

This is the Author's Preprint version of the following article: GENETICS, 208 (2017) 113-128; DOI 10.1534/genetics.118.301202 © 2018 Genetics Society of America. The published article is available at www.genetics.org

1 **Chromatin loop formation induced by a subtelomeric protosilencer represses *EPA* genes**
2 **in *Candida glabrata***

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22 Key words: *Candida glabrata*, cis-elements, transcriptional regulation, protosilencer, EPA
23 genes, Rap1, chromatin loop

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25 Running title: Sil2126 of *C. glabrata* forms a DNA loop

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45 **Abstract**

46 Adherence, an important virulence factor, is mediated by the *EPA* (Epithelial Adhesin)
47 genes in the opportunistic pathogen *Candida glabrata*. Expression of adhesin-encoding
48 genes requires tight regulation in order to respond to harsh environmental conditions
49 within the host. The majority of *EPA* genes are localized in subtelomeric regions regulated
50 by subtelomeric silencing, which depends mainly on Rap1 and the Sir proteins. *In vitro*
51 adhesion to epithelial cells is primarily mediated by Epa1. *EPA1* forms a cluster with *EPA2*
52 and *EPA3* in the right telomere of chromosome E (E_R). This telomere contains a *cis*-acting
53 regulatory element, the protosilencer Sil2126 between *EPA3* and the telomere.
54 Interestingly, Sil2126 is only active in the context of its native telomere. Replacement of
55 the intergenic regions between *EPA* genes in E_R revealed that *cis*-acting elements
56 between *EPA2* and *EPA3* are required for Sil2126 activity when placed 32 kb away from
57 the telomere (Sil@-32kb). Sil2126 contains several putative binding sites for Rap1 and
58 Abf1 and its activity depends on these proteins. Indeed, Sil2126 binds Rap1 and Abf1 at its
59 native position and also when inserted at -32 kb, a silencing-free environment in the
60 parental strain. In addition, we found that Sil@-32kb and Sil2126 at its native position can
61 physically interact with the intergenic regions between *EPA1-EPA2* and *EPA2-EPA3*
62 respectively by Chromosome Conformation Capture assays (3C). We speculate that Rap1
63 and Abf1 bound to Sil2126 can recruit the SIR complex and together mediate silencing in
64 this region, probably through the formation of a chromatin loop.

65

66 **Introduction**

67 Regulation of transcription, DNA replication, recombination and DNA damage repair in
68 eukaryotes depend critically on the chromatin structure. The nucleus is organized in
69 different subcompartments in which the chromosomes are non-randomly positioned
70 adopting *ad-hoc* conformations for each process (DUAN *et al.* 2010). Regulatory *cis*-acting
71 DNA regions for gene expression distantly localized in chromosomes are thought to be
72 brought into physical proximity with their gene targets through DNA loop formation. It is
73 proposed that chromatin loops associate in space and lead to the organization of
74 chromatin into functionally-related topological domains (BONEV AND CAVALLI 2016). In
75 addition, the telomeres, which are specialized structures at the ends of the chromosomes,
76 are generally found in clusters around the nucleus at the nuclear periphery (PALLADINO *et*
77 *al.* 1993), and excluded from the nucleolus (THERIZOLS *et al.* 2010). The adjacent sequences,
78 called subtelomeres, are also mostly found near the nuclear periphery (GOTTA *et al.* 1996;
79 HEDIGER *et al.* 2002). In the baker's yeast *Saccharomyces cerevisiae*, there are several
80 proteins that interact with telomeres and subtelomeres, which are enriched at the nuclear
81 periphery, such as the Silent Information Regulator (SIR) complex, (formed by the Sir2,
82 Sir3 and Sir4 proteins) (ANDRULIS *et al.* 1998), and the repressor-activator protein 1, Rap1
83 (GOTTA *et al.* 1996). The interaction of these proteins with the telomeres and subtelomeres
84 leads to the formation of a repressive form of chromatin called heterochromatin.

85 Heterochromatin in *S. cerevisiae* is found at the ribosomal DNA (rDNA) tandem array, the
86 silent mating loci and the telomeres. Transcriptional silencing close to the telomeres is
87 also called telomere position effect (TPE) and is found in many organisms in addition to *S.*

88 *cerevisiae*, such as, fission yeast (*Schizosaccharomyces pombe*), the fruit fly *Drosophila*
89 *melanogaster*, the sleeping sickness parasite *Trypanosoma brucei*, the malaria parasite
90 *Plasmodium falciparum*, plants and humans (GOTTSCHLING *et al.* 1990; LEVIS *et al.* 1993;
91 NIMMO *et al.* 1994; HORN AND CROSS 1995; SCHERF *et al.* 1998; BAUR *et al.* 2001).

92 Transcriptional silencing is propagated from the telomere to the centromere, spanning the
93 subtelomeric regions. Genes naturally located in the subtelomeric region are repressed in
94 a promoter-independent fashion, although silencing at subtelomeric regions varies from
95 telomere to telomere in fungi such as *S. cerevisiae* (PRYDE AND LOUIS 1999) and in the
96 opportunistic fungal pathogen *Candida glabrata* (ROSAS-HERNANDEZ *et al.* 2008).
97 Notoriously, in some pathogenic organisms several genes encoding known or suspected
98 virulence factors are localized at subtelomeric regions. For example, in the case of
99 unicellular parasites, the *var* genes of *Plasmodium falciparum* (GARDNER *et al.* 2002) and
100 the single variant-specific surface glycoprotein gene (*VSG*) of *Trypanosoma brucei* are
101 located adjacent to a telomere (HORN AND CROSS 1995); and in the pathogenic fungus,
102 *Pneumocystis carinii*, the major surface glycoprotein (*MSG*) gene family is located near
103 chromosomes ends (KEELY *et al.* 2005).

104 *Candida glabrata* is a haploid budding yeast, which has emerged as an important
105 nosocomial fungal pathogen associated with an attributable mortality of ~30% (KLEVAY *et*
106 *al.* 2009). It normally resides as a commensal in the flora of healthy human mucosal
107 tissues to which it adheres tightly, but can cause infections in immunocompromised
108 patients (PFALLER AND DIEKEMA 2007).

109 In *C. glabrata* most of the EPA (Epithelial adhesin) genes encoding adhesins, are located in
110 subtelomeric regions. The Epa family is the largest family of cell wall proteins in *C.*
111 *glabrata*, with at least 17 and up to 23 paralogues, depending on the strain. Epa1
112 mediates almost all the adherence to epithelial cells *in vitro* (CORMACK *et al.* 1999), and
113 Epa6 and Epa7 are also functional adhesins involved in kidney colonization (CASTANO *et al.*
114 2005).

