A Protosilencer of Subtelomeric Gene Expression in *Candida glabrata* with Unique Properties

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ABSTRACT Adherence to host cells is an important step in the pathogenicity of the opportunistic fungal pathogen *Candida glabrata*. This adherence is mediated by some members of the large family of cell wall proteins encoded by the *EPA* (**Ep**ithelial **A**dhesin) genes present in the *C. glabrata* genome. The majority of the *EPA* genes are localized close to different telomeres in *C. glabrata*, resulting in a negative regulation of transcription of these genes through chromatin-based subtelomeric silencing. *In vitro*, adherence to epithelial cells is mainly mediated by Epa1, the only member of the *EPA* family that is expressed *in vitro*. *EPA1* forms a cluster with *EPA2* and *EPA3* at the subtelomeric region of telomere E_{-R}. *EPA2* and *EPA3* are subject to silencing that propagates from this telomere in a process that depends on the Sir2, -3, -4, and Rif1 proteins, but surprisingly not on the yKu70 and yKu80 proteins. Here we describe that the yKu70/yKu80-independent silencing of telomere E_{-R} is due to the presence of a *cis*-acting protosilencer (Sil2126) located between *EPA3* and the telomere. This element can silence a reporter gene when placed 31.9 kb away from this telomere, but not when it is removed from the telomere context, or when it is placed near other telomeres, or inverted with respect to the reporter. Importantly, we show that the *cis*-acting Sil2126 element is required for the yKu70/80-independent silencing of this telomere, underscoring the importance of *cis*-elements for repressive chromatin formation and spreading on some telomeres in *C. glabrata*.

THE fungal pathogen *Candida glabrata* is able to adhere to host epithelial cells *in vitro*, a property thought to be important for the virulence. This ability is mediated primarily by Epa1, the founding member of a large family of cell wall proteins, some of which have been shown to be functional adhesins (Cormack *et al.* 1999; De Las Penas *et al.* 2003; Castano *et al.* 2005). Most of the genes encoding these adhesins, the *EPA* genes, are localized to subtelomeric regions of the *C. glabrata* genome, where they are subject to chromatin-based silencing.

Subtelomeric silencing in *C. glabrata* is a form of transcriptional repression that depends on Sir2, Sir3, Sir4, Rap1, and to different extents on yKu70, yKu80 (encoded by the *HDF1* and *HDF2* genes), and Rif1, depending on the particular subtelomeric region (De Las Penas *et al.* 2003; Castano *et al.* 2005; Rosas-Hernandez *et al.* 2008). This form of repression can propagate over long distances from the telomere (up to 20 kb), silencing native genes as well as reporter genes inserted at these regions. *EPA1* is localized 21 kb from the right telomere of chromosome E (E_{-R}) and is the only *EPA* gene expressed *in vitro* (De Las Penas *et al.* 2003; Castano *et al.* 2005). *EPA1* forms a cluster with *EPA2* and *EPA3*, and silencing at this telomere can propagate toward the centromere, resulting in a lack of expression of both *EPA2* and *EPA3 in vitro*. This particular telomere is different from three other telomeres studied because it is the only example so far in *C. glabrata* of yKu70 and yKu80-independent subtelomeric silencing (Rosas-Hernandez *et al.* 2008).

Compared to *C. glabrata*, subtelomeric silencing in *Saccharomyces cerevisiae*, to which *C. glabrata* is closely related phylogenetically, generally propagates relatively short distances from the telomeric repeats (4–8 kb from the telomere) and depends on Sir2, Sir3, Sir4, Rap1, yKu70, and yKu80 (Pryde and Louis 1999). Telomeres in *S. cerevisiae* consist of short heterogeneous tandem repeats with a consensus sequence $T(G)_{2\text{-}3}(TG)_{1\text{-}6}$ (McEachern and Blackburn

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1994) ~350 bp in length forming nonhistone nucleoprotein complexes with Rap1 and yKu proteins. Additionally, adjacent to the ends, the subtelomeric regions contain two types of repeats, the Y′ and X elements that are organized into nucleosomes. About half of the chromosomes in *S. cerevisiae* contain Y′ elements (one to four copies per subtelomeric region) and all telomeres contain the X sequence, which is very heterogeneous. The only highly conserved sequence within X is the ~500-bp sequence called core X, which contains an origin of replication sequence or autonomously replicating sequence (ARS), which contributes to subtelomeric silencing; this sequence is present in some form at all telomeres of the sequenced strain (Louis 1995; Tham and Zakian 2002).

Silencing at other chromosomal loci such as the silent mating loci *HML* and *HMR* of *S. cerevisiae* is achieved through *cis*-acting elements known as silencers, which recruit a subset of the silencing proteins that condense the chromatin and results in very efficient transcriptional silencing. Silencers function autonomously to repress nearby genes (or ectopically inserted genes), resulting in a regional, rather than gene-specific repression. In *S. cerevisiae*, the silencers studied are composed of different arrangements of binding sites for the sequence-specific DNA-binding proteins Abf1, Rap1, and origin recognition complex (ORC) for DNA replication and exert their effect over several kilobases with no requirement for additional silencer elements to repress expression of the target genes (reviewed in Fourel *et al.* 2002; Fox and McConnell 2005; Buhler and Gasser 2009). Protosilencers on the other hand, are repressive elements that enhance the action of silencers but do not act by themselves; instead they strongly depend on the presence of *bona fide* silencers to bring about repression (Fourel *et al.* 1999; Lebrun *et al.* 2001). Protosilencers include several different types of sequences that can enhance and extend repression in regions where silencing is already present (silencing promoting environments). These elements may be as simple as single binding sites for silencer proteins (Rap1, Abf1, and ORC), or complex repetitive sequences like the core X sequences in subtelomeric regions in *S. cerevisiae* (Brand *et al.* 1985; Boscheron *et al.* 1996). The presence of these protosilencers at the subtelomeric regions thus can act as relays and propagate silencing over longer distances in a discontinuous manner (Fourel *et al.* 1999; Lebrun *et al.* 2001).

Silenced chromatin or heterochromatin is correlated with histone hypoacetylation and relatively lower gene density (reviewed in Rusche *et al.* 2003; Ottaviani *et al.* 2008; Rusche and Lynch 2009). Heterochromatin has the capacity to propagate and this is thought to be mediated by the histone deacetylase activity of Sir2 and subsequent binding of Sir3 and Sir4 to deacetylated histones H3 and H4 in neighboring nucleosomes. Sir3 and Sir4 in turn recruit more Sir2 molecules, resulting in propagation of the silenced structure (Hoppe *et al.* 2002; Rusche *et al.* 2002). Propagation of silenced chromatin is limited by the presence of other *cis*-acting elements called boundary or barrier elements that block spreading of the silenced chromatin, thereby separating transcriptionally active from inactive domains (Bi and Broach 1999; Rusche and Lynch 2009). Some boundary elements are associated with strong promoters that assemble RNA polymerase transcription complexes that can in turn recruit chromatin-remodeling complexes with histone acetyltransferase activity, like the tRNA gene on the telomere side of *HMR*. This transcription complex assembly can physically interfere with propagation of the silent chromatin or can compete with the histone deacetylase activity of the Sir complex (Donze *et al.* 1999; Donze and Kamakaka 2001), since transcription *per se* is not required for boundary activity, but recruitment of histone acetyltransferases is (Fox and McConnell 2005; Rusche and Lynch 2009). At *HMR*, repression of the native or reporter genes inserted between the silencers is almost complete, while silencing beyond the elements decreases sharply (McNally and Rine 1991; Bi *et al.* 1999; Rusche *et al.* 2003).

The level and degree of propagation of silencing at the subtelomeric regions of both *C. glabrata* and *S. cerevisiae* depend on the particular subtelomeric context (Pryde and Louis 1999; Rosas-Hernandez *et al.* 2008). In the E_{-R} telomere in *C. glabrata*, we have found a *cis*-acting element adjacent to *EPA3* that can silence a reporter gene when inserted 31.9 kb from the telomere. In this article we present evidence that indicates that this element, called Sil2126, is a protosilencer with unique properties: it contributes to silencing only at this telomere and not others, and it is responsible for the yKu proteins' independent silencing observed at this telomere.

Materials and Methods

Strains, plasmids, and primers

All strains, plasmids, and oligonucleotides used are listed in Supporting Information, Table S1, Table S2, and Table S3, respectively.

Media

Yeast were grown in standard yeast media as described previously (Sherman *et al.* 1986) with 2% agar added for plates. Synthetic complete (SC) contains 1.7 g/liter yeast nutrient base (without NH_2SO_4 and amino acids), 5 g/liter NH_2SO_4 and supplemented with 0.6% casamino acids and 2% glucose. When needed, SC was supplemented with 25 mg of uracil/liter. To score for resistance to 5-fluoroorotic acid (5-FOA; Toronto Research Chemicals), 0.9 g of 5-FOA and 25 mg of uracil/liter were added to the SC. Yeast extract-peptone-dextrose (YPD) medium contains 10 g/liter yeast extract, 20 g/liter peptone, and is supplemented with 2% glucose. When required, YPD plates were supplemented with hygromycin (Invitrogen) at 440 μg/ml.

Bacteria were grown in LB medium as described previously (Ausubel *et al.* 2001). LB medium contained 5 g/liter yeast extract, 10 g/liter tryptone, 5 g/liter NaCl. All plasmid constructs were introduced into strain DH10 by electroporation, and 50 μg/ml carbenicillin (Invitrogen) was added to select for plasmids. For plates, 1.5% agar was used.

Yeast transformation

Yeast transformations with digested plasmids were performed as previously described (Castano *et al.* 2003).

Reporter URA3 gene expression assays (5-FOA sensitivity assays)

The level of silencing of the *URA3* gene inserted at different positions throughout the telomeres was assessed using a plate growth assay as described previously (De Las Penas *et al.* 2003; Castano *et al.* 2005). Briefly, strains containing the different *URA3* insertions were grown in YPD for 36 hr to stationary phase. The cultures were adjusted to an optical density of 1 with sterile water at 600 nm, and 10-fold serial dilutions were made in 96-well plates. A total of 5 μl of each dilution was spotted onto YPD, SC lacking uracil (SC −Ura), and SC +5-FOA plates, then incubated for 48 hr at 30°, and then photographed.

Construction of C. glabrata strains with different Sil2126-URA3 integrations

To generate all integration strains containing the Sil2126-*URA3* reporter system, we first constructed a starting plasmid on integrative vector pYIp*lac*211 containing an integration region and the complete Sil2126 region as follows. A 0.671-kb PCR product containing the intergenic region between *ISC1* and *HYR1* with *Pst*I and *Sal*I ends, and a 2.126-kb PCR product containing the Sil2126 region with *Sal*I and *Bam*HI ends were cloned into the same sites in pYIp*lac*211, which contains the *S. cerevisiae URA3* gene with its own promoter that is used as the reporter gene. In this construct (pAP430) the *URA3* gene and the Sil element are 511 bp apart (Figure 1). pAP430 plasmid was linearized with *Spe*I and integrated by homologous recombination in *C. glabrata* at −32 kb from telomere E_{-R} (between *ISC1* and *HYR1* genes and the *URA3* reporter start codon lies 35 kb away from the telomere). The series of *C. glabrata* strains with 5′ and 3′ serial deletions of Sil2126 were constructed by transforming *C. glabrata* with the appropriate plasmids inducing homologous recombination. The serial deletion plasmids were constructed by double digestion of the starting plasmid using internal sites within the Sil2126 region and an external site or replacing Sil2126 fragment by PCR products of progressively smaller fragments of the Sil2126 region. To construct the strains with Sil2126-*URA3* integrated at different genomic loci, the *Pst*I–*Sal*I integration fragment on starting plasmid was replaced by PCR products of different genomic locations containing a unique restriction enzyme site to linearize and integrate at the homologous genomic region in *C. glabrata*.

Construction of HDF1, HDF2, and Sil2126 deletion strains

To obtain the *HDF1* and *HDF2* deletion strains, fragments >800 bp from the 5′ and 3′ flanking intergenic regions of each gene were cloned into pAP599 flanking the hygromycin expression cassette. The plasmids generated (pAJ27 and pAJ28, respectively) (Table S2) were used to obtain allele replacements of each gene to be deleted by homologous recombination in a one-step gene replacement procedure. Briefly, each plasmid was digested with enzymes cutting at both ends and within the cloned 5′ and 3′ flanking fragments, generating ends homologous to each specific gene to be deleted in the *C. glabrata* genome. The released fragment was used to transform *C. glabrata*, selecting on plates supplemented with 440 μg of hygromycin/ml. Homologous recombination and allele replacement of each locus were verified by PCR analysis using a primer that anneals in the sequences external to the cloned fragments and a primer annealing within the hygromycin cassette. We also verified the absence of each gene deleted by the inability to PCR amplify an internal fragment from each deleted gene.

A similar strategy was used to construct a vector to delete the Sil2126 region; the resulting plasmid (pAJ25) was digested and transformed into *C. glabrata*. Allele replacement was verified as described above.

