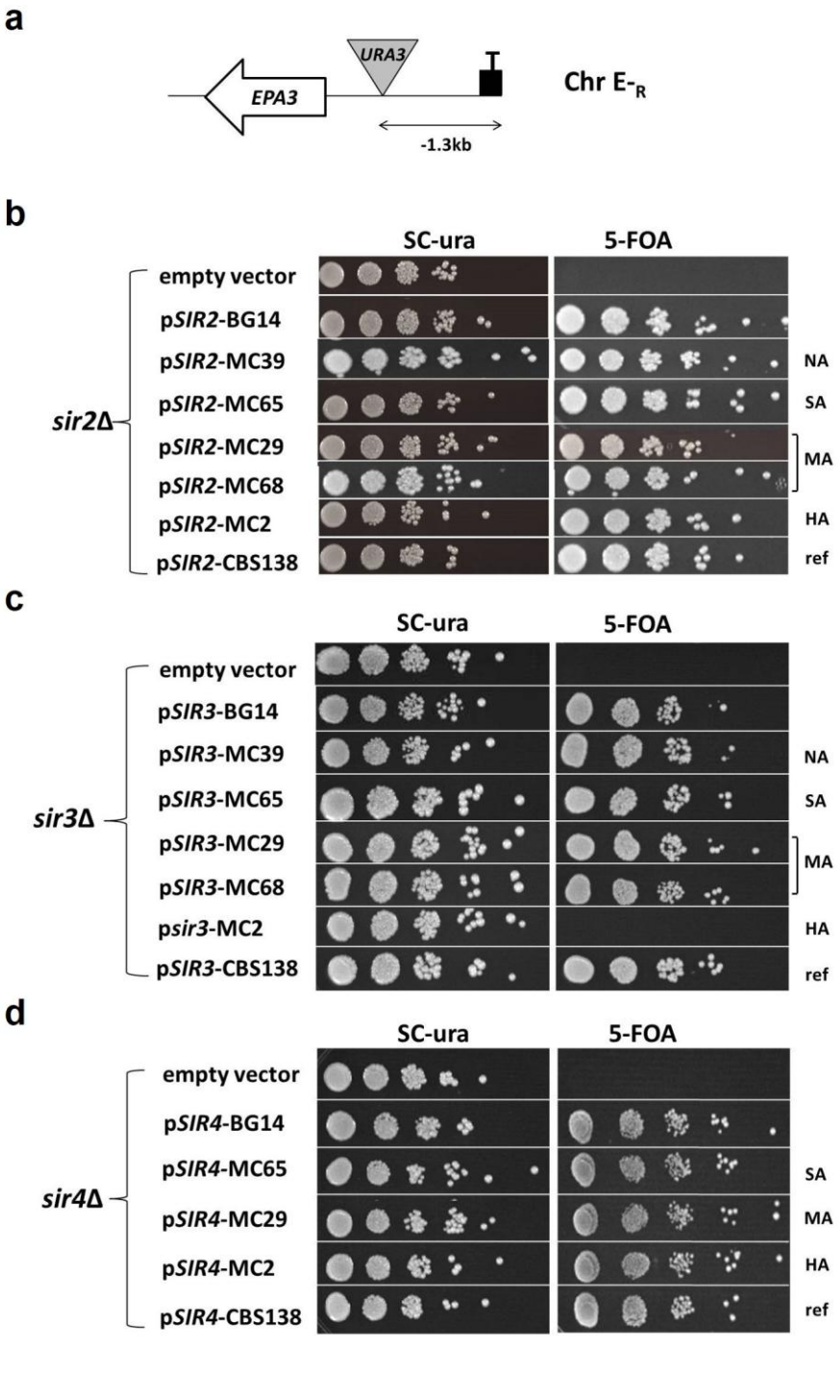
Fig. 3 The *sir3*-MC2 allele does not complement the *sir3*Δ strain for silencing the *URA3* reporter inserted at different telomeres. **a** The *sir3* allele from MC2 isolate does not silence the *URA3* reporter gene inserted 23.69 kb from telomere I_{-R} in the *sir3*Δ strain (BG14 background). The *sir3*-MC2 allele as well as that of the control strains BG14 and CBS138 were introduced independently into the *sir3*Δ strain and the expression of the *URA3* reporter was assesed by growth on 5-FOA. **b** The *sir3* allele from MC2 clinical isolate cannot complement the *sir3*Δ strain to silence the *URA3* reporter inserted 2.23 kb from telomere C_{-R}. Cells were grown and treated as described in Fig. 2.