115 The variant gene families located in subtelomeric regions are not restricted to pathogenic
116 species, for example *S. cerevisiae* contains four of the five members of the *FLO* gene
117 family of cell wall proteins in subtelomeric regions (Guo *et al.* 2000). The expression of
118 some subtelomeric genes in *S. cerevisiae* is regulated by transcriptional silencing (ELLAHI *et*
119 *al.* 2015), which requires different proteins, such as, Rap1, which binds to telomeric
120 repeats, yKu70, yKu80, the SIR complex, Rif1 and other proteins (KYRION *et al.* 1993; LUO *et*
121 *al.* 2002; THURTLIE AND RINE 2014; GARTENBERG AND SMITH 2016). In addition, *cis*-acting
122 elements called silencers and protosilencers aid in transcriptional silencing by binding
123 sequence-specific factors that lead to the recruitment of the SIR complex. Silencers are
124 negative regulatory elements composed of a combination of binding sites for various
125 silencing factors (FOUREL *et al.* 1999). At telomeres, the terminal repeated TG₁₋₃ sequences
126 serve as silencers. Protosilencers may act in synergy with silencers or other protosilencers
127 to stabilize and extend the propagation of heterochromatin (FOUREL *et al.* 2002).

128 In *C. glabrata*, subtelomeric silencing requires the SIR complex, as well as the Rif1, Rap1
129 and the yKu proteins, and can extend >20 kb toward the centromere (DE LAS PENAS *et al.*
130 2003; DOMERGUE *et al.* 2005; ROSAS-HERNANDEZ *et al.* 2008). Different telomeres in *C.*

131 *glabrata* have different protein requirements for silencing. For instance, the proteins
132 yKu70 and yKu80 are not required in the right telomere of the chromosome E (E_R) where
133 *EPA1* forms a cluster with *EPA2* and *EPA3* genes. This independence of yKu proteins is due
134 to a *cis*-acting element, the protosilencer Sil2126, which has overlapping functions with
135 the yKu proteins (JUAREZ-REYES 2012) (Fig. 1A). The Sil2126 element can mediate silencing
136 of the *URA3* reporter when both are inserted 32 kb away from the telomere in the right
137 telomere of the chromosome E, but not when they are placed at similar distances in other
138 telomeres. Sil2126 contains a putative binding site for Rap1 and another for the ARS
139 binding factor (Abf1) in the 5' fragment (JUAREZ-REYES 2012). In addition to Sil2126, we
140 have identified another *cis*-acting element 300 bp downstream from *EPA1*, called negative
141 element (NE) (Fig. 1A), which negatively regulates *EPA1* expression in a promoter-specific
142 fashion (GALLEGOS-GARCIA *et al.* 2012).

143 In this work, we wanted to understand the mechanism by which Sil2126 extends gene
144 silencing in the subtelomeric region of telomere E_R and uncover elements in this region
145 that are required for its telomere E_R specific activity. We show that the protosilencer
146 Sil2126 recruits Rap1 and Abf1 both, when it is located in its original position between
147 *EPA3* and telomere E_R and when moved 32 kb away from the telomere (Sil@-32kb),
148 where there is normally no silencing. In addition, we observe that Sil@-32kb interacts with
149 the *EPA1-EPA2* intergenic region by 3C assay (Chromosome Conformation Capture).
150 Furthermore, Sil2126 at its native locus strongly interacts with *cis*-acting elements
151 between *EPA2* and *EPA3*. We propose that Sil2126 induces the formation of alternative

152 chromatin loops mediated by protein-protein interactions between silencing proteins
153 recruited to Sil2126 and these intergenic regions to extend the silencing.

154

155 **Materials and Methods**

156 **Strains**

157 All strains and plasmids used are listed in Table S1 and S2, respectively.

158 **Media**

159 *Candida glabrata* strains were grown at 30° in plates with YPD medium which contains 10
160 g/L of yeast extract and 20 g/L of peptone, supplemented with 2% glucose and 2% agar. If
161 necessary, culture plates were supplemented with Hygromycin (Invitrogen) 440 µg/mL or
162 Nourseothricin 100 µg/mL (Streptothricin Sulfate, NTC, cloNAT, CAT#N-500-1). We used
163 synthetic complete (SC) medium for the plate growth assays. This medium contains 1.7 g/L
164 yeast nutrient base (without (NH₄)₂SO₄ and amino acids), 5 g/L (NH₄)₂SO₄ and is
165 supplemented with 0.6% casaminoacids and 2% glucose. In order to test the silencing
166 level, 5-fluoroorotic acid (5-FOA; Toronto Research Chemicals), 0.9 g/L 5-FOA and 25 mg/L
167 uracil were added to the SC medium. Minimal medium was used for the ChIP and 3C
168 assays. This medium contains 1.7 g/L yeast nutrient base, 5 g/L (NH₄)₂SO₄ and is
169 supplemented with 2% glucose and 25 mg/L uracil.

170 Bacteria were grown at 30° in LB medium as described previously by (AUSUBEL 2001). LB
171 medium contains 5 g/L yeast extract, 10 g/L tryptone and 5 g/L NaCl. If necessary, 1.5%

172 agar was added. All plasmid constructs were introduced via electroporation into the DH10
173 strain. 50 mg/mL carbenicillin (Invitrogen) was added for plasmid selection.

174 **Yeast transformation**

175 Yeast transformation was performed using the lithium acetate protocol as described
176 previously by (CASTANO *et al.* 2003).

177 **Plate growth assays**

178 The level of silencing or expression of the *URA3* reporter was assessed using a plate
179 growth assay as described previously (DE LAS PENAS *et al.* 2003; CASTANO *et al.* 2005). Briefly,
180 strains containing the different *URA3* insertions were grown at 30° in YPD for 48 hr to
181 stationary phase. The cultures were adjusted to an optical density of 1 at 600 nm with
182 sterile water. 10-fold serial dilutions were made in 96-well plates. A total of 5 µL of each
183 dilution was spotted onto YPD, SC-Ura and SC +5-FOA plates, and plates were incubated
184 for 48 hrs at 30° and photographed.

185 **GFP expression by flow cytometry**

186 Strains were grown for 48 hr at 30° in SC medium supplemented with uracil when it was
187 necessary. Cultured cells were diluted into fresh media to induce *EPA1* expression and
188 samples were taken every 2 hr. Activity of the *EPA1* promoter was measured by
189 determining fluorescence of the GFP reporter by FACS analysis using a BD FACSCalibur
190 flow cytometer with Cell Quest Pro software and results analyzed with FlowJo software.