The double mutant strains carrying deletions in *HDF1* and *HDF2* and a Sil2126 insertion at −2.1 kb of telomere I_{-R} were constructed by transforming *C. glabrata hdf1*Δ or *hdf2*Δ strains with the appropriate linearized plasmids to induce homologous recombination.

Construction of double mutants

To generate double mutants (silΔ *hdf1*Δ or silΔ *hdf2*Δ), single *hdf1*Δ::*hph* or *hdf2*Δ::*hph* mutants that contain an insertion of the *hph* cassette flanked by the **F**lp1 **r**ecognition **t**arget (FRT) sites, were transformed with plasmid pLS9 (Table S2). This plasmid expresses Sc*FLP1* recombinase that recognizes two direct repeats, the FRT sites, under the control of an inducible promoter. A total of 50 μl of stationary phase cells harboring this plasmid were resuspended in fresh YPD and were grown for 2 hr. The Flp1 recombinase induces recombination between FRT sites and the hygromycin marker is excised from the chromosome, resulting in an unmarked deletion of the corresponding gene and leaving one copy of the FRT site. Dilutions of the cell suspensions were plated on YPD, and replica plates were made on YPD hygromycin plates. Unmarked deletion strains (*hdf1*Δ or *hdf2*Δ) are identified as Hyg^S colonies and the deletion is confirmed by PCR, using primers annealing in the chromosome outside the 5′ and 3′ regions used for the disruption.

To construct double mutants, the *hdf1*Δ and *hdf2*Δ unmarked deletion strains described above were transformed with the deletion/insertion construct to delete Sil2126 (pAJ25, see Table S2). Transformants were screened to verify integration of the deletion/insertion plasmid at the correct chromosomal location by PCR as previously described, and the *hph* cassette was removed by expressing the Flp1 recombinase as above.

To test whether the silencing proteins are required for Sil2126 activity, we used deletion alleles of *RIF1*, *SIR2*, *SIR3*, *HDF1*, *HDF2*, and the *rap1-21* allele (since *RAP1* is

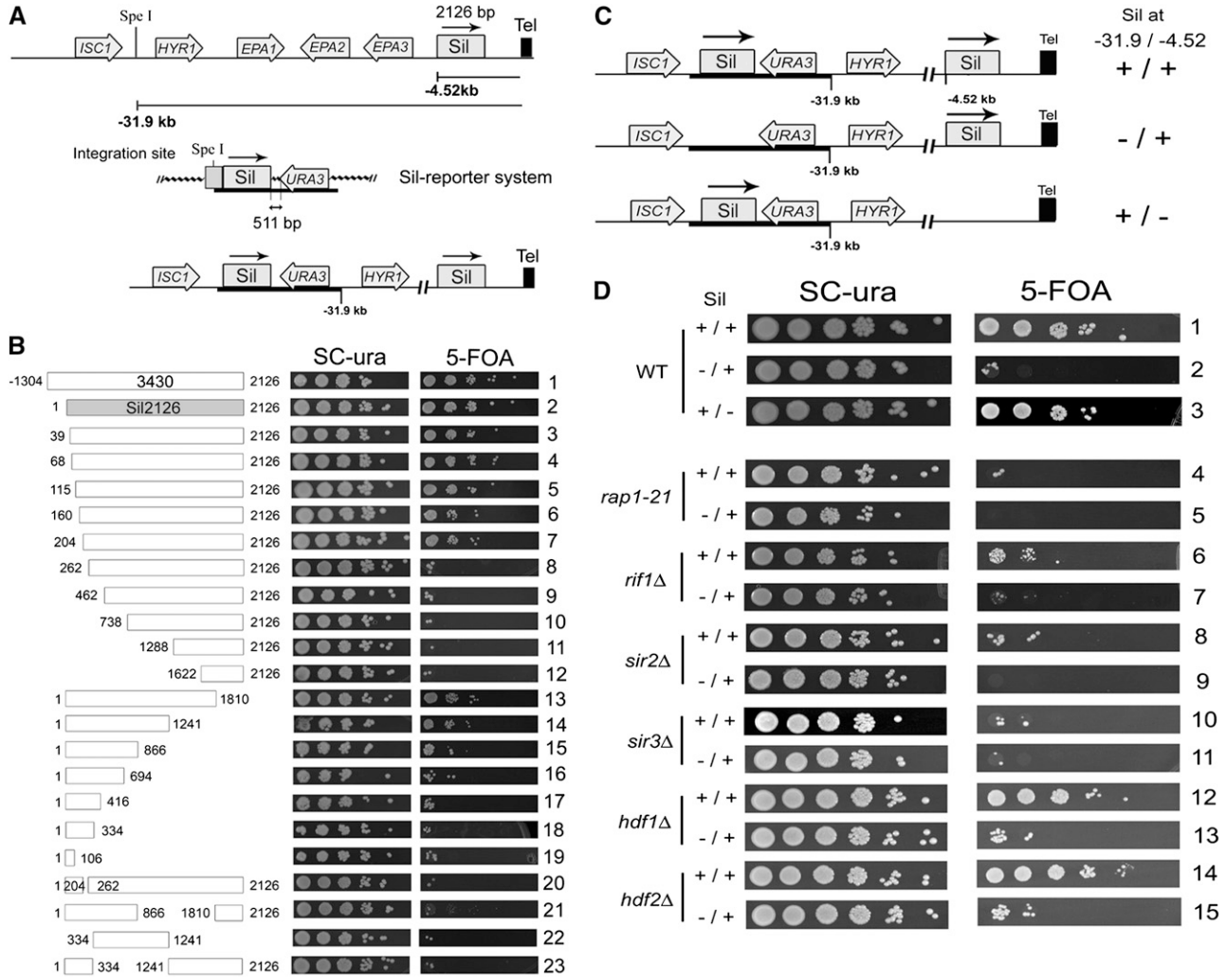

Figure 1 The *cis*-acting silencing element Sil2126 is composed of at least two functional modules and requires Sir2, Sir3, Rap1, Rif1, but not the yKu70/80 heterodimer. (A, top) Schematic representation of the E_{-R} telomere showing the Sil2126 element (represented by a shaded rectangle labeled 2126), and the nucleotide position of its 5′ end (4.52 kb from the telomere). Arrowheads indicate 5′ to 3′ direction of Sil2126. Lightly shaded arrows represent the different subtelomeric *EPA* genes and indicate their direction of transcription. (Middle) Map of the Sil-reporter system used to integrate at the *Spe*I site between *ISC1* and *HYR1*, consisting of a PCR fragment containing the integration region, cloned immediately adjacent to the 5′ end of the Sil2126 element followed by the *URA3* reporter gene. The *Spe*I recognition site (at −31.9 kb) used to linearize and integrate the vector is indicated. (Bottom) Schematic representation of the Sil2126-reporter system integrated between *ISC1* and *HYR1* (indicated by a thick solid line). The telomere is represented by solid rectangles labeled "Tel." (B) Assessment of the level of silencing of serial 5′ and 3′ deletions of the Sil2126 element and precise deletions of the two modules identified. On the left side is shown the different constructs represented by open rectangles, used to assay the level of silencing of the *URA3* gene conferred by the truncated versions of the Sil2126 element. Numbers on either side of the rectangles indicate the nucleotide position of the 5′ and 3′ ends of the truncated versions. The complete, original Sil2126 element is indicated by the shaded rectangle. On the right side is shown the level of silencing conferred by the truncated Sil elements tested as measured by growth on SC −ura and 5-FOA plates. Strains of *C. glabrata* containing the different truncated versions of the Sil element integrated between *ISC1* and *HYR1* were grown to stationary phase in YPD, and 10-fold serial dilutions in sterile water were made. Equal numbers of cells of each dilution were spotted onto SC −ura and SC plates containing 5-FOA. Plates were incubated at 30° for 48 hr and photographed. (C) Schematic representation of different combinations of Sil2126 elements at telomere E_R. (Top) Map of the E_{-R} telomere showing the original Sil2126 element at its native position (−4.52 kb from the telomere repeats) plus the integrated Sil2126-reporter system integrated at −31.9 kb (*Spe*I site) generating a duplication of Sil2126 element, indicated by Sil +/+ on the right side. (Middle) Integration of the negative control used consisting of the *URA3* reporter integrated at −31.9 kb without Sil2126. This construct conserves the original Sil2126 at −4.52 kb and is indicated by sil −/+. (Bottom) Map of the construct containing the Sil2126-reporter system integrated at −31.9 kb of telomere E_{-R} and deletion of the original Sil element at −4.52 kb, indicated by Sil +/−. (D) Level of silencing of the Sil-*URA3* reporter constructs (indicated to the left) in different mutants of the silencing machinery. The experiments were made as indicated in B.

an essential gene), to introduce into the strain containing the Sil-reporter system. The *rap1-21* allele is a deletion of the last 28 amino acids of Rap1, which has been shown in *S. cerevisiae* to be the domain that interacts with Sir3, resulting in a viable strain that is defective for subtelomeric silencing (Liu *et al.* 1994), and we have shown that it has the same phenotype in *C. glabrata* (De Las Penas *et al.* 2003).

RT-PCR

RNA was extracted from stationary-phase cells (36 hr in YPD) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Sigma-Aldrich). Synthesis of cDNA and PCR was carried out using the AccessQuick reverse transcription–PCR system (Promega). The RT primers used for *EPA3* and *ACT1* gene are listed in Table S3. The cDNA synthesis reaction was carried out at 45° for 45 min for all of the genes. The PCR was carried out at 57° for *EPA3* and *ACT1* genes. A reaction mixture without reverse transcriptase was included as a negative control in all of the RNA samples and with every pair of primers. No bands were obtained, indicating that the RNA preparations had no DNA contamination. RT–PCR experiments were performed three separate times with different RNA extractions. Results in Figure 4C are reproducible and show a representative experiment.

Results

Telomere E_{-R} of *C. glabrata* contains a cis-acting protosilencer element

We have previously identified a *cis*-acting element localized close to the right telomere of chromosome E (E_{-R}) of *C. glabrata* where *EPA1*, *EPA2*, and *EPA3* reside. This element, called Sil2126, is a 2.126-kb DNA fragment that extends from nucleotide positions 684,673–686,798 (accession no. CR380951) and can silence a *URA3* reporter gene when placed 31.9 kb away from this telomere in a region not normally subject to subtelomeric silencing (Rosas-Hernandez *et al.* 2008).

The *C. glabrata* genome contains only one more sequence very similar to Sil2126 at position 8061–10,185, from the left telomere of chromosome A (A_{-L}) (accesion no. CR380947.2). A smaller fragment of this element (nucleotides 120–872 of Sil2126) is also present between *EPA5* and *EPA4* at position 15,040–15,850 from telomere I_{-R} (accession no. AY344225.1), Figure S1.

To finely map the Sil2126 element at the E_{-R} telomere, we first constructed a reporter-Sil system to assay silencing activity by the Sil2126 element (Figure 1A, middle line). This system consists of an integrative plasmid that contains a PCR fragment containing a 671-bp fragment of the intergenic region between *ISC1* (CAGL0E06556g) and *HYR1* (CAGL0E06600g), used as the integration site (*Spe*I site at −31.9 kb from telomere E_{-R}), and the Sil fragment (or the deletion derivatives) cloned immediately adjacent to it, followed by the *URA3* reporter gene in the vector. Silencing activity by the Sil2126 element (and the deletion derivatives) was assayed by the ability of the strains carrying each construct to grow on plates lacking uracil (SC −ura plates) where only cells expressing the *URA3* reporter can grow, and on plates containing 5-fluoorotic acid (5-FOA plates) where cells expressing *URA3* convert 5-FOA into a toxic compound and do not grow.