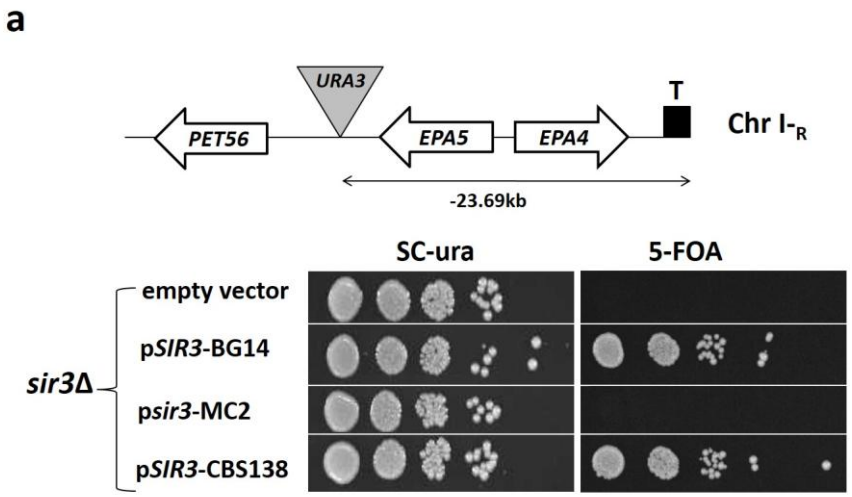
Fig. 4 The *sir3* allele from MC2 cannot complement the hyper-adherent phenotype of the *sir3*Δ strain. **a** The *SIR3* alleles from MC2 and control strains were introduced in replicative plasmids into the BG14 *sir3*Δ and the level of adhesion to HeLa cells was determined. The experiment was performed as described in Fig. 1. Statistical analysis (ANOVA, Dunnett´s Multiple Comparison Test $P<0.05$) shows significant differences only between the strain transformed with the empty vector (pMJ22) and the parental strain (BG14) and the strain transformed with *SIR3*-BG14 allele. **b** An extra copy of the *sir3*-MC2 allele does not change the level of adherence of the BG14 strain under repressing conditions for *EPA1* (stationary phase). The *SIR3* alleles from MC2 and control strains were introduced into the parental strain BG14 and the ability of adhesion to HeLa cells was determined. Strains were grown and processed as described in Fig. 1. Data represent the mean of three independent biological replicates. Statistical analysis (ANOVA, Dunnett´s Multiple Comparison Test $P<0.05$) shows there were no significant differences among the tested strains in the BG14 background. **c** An extra copy of the *SIR3* allele from BG14 reduces the hyper-adherent phenotype of the MC2 clinical isolate. The *SIR3* alleles from MC2 and control strains were