191 **Western blot assay**

192 We constructed epitope-tagged versions of each protein tested. Rap1 and Sir3 were
193 tagged with Fag epitope at the C-terminus and integrated in their native loci, respectively.
194 To test Abf1, we constructed a plasmid containing an N-terminal fusion of cMyc with Abf1
195 and under the inducible promoter P_{MT1} . The strains were grown in YPD at 30° and
196 harvested in stationary phase. The protein extraction and western blot assays were done
197 as described with minor modifications (Orta-Zavalza *et al.* 2013; Robledo-Márquez *et al.*
198 2016). Briefly, cells were resuspended in lysis buffer (45 mM HEPES, 400 mM Potassium
199 acetate, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1X Complete protease
200 inhibitors cocktail ROCHE®), 100 µL of zirconia beads were added and cells were broken
201 using a FastPrep®-24 (MP Biomedicals) equipment, with three pulses for 60 s at 6 m/s. The
202 cells were centrifuged at 15000 rpm for 40 min at 4°, the supernatant was recovered and
203 the protein content was determined by Bradford assay. 50 µg of total protein were mixed
204 with 2X SDS loading buffer were preheated (95° for 8 min) and then loaded onto a 10%
205 SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted onto PVDF
206 membranes (BIO-RAD®) and probed overnight with anti-Flag (Sigma®) at final
207 concentration of 3 µg/mL. After washing, the membrane was probed with a goat-mouse
208 horseradish peroxidase-conjugated secondary antibody (MERCK®). The signal was
209 detected by ECL chemiluminescence reagents (Pierce®) and recorded using a BioRad
210 ChemiDoc MP System equipped with chemiluminescence.

211 **Chromatin immunoprecipitation (ChIP) assay**

212 Yeast cultures (150 mL) were grown in minimal medium to an OD₆₀₀ of 1 at 30°. Cells were
213 fixed with 1% formaldehyde for 15 min at 25°. Cross-linking was quenched by the addition

214 of glycine to 125 mM and incubated for 5 min. The cells were harvested, washed twice
215 with TBS buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and transferred to 1.5 mL
216 centrifuge tubes; yeast pellets were frozen at -80°. The cells were lysed with 500 µL lysis
217 buffer (10 mM EDTA [pH 8], 50 mM Tris-HCl [pH 8], 1% SDS and, 1 mM PMSF and protease
218 inhibitor ULTRA Tablet Mini/10 mL EASYpack [ROCHE®]) added just before use, 500 µL
219 glass beads were added and cells were disrupted by vortexing for 30 s and placed on ice
220 for 1 minute (repeated ten times). The chromatin in the lysates was sheared by sonication
221 with 30 cycles (effective sonication time: 3 min 45 s) at 20% amplitude in Episonic multi-
222 functional bioprocessor Model Oasis 180. The DNA was sheared to an average size ~ 500
223 bp. Tagged proteins were immunoprecipitated with 5µg mouse anti-Flag (Sigma®) or anti-
224 cMyc (Millipore®) bound to Dynabeads® Protein G for immunoprecipitation (Invitrogen).
225 Dynabeads with the immunoprecipitates were washed with Dilution buffer (2 mM EDTA
226 [pH 8], 20 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Triton) twice and washed with Wash
227 buffer (2 mM EDTA [pH 8], 20 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Triton, 0.1% SDS) four
228 times. Protein and cross-linked DNA were eluted in 100 µL of Elution buffer (1% SDS, 0.1M
229 NaHCO₃) at 65° for 10 min. To reverse the crosslinking, the mixture was incubated at 65°
230 overnight with 50 µg/mL proteinase K. DNA was extracted with
231 phenol:chloroform:isoamyl alcohol 25:24:1 and precipitated with 5 M NaCl, glycogen and
232 ethanol. The IPs were resuspended in 30 µL of TE (10 mM Tris-Cl [pH 8], 1 mM EDTA)
233 containing 2 µg/mL RNase cocktail (Ambion). Input DNA was prepared by mixing 20% of
234 the starting lysate (after sonication) with 200 µL TE. The lysate was processed in the same
235 way as the immunoprecipitates, proteinase K was added, the crosslinking was reversed

236 and the DNA was extracted. The immunoprecipitated DNA and the input were used as
237 templates for qPCR reactions conducted with ABI 7500 instrumentation (Applied
238 Biosystems) and SYBR Green PCR Master Mix (Life Technologies). The primers used are
239 listed in Table S3. The results shown represent the average of duplicate biological samples
240 and three technical replicates and are expressed as percent enrichment of input relative
241 to binding at *ISC1* for Rap1 and Sir3 and percent enrichment relative to binding at the
242 telomere repeats for Abf1, since these are the loci where there is the least binding for
243 each protein and is considered the negative control. The percentage of input was
244 calculated by the percent input method, using the formula $100 \cdot 2^{(\text{Adjusted input to}$
245 $100\% - \text{Ct (IP)})}$ and the data are presented as the mean \pm SD. Statistical analysis was
246 performed using unpaired t-test two-tailed with $p < 0.001$. Statistical significance was
247 calculated for the percent input for each target, compared to the negative control. We
248 also used untagged strains as negative controls calculating the percentage of input (Fig.
249 S1).

250

251 **Chromosome Conformation Capture (3C) assay**

252 Chromosome Conformation Capture (3C) was performed as described in (BELTON AND
253 DEKKER 2015a). Briefly, cells were grown in SC medium to an OD_{600} of 1. Cells were fixed
254 with 3% formaldehyde for 20 min at 25°. The crosslinking was quenched by adding 2.5 M
255 glycine at 2X the volume of formaldehyde used in the previous step and the culture was
256 shaken for 5 min at 25°. Cross-linked cells were washed with water and resuspended in
257 the appropriate 1X restriction enzyme buffer. The sample was frozen and ground with

258 liquid nitrogen for 10 min. The ground sample was resuspended in 1X restriction enzyme
259 buffer and adjusted to OD₆₀₀ of 10. Cells were distributed into a 96-well PCR plate.
260 Chromatin was solubilized by the addition of SDS (0.1% final) and incubated for 10
261 minutes at 65°. Triton X-100 was added to a final concentration of 1% to sequester the
262 SDS. Chromatin was digested with 100U of *HindIII* and incubated overnight at 37°. The
263 restriction enzyme was denatured by adding SDS (1.67% final) and incubating for 20 min
264 at 65°. Chromatin fragments were ligated in dilute (12X) conditions assembling the ligation
265 reaction (1% Triton X-100, 1X Ligation buffer, 0.1 mg/mL BSA, 1mM ATP, 4.8 U/mL T4 DNA
266 ligase and water) and incubating 4 h at 16°. Cross-links were reversed by incubating the
267 samples for 4 h at 65° in the presence of 0.0625 mg/mL proteinase K, followed by adding
268 again 0.0625 mg/mL proteinase K and incubating overnight at 65°. DNA was purified by a
269 series of phenol-chloroform extractions followed by ethanol precipitation. The resulting
270 template was then treated with RNase cocktail (Ambion) and incubated 1 h at 37° yielding
271 the “3C template”. In addition to the 3C template, a randomized ligation control template
272 was generated (BELTON AND DEKKER 2015b) which was used to determine the PCR
273 amplification efficiency of specific ligation products. This template was generated by
274 digesting naked, non-crosslinked yeast genomic DNA with *HindIII* and ligating it in
275 concentrated conditions to maximize the formation of random inter-molecular
276 combinations of chimeric ligation products. The resulting template was purified by a series
277 of phenol-chloroform extractions and ethanol precipitations and treated with RNase
278 cocktail (Ambion).