We constructed 5′ and 3′ end deletion series of the Sil2126 element and integrated each one 31.9 kb away from telomere E_{-R} (the *URA3* promoter is positioned at −35 kb). We initially determined whether the complete Sil2126 could extend on the 5′ side up to the start codon of *EPA3*. As shown in Figure 1B the entire region between *EPA3* and Sil2126 (3430 bp, line 1) confers essentially the same level of silencing as the previously described Sil2126 (Rosas-Hernandez *et al.* 2008) (Figure 1B, compare lines 1 and 2) and therefore Sil2126 comprises the entire element. As shown in Figure 1B, deletions from the 5′ essentially show the same silencing activity as the entire Sil2126 element, when the first 204 bp have been removed (Figure 1B, lines 2–7). Deleting the next 58 nucleotides results in almost complete loss of silencing by this element, indicating that the sequence from nucleotides 204 to 262 is essential for the silencing activity of Sil2126 (Figure 1B, compare line 7 with line 8). The 3′ end deletion series on the other hand, shows a gradual decrease in silencing by the removal of up to nucleotide 1241 of Sil2126 (removal of the last 885 bp of Sil2126) (Figure 1B, lines 13 and 14). The next deletion from the 3′ end up to nucleotide 866 (removal of 1260 nucleotides) dramatically decreases silencing (Figure 1B, line 15), suggesting that between nucleotides 866 and 1810 resides an important module of the Sil2126 element. Progressive deletions from the 3′ end from this point very gradually decrease the remaining silencing effect until there is no detectable silencing activity (deletion to nucleotide 334; Figure 1B, line 18), and does not decrease with further deletion to nucleotide 106 (Figure 1B, line 19). Therefore the fragment from nucleotide 1 to 334 has no silencing activity by itself despite containing nucleotides 204–262, identified as essential in the 5′ deletion series. This suggests that nucleotides 204–262 are essential but not sufficient for silencing activity by the Sil2126 element. We made two additional constructs in which we deleted precisely the two fragments that appeared to contain important elements for silencing activity. First a deletion of 58 nucleotides from position 204–262 (region 1) and second, a precise deletion of 944 bp from nucleotides 866–1810 (region 2). We found that silencing in both of these constructs is almost completely lost (Figure 1B, lines 20 and 21), supporting the notion that both of these fragments are required for silencing activity. The last two constructs tested did not silence the reporter either, and include an internal fragment from nucleotides 334–1241 (Figure 1B, line 22) and a combination of the 5′ end of the element (from nucleotides 1–334) cloned adjacent to a fragment from nucleotide 1241 to the end of the element (nucleotide 2126). Taken together, these data indicate that Sil2126 is a *cis*-acting element composed of at least two distinct modules important for silencing activity: the first element (region 1) comprises nucleotides 204–262 (Figure 1B, compare line 2 with lines 7, 8, and 20), and the other (region 2) extends from nucleotides 866–1810 (Figure 1B, compare line 2 with lines 13–15 and 21).

Sil2126 element depends on Rap1, Sir2, and Sir3, partially on Rif1 but not on yKu70/80

Insertion of the Sil-reporter system at −31.9 kb from telomere E_{-R} generates a duplication of the Sil2126 at this chromosome end (the original Sil2126 is localized 4.52 kb

upstream from the telomere; see Figure 1C, top line); therefore, we wondered whether the silencing activity of Sil2126 depends on the presence of the original copy near the telomere. We deleted the original copy of Sil2126, leaving only one copy of the Sil-reporter system at −31.9 kb (Figure 1C, compare bottom and top lines, +/− and +/+), and found that this element silences the *URA3* reporter efficiently in the absence of the original copy (Figure 1D, lines 1–3).

We have previously shown that the Sil2126 element depends on Sir3 and partially on Rif1, but not on yKu70/80 (Rosas-Hernandez *et al.* 2008). We have now extended these results by testing the dependence on Sir2 and Rap1. The Rap1-21 allele used is defective in subtelomeric silencing in *C. glabrata* (De Las Penas *et al.* 2003). The results confirmed previous data (Rosas-Hernandez *et al.* 2008) and in addition we show that Sil2126 also depends on Rap1 and Sir2 to silence the reporter at −31.9 kb from the telomere E_{-R} (Figure 1D, compare line 1 with lines 4 and 8).

Sil2126 requires the telomere E_{-R} context and is orientation dependent

The Sil2126 element mediates silencing of a *URA3* gene integrated at −35 kb away from the telomere. To test whether this element is a *bona fide* silencer or a protosilencer that requires the telomere context, we first integrated the Sil-reporter system further away from the same telomere at a distance of −50 kb from telomere E_{-R}. We found that increasing the distance from 35 to 50 kb from the same telomere, completely abolishes the silencing activity of Sil2126 (Figure 2A, compare lines 1 and 2 with 3 and 4). We also moved the Sil-reporter system to three different internal locations in different chromosomes, >100 kb from either telomeres or centromeres, structures where chromatin is normally assembled into a repressive conformation. We chose a large intergenic region in chromosome F between CAGL0F04015g and CAGL0F04081g genes 527 kb away from F_{-R} telomere, or an intergenic region in chromosome M between CAGL0M01870g and CAGL0M01892g localized 224 kb from the left telomere, and in an intergenic region in chromosome L between CAGL0L06072g and CAGL0L06094g, 678 kb from the telomere L_{-L}. Figure 2A shows that the Sil-reporter system does not display silencing activity at any of the three internal chromosomal locations tested, suggesting that Sil2126 requires the telomere context, thus behaving as a protosilencer (Figure 2A, compare lines 5–9 with lines 1 and 2).

To test whether any telomere can serve as a nucleating center for the silencing activity for Sil2126, we integrated the Sil-reporter system close to four other telomeres. We introduced the system in both orientations, 34 kb away from the left telomere on chromosome E (E_L), a distance similar to where we detect silencing activity by Sil2126 at the other telomere (E_{-R}). Interestingly, we found that Sil2126 cannot mediate silencing of the reporter at this position in either orientation (Figure 2B, compare lines 10–12 with 1 and 2). We also integrated the Sil-reporter system at position −26 kb from telomere C_{-L}, at position −23 kb from telomere I_{-L} and at position −19.4 kb from telomere K_R. Even though all of these subtelomeric regions have a similar relatively low gene density to that of subtelomeric region E_{-R} (from 3 to 6 genes), and most of these genes encode either *EPA* genes or other cell wall proteins (like telomere E_{-R}), the Sil2126 element does not mediate silencing at any of these other subtelomeric positions, indicating that telomeres are not equivalent at propagating and or nucleating silencing (Figure 2B, compare lines 13–18 with lines 1 and 2).

Because Sil2126 behaves as a protosilencer rather than a silencer, we also tested whether its activity is orientation dependent like some other protosilencers (McNally and Rine 1991; Shei and Broach 1995; Bi *et al.* 1999; Zou *et al.* 2006). We inverted Sil2126 with respect to the *URA3* gene and integrated it at the original location near telomere E_{-R} (*URA3* promoter is at −35 kb from the telomere repeats). As shown in Figure 2C, line 21, inverting Sil2126 completely abolishes silencing activity of the element, further indicating that Sil2126 behaves as a protosilencer. Furthermore, inverting the whole Sil-reporter system with respect to the telomere E_{-R} (but maintaining the relative orientation between Sil2126 and the *URA3* reporter) and inserting it at −31.9 kb, also results in loss of silencing mediated by the Sil2126 element (Figure 2B, line 22).

Subtelomeric silencing at telomere E_{-R} is strongly diminished by a strong promoter inserted close to the telomeric repeats

A strong promoter close to a silenced region can work as a barrier to prevent spreading of the silent chromatin nucleated at a telomere or a silencer located close by (Bi and Broach 1999; Donze *et al.* 1999; Donze and Kamakaka 2001; Fourel *et al.* 2001). To test whether a strong promoter can interrupt silencing started at the E_{-R} telomere, we used the set of strains containing insertions of the *URA3* gene at different positions throughout the E_{-R} telomere, and replaced in each of them the Sil2126 element with the hygromycin-resistance cassette containing the strong promoter from the *PGK1* gene driving the *hph* gene. We also constructed a simple deletion of Sil2126 in each reporter strain by removing the hygromycin cassette by recombination of the flanking FRT sites with Flp1 leaving only a 35-bp FRT "scar" (*Materials and Methods*). As shown in Figure 3 the presence of the *PGK1* promoter dramatically diminishes silencing nucleated at the telomere of different *URA3* reporter insertions located at increasing distances from telomere E_{-R} (Figure 3B, compare lines 1 and 2 with line 3; lines 4 and 5 with line 6; and lines 7 and 8 with line 9). Interestingly, silencing of the reporter insertions throughout this telomere is achieved even in the absence of the original copy of Sil2126 (silΔ) in those constructs where the *PGK1* promoter has been deleted (Figure 3, compare lines 1, 4, and 7 with lines 2, 5, and 8).

yKu70/80 independent silencing of telomere E_{-R} is due to the Sil2126 element

We next considered that the presence of the Sil2126 at this telomere might contribute to the silencing nucleated at the

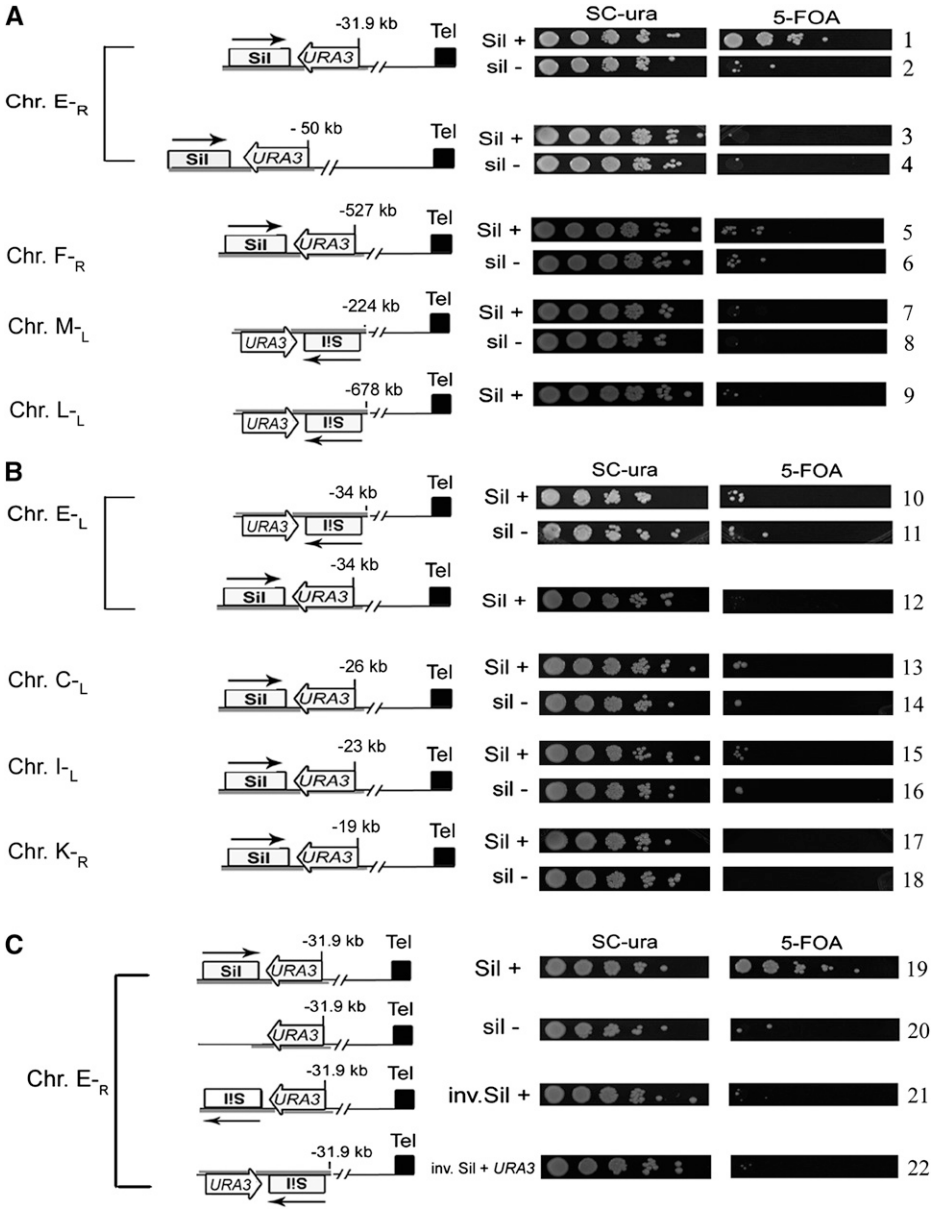

Figure 2 Sil2126 requires the telomere E_{-R} context and is orientation dependent. (A) Dependence of the Sil element on the proximity to the telomere. Schematic representation of the Sil2126-reporter system is shown on the left side (Sil+). The schematic map of the negative control (sil−) consisting only of the *URA3* reporter with no Sil2126 is not shown. Both of these constructs are integrated at two positions in telomere E_{-R}: at −31.9 kb (the *URA3* gene starts at position −35 kb), lines 1 and 2; and integration at −50 kb (lines 3 and 4). Integration of the Sil + or sil− constructs at an internal position in chromosome F (527 kb from the telomere F_R, lines 5 and 6). Integration of the constructs at an internal position in chromosome M (224 kb from the telomere M_{-L}, lines 7 and 8) and integration of the Sil+ at an internal site in chromosome L at 678 kb from telomere L_{-L} (line 9). On the right side, the growth phenotype on SC −ura and on 5-FOA plates is shown for each construct to assess the level of silencing. Strains carrying the corresponding Sil-reporter constructs integrated at the indicated chromosomal locations were grown to stationary phase in YPD and diluted and spotted on SC −ura and 5-FOA plates as described in Figure 1B. (B) Sil2126 is functional only in the context of telomere E_{-R}. Insertion of Sil+ and sil− reporter constructs in both orientations at −34 kb from telomere E_{-L} (lines 10–12) or at various distances from three other telomeres: C_{-L}, I_{-L}, and K_{-R} abolishes silencing activity of the element (lines 13–18). (C) Silencing activity of Sil2126 is orientation dependent. The orientation of the Sil2126 was inverted with respect to the *URA3* reporter and integrated at −31.9 kb from the telomere E_{-R} (lines 19 and 21). Orientation of the entire module Sil2126-*URA3* was inverted, resulting in inversion of both elements of the system with respect to the original orientation but maintaining the same relative position to each other (line 22). All the telomeres were drawn to the right of the sequences. The *URA3* reporter and Sil2126 were flipped accordingly to show the correct relative orientation and order between each element and the telomere. Arrowheads indicate the 5′ to 3′ direction of Sil2126.

telomere and could account for the relative independence of this telomere to the silencing proteins yKu70 and yKu80, which are essential for subtelomeric silencing in *S. cerevisiae* and at least three other telomeres tested in *C. glabrata*. We introduced deletion alleles of Sil2126 (silΔ), yKu70 (*hdf1*Δ), yKu80 (*hdf2*Δ), and double deletion mutants (*hdf1*Δ silΔ and *hdf2* silΔ) into the four *URA3* reporter strains. As shown in Figure 4A, single deletions of Sil2126 or either of the *HDF1* or *HDF2* genes has almost no effect on the level of silencing on each of the *URA3* reporters (Figure 4A, compare line 1 with lines 2–4). However, double deletions of Sil2126 and either *HDF1* or *HDF2* results in complete loss of silencing of the four reporter genes assayed at this telomere (Figure 4A, compare line 1 with lines 5 and 6), indicating that Sil2126 and yKu70/80 perform overlapping functions for silencing at this telomere.