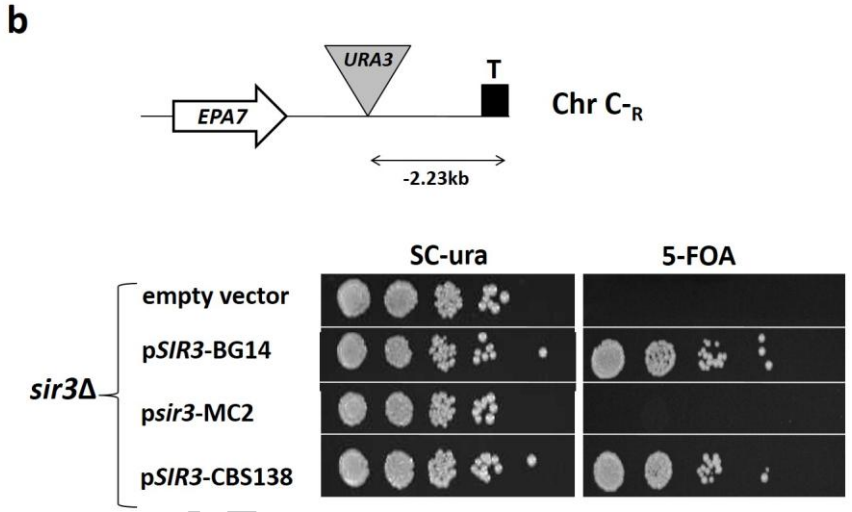
introduced into the MC2 clinical isolate and the ability of adhesion to HeLa cells was determined. The experiment was done as described in Fig. 1. Statistical analysis (ANOVA, Dunnett´s Multiple Comparison Test $P<0.05$) shows significant differences among the strain transformed with the empty vector (pMJ22) and the untransformed parental strain (BG14) as well as the ones transformed with the *SIR3* allele from BG14, MC2 and CBS138.

Fig. 5 The combination of all five polymorphisms present in the *sir3*-MC2 allele are required to lose its silencing activity. **a** Schematic representation of Chimera 1 and Chimera 2. **b (left):** Schematic representation of the *URA3* reporter location. **b (right):** Chimeras 1 and 2 containing only either the first 3 or the last 2 polymorphisms from *sir3*-MC2 can silence the *URA3* reporter gene in the *sir3*Δ strain. The plasmids containing the corresponding chimeras or control Sir3 alleles were introduced into the *sir3*Δ strain and the expression of the *URA3* reporter was assessed by growh on 5-FOA containing plates as described in Fig. 2b. . **c** Only chimera 1 can complement the *sir3*Δ strain in the BG14 background, while chimera 2 behaves like the identical allele Sir3-CBS138. The *SIR3* chimeras were introduced into the *sir3*Δ background and the level of adhesion to HeLa cells was determined. The experiment was performed as described in Fig. 1. Statistical analysis (ANOVA, Dunnett´s Multiple Comparison Test $P<0.05$) shows significant differences among the strain transformed with the empty vector (pMJ22) and the ones transformed with the *SIR3* allele from BG14, Chimera 1 and Chimera 2.

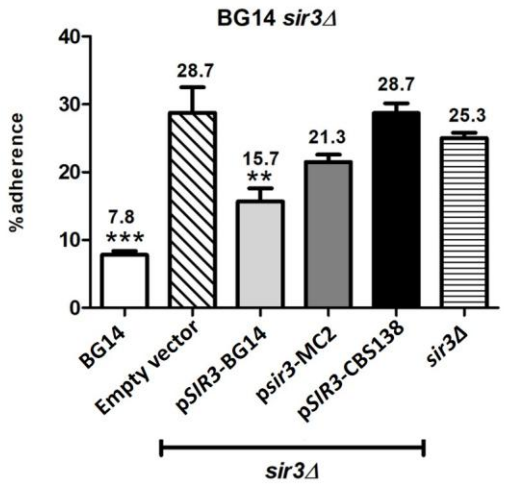



a
URA3
EPA3
Chr E-R
-1.3kb
b
SC-ura
5-FOA
empty vector
pSIR2-BG14
pSIR2-MC39
pSIR2-MC65
pSIR2-MC29
pSIR2-MC68
pSIR2-MC2
pSIR2-CBS138
sir2Δ
NA
SA
MA
HA
ref
c
SC-ura
5-FOA
empty vector
pSIR3-BG14
pSIR3-MC39
pSIR3-MC65
pSIR3-MC29
pSIR3-MC68
psir3-MC2
pSIR3-CBS138
sir3Δ
NA
SA
MA
HA
ref
d
SC-ura
5-FOA
empty vector
pSIR4-BG14
pSIR4-MC65
pSIR4-MC29
pSIR4-MC2
pSIR4-CBS138
sir4Δ
SA
MA
HA
ref