279 Once the 3C samples were generated, DNA concentration was determined by SybrGreen
280 quantitative PCR (qPCR) using an internal primer set. 3C samples were adjusted to 50
281 ng/ μ L and the concentrations were verified once again by qPCR. Quantification of ligation
282 products was performed with qPCR using Applied Biosystems™ TaqMan® MGB probes and
283 PerfeCTa FastMix II Low ROX (Quanta Biosciences Inc.). The qPCR reactions contain an
284 anchor primer (anchor H), a TaqMan probe (probe H) and one of the test primers (primers
285 H1 through H9). The probe and primers used are listed in Table S3. A standard curve was
286 performed with each pair of primers using serial dilutions of a random ligation control
287 (Table S4). The conditions used for qPCR were: 15 min at 95° (cycle 1) and 10 s at 95°, 1
288 min at 60° (cycles 2-40) conducted with ABI 7500 instrumentation (Applied Biosystems).
289 3C experiments were performed once for all the strains shown except for the parental
290 strain with Sil2126 in its natural position, which was performed in two biological
291 replicates. All experiments were done in technical triplicates for each oligonucleotide pair.
292 Data shown in Fig. 7 and Fig. S8 represent the mean of the three technical replicas and
293 each data point normalized to its standard curve with the random ligation. Statistical
294 analysis was performed using two-way ANOVA with $p < 0.0001$. Statistical significance was
295 calculated by comparing the crosslinking frequencies at each point with the Sil@-32kb
296 strain.

297

298 **Data availability**

299 All strains and plasmids are available upon request. Strains are listed in Table S1, plasmids
300 in Table S2 and primers in Table S3. Table S4 shows data analysis of the interaction

301 between Sil@-32kb or Sil2126 at its native position and the intergenic regions of telomere
302 E_R by 3C. In addition there are 9 supplementary Figures. All supplementary information is
303 available and has been uploaded in the GSA Figshare portal.

304

305 **Results**

306 **Sil2126 requires the telomere E_R context**

307 We have previously identified a *cis*-acting element located in the right telomere of
308 chromosome E (E_R) between *EPA3* and the telomere. This element, called Sil2126, is a
309 2.126 kb DNA fragment that comprises nucleotide positions 684,673–686,798 (accession
310 no. CR380951) (JUAREZ-REYES 2012). Sil2126 can silence the *URA3* reporter gene integrated
311 at -32kb in telomere E_R (hereafter called Sil@-32kb). However, it does not display
312 silencing activity in other telomeres at similar distances suggesting that Sil2126 is only
313 functional in its native telomere (E_R) (Fig. 1 and (JUAREZ-REYES 2012)).

314

315 **The NE is not required for silencing activity when Sil2126 is inserted 32 kb away from** 316 **telomere E_R**

317 In this subtelomeric region (E_R) there is another *cis*-acting element, the NE (Negative
318 Element), localized 300 bp downstream of the *EPA1* stop codon. The NE negatively
319 regulates *EPA1* expression, in a promoter specific manner and its activity depends on the
320 yKu proteins (GALLEGOS-GARCIA *et al.* 2012). Due to the fact that Sil2126 is only active in this
321 particular subtelomeric region, we decided to test whether the NE is required for Sil@-
322 32kb activity. We used two parental strains, one with Sil@-32kb (Fig. 2A and 2B, line 1)

323 and the second with just the reporter *URA3* (with no Sil@-32kb; Fig. 2B, line 2); both
324 constructs were integrated at -32 kb from telomere E_R (Fig. 2A). In each of these parental
325 strains, we deleted the NE and tested the silencing level of the reporter. We found that
326 the NE is not required for Sil@-32kb activity when Sil2126 is still at its native position (Fig.
327 2B, compare line 1 with line 3). Even in the absence of both, the original copy of Sil2126
328 (*silΔ*) and the NE (*neΔ*), Sil@-32kb is still functional and can silence the reporter (Figure 2B,
329 compare line 5 with line 6). Therefore, the NE is not required for Sil@-32kb activity at the
330 ChrE_R telomere.

331

332 **The intergenic region between *EPA2* and *EPA3* and/or Sil2126 in its original position are**
333 **required for Sil@-32kb activity**

334 Since the NE located in the *EPA1-EPA2* intergenic region is not required for Sil@-32kb
335 activity, we wanted to determine whether the intergenic regions between the *EPA* genes
336 at this telomere and the original copy of Sil2126 are responsible for the telomere E_R
337 specific activity of Sil@-32kb. We replaced the *EPA1-EPA2* and *EPA2-EPA3* intergenic
338 regions by vector sequences maintaining the corresponding genomic distances between
339 the genes and evaluated the Sil@-32kb activity. We tested different combinations of the
340 intergenic region replacements in two backgrounds, a strain lacking Sil2126 (*silΔ*, Fig. 3A,
341 line 1) and a strain with Sil2126 in its original position (Fig. 3B, line 1). In the absence of
342 Sil2126 (*silΔ*), replacement of the *EPA1-EPA2* intergenic region by vector sequences did
343 not have an effect in Sil@-32kb activity (Fig. 3A, compare line 1 with line 2). This is
344 consistent with the fact that the NE element is not required for Sil@-32kb activity (Fig. 2B,

345 lines 5 and 6). However, replacement of the *EPA2-EPA3* intergenic region in this
346 background resulted in loss of silencing of the reporter by Sil@-32kb (Fig. 3A, line 4). As
347 expected, simultaneous replacement of both intergenic regions has the same effect as
348 replacement of only the *EPA2-EPA3* region (Fig. 3A, compare line 4 with line 6).
349 In the strain where the original copy of Sil2126 is present, we observed no effect on Sil@-
350 32kb activity when replacing the *EPA1-EPA2* or *EPA2-EPA3* intergenic regions, or
351 simultaneous deletion of both intergenic regions (Fig. 3B, compare line 3, line 5 and line
352 7), that is, Sil@-32kb does not require the *EPA1-EPA2* or *EPA2-EPA3* intergenic regions for
353 silencing a reporter gene if the original copy is also present close to telomere E_R. Taken
354 together, these results indicate that there are *cis*-acting elements present in the *EPA2*-
355 *EPA3* intergenic region that are required for Sil@-32kb silencing activity. Also, these data
356 suggest that the presence of the original copy of Sil2126 can compensate for the absence
357 of the *EPA2-EPA3* intergenic region elements.