We tested whether silencing of the native gene *EPA3* also depends on both Sil2126 and yKu70/80, by assaying *EPA3* expression by RT–PCR in different deletion strains. We found that even though there is no detectable expression of *EPA3* in stationary phase cultures of the wild-type and silΔ strains (Figure 4C, lanes 2 and 4), *EPA3* transcription is strongly derepressed in two types of strains: in the double *hdf2*Δ silΔ as well as in the strain where the *PGK1* promoter has been inserted between the telomere and *EPA3* (Figure 4C, lanes 5 and 9). These results indicate that the apparent independence of the yKu proteins for silencing of the native *EPA3* gene, and the reporter *URA3* inserted at different distances from telomere E_{-R}, requires the presence of Sil2126 in this telomere, and that yKu proteins and Sil2126 perform overlapping functions to bring about silencing at this telomere.

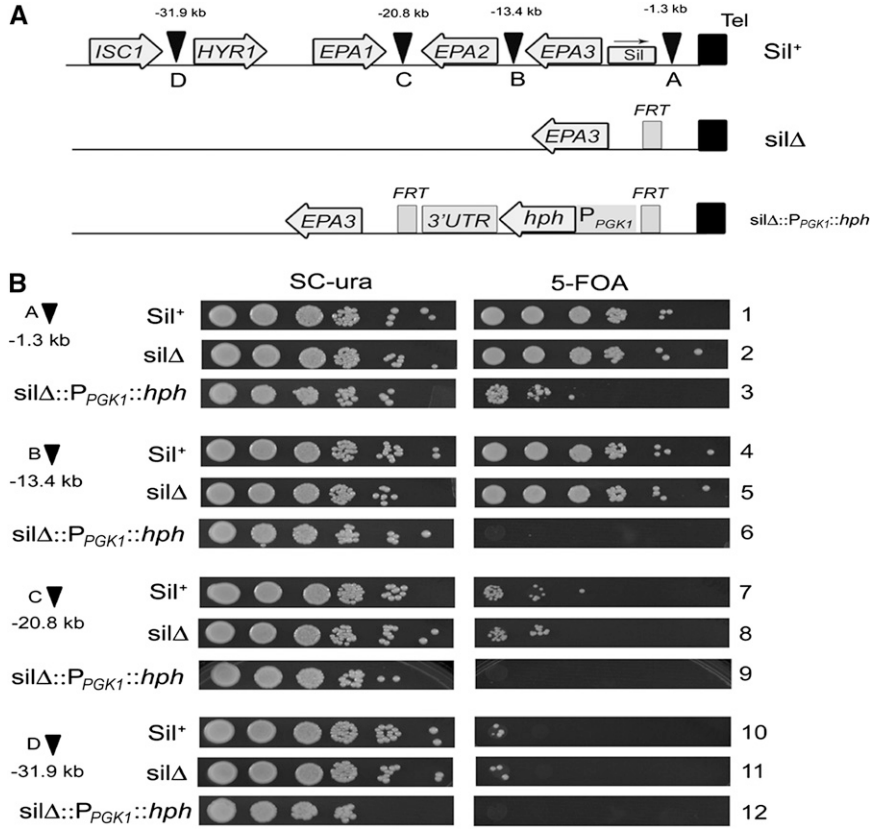

Figure 3 Subtelomeric silencing in telomere E_{-R} is abolished by the presence of a strong promoter between the telomere and the *URA3* reporter. (A, top) Schematic representation of the subtelomeric region of chromosome E_{-R}. Open, shaded arrows indicate the five native genes present in this region and their respective direction of transcription. Inverted solid triangles indicate *URA3* insertions at the positions shown with respect to the telomere. The shaded rectangle indicates the Sil2126 element at its original position. Arrowhead indicates the 5′ to 3′ direction of the element. (Middle) The hph cassette is excised from the deletion/insertion allele leaving one copy of the 35-bp FRT site. (Bottom) Sil deletion/insertion allele was integrated in each strain carrying the corresponding *URA3* reporter insertions at different positions throughout telomere E-R. The *hph* gene driven by the strong promoter from *PGK1* is inserted replacing Sil2126 thus placing a strong promoter close to the telomere. (B) Plate growth assay of strains containing the indicated *URA3* reporter insertions at increasing distances from the telomere in the wild-type Sil2126 genetic background (Sil+), the sil simple deletion (silΔ), and sil deletion/insertion allele (silΔ::P_{PGK1}::*hph*) as indicated. Strains containing each of the *URA3* reporter insertions in each of the three backgrounds were grown to stationary phase in YPD and spotted onto the indicated plates as described in Figure 1B.

Sil2126 alone is not sufficient to confer yKu70 and yKu80 independence to a telomere

To test whether the Sil2126 element can confer yKu independence to a yKu-dependent telomere, we inserted the Sil-reporter system at −2.1 kb from the I_{-R} telomere, which we have shown to be absolutely dependent on both Ku proteins for subtelomeric silencing (Rosas-Hernandez *et al.* 2008). As shown in Figure 4B, we confirmed previous results in which the reporter *URA3* inserted at this position (I_{-R} telomere) is silenced and the level of silencing is comparable in the presence or absence of Sil2126 (Figure 4B, lines 7 and 8). Interestingly, deleting either *HDF1* or *HDF2*, completely abolishes silencing of the reporter in the presence (or absence) of the Sil2126 element (Figure 4B, compare lines 9–12 with lines 7 and 8), indicating that Sil2126 is required but not sufficient to confer yKu independence silencing to a given telomere in *C. glabrata*.

Discussion

Adherence of *C. glabrata* to host epithelial cells depends on the expression of some members of the *EPA* family of adhesins, in particular Epa1, which is the major protein mediating adherence to epithelial cells *in vitro* (Cormack *et al.* 1999). The *C. glabrata* genome contains ∼23 *EPA* genes (depending on the strain), the majority of which are encoded in subtelomeric regions where they are subject to chromatin-based silencing. Of particular interest is the fact that the subtelomeric silencing of *EPA2* and *EPA3* genes, which are localized in a cluster close to the right telomere on chromosome E (E_{-R}), does not depend on yKu70 and yKu80 proteins (Rosas-Hernandez *et al.* 2008). In this article, we characterized the *cis*-acting element Sil2126 localized between *EPA3* and the telomere E_{-R}. The data presented suggest first that Sil2126 is a unique protosilencer element that contributes to silencing only at this telomere, and second that Sil2126 and the Ku proteins perform overlapping functions to bring about silencing, so that the presence of Sil2126 in this telomere results in the apparent lack of dependence of this telomere on the yKu proteins.

Sil2126 contains two sequences that are necessary for silencing activity

Detailed deletion analysis presented in Figure 1B shows that the two sequences from nt 204–262 (region 1) and from nt 866–1810 (region 2) are essential for silencing function of Sil2126. We found a single, putative Abf1 binding site, located precisely within region 1, at nt position 212–224 (Figure S2). *C. glabrata* Abf1 is 80 and 77% identical in each of the bipartite DNA binding domains of *S. cerevisiae* Abf1, suggesting that *Cg* Abf1 could recognize similar consensus sequences in the DNA. We also found a possible binding site for Rap1 localized 5′ to the putative Abf1 site at nt position 154–165 (Figure S2), but deletion of this fragment did not result in loss of silencing. The presence of these putative binding sites suggests that Sil2126 might function by recruiting Abf1 and possibly Rap1 silencing proteins.

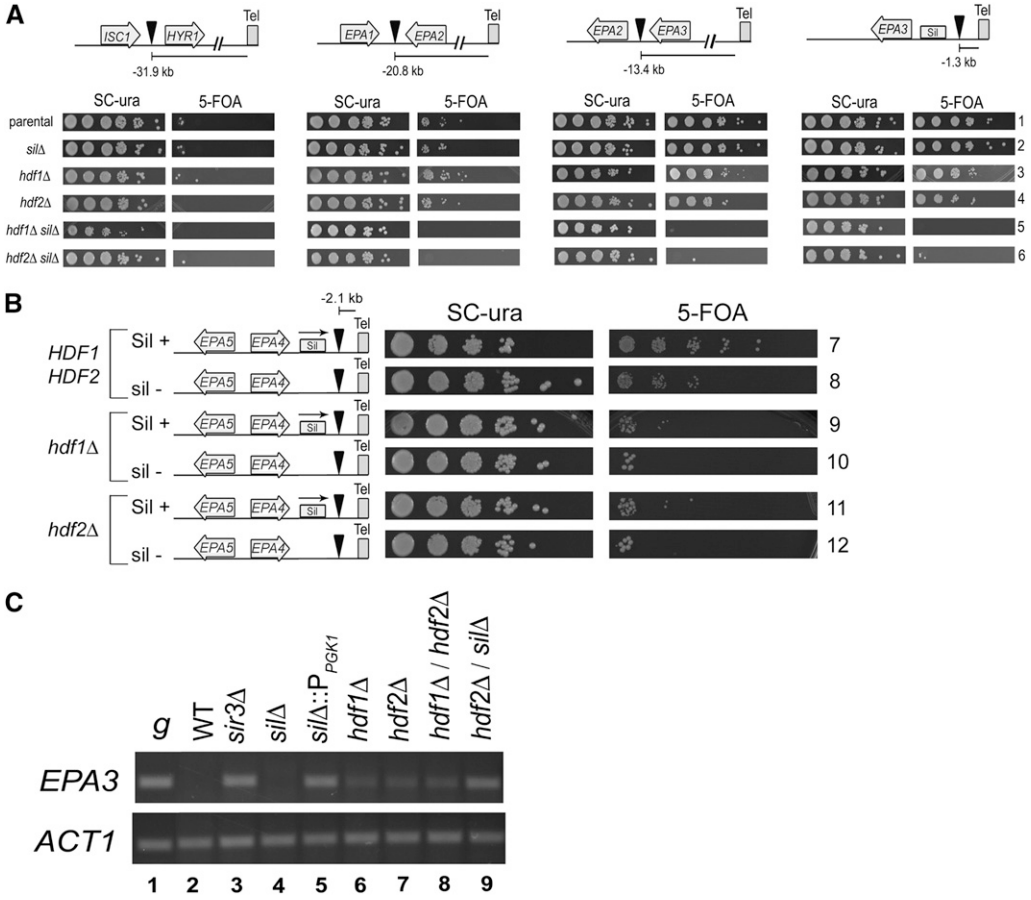

Figure 4 yKu70/80 independence of subtelomeric silencing on chromosome E_{-R} requires the Sil2126 element. (A) Silencing of four different *URA3* reporters (solid triangles) in the E_{-R} telomere as measured by the ability to grow on 5-FOA plates. Strains containing the indicated reporter insertions throughout the E_{-R} telomere (shown at the top of each panel) were used as recipients to introduce the following deletion alleles: silΔ, *hdf1*Δ (yKu70), *hdf2*Δ (yKu80), or double deletions *hdf1*Δ *sil*Δ or *hdf2*Δ *sil*Δ. All of these strains were grown to stationary phase and spotted onto the indicated plates as described in Figure 1B. (B) Sil2126 is not sufficient to confer yKu independence to the yKu-dependent telomere I_{-R}. The Sil-reporter system was integrated at position −2.1 kb from telomere I_{-R} where subtelomeric silencing depends on both yKu proteins. These strains were used as recipients to introduce *hdf1*Δ (yKu70), *hdf2*Δ (yKu80) alleles. The level of silencing was assessed in the same way as described in Figure 1B. (C) *EPA3* transcription is strongly derepressed both when the Sil2126 element is deleted as well as one of the *HDF* genes (*hdf1*Δ or *hdf2*Δ) or when the strong promoter from *PGK1* is inserted between the telomere and *EPA3*. We used a *sir3*Δ strain as control since we have previously shown that *EPA3* is derepressed in the absence of Sir3. All strains were grown to stationary phase and total RNA was isolated and used for RT–PCR (see *Materials and Methods*). Lane 1 shows genomic DNA used as positive control for PCR. Actin-specific primers were used as internal control for RT–PCR. A negative control without RT was also made and no bands were obtained (not shown).