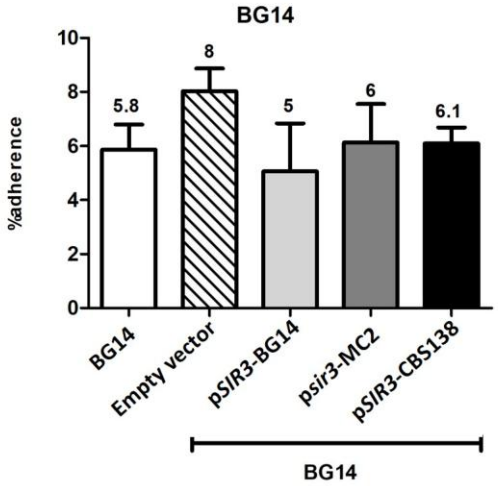




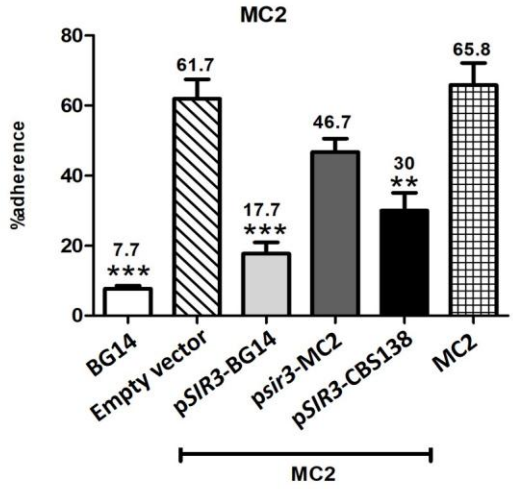
a

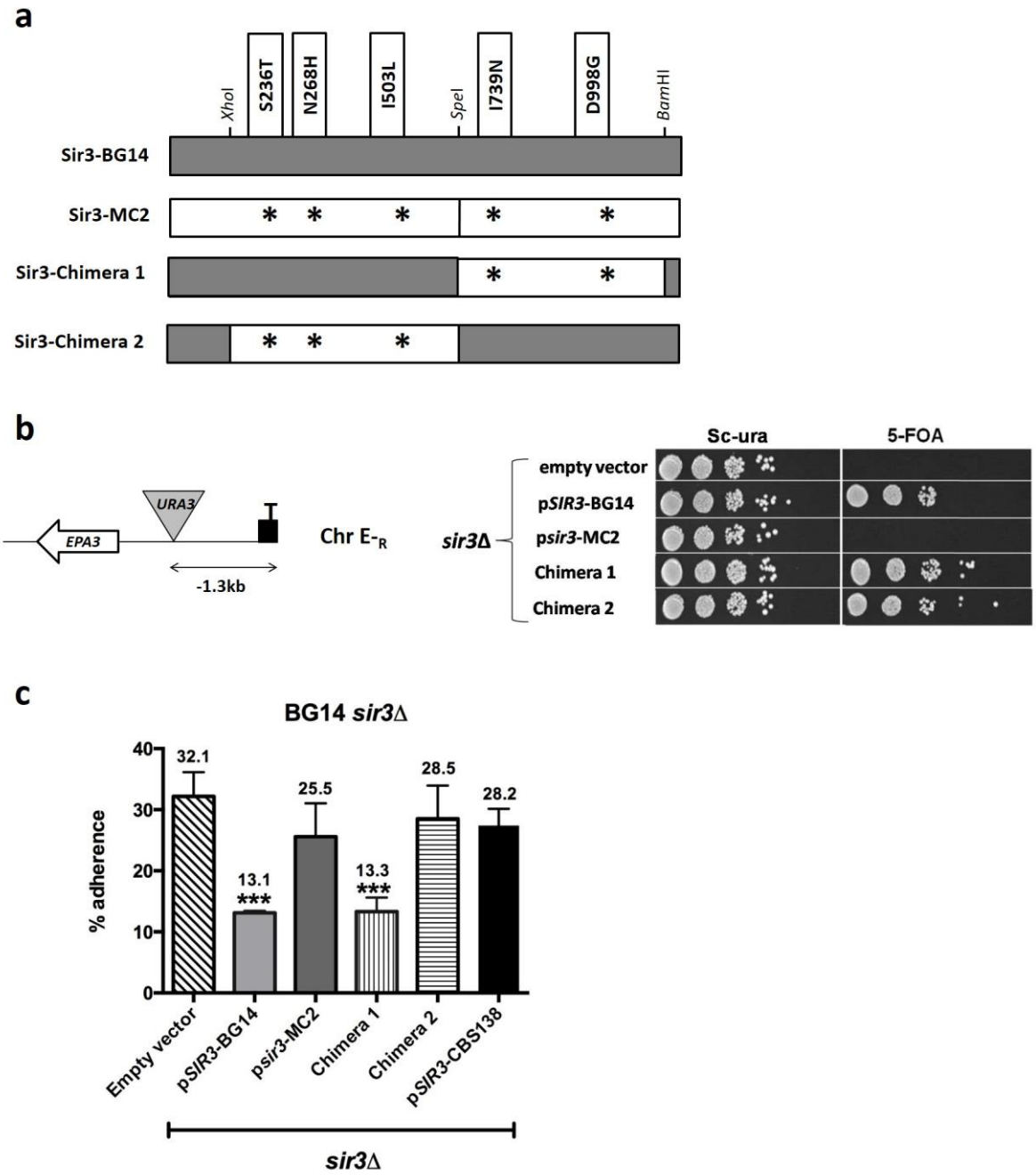

BG14 sir3Δ
% adherence
40
30
20
10
0
7.8
***
28.7
15.7
**
21.3
28.7
25.3
BG14
Empty vector
pSIR3-BG14
psir3-MC2
pSIR3-CBS138
sir3Δ
sir3Δ

b


BG14
%adherence
10
8
6
4
2
0
5.8
8
5
6
6.1
BG14
Empty vector
pSIR3-BG14
psir3-MC2
pSIR3-CBS138
BG14

c


MC2
%adherence
80
60
40
20
0
7.7
***
61.7
17.7
***
46.7
30