358

359 **Rap1 and Abf1 putative binding sites are required for Sil@-32kb activity**

360 Sil2126 has several putative binding sites for Rap1 and Abf1 as predicted by JASPAR 2016
361 server [(MATHÉLIER *et al.* 2016), Fig. 5A] and we have shown that Sil@-32kb activity
362 depends on Rap1 to silence the reporter (JUAREZ-REYES 2012). In addition, we found that
363 Sil@-32kb activity also depends on Abf1 since, in a strain containing a C-terminal end
364 truncated version of Abf1 (Abf1-43), silencing of the reporter by Sil@-32kb is greatly
365 diminished (Fig. S2). This is the first study in which Abf1 has been found to have a role in
366 the subtelomeric silencing in *C. glabrata* (Castanedo, Hernández-Hernández and Castaño,

367 manuscript in preparation). We wondered whether the activity of Sil@-32kb is dependent
368 on the presence of Rap1 and Abf1 putative binding sites. We tested the level of silencing
369 of the *URA3* reporter in Sil@-32kb precise internal deletions in a *silΔ* background (Fig. 4A).
370 We found that in the absence of the 5' putative binding sites for Rap1 and Abf1 [*sil* (nt1-
371 262)Δ], Sil@-32kb cannot silence the reporter (Fig. 4B, line 3). When we deleted only the
372 putative binding site for Rap1 [*sil* (nt1-204)Δ] but leaving the putative binding site for Abf1
373 (Fig. 4 line 4), or deleted the putative Abf1 binding site and leaving the Rap1 putative
374 binding site (Fig. 4, line 5) in the 5' region of Sil, the level of silencing of the reporter was
375 reduced but was not eliminated (Fig. 4B, compare line 1 with lines 4 and 5). In addition,
376 we found that a 334bp construct containing the combination of just the first binding sites
377 for Rap1 and Abf1 (Sil@-32kb fragment from nt 1-334) cannot mediate the silencing of the
378 reporter, indicating that the other binding sites throughout Sil@-32kb are also required
379 (Fig. 4B, line 6).

380

381 **Rap1 and Sir3 bind to Sil2126 in its original position**

382 To understand the mechanism of action of Sil2126 and whether this element can recruit
383 silencing proteins, in particular Rap1 and Sir3, we performed a ChIP assay using Rap1
384 tagged with the Flag epitope at the C-terminus and integrated this construct in the native
385 *RAP1* locus (Rap1-Flag, Fig. 5A, bottom). We confirmed that the fusion protein is
386 appropriately synthesized by Western blot (Fig. S3A) and we determined its functionality
387 by a silencing assay. As shown in Supplementary Fig. S4, the *URA3* reporter was silenced in
388 the strain containing the Rap1-Flag fusion, although at a decreased level compared to the

389 wild-type, untagged strain (Fig. S4, lines 2 and 3). We then examined the binding profile of
390 Rap1 at the subtelomeric region of telomere E_R (Fig. 5B) by ChIP-qPCR in the parental
391 strain with Sil2126 at its native position. We found that Rap1 is bound only to Sil2126
392 between the *EPA3* and the telomere, and not elsewhere in this subtelomeric region,
393 except at the telomeric repeats where Rap1 enrichment is very high (Fig. 5B, left, columns
394 4 and 5). In addition, we also determined the distribution of the SIR complex throughout
395 the telomere E_R . We tagged Sir3 at the C-terminal end with the Flag epitope and
396 confirmed that it is expressed and functional (Fig. S3A and S4A, line 5), and then
397 performed ChIP-qPCR assays. In the parental strain, we found that Sir3 is highly enriched
398 at Sil2126 in its original position (Fig. S5A, columns 4 and 5), but it is also enriched, albeit
399 to a lesser extent, at longer distances from the telomere i.e. at the *EPA2-EPA3* intergenic
400 regions and at the NE (Fig. S5A, columns 2, 3).

401

402 **In the absence of Sil2126, Rap1 binding in the intergenic region between *EPA2* and *EPA3***
403 **increases**

404 In order to determine if the binding profiles of Rap1 and Sir3 are affected by the presence
405 of Sil2126 in this subtelomeric region, we conducted a ChIP assay in the *silΔ* strain (Fig. 5C,
406 top). We found that while Rap1 is still highly enriched at the telomere (Fig. 5C left column
407 6), Rap1 binding to a region between *EPA2* and *EPA3* (Fig. 5C, left, column 3) is increased
408 when compared to the parental strain with Sil2126 is at its native locus (compare Fig. 5B
409 left, column 3, with Fig. 5C left, column 3). Instead, Sir3 enrichment throughout this region

410 in the *silΔ* strain did not change significantly compared to the parental strain (compare Fig.
411 S5 B, columns 2 and 3 with Fig. S5 A columns 2 and 3).

412

413 **Sil2126 can recruit Rap1, Sir3 and Abf1 when inserted 32 kb away from telomere E_R**

414 The subtelomeric region of Chr E_R contains several putative binding sites for Rap1 and
415 Abf1 (Fig. 5A). We have shown that Sil@-32kb activity also depends on Abf1 to silence the
416 reporter (Fig. S2). In order to determine the binding profile of Rap1, Abf1 and Sir3
417 throughout the subtelomeric region with Sil@-32kb, we analyzed the enrichment of these
418 proteins at several regions in the Chr E_R by ChIP assays. We generated a tagged version of
419 Abf1 at the amino-terminal end, which is expressed from a replicative plasmid under the
420 inducible promoter P_{MT1} (Fig. 5A, bottom). We showed that this cMyc-Abf1 fusion protein
421 is expressed (Fig. S3 B) and functional for silencing (Fig. S4 B, data not shown). First we
422 determined by ChIP-qPCR that Abf1 is enriched at the NE, between *EPA1* and *EPA2*, both
423 in the parental strain with Sil2126 at its native locus (Fig. 5B right column 2) and also in
424 the *silΔ* strain (Fig 5C right, column 2). We then used the *silΔ* strain with Sil@-32kb (Fig.
425 6A, top) and found that Rap1 is bound to Sil@-32kb (Fig. 6A left, columns 7, 4 and 5), to
426 the *EPA2-EPA3* intergenic region (Fig. 6A left, column 3) and to the region immediately
427 adjacent to the telomeric repeats as reported for *S. cerevisiae* (Fig. 6A left, column 6).
428 However, Abf1 localization shows a different distribution from that of Rap1. Sil@-32kb can
429 also recruit Abf1 (Fig. 6A right, columns 7, 4 and 5), but in contrast to Rap1, Abf1 also
430 binds to the NE in this strain (Fig. 6A right, column 2). Sil@-32kb can also recruit Sir3 at
431 that distance from the telomere (Fig. S5C columns 7, 4 and 5). These results show that

432 Rap1, Sir3 and Abf1 are recruited to Sil@-32kb, suggesting that the protosilencer can
433 nucleate a compact chromatin structure at this distance from the telomere to mediate
434 silencing of the reporter.

435

436 **Sil2126 recruits Rap1 and Abf1 in the absence of the intergenic region between *EPA2***
437 **and *EPA3*.**

438 Since the *EPA2-EPA3* intergenic region is required for silencing activity of Sil@-32kb in the
439 absence of the original copy of Sil2126 (Fig. 3A line 4), we decided to determine whether
440 Rap1 and Abf1 can be recruited to Sil@-32kb in a strain where the *EPA2-EPA3* intergenic
441 region has been replaced by vector sequences (Fig. 6B, Top). The results show that Rap1
442 and Abf1 are bound at the same positions within Sil@-32kb, even though neither the
443 *EPA2-EPA3* intergenic region, nor Sil2126 are present in this strain (Fig. 6B). It is
444 noteworthy that the rest of the binding profile of Rap1 and Abf1 throughout this region
445 remains unchanged with respect to the strain that has the native *EPA2-EPA3* intergenic
446 region (compare Fig. 6A with 6B), i.e. Rap1 is highly enriched at the telomere (Fig. 6A left,
447 column 6) and Abf1 at the NE (Fig. 6B right, column 2, and compare Fig. 5B with Fig. S6A,
448 Fig. 5C with Fig. S6B). This pattern is also observed in the strain that contains Sil2126 at its
449 native locus (compare Fig. S7A with Fig. S7B).