Sil2126 behaves as a protosilencer with unique properties

The *cis*-acting Sil2126 region described in this article behaves as an atypical protosilencer with the unique property of depending on a specific subtelomeric context: the chromosome E_{-R} subtelomeric region. In this regard, Sil2126 is different from the protosilencers described in *S. cerevisiae* that can enhance silencing nucleated at silencing conducive environments like telomeres or *bona fide* silencers (Lebrun *et al.* 2001). The conclusion that Sil2126 is a unique type of protosilencer is based on several pieces of evidence: (a) The element does not mediate silencing of the reporter when it is moved to internal regions in three different chromosomes far from a telomere or centromere; (b) the silencing activity of Sil2126 is sensitive to the distance from its natural telomere, although it is also possible that there may exist a boundary element in this region that could cause the same effect; (c) Sil2126 activity is orientation dependent; and (d) only telomere E_{-R} provides the appropriate context for the silencing activity of Sil2126.

The additional telomeres we used to integrate the Sil-reporter system were chosen so that genes at the different subtelomeric regions were similar to those in telomere E_{-R} and that have relatively low gene density (only 4–6 genes in the ~20-kb fragment to the respective telomere). We also used shorter distances than the one used in telomere E_{-R}, but Sil2126 did not function at any of these other regions (Figure 2B). We propose that in telomere E_{-R} there are other structural elements that are required for the protosilencer activity that we have not identified yet. Therefore, the particular combinations of *cis*-elements at each subtelomeric region, as well as distance from the telomere, are both important determinants of the final silencing efficiency at each telomere.

Sil2126 is responsible for the apparent yKu70 yKu80 independent silencing at telomere E_{-R}, but it is not sufficient to confer yKu-independent subtelomeric silencing to the yKu-dependent telomere I_{-R}

We have shown that the presence of Sil2126 in the subtelomeric region of chromosome E_{-R} renders silencing at this telomere seemingly independent of yKu70 and yKu80 (Figure 4A). However, the participation of these proteins in silencing is uncovered in the strain carrying a deletion of Sil2126.

Surprisingly, we found that insertion of Sil2126 close to the telomere I_{-R} does not confer yKu70 and yKu80 independence to this telomere (Figure 4B). This is consistent, however, with the fact that Sil2126 can only function in the context of telomere E_{-R} and not at other subtelomeric regions, perhaps because of the requirement for an additional unidentified element present only at telomere E_{-R}.

Silencing mediated by the combination of *bona fide* silencers and protosilencers can propagate discontinuously by the formation of chromatin loops between the telomere and the protosilencers present at subtelomeric loci or other silencing conducive environments (Fourel *et al.* 1999, 2001; Sun and Elgin 1999; Lebrun *et al.* 2001). Such loops could explain the repression observed "at a distance" in *S. cerevisiae* when the repressor Tup1 is tethered to the DNA near a telomere and far from the target promoter (Zaman *et al.* 2002) and the presence of Rap1 and yKu at positions far away from the telomeric repeats and chromosome ends, where they normally bind (Strahl-Bolsinger *et al.* 1997; Martin *et al.* 1999; Zaman *et al.* 2002).

In the E_{-R} subtelomeric region, silencing is propagated over long distances and the combination of the telomere environment, the Sil2126, and presumably other structural elements at the subtelomeric region, provides strong silencing of this locus. yKu70 and yKu80 must be normally contributing to silencing of this telomere because they become essential in the absence of Sil2126. Similarly, in *S. cerevisiae* *hdf1*Δ mutants, loss of silencing at truncated telomeres that contain core X element is less severe than in truncated telomeres not containing core X (Fourel *et al.* 1999); and in *hdf1*Δ mutants the presence of an ARS consensus sequence (ACS) or Abf1 binding site at a truncated telomere can mediate partial silencing that compensates for the loss of yKu70 (Lebrun *et al.* 2001). We are currently investigating whether the distribution of the Sir and yKu proteins along the E_{-R} telomere is continuous or discontinuous and what proteins are required or bind to Sil2126. In particular, we are testing whether Abf1 and Rap1 bind to the element.

The *EPA1* gene, encoding the major adhesin in *C. glabrata*, is close to the E_{-R} telomere and subject to a complex transcriptional regulation. In this work we have provided evidence to suggest that there are significant differences in the silencing mechanism at different telomeres in *C. glabrata*, and that the particular combination of *cis*-acting sequences at each telomere determines the level of silencing at each position in different telomeres. This could result in a particular pattern of *EPA* gene expression depending on the specific context where the genes reside. In addition it is possible that some of the proteins involved in silencing at the subtelomeric regions respond to environmental signals, which modulate the level of repression differently at each telomere. The ability to express different *EPA* genes could allow *C. glabrata* to adhere to different surfaces and this could be important for the persistence of *C. glabrata* in different environments within the mammalian host.

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GENETICS

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A Protosilencer of Subtelomeric Gene Expression in *Candida glabrata* with Unique Properties

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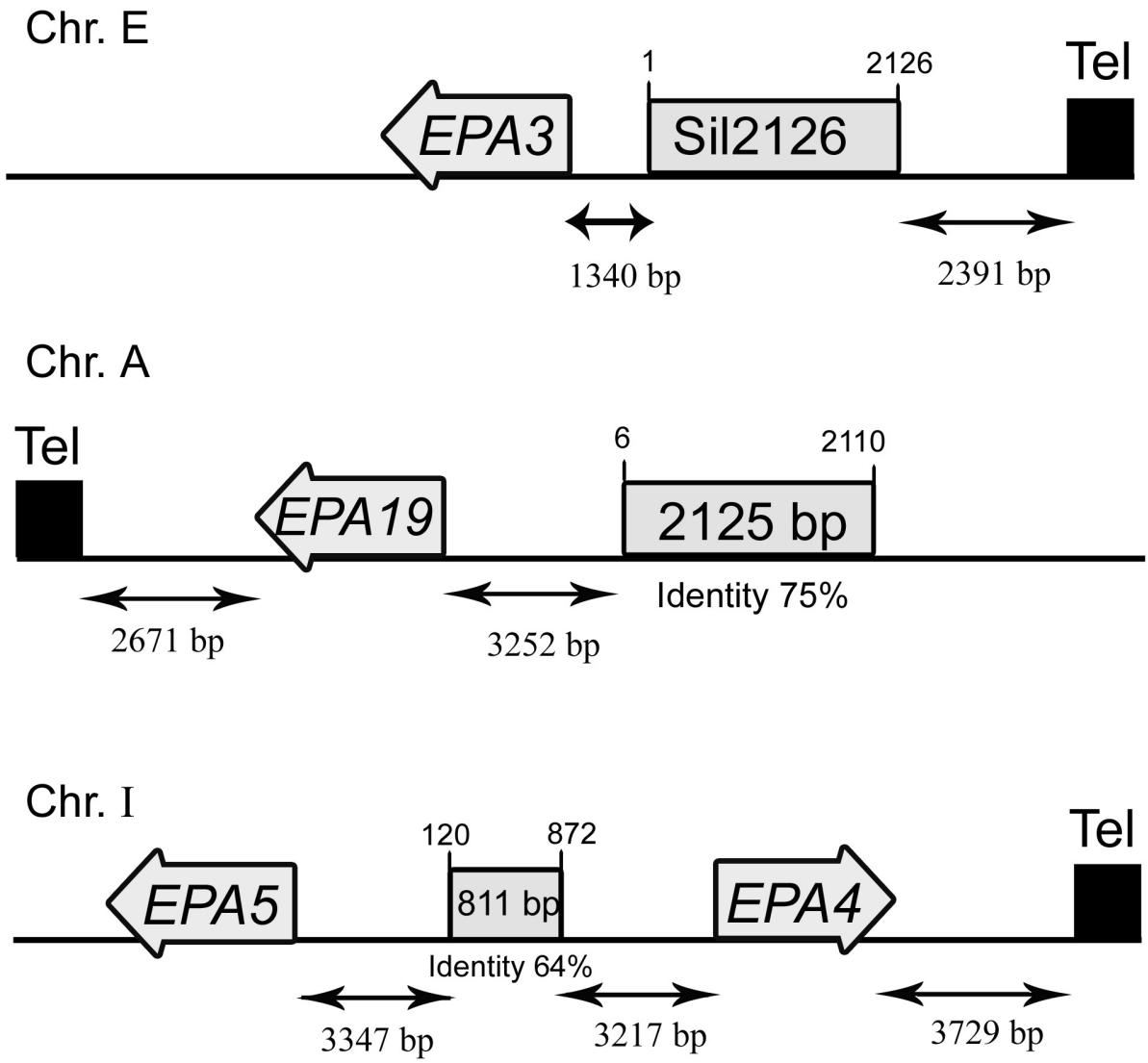

Figure S1 The *C. glabrata* genome contains one additional copy of Sil2126 close to telomere A_{-L}. Only one sequence of almost the same size and with high similarity to Sil2126 (75% identity) was found near telomere A_{-L}. A small fragment (811 bp long) with 65% identity to Sil 2126 was also found between *EPA4* and *EPA5*. Numbers above the boxes of the elements with similarity indicate the base positions where similarity starts with respect to Sil2126.

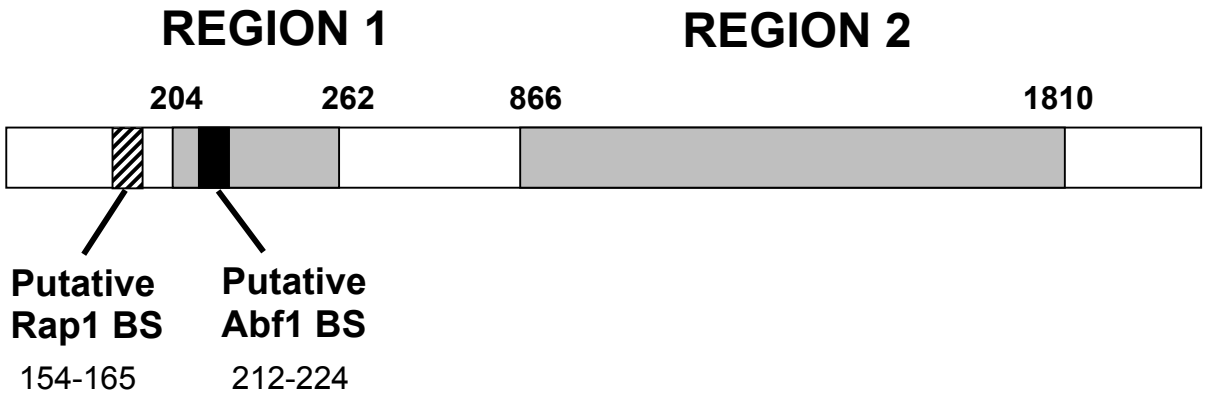

Figure S2 The Sil2126 element contains two regions essential for silencing activity and putative binding sites for Abf1 and Rap1. Schematic representation of Sil2126, the two gray boxes labeled region 1 and region 2 represent the sequences identified as necessary for silencing function. The numbers above the boxes indicate the nucleotide positions of the regions. The black box within region 1 indicates the position of the putative binding site for Abf1. The small hatched box indicates the putative Rap1 binding site and its position.