**
65.8
BG14
Empty vector
pSIR3-BG14
psir3-MC2
pSIR3-CBS138
MC2
MC2

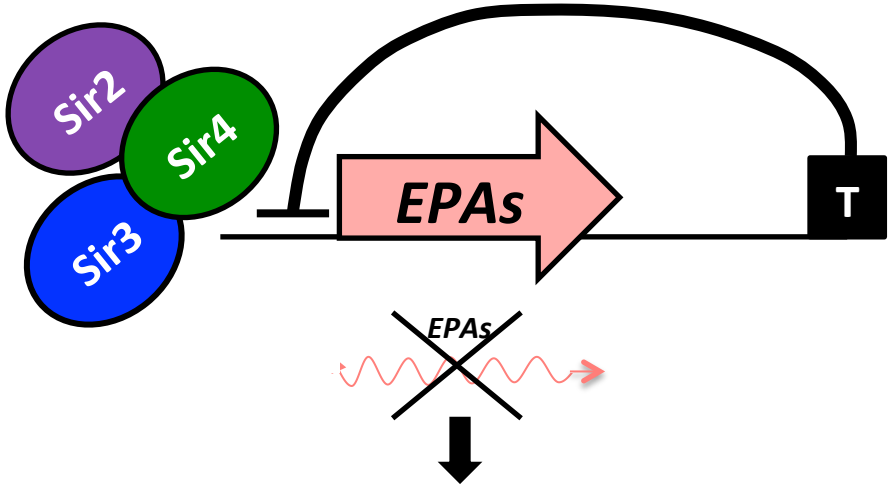

a
XhoI
S236T
N268H
I503L
SpeI
I739N
D998G
BamHI
Sir3-BG14
Sir3-MC2
Sir3-Chimera 1
Sir3-Chimera 2
b
URA3
EPA3
-1.3kb
Chr E-R
sir3Δ
Sc-ura
5-FOA
empty vector
pSIR3-BG14
psir3-MC2
Chimera 1
Chimera 2
c
BG14 sir3Δ
% adherence
32.1
13.1
***
25.5
13.3
***
28.5
28.2
Empty vector
pSIR3-BG14
psir3-MC2
Chimera 1
Chimera 2
pSIR3-CBS138
sir3Δ