450

451 **A 5' fragment of Sil2126 (334 bp) efficiently recruits Rap1 and Abf1 when integrated 32**
452 **kb away from telomere E_R**

453 We have shown that the 5' fragment of Sil2126 (334 bp) containing the putative Abf1 and
454 Rap1 binding sites is not sufficient to mediate silencing of the reporter *URA3* (Fig. 4B).
455 ChIP assays in the strain containing this 5' fragment of Sil2126 inserted at -32kb (Fig. 6C,
456 top), showed a strong enrichment of Rap1 and Abf1 binding to this 5' fragment (Fig. 6C,
457 bottom). In contrast, the enrichment of Rap1 in the *EPA2-EPA3* intergenic region is
458 decreased relative to the enrichment at this site in the strain with full length Sil@-32kb
459 (Fig. 6C, left, column 3). The 5' fragment of Sil recruits even more efficiently Abf1 and
460 Rap1 than the full length Sil@-32kb, which might suggest that the distribution of Rap1 and
461 Abf1 is rearranged depending on the particular *cis*-acting elements present in this region.

462

463 **Sil2126 inserted 32 kb away from the telomere interacts with the intergenic region**
464 **between *EPA1* and *EPA2* to form a loop and establish silencing**

465 Since Sil@-32kb can silence the adjacent reporter *URA3* and recruits silencing proteins
466 such as Rap1 and Abf1, we wondered whether a loop can be formed between Sil@-32kb
467 and other *cis*-elements in this subtelomeric region, which would allow the propagation of
468 silencing. First, we performed a 3C assay (Chromosome Conformation Capture) in two
469 strains where the original copy of Sil2126 has been deleted (*sil*Δ); one with Sil@-32kb and
470 the other containing at this position a 3' fragment of Sil from nucleotide 262 – to 2126
471 [*sil*(1-262)Δ] that lacks the 5' end Abf1 and Rap1 putative binding sites, and cannot
472 mediate silencing (Fig. 4, line 3). We determined the crosslinking frequency by qPCR using
473 an anchor primer (anchor H) and a Taqman probe (probe H), which anneal at Sil@-32kb

474 (Fig. 7A, bottom). We detected a DNA looping interaction between the full length Sil@-
475 32kb and the *EPA1-EPA2* intergenic region (a 1647 bp fragment that contains the 3' and
476 downstream region of *EPA1*, including the NE, Fig. 7A, purple line). In contrast, the strain
477 with *sil(nt1-262)*Δ did not show any interactions across the subtelomeric region E_R (Fig.
478 7A, green line). These data suggest that Sil@-32kb can induce the formation of a
479 chromatin loop that can propagate silencing. Furthermore, loop formation requires the
480 Rap1 and Abf1 putative binding sites present in the first 262 nucleotides of the
481 protosilencer.

482

483 **DNA loop formation between Sil@-32kb and the intergenic region between *EPA1* and**
484 ***EPA2* depends on silencing proteins**

485 To determine if the interaction observed between Sil@-32kb and the *EPA1-EPA2*
486 intergenic region depends on silencing proteins, we performed a 3C assay using derivative
487 strains from the 3C assay above (*sil*Δ containing Sil@-32kb) but introducing either the
488 *rap1-21* allele, which is a deletion of the last 21 amino acids of Rap1 and is completely
489 defective for silencing, or the *sir3*Δ allele (Table S1). We found that the interaction
490 between Sil2126 and the *EPA1-EPA2* intergenic region is lost in the absence of silencing
491 activity of Rap1 (*rap1-21*Δ) or Sir3 (*sir3*Δ) strain (Fig. 7A, red line and Fig. S8, blue line).
492 These data suggest that at least Rap1 and Sir3 silencing proteins are necessary for the
493 interaction between these two loci, possibly by favoring a compact, structure through
494 protein-protein interactions.

495

496 **Sil2126 in its native position interacts with the region between *EPA2* and *EPA3***

497 We next asked whether Sil2126 in its native position is able to interact with the elements
498 that are required for its activity at -32 kb. We performed a 3C assay to determine the
499 crosslinking frequencies in the parental strain where Sil2126 is in its native position using
500 the anchor primer H and the Taqman probe (probe H) aligned within Sil2126 (Fig. 7B,
501 bottom). We detected a strong interaction between Sil2126 and the *EPA2-EPA3* intergenic
502 region (primers H7 and H8). This is in agreement with our previous data in which Sil@-
503 32kb requires the *EPA2-EPA3* intergenic region for its silencing activity. In addition, we
504 observed weaker interactions between Sil2126 with the flanking intergenic regions of
505 *EPA1* (Fig. 7B). These data suggest that the subtelomeric region of Chr E_R is able to form
506 different three-dimensional structures between the various *cis*-acting elements.

507

508 **Formation of a DNA loop between Sil@-32kb and the region between *EPA1* and *EPA2***
509 **results in repression of the *EPA1* promoter**

510 Since Sil@-32kb forms a loop with the region downstream from *EPA1*, and this loop allows
511 propagation of silencing up to 32kb away from the telomere, we wondered whether this
512 interaction allows a heterochromatin structure that would result in repression of *EPA1*,
513 which forms part of this loop. To test this, we measured activity of *EPA1* promoter using a
514 transcriptional fusion of P_{*EPA1*} with *GFP* by flow cytometry in a strain that only contains

515 Sil@-32kb. We have previously shown that dilution of cells into fresh media from
516 stationary phase cultures results in induction of *EPA1*. We used stationary phase cultures
517 diluted into fresh media and found that *GFP* could not be induced under this condition,
518 which results in *EPA1* induction in the strain that does not contain Sil@-32kb (Fig. 8). This
519 data suggests that Sil@-32kb forms a three dimensional structure which does not allow
520 induction of P_{EPA1} upon dilution into fresh medium.