Table S1 ***Escherichia coli*** **and** ***Candida glabrata*** **strains used in this study**

E. coli strains	Genotype	Reference
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) f80d*lacZ*Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara,leu*)7697 *galU galK* I$^-$ *rpsL nupG*	(CALVIN and HANAWALT 1988)

Transposon		
Tn*7*	Tn*7* R6Kg ori *URA3 npt* (Km^R)	(CASTANO *et al.* 2003)

Candida glabrata strains			
Strain	Parent	Genotype	
BG2		Clinical isolate	(FIDEL *et al.* 1996)
BG14	BG2	*ura3Δ*::Tn*903* G418^R	(CORMACK and FALKOW 1999)
Tn*7* insertions at *EPA* loci			
BG684	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *ISC1* and *HYR1* (pAP540 *Bcg* I). Insertion 1	(DE LAS PENAS *et al.* 2003)
BG646	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *EPA1* and *EPA2* (pAP508 *Spe* I/*Bcg* I). Insertion 2	(DE LAS PENAS *et al.* 2003)
BG833	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *EPA2* and *EPA3* (pAP559 *Bsr*G I/*Sph* I). Insertion 3	(DE LAS PENAS *et al.* 2003)
BG432	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *EPA3* and telomere (pAP553 *Pst* I/*Eco*R I). Insertion 4	(DE LAS PENAS *et al.* 2003)
Deletion analysis of Sil2126-*URA3* reporter system at -31.9 kb			
Strain	Parent	Genotype	
CGM399	BG14	*ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	(ROSAS-HERNANDEZ *et al.* 2008)
CGM397	BG14	*ura3Δ*::Tn*903* G418^R pAP430 *Spe* I integrated in the chromosome	(ROSAS-HERNANDEZ *et al.* 2008)
CGM435	BG14	*ura3Δ*::Tn*903* G418^R pAP566 *Spe I* integrated in the chromosome	This work
CGM401	BG14	*ura3Δ*::Tn*903* G418^R pAP567 *Spe I* integrated in the chromosome	This work
CGM403	BG14	*ura3Δ*::Tn*903* G418^R pAP568 *Spe I* integrated in the chromosome	This work
CGM405	BG14	*ura3Δ*::Tn*903* G418^R pAP569*Spe I* integrated in the chromosome	This work
CGM407	BG14	*ura3Δ*::Tn*903* G418^R pAP570 *Spe I* integrated in the chromosome	This work
CGM409	BG14	*ura3Δ*::Tn*903* G418^R pAP571 *Spe I* integrated in the chromosome	This work
CGM411	BG14	*ura3Δ*::Tn*903* G418^R pAP572 *Spe I* integrated in the chromosome	This work
CGM437	BG14	*ura3Δ*::Tn*903* G418^R pAJ1 *Spe I* integrated in the chromosome	This work
CGM438	BG14	*ura3Δ*::Tn*903* G418^R pAJ2 *Spe I* integrated in the chromosome	This work
CGM984	BG14	*ura3Δ*::Tn*903* G418^R pAJ29 *Spe I* integrated in the chromosome	This work

CGM960	BG14	*ura3Δ*::Tn*903* G418^R pAJ30 *Spe I* integrated in the chromosome	This work
CGM1009	BG14	*ura3Δ*::Tn*903* G418^R PAJ31 *Spe I* integrated in the chromosome	This work
CGM1019	BG14	*ura3Δ*::Tn*903* G418^R pAJ32 *Spe I* integrated in the chromosome	This work
CGM962	BG14	*ura3Δ*::Tn*903* G418^R pAJ33 *Spe I* integrated in the chromosome	This work
CGM449	BG14	*ura3Δ*::Tn*903* G418^R pAJ7 *Spe I* integrated in the chromosome	This work
CGM447	BG14	*ura3Δ*::Tn*903* G418^R pAJ6 *Spe I* integrated in the chromosome	This work
CGM445	BG14	*ura3Δ*::Tn*903* G418^R pAJ5 *Spe I* integrated in the chromosome	This work
CGM443	BG14	*ura3Δ*::Tn*903* G418^R pAJ4 *Spe I* integrated in the chromosome	This work
CGM441	BG14	*ura3Δ*::Tn*903* G418^R pAJ3 *Spe I* integrated in the chromosome	This work
CGM839	BG14	*ura3Δ*::Tn*903* G418^R pAJ26 *Spe I* integrated in the chromosome	This work
CGM1409	BG14	*ura3Δ*::Tn*903* G418^R pAJ59 *Spe I* integrated in the chromosome	This work
CGM1412	BG14	*ura3Δ*::Tn*903* G418^R pAJ67 *Spe I* integrated in the chromosome	This work

Mutants in silencing proteins

Strain	Parent	Genotype	Reference
CGM312	BG14	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase).	(ROSAS-HERNANDEZ *et al.* 2008)
CGM481	CGM312	*ura3Δ*::Tn*903* G418^R. *sir3Δ::hph* pAP430 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et al.* 2008)
CGM483	CGM312	*ura3Δ*::Tn*903* G418^R *sir3Δ*::*hph* pAP509 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et al.* 2008)
CGM71 (BG1048)	BG14	*ura3Δ*::Tn*903* G418^R *sir2Δ*::*hph* (pAP596/*Sac* I-*Kpn* I).	(5)
CGM579	CGM71	*ura3Δ*::Tn*903* G418^R *sir2Δ*::*hph* pAP430 *SpeI* integrated between *ISC1* and *HYR1.*	This work
CGM571	CGM71	*ura3Δ*::Tn*903* G418^R *sir2Δ*::*hph* pAP509 *SpeI* integrated between *ISC1* and *HYR1.*	This work
BG592	BG14	*ura3Δ*::Tn*903* G418^R *rap1-21*	(DE LAS PENAS *et al.* 2003)
CGM587	BG592	*ura3Δ*::Tn*903* G418^R *rap1-21* pAP430 *SpeI* integrated between *ISC1* and *HYR1.*	This work
CGM589	BG592	*ura3Δ*::Tn*903* G418^R *rap1-21* pAP509 *SpeI* integrated between *ISC1* and *HYR1.*	This work
CGM314	BG14	*ura3Δ*::Tn*903* G418^R *rif1Δ::hph* Hyg^R (pSP166 *Bsg* I).	(ROSAS-HERNANDEZ *et al.* 2008)
CGM485	CGM314	*ura3Δ*::Tn*903* G418^R *rif1Δ::hph* pAP430 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et al.* 2008)
CGM487	CGM314	*ura3Δ*::Tn*903* G418^R *rif1Δ::hph* pAP509 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et al.* 2008)
BG1080	BG14	*ura3Δ*::Tn*903* G418^R *hdf1Δ::hph* Hyg^R (pAP611 *Bcg* I).	(ROSAS-HERNANDEZ *et al.* 2008)
CGM489	BG1080	*ura3Δ*::Tn*903* G418^R *hdf1Δ::hph* pAP430 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et*

CGM491	BG1080	*ura3Δ*::Tn*903* G418^R *hdf1Δ::hph* pAP509 *SpeI* integrated between *ISC1* and *HYR1.*	*al.* 2008) (ROSAS-HERNANDEZ *et al.* 2008)
CGM79 (BG1081)	BG14	*ura3Δ*::Tn*903* G418^R *hdf2Δ::hph* Hyg^R (pAP612 *Bcg* I).	(ROSAS-HERNANDEZ *et al.* 2008)
CGM493	CGM79	*ura3Δ*::Tn*903* G418^R *hdf2Δ::hph* pAP430 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et al.* 2008)
CGM495	CGM79	*ura3Δ*::Tn*903* G418^R *hdf2Δ::hph* pAP509 *SpeI* integrated between *ISC1* and *HYR1.*	(ROSAS-HERNANDEZ *et al.* 2008)

Sil2126-*URA3* integration at different genomic loci

Strain	Parent	Genotype	Reference
CGM570	BG14	*ura3Δ*::Tn*903* G418^R pAJ8 *Mfe I* integrated at -160kb of centromere on chromosome F	This work
CGM572	BG14	*ura3Δ*::Tn*903* G418^R pAJ9 *Msc I* integrated at -263kb of centromere on chromosome L	This work
CGM574	BG14	*ura3Δ*::Tn*903* G418^R pAJ10 *Xho I* integrated at -224kb of left arm telomere on chromosome M	This work
CGM575	BG14	*ura3Δ*::Tn*903* G418^R pAJ19 *Mfe I* integrated at -160kb of centromere on chromosome F	This work
CGM577	BG14	*ura3Δ*::Tn*903* G418^R pAJ20 *Xho I* integrated at -224kb of left arm telomere on chromosome M	This work
CGM1011	BG14	*ura3Δ*::Tn*903* G418^R, pAJ35 MfeI integrated at -34kb of telomere E-$_L$	This work
CGM1015	BG14	*ura3Δ*::Tn*903* G418^R, pAJ40 MfeI integrated at -34kb of telomere E-$_L$	This work
CGM1027	BG14	*ura3Δ*::Tn*903* G418^R, pAJ34 Cla I integrated at -50kb of telomere E-$_R$	This work
CGM1013	BG14	*ura3Δ*::Tn*903* G418^R, pAJ39 Cla I integrated at -50kb of telomere E-$_R$	This work
CGM1414	BG14	*ura3Δ*::Tn*903* G418^R, pAJ51 Bst XI integrated at -26 kb of telomere C-$_L$	This work
CGM1415	BG14	*ura3Δ*::Tn*903* G418^R, pAJ61 Bst XI integrated at -26 kb of telomere C-$_L$	This work
CGM1417	BG14	*ura3Δ*::Tn*903* G418^R, pAJ53 Bst XI integrated at -23 kb of telomere I-$_L$	This work
CGM1419	BG14	*ura3Δ*::Tn*903* G418^R, pAJ63 Bst XI integrated at -23 kb of telomere I-$_L$	This work
CGM1421	BG14	*ura3Δ*::Tn*903* G418^R, pAJ55 Mfe I integrated at -19 kb of telomere K-$_R$	This work

CGM1423	BG14	*ura3Δ*::Tn*903* G418^R , pAJ64 Mfe I integrated at -19 kb of telomere K-$_R$	This work
CGM1425	BG14	*ura3Δ*::Tn*903* G418^R , pAJ57 Mfe I integrated at -2.1 kb of telomere I-$_R$	This work
CGM1433	BG14	*ura3Δ*::Tn*903* G418^R , pAJ66 Mfe I integrated at -2.1 kb of telomere I-$_R$	This work
CGM1428	CGM708	*ura3Δ*::Tn*903* G418^R , *hdf1Δ,* pAJ57 Mfe I integrated at -2.1 kb of telomere I-$_R$	This work
CGM1434	CGM708	*ura3Δ*::Tn*903* G418^R , *hdf1Δ,* pAJ66 Mfe I integrated at -2.1 kb of telomere I-$_R$	This work
CGM1430	CGM710	*ura3Δ*::Tn*903* G418^R , *hdf2Δ,* pAJ57 Mfe I integrated at -2.1 kb of telomere I-$_R$	This work
CGM1432	CGM710	*ura3Δ*::Tn*903* G418^R , *hdf2Δ,* pAJ66 Mfe I integrated at -2.1 kb of telomere I-$_R$	This work

*hdf1*Δ, *hdf2*Δ, *sil2126*Δ mutants in *URA3* reporter strains

Strain	Parent	Genotype	Reference
CGM399	BG14	*ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	(ROSAS-HERNANDEZ *et al.* 2008)
BG646	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *EPA1* and *EPA2* (pAP508 *Spe* I/*Bcg* I). Insertion 2	(DE LAS PENAS *et al.* 2003)
BG833	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *EPA2* and *EPA3* (pAP559 *Bsr*G I/*Sph* I). Insertion 3	(DE LAS PENAS *et al.* 2003)
BG432	BG14	*ura3Δ*::Tn*903* G418^R Tn*7* at intergenic region between *EPA3* and telomere (pAP553 *Pst* I/*Eco*R I). Insertion 4	(DE LAS PENAS *et al.* 2003)
CGM903	CGM399	sil2126Δ. pAJ25-Bcg I hyg^s *ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	This work
CGM890	CGM399	*hdf1*Δ::FRT *ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	This work
CGM897	CGM399	*hdf2*Δ::FRT *ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	This work
CGM1043	CGM399	sil2126Δ , *hdf1*Δ::FRT *ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	This work
CGM1051	CGM399	Sil2126Δ , *hdf2*Δ::FRT *ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	This work
CGM899	BG646	sil2126Δ . pAJ25-Bcg I *ura3Δ*::Tn*903* G418^R *hdf1*Δ	This work

		Tn*7* at intergenic region between *EPA1* and *EPA2*	
CGM885	BG646	*ura3Δ*::Tn*903* G418^R *hdf1*Δ	This work
		Tn*7* at intergenic region between *EPA1* and *EPA2*	
CGM892	BG646	*ura3Δ*::Tn*903* G418^R *hdf2*Δ	This work
		Tn*7* at intergenic region between *EPA1* and *EPA2*	
CGM1045	BG646	*ura3Δ*::Tn*903* G418^R sil2126Δ, *hdf1*Δ::FRT	This work
		Tn*7* at intergenic region between *EPA1* and *EPA2*	
CGM1053	BG646	*ura3Δ*::Tn*903* G418^R sil2126Δ, *hdf2*Δ::FRT	This work
		Tn*7* at intergenic region between *EPA1* and *EPA2*	
CGM900	BG833	*sil2126*Δ. pAJ25-Bcg I	This work
		ura3Δ::Tn*903* G418^R *hdf1*Δ	
		Tn*7* at intergenic region between *EPA2*and *EPA3*	
CGM888	BG833	*ura3Δ*::Tn*903* G418^R *hdf1*Δ	This work
		Tn*7* at intergenic region between *EPA2* and *EPA3*	
CGM894	BG833	*ura3Δ*::Tn*903* G418^R *hdf2*Δ	This work
		Tn*7* at intergenic region between *EPA2* and *EPA3*	
CGM1047	BG833	*ura3Δ*::Tn*903* G418^R sil2126Δ, *hdf1*Δ::FRT	This work
		Tn*7* at intergenic region between *EPA2*and *EPA3*	
CGM1055	BG833	*ura3Δ*::Tn*903* G418^R sil2126Δ, *hdf2*Δ::FRT	This work
		Tn*7* at intergenic region between *EPA2* and *EPA3*	
CGM901	BG432	sil2126Δ. pAJ25-Bcg I	This work
		ura3Δ::Tn*903* G418^R Tn*7* at intergenic region between *EPA3* and telomere	
CGM1213	BG432	*ura3Δ*::Tn*903* G418^R *hdf1*Δ	This work
		Tn*7* at intergenic region between *EPA3* and telomere	
CGM895	BG432	*ura3Δ*::Tn*903* G418^R *hdf2*Δ	This work
		Tn*7* at intergenic region between *EPA3* and telomere	
CGM1049	BG432	*ura3Δ*::Tn*903* G418^R *sil2126*Δ, *hdf1*Δ	This work
		Tn*7* at intergenic region between *EPA3* and telomere	
CGM1057	BG432	*ura3Δ*::Tn*903* G418^R *sil2126*Δ, *hdf2*Δ	This work
		Tn*7* at intergenic region between *EPA3* and telomere	