Molecular characterization of the Silencing complex SIR in *Candida glabrata* hyperadherent clinical isolates

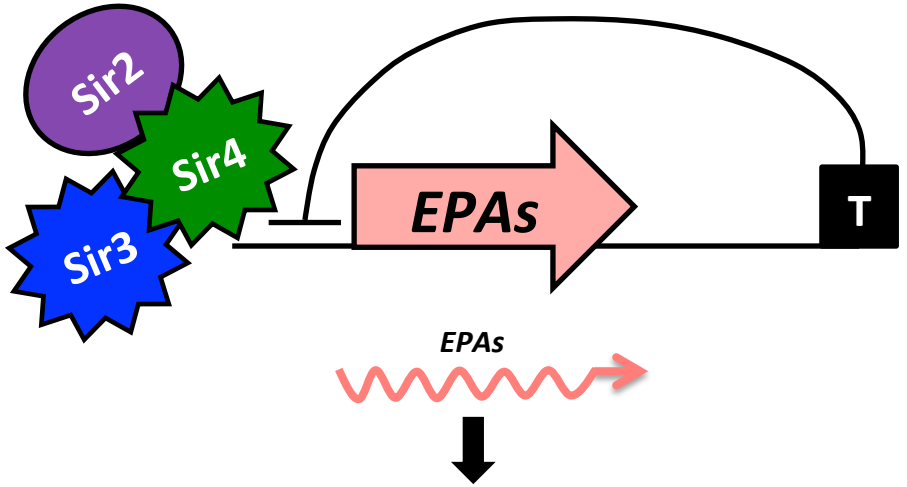
Osney Leiva-Peláez, Guadalupe Gutiérrez-Escobedo, Alejandro De Las Peñas and Irene Castaño

Highlights

- *C. glabrata* clinical isolates can be non-adherent or highly adherent to epithelial cells.
- The SIR complex is required for subtelomeric silencing of *EPA* genes
- Sir3 and Sir4 from hyperadherent isolates contain multiple polymorphisms
- The most adherent isolate MC2, contains a non-functional, recessive Sir3 allele

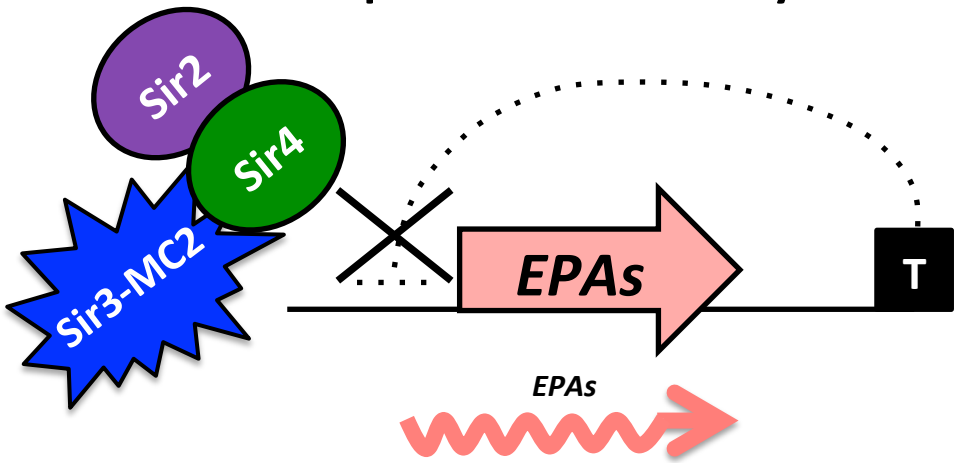


non-adherent
clinical isolates



hyper-adherent
clinical isolates

Complementation analysis



sir3-MC2 non-functional allele