521

522 **Discussion**

523 Members of a large family of cell wall protein genes called the *EPA* family, some of which
524 have been shown to function as adhesins (*EPA1*, *EPA6* and *EPA7*) (CORMACK *et al.* 1999; DE
525 LAS PENAS *et al.* 2003; CASTANO *et al.* 2005), are encoded in the subtelomeric regions of
526 chromosomes of the fungal pathogen *Candida glabrata*. In the BG2 strain background
527 (CORMACK AND FALKOW 1999), the expression of most of the *EPA* genes is repressed by
528 chromatin-based silencing due to their localization near the telomeres. In particular *EPA1*,
529 which encodes the major adhesin in *C. glabrata* and is localized ± 20 kb from the telomere
530 E_{-R}, is tightly regulated by several layers of regulation, including subtelomeric silencing
531 (GALLEGOS-GARCIA *et al.* 2012). The presence of telomere-specific *cis*-acting elements might
532 explain the significant differences found in the requirement for some silencing proteins at
533 different telomeres, which result in a complex and unique transcriptional regulation of
534 native subtelomeric genes. For example, *EPA1* at the telomere E_{-R} is subject to a
535 promoter-specific repression independent of the subtelomeric silencing, which is

536 mediated by a *cis*-acting element called the negative element, NE (GALLEGOS-GARCIA *et al.*
537 2012). In addition to the NE, telomere E_R contains the *cis*-acting Sil2126 protosilencer
538 between *EPA3* and the telomere repeats, which contributes to silence the *EPA* genes
539 present at this region.

540 In this work, we showed that the protosilencer Sil2126, can recruit silencing proteins, such
541 as Rap1, Sir3 and Abf1, both when present at its native position or when inserted 32 kb
542 away from the telomere. We propose that Sil2126 can induce the formation of a DNA loop
543 in this subtelomeric region by interacting with an intergenic region in the *EPA1-3* cluster
544 probably through protein-protein interactions between silencing proteins recruited to
545 Sil2126 and the intergenic regions involved. This results in remodeling of the chromatin
546 structure close to the telomere E_R leading to the formation of heterochromatin.

547

548 ***Cis*-acting elements present in the intergenic region between *EPA2* and *EPA3* are**
549 **required for Sil2126 at -32 kb**

550 We have previously shown that the protosilencer Sil@-32kb is only functional in its native
551 telomere (JUAREZ-REYES 2012) and in this work we found that in the absence of the native
552 copy, it requires *cis*-acting elements located in the *EPA2-EPA3* intergenic region, but not
553 the NE or the entire *EPA1-EPA2* intergenic region, for its activity (Fig. 3A, line 4). The *EPA2-*
554 *EPA3* region contains several putative binding sites for Rap1 and Abf1 (Fig. 5A), which
555 could have a role in the spreading of silencing at the subtelomere E_R to up to 20 kb. Since
556 it is thought that silencing can propagate by the formation of loops between silencers and
557 protosilencers or between distant protosilencers (LEBRUN *et al.* 2001; FOUREL *et al.* 2002),

558 Sil2126 and the *cis*-acting elements in the *EPA2-EPA3* intergenic region could work
559 synergistically to extend silencing. This could explain the specificity of Sil2126 for the E_R
560 telomere. Sil2126 in its native locus can compensate for the absence of the *EPA2-EPA3*
561 intergenic region (compare Fig. 3A, line 4 with Fig. 3B, line 5), probably because Sil2126
562 recruits silencing proteins and both copies of Sil2126 could interact through protein-
563 protein interactions.

564

565 **Rap1, Abf1 and Sir3 bind at several positions throughout the subtelomeric region of Chr**
566 **E_R and are recruited to Sil2126 when inserted 32 kb away from the telomere E_R**

567 In this work we showed that Sir3 and Rap1 are clearly bound to Sil2126 at its native
568 position, close to the telomere (Fig. 5B left and Fig. S5A). Furthermore, this protosilencer
569 can recruit Rap1, Sir3 and Abf1 when inserted in a silencing-free environment (32 kb away
570 from the telomere; Fig. 6A and Fig. S5C). These results suggest that the mechanism of
571 silencing of Sil2126 is through recruitment of Rap1 and Abf1. In turn, these proteins
572 recruit the SIR complex to establish a silent domain in a similar way to the interactions
573 between Rap1 with Sir3 and Sir4 reported in *S. cerevisiae* (MORETTI *et al.* 1994; CHENG AND
574 GARTENBERG 2000; MORETTI AND SHORE 2001). Rap1 and/or Abf1 could bind to the *cis*-acting
575 elements with different affinities or even cooperatively, so that the equilibrium could be
576 driven toward the formation of a compact silent chromatin structure.

577

578 **Sil2126 at its natural position strongly interacts with the intergenic region between**
579 ***EPA2* and *EPA3***

580 In its normal context between *EPA3* and the telomere, Sil2126 strongly interacts with the
581 *EPA2-EPA3* intergenic region (Fig. 7B, orange line) resulting in a loop schematically shown
582 in Fig. 9B. We propose that in the parental strain, Sil2126 can in fact form alternative
583 loops with *cis*-acting elements across the subtelomeric region E_R. The most frequent loop
584 is with the *EPA2-EPA3* intergenic region, but also to a lesser extent, a loop can be formed
585 with the NE region. We think this compact structure results in the strong repression of
586 *EPA3*, *EPA2* and also *EPA1* observed in the parental strain under most *in vitro* conditions
587 (CASTANO *et al.* 2005; GALLEGOS-GARCIA *et al.* 2012). Another possibility is that the strong
588 signal detected between Sil2126 and its immediate vicinity (Fig. 7B probes H8 and H9),
589 could be due to an alternative chromatin conformation at this site and not to a loop *per*
590 *se*.

591

592 **Sil@-32kb propagates silencing by interacting with the intergenic region between *EPA1***
593 **and *EPA2***

594 We showed that the *cis*-acting element Sil@-32kb (in the *silΔ* strain) interacts with a
595 fragment in the *EPA1-EPA2* intergenic region between *EPA1* and *EPA2* through a DNA loop
596 formation (Fig. 7, purple line). This interaction is significantly more frequent than with any
597 other fragment in this subtelomeric region in the absence of Sil at its native position and
598 thus suggests that the interaction is specific. Importantly, we showed that loop formation
599 in this strain critically depends on both Rap1 and Sir3 (Fig. 7A, red line and Fig. S8, blue
600 line). Besides, the deletion of the first 262bp of Sil2126, which contain the 5' end Rap1 and
601 Abf1 binding sites, results in the loss of this interaction (Fig. 7, green line). The loop

602 formation allows propagation of silencing up to 32kb away from the telomere and forms a
603 heterochromatin domain that includes *EPA1* as assessed by lack of induction of the P_{EPA1}
604 upon dilution of stationary phase cells into fresh medium (Fig. 8). It should be pointed out
605 that the fragment that interacts with Sil@-32kb contains the NE and we showed that the
606 NE is not required for Sil@-32kb activity (Fig. 2). It is possible that when Sil2126 is inserted
607 at -32 kb, it can also promote less strong interactions with another *cis*-acting element,
608 possibly the *EPA2-EPA3* intergenic region. We speculate that this proposed, less frequent
609 loop between Sil@-32kb and *EPA2-EPA3* intergenic region, might be more efficient at
610 silencing of the reporter integrated with Sil@-32kb. We think this is possible because
611 replacement of the *EPA2-EPA3* intergenic region, and therefore loss of this alternate loop
612 with the *EPA2-EPA3* intergenic region, completely abolishes silencing of *URA3* (Fig. 3A, line
613 4). Recently, 3C assays have been used to find potentially new *cis*-acting elements (LIU AND
614 GARRARD 2005), we are currently testing other regions of interaction using different
615 oligonucleotides throughout this region.