Insertion of P_{PGK1} promoter at the subtelomere E_{-R}

Strain	Parent	Genotype	Reference
CGM687	BG646	silΔ::(FRT-P_{PGK1}::*hph*::3'UTR_{HIS3}-FRT)	This work
		ura3Δ::Tn*903* G418^R Tn*7* at intergenic region between *EPA1* and *EPA2*	
CGM689	BG833	silΔ::(FRT-P_{PGK1}::*hph*::3'UTR_{HIS3}-FRT)	This work
		ura3Δ::Tn*903* G418^R Tn*7* at intergenic region between *EPA2* and *EPA3*	
CGM691	BG432	silΔ::(FRT-P_{PGK1}::*hph*::3'UTR_{HIS3}-FRT)	This work
		ura3Δ::Tn*903* G418^R Tn*7* at intergenic region between *EPA3*and telomere	

CGM693	CGM399	silΔ::(FRT-P_{PGK1}::*hph*::3'UTR_{HIS3}-FRT) *ura3Δ*::Tn*903* G418^R pAP509 *Spe* I integrated between *ISC1* and *HYR1*.	This work

EPA3 RT-PCR strains

Strain	Parent	Genotype	Reference
BG14	BG2	*ura3Δ*::Tn*903* G418^R	(CORMACK and FALKOW 1999)
BG676	BG14	*ura3Δ*::Tn*903* G418^R *sir3*Δ	(DE LAS PENAS *et al.* 2003)
CGM686	BG14	*ura3Δ*::Tn*903* G418^R *(pAJ25-Bcg I)* silΔ::(P_{PGK1}::hph::3'UTR_{HIS3}::FRT)	This work
CGM743	BG686	*ura3Δ*::Tn*903* G418^R *silΔ*	This work
CGM709	BG14	*ura3Δ*::Tn*903* G418^R *hdf1Δ*	This work
CGM710	BG14	*ura3Δ*::Tn*903* G418^R *hdf2Δ* colony 4	This work
CGM711	BG14	*ura3Δ*::Tn*903* G418^R *hdf2Δ* colony 8	This work
CGM792	CGM710	*ura3Δ*::Tn*903* G418^R *hdf1Δ/ hdf2Δ*	This work
CGM1052	BG14	*ura3Δ*::Tn*903* G418^R *hdf2Δ/ silΔ*	This work

Strains to study properties of Sil2126-*URA3* at -31.9 kb of telomere

Strain	Parent	Genotype	Reference
CGM685	BG14	*ura3Δ*::Tn*903* G418^R pAJ25/Bcg I -- silΔ::(P_{PGK1}::hph::3'UTR_{HIS3}::FRT)	This work
CGM742	CGM685	*ura3Δ*::Tn*903* G418^R silΔ::FRT	This work
CGM815	CGM685	*ura3Δ*::Tn*903* G418^R silΔ::(P_{PGK1}::hph::3'UTR_{HIS3}::FRT) pAP430/Spe I integrated between *ISC1* and *HYR1*	This work
CGM817	CGM742	*ura3Δ*::Tn*903* G418^R silΔ::FRT pAP430/Spe I integrated between *ISC1* and *HYR1*	This work
CGM1077	BG14	*ura3Δ*::Tn*903* G418^R pAJ41/SpeI integrated between *ISC1* and *HYR1*	This work
CGM1300	BG14	*ura3Δ*::Tn*903* G418^R pAJ47/SpeI integrated between *ISC1* and *HYR1*	This work

Table S2 Plasmids used in this study

Plasmid	Relevant genotype	Reference
Cloning or marker removal vectors		
pGRB2.0	Cloning replicative vector *URA3* Ap^R pRS406::*C.g*. *CEN* ARS	(De Las Penas *et al.* 2003)
pYIp*lac*211	Cloning, integrative vector *URA3* Ap^R	(Gietz and Sugino 1988)
pGEM-T	Cloning vector, Amp^R	Promega
pAP599	Cloning, integrative vector with 2 FRT direct repeats flanking a hygromycin resistance cassette [FRT-P_{PGK1}::*hph*::3'UTR_{HIS3}-FRT] for construction of multiple rounds of knock-out mutants, Amp^R, Hyg^R, *URA3*	(Domergue *et al.* 2005)
pMZ18	Replicative vector expressing Sc*FLP1* (recombinase gene) for removing the hygromycin marker, P_{EPA1}::*FLP1*::(3' UTR of *HIS3*) Cg *CEN ARS*, Amp^R, *URA3*	Cormack lab collection
pLS9	Replicative vector expressing Sc*FLP1* (recombinase gene) for removing the hygromycin marker, P_{EPA1}::*FLP1*::(3' UTR of *HIS3*) Cg *CEN ARS*, Amp^R, *URA3, nat1*	Lab collection
Plasmids for Tn7 (*URA3*) insertions at *EPA* loci		
pAP540 Insertion 1	A 1.241kb *Bam*H I PCR fragment carrying a Tn*7* in the intergenic region between *ISCI* and *HYRI* cloned into pAP502. Insertion 1	(De Las Penas *et al.* 2003)
pAP508 Insertion 2	A 1.6 kb *Hin*d III PCR fragment carrying a Tn*7* in the intergenic region between *EPA1* and *EPA2* cloned into pBR322. Insertion 2	(De Las Penas *et al.* 2003)
pAP559 Insertion 3	A 2.9 kb *Eco*R I fragment carrying a Tn*7* in the intergenic region between *EPA2* and *EPA3* cloned into pUC19. Insertion 3	(De Las Penas *et al.* 2003)
pAP553 Insertion 4	A 3.2 kb *Hin*d III /*Eco*R I fragment carrying a Tn*7* in the intergenic region between *EPA3* and telomere cloned into pUC19. Insertion 4	(De Las Penas *et al.* 2003)
Plasmids for protosilencer integration at -31.9 kb of telomere $E\text{-}_R$		
pAP430	Plasmid to integrate the protosilencer Sil2126 and *URA3* at the intergenic region between *C.g. ISC1* and *C.g.HYR1*. Cloned into pYIp*lac*211. Ap^R	(De Las Penas *et al.* 2003)
pAP509	Vector to integrate *URA3* at the intergenic region between *C.g. ISC1* and *C.g. HYR1*. A 0.686 kb *Pst* I-*Sal* I PCR fragment (Primers #962, #963) carrying the intergenic region between *C.g. ISC1* cloned into pYIp*lac*211. Amp^R	(Rosas-Hernandez *et al.* 2008)

Plasmids for protosilencer deletion analysis at -31.9 kb of telomere $E\text{-}_R$		
pAP566	A 316pb deletion (nucleotide 1810 to 2126) from the 3' end of Sil2126 in pAP430	This work
pAP567	A 885pb deletion (nucleotide 1241 to 2126) from the 3' end of Sil2126 in pAP430	This work
pAP568	A 1260pb deletion (nucleotide 866 to 2126) from the 3' end of Sil2126 in pAP430	This work
pAP569	A 1432pb deletion (nucleotide 694 to 2126) from the 3' end of sil2126 in pAP430	This work
pAP570	A 1710pb deletion (nucleotide 416 to 2126) from the 3' end of Sil2126 in pAP430	This work
pAP571	A 1792pb deletion (nucleotide 334 to 2126) from the 3' end of Sil2126 in pAP430	This work
pAP572	A 2020 deletion (nucleotide 106 to 2126) from the 3' end of Sil2126 in pAP430	This work
pAJ29	A 39pb deletion from the 5' end of Sil2126 (nucleotides 1-39) in pAP430.	This work
pAJ30	A 68pb deletion from the 5' end of Sil2126 (nucleotides 1-68) in pAP430	This work
pAJ31	A 115pb deletion from the 5' end of Sil2126 (nucleotides 1-115) in pAP430	This work
pAJ32	A 160pb deletion from the 5' end of Sil2126 (nucleotides 1-160) in pAP430	This work
pAJ33	A 204pb deletion from the 5' end of Sil2126 (nucleotides 1-204) in pAP430	This work
pAJ7	A 262pb deletion from the 5' end of Sil2126 (nucleotides 1-262) in pAP430	This work
pAJ6	A 462pb deletion from the 5' end of Sil2126 (nucleotides 1-462) in pAP430	This work
pAJ5	A 738pb deletion from the 5' end of Sil2126 (nucleotides 1-738) in pAP430	This work
pAJ4	A 1288pb deletion from the 5' end of Sil2126 (nucleotides 1-1288) in pAP430	This work
pAJ3	A 1622 deletion from the 5' end of Sil2126 (nucleotides 1-1622) in pAP430	This work
pAJ2	An internal deletion of 907pb from nucleotide 334 to 1241 of Sil2126 in pAP430	This work
pAJ1	An internal fragment of 907pb from nucleotide 334 to 1241 of Sil2126 replacing Sil2126 in pAP430	This work
pAJ26	A 3430 PCR fragment containing the entire Sil2126 and the remaining 1304pb next to 5' of this region to the ATG of the	This work

	contiguos gene EPA3, cloned in pAP430 BamHI-SalI sites	
pAJ59	An internal deletion of 58pb from nucleotide 204 to 262 of Sil2126 in pAP430	This work
pAJ67	An internal deletion of 944pb from nucleotide 866 to 1810 of Sil2126 in pAP430	This work

Plasmids for protosilencer integration at internal locations on chromosomes F,L,M		
pAJ8	Vector to integrate Sil2126-*URA3* at 160 kb from centromere of chromosome F. A 789pb *PstI-SalI* PCR fragment (Primers #252,#253) of an intergenic region of chromosome F, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ9	Vector to integrate Sil2126-*URA3* at 263 kb from centromere of chromosome L. A 628pb *PstI-SalI* PCR fragment (Primers #254,#255) of an intergenic region of chromosome L, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ10	Vector to integrate Sil2126-*URA3* at 224 kb from left arm of telomere on chromosome M. A 556pb *PstI-SalI* PCR fragment (Primers #256,#257) of an intergenic region of chromosome M, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ19	A deletion of Sil2126 protosilencer region from pAJ8. sil– control, AmpR	This work
pAJ20	A deletion of Sil2126 protosilencer region from pAJ10. sil– control, AmpR	This work
pAJ51	Vector to integrate Sil2126-*URA3* at 26 kb from left telomere of chromosome C. A 587 pb *PstI-SalI* PCR fragment (Primers #867,#866) of a region of chromosome C, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ53	Vector to integrate Sil2126-*URA3* at 23 kb from left telomere of chromosome I. A 475 pb *PstI-SalI* PCR fragment (Primers #871,#870) of a region of chromosome I, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ55	Vector to integrate Sil2126-*URA3* at 19 kb from right telomere of chromosome K. A 658 pb *PstI-SalI* PCR fragment (Primers #874,#875) of a region of chromosome K, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ57	Vector to integrate Sil2126-*URA3* at 2.1 kb from right telomere of chromosome I. A 676 pb *PstI-SalI* PCR fragment (Primers #862,#863) of a region of chromosome I, replacing *Pst I-Sal I* integration sequence of pAP430. Amp^R	This work
pAJ61	A deletion of Sil2126 protosilencer region from pAJ51. sil– control, AmpR	This work

pAJ63	A deletion of Sil2126 protosilencer region from pAJ53. sil– control, AmpR	This work
pAJ64	A deletion of Sil2126 protosilencer region from pAJ55. sil– control, AmpR	This work
pAJ66	A deletion of Sil2126 protosilencer region from pAJ57. sil– control, AmpR	This work
*hdf1*Δ*::hph* and *hdf2*Δ*::hph*		
pAJ27	*HDF1* deletion vector. A 804pb *KpnI HindIII HDF1 5'* fragment and a 826pb *SacI-BglII HDF1* 3' fragment, cloned into pAP599 at both sides of *hph* marker	This work
pAJ28	*HDF2* deletion vector. A 905pb *KpnI HindIII* 5' *HDF1* fragment and a 813pb *SacI-BglII* 3' *HDF1* fragment, non-coding regions cloned into pAP599 on either side of *hph* marker	This work
Plasmid for deletion of Sil2126 region		
pAJ25	Sil2126 deletion vector. A 1025pb KpnI-XhoI fragment (Primers #339, #340) and a 935pb BamHI-SacI fragment (Primers #337, #338), cloned into pAP599 on either side of *hph* marker	This work
Plasmids to study location and orientation properties of Sil2126		
pAJ34	Vector to integrate Sil2126-*URA3* reporter system at 50Kb from telomere $E\text{-}_R$. A 742pb *Pst I-Sal I* PCR fragment (Primers #654, #655), replacing *Pst I-Sal I* integration sequence in pAP430	This work
pAJ35	Vector to integrate Sil2126-*URA3* reporter system at ~ 34Kb from telomere $E\text{-}_L$. A 622 *Pst I-Sal I* PCR fragment (Primers #658, #659), replacing *Pst I-Sal I* integration sequence in pAP430.	This work
pAJ39	A *BamH I-Sal I* deletion on pAJ34 which eliminates Sil2126. Amp^R	This work
pAJ40	A *BamH I-Sal I* deletion on pAJ35 which eliminates Sil2126. Amp^R	This work
pAJ41	Plasmid to integrate the protosilencer Sil2126 and *URA3* at the intergenic region between *C.g. ISC1* and *C.g. HYR1*. A Sil2126 inverted orientation respect to *URA3* on pAP430	This work
pAJ47	Plasmid to integrate the protosilencer Sil2126 and *URA3* at the intergenic region between *C.g. ISC1* and *C.g. HYR1*. The (Sil2126-*URA3*) cassette is in inverted orientation with respect to pAP430	This work
pAJ49	Plasmid to integrate the protosilencer Sil2126 and *URA3* at ~ 34Kb from telomere $E\text{-}_L$. The (Sil2126-*URA3*) cassette is in inverted orientation with respect to pAJ35	This work

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Table S3 Oligonucleotides used in this study.

Primer	Name	Sequence (5'-3')	Site(s) added
Primers used to construct vectors with progressive 5' deletions of Sil2126			
237	EPA3@-1609 Sal Fw	CGCGTCGACGCTTTCCTCTCAAGAGGGTGC	*Sal* I
238	EPA3@-1808 Sal Fw	AGTGTCGACGACACTTGTAACAGATATTGG	*Sal* I
239	EPA3@-2086 Sal Fw	AGTGTCGACCATCCAACAAGTCTTTTACCG	*Sal* I
240	EPA3@-2967 Sal Fw	CTAGTCGACGAATCGAAGATGAAAGACC	*Sal* I
241	EPA3@-2575 Sal Fw	TGAGTCGACGTATCACTGTACAAAGTAACC	*Sal* I
564	EPA3@-1544 SalI Fw	TAGGTCGACCCCTTAATATCACTTTCAACG	*Sal* I
565	EPA3@-1500 SalI Fw	GTAGTCGACCTGGATGACAAAATACACCCC	*Sal* I
566	EPA3@-1455 SalI Fw	GCGGTCGACATAAGAAAAACAACTTTTACC	*Sal* I
567	EPA3@-1408 SalI Fw	TCAGTCGACCAAAAATATACATTTCTCCCC	*Sal* I
568	EPA3@-1379 SalI Fw	CTCGTCGACTAGATTGCTGAAATATTCTGG	*Sal* I
297	EPA3@-36Sal Fw	GACGTCGACATTTAATTCGATTATGATGGG	*Sal* I
964	EPA3@-1340 Fw	AGAATAGTCGACTGTTGCAGCAAGAACCA	*Sal* I
236	EPA3@-3466 BamHI Rv	AGTGGATCCATAGATGCATGTAGCCATATC	*BamH* I
878	Sildelet 204-262 PstI Fw	CTTGCATGCCTGCAGCTATACAAGC	*Pst* I
879	EPA3@-1537 XhoI Rv	GTACTCGAGTTTTTGCCTTCTCTTAAGGGG	*Xho* I
Primers to diagnose integration of these vectors at -31.9 kb from telomere $E\text{-}_R$ in *C. glabrata*			
13	Primer 13	GGCGATTAAGTTGGGTAACGCCAGGG	
14	Primer 14	TATGTTGTGTGGAATTGTGAGCGGA	
187	ISC1@1232Fw	GGTCTGAAATCAGAGCATTATGGGAGG	
188	HYR1@-301Rv	GCATCAAATAATGGATAGGTTGAAATCCCC	
Primers used to construct integrative vectors for (Sil2126-*URA3*) at chromosome F, L, M loci			
252	IR Chr F Fw@399784 Pst	CGCCTGCAGTAAACCTTCTTGAACCCTGCCAGC	*Pst* I
253	IR Chr F Rv@400572 Sal	CGCGTCGACTCAATCATCATCACCAATACGAGC	*Sal* I
254	IR Chr L Fw@686898 Pst	CGCCTGCAGACATGTCTCCCTGCTAAGGTATCC	*Pst* I
255	IR Chr L Rv@687525 Sal	CGCGTCGACAGTGGAGGATCAAGAGTAGATTGG	*Sal* I
256	IR Chr M Fw@224158 Pst	CGCCTGCAGAGAGACTGTCTGTACAAATAGCGG	*Pst* I
257	IR Chr M Rv@224713 Sal	CGCGTCGACAATATCTGTTGCCGTTTGTGCAGG	*Sal* I
Primers to diagnose integration of vectors at chromosome F, L, M			
258	IR Chr F Fw@399593	GTAACTGGTACATTATAATAGGAGCGGG	
259	IR Chr F Rv@400657	CGTGTTTTGGTATCGCATATTTCCC	
260	IR Chr L Fw@686777	CAACGGGAGGAATATTTGCTCAGGTGC	
261	IR Chr L Rv@687711	TTAAGGACATGCGCGTAAACATGGG	
262	IR Chr M Fw@223971	CCCTCTCTCTCTCCTTCTCTTTGTCCC	
263	IR Chr M Rv@224884	TGACTACCATGTTTCCTTTGCCAGC	
Primers used to construct integrative vectors for (Sil2126-*URA3*) at chromosome $I\text{-}_R$, $C\text{-}_L$, $I\text{-}_L$, $K\text{-}_R$ loci			

862	EPA4@+1177 PstI Fw	CACCTGCAGTTTCTTATAAAATCTTGGTCAGCC	*Pst* I
863	EPA4@+1834 SalI Rv	GCTGTCGACTTCTGTGTTCATGTGTATATTTCG	*Sal* I
866	CAGL0C00275@-2460 SalI Fw	ATCGTCGACAGTAATAGTAGGGATGTTTGCAGC	*Sal* I
867	CAGL0C00275g@-1891 PstI Rv	ATCCTGCAGCAACCTCAGCAGTATATGTAAAGG	*Pst* I
870	CAGL0I00286@-564 SalI Fw	ATCGTCGACGTACATAATTTTCCTTGCCCC	*Sal* I
871	CAGL0I00286@-1021 PstI Rv	ATCCTGCAGTTTCCGTGTTTCTCCGAACTG	*Pst* I
874	CAGL0K0012958@-491 PstI Fw	TCTCTGCAGTAATAATTGCACTTATACCTACGG	*Pst* I
875	CAGL0K0012958@-1131 SalI Rv	ATCGTCGACTCTATGGAGGTTTTATATGTATGG	*Sal* I

Primers to diagnose integration of vectors at chromosome I_{-R}, C_{-L}, $I_{-L,}$ K_{-R} loci			
864	Diag EPA4@+965 Fw	CATCTATATTTCATGTTTGTAATTTGGGGC	
865	Diag EPA4@+1987 Rv	ATCTCAGACAGGGCTTTTTCTGTAGGAG	
868	Diag CAGL0C00275@-2600 Fw	GTGCGAATAAGAGTTCCTTTAGTTACCGG	
869	Diag CAGL0C00275@-1695 Rv	TTGCCAATTATATCCAACAATATGGTG	
872	Diag CAGL0I00286@-245 Fw	CAAAAAGAAATTGAAGTGGGGTACGG	
873	Diag CAGL0I00286@-1327 Rv	GCATCAGCCACCATGGATCACCTC	
876	Diag CAGL0K0012958@-359Fw	GTTCGCATACTCTTCAGCAGAGATTCCC	
877	Diag CAGL0K0012958@-1224Rv	TGGATGAACATTTTATGTGTTCATACCG	

Primers used to construct Sil2126 deletion vector			
337	EPA3@-3460 Sac Bcg Rev	GGGGAGCTCGTTCGATATAAGTGCGCATGTAGCCATATCTGTGAGAGAG	*Sac* I, *Bcg* I
338	EPA3@-2575 Bam Fwd	GGGGGATCCGTACAAAGTAACCACTTTTAAGATGCG	*BamH* I
339	EPA3@977 Kpn Bcg Fwd	GGCGGTACCTTCGACTTGGTTGCGGATAGTAGCATGTATTTTTCAATGG	*Kpn* I, *Bcg* I
340	EPA3@-4 Xho Rev	GCGCTCGAGCAATATGTTCAACTGGATTATCGTATG	*Xho* I
	Primers for diagnosis of integration of this vector in *C. glabrata*		
342	Diag EPA3@1014 Rv	CAGGTTTTGGGTCCTCAGGTTTTGG	
343	Diag EPA3@-3528 fw	GTGTAATGATAGCCAGTACTAGTG	

Primers used to construct integrative vector containing *PGK1* promoter driven *hph* between Sil2126 and Telomere E_{-R}			
648	EPA3@-3493 KpnI BcgI Fw	GGCGGTACCCGACTTGGTTGCAACACTAAGAAACACTAGTACTGGC	*Kpn* I, *Bcg* I
649	EPA3@-4158 XhoI Rv	GCGCTCGAGTTCCATTTTCGACAATGAGGATGCG	*Xho* I
650	EPA3@-4361 BamHI Fw	GGGGGATCCGCACAAGAACTCCAATTCAGGTCTTGG	*BamH* I

651	EPA3@-4930 SacI BcgI Rv	TAGGAGCTCCGATATAAGTGCGAACTTGTCCACGGCATTTGTTTCC	*Sac* I, *Bcg* I
Primers for diagnosis of integration of this vector in *C. glabrata*			
652	Diag 3' EPA3@-4962 Rv	CCCTAAACGAGAAAATCTTAAACG	
653	Diag 5' EPA3@-3436 Fw	CTCTCTCACAGATATGGCTACATGC	

Primers used to construct Sil2126-*URA3* integrative vector at -34kb in chromosome $E\text{-}_L$		
658	OCA4@-112 Pst I Fw	agactgcagAGGGCGATCATTATTAAATCGAGTCC
659	OCA4@510 SalI Rv	cgagtcgacTTACCAAAGAAGTTTTGTCCACAAGC
Primers for diagnosis of integration of this vector in *C. glabrata*		
660	Diag 5' OCA4@-162 Fw	GTGACGGTTGCTTACCCATAGCGTGC
661	Diag 3' OCA4@740 Rv	TTCAACGGGATTCGACTTGTGTTCG

Primers used to construct Sil2126-*URA3* integrative vector at -50 kb of chromosome $E\text{-}_R$		
654	ELF1@-2291 SalI Rv	gtagtcgacTTGAAAGAGTACTGTCCCATCTTCC
655	ELF1@-1549 PstI Fw	agactgcagTGTTACGTTTACAGCACCTTAAAGC
Primers for diagnosis of integration of this vector in *C. glabrata*		
656	Diag ELF1@-1376 Fw	GCTTAGAAGTTGATTGTTCAATTGCC
657	Diag ELF1@-2339 Rv	GACCCGGTTTGTAAAACACCAGACC

EPA3 RT-PCR primers		
219	ACT1Fw@70 RT	CGCCGGTGACGATGCTCC
220	ACT1Rv@210 RT	CTTGGATTGAGCTTCGTC
38	EPA3 RT-PCR Fw	GCATGTTGATAGTTCCAAAA
24	EPA3 RT-PCR Rev	TAATTTGATCAGTAGCACCG

Primers to diagnose *hdf1Δ* and *hdf2Δ*		
2242	HDF1@-1034Fw	AGCGAGGTACGAAGATATAGAACGC
2243	HDF1@+971 Rev	CCCGTACTACAACTAACAATGCAGCT
2248	HDF2@-1148 Fw	AGCCGCTGTATCGGGATCAAC
2249	HDF2@+988 Rev	CGCACAACAAGAACAGCACCCTTGG

Primers to diagnose (P_{PGK1}::*hph*::FRT) cassette deletion		
325	PGKp RI Rv	GGGAATTCTGTTTTATATTTGTTGTAAAAAGTAG
326	PGKp Sac Fw	GGGGAGCTCCATAAAGCACGTGGCCTCTTATCG