616 The proteins involved in bridging interactions between these *cis*-acting elements might be
617 the SIR complex recruited by Rap1 and Abf1 bound to Sil2126. It is thought that in order to
618 attain a repressed domain, the SIR complex bound to nucleosomes needs to compact the
619 chromatin into a higher order structure, probably by folding the telomere and generating
620 a compact domain. Interactions at a distance between silencers or protosilencers and the
621 nucleation sites like the telomeres, could promote the initial recruitment of the SIR
622 complex or the maintenance of the compact silent chromatin (KUENG *et al.* 2013; THURTLIE
623 AND RINE 2014). Indeed, in the heterochromatin regions in *S. cerevisiae* like the mating loci,

624 silencer elements (*HMR-E* and *HMR-I*) can interact with each other to silence *HMR* and the
625 SIR complex is required (VALENZUELA *et al.* 2008; MIELE *et al.* 2009).

626

627 We propose that a 3D structure is necessary for spreading of the subtelomeric silencing
628 and requires a repertoire of *cis*-acting elements and silencing proteins bound to these
629 elements (Fig. 9A). When Sil2126 is integrated 32 kb away from the telomere in a *silΔ*
630 background, we propose a model where Sil@-32kb can induce the formation of a loop in
631 this subtelomeric region by interacting with an *EPA1-EPA2* intergenic region probably
632 through protein-protein interactions between silencing proteins recruited to Sil2126 and
633 the intergenic region involved. This results in remodeling of the chromatin structure close
634 to the telomere E_R , leading to the formation of heterochromatin and spreading of
635 silencing. In fact it is possible that there are alternate loops that can be formed between
636 Sil2126 (at its native position or at -32 kb) and the various *cis*-acting elements throughout
637 this region. This in turn depends on the binding of Rap1 and Abf1 and subsequent
638 recruitment of the SIR complex. The nucleation mechanisms of the SIR complex at
639 increasing distances from the telomere is not known, it might be achieved by propagating
640 from the telomeric repeats recruited by Rap1 and/or from the other *cis*-elements that
641 bind Rap1. In this regard, it is interesting to note that Rap1 can associate with distal sites
642 and loop out intervening DNA (HOFMANN *et al.* 1989). This model is supported by the
643 recent finding in *S. cerevisiae* that the spreading of the SIR complex on chromatin is
644 through pairs of nucleosomes lacking histone H4K16 acetylation and H3K79 methylation

645 and this propagation can occur across non-neighboring nucleosomes, which can promote
646 loop formation in the heterochromatin (BEHROUZI *et al.* 2016).

647 The fact that most of the *EPA* genes are located in subtelomeric regions and regulated by
648 subtelomeric silencing at least in some strains of *C. glabrata*, would seem to imply that all
649 *EPA* genes are regulated in a similar way. However, each telomere contains different *cis*-
650 acting elements and different requirements for silencing proteins, this allows for flexibility
651 in the regulation of individual *EPA* genes, which would allow the cell to respond to
652 different environmental conditions expressing the appropriate *EPA* gene for each host
653 niche.

654 **Acknowledgments**

655 The authors would like to thank Verónica Zárata and LANBAMA for expert technical
656 assistance with sample sequencing and Nicolás Gómez for excellent technical assistance
657 with ChIP assay. E.L.F., G.H.H. and L.C. were supported by CONACyT fellowships No.
658 261740, No. 590366 and No. 448801 respectively. This work was supported by CONACyT
659 grant No. CB-2014-239629 to I.C.N.

660

661 **Author Contributions**

662 E.L.F. and I.C. designed and performed the experiments. G.H.H. and L.C. tagged the Rap1
663 and Abf1 proteins. G.G.E. gave technical assistance. K.O. supervised the 3C experiments.
664 A.D.L.P. edited the manuscript and all authors were involved in the final preparation of
665 the manuscript.

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780

781

782 **Figure Legends**

783 **Figure 1.** Sil2126 requires the context of telomere E_{-R}.

784 **(A, top)** Map of the telomere E_{-R} showing the relevant *cis*-acting elements and the proteins required
785 for subtelomeric silencing. This region contains the *EPA1*, *EPA2* and *EPA3* genes indicated by
786 arrows. The protosilencer Sil2126 is drawn as an orange arrow between *EPA3* and the telomere (T).
787 Rap1 (red circle), binds to the telomere repeats and recruits the SIR complex (Sir2, Sir3 and Sir4)
788 and Rif1 (green rectangle). A second *cis*-acting element called the negative element (NE,
789 represented as a pink rectangle), represses *EPA1* expression in a promoter-dependent way, and
790 requires the yKu proteins (yKu70 and yKu80). Silencing can spread from the telomere with the
791 contribution of the protosilencer Sil2126, to up to > 20 kb to the *EPA1* gene.

792 **(B, top)** Schematic representation of the Sil-reporter system consisting of a PCR product containing
793 a 665 bp integration region (gray box), cloned immediately adjacent to the 5' end of the Sil2126
794 element followed by the *URA3* reporter gene with its own promoter. **(B, middle)** Sil2126 integrated
795 between *ISC1* and *HYR1*, which is 32 kb from the right telomere of chromosome E (E_{-R}, Sil@-32kb),
796 the *SpeI* site used to linearize and integrate the vector, is indicated. Only the genes from *ISC1* to
797 *EPA1* are shown. Note the discontinuity from the NE close to the 3' UTR of *EPA1* up to the native
798 Sil2126 element near the telomere. **(B, bottom)** The Sil-reporter system was integrated in different
799 chromosomes at similar distances from the indicated telomere (shown to the left of each line). In
800 Chr E_{-R} the Sil-reporter system (line 1) and the negative control (*sil*) consisting only of the *URA3*
801 reporter (line 2) was integrated at -32 kb. The Sil-reporter system in Chr C_{-L} was integrated at -26 kb
802 from the telomere (line 3); in Chr I_{-L}, at -23 kb (line 4) and in Chr K_{-R}, at -19 kb (line 5) from the
803 telomere. The level of silencing of *URA3* reporter was tested using a growth plate assay on SC -ura
804 or SC +5-FOA plates. The number of viable cells used for each experiment is estimated by the
805 growth on rich media YPD. Strains were grown to stationary phase in YPD after which 10-fold serial
806 dilutions were made in sterile water and equal numbers of cells were spotted onto the indicated
807 plates. Plates were incubated 48 h at 30° and photographed.

808

809 **Figure 2.** The NE is not required for Sil@-32kb activity at the right telomere of chromosome E.

810 (A) Schematic representation of the Sil@-32kb and the *URA3* reporter integrated in the right
811 telomere of the chromosome E (Chr E_R) between *ISC1* and *HYR1* genes in the parental strain. The
812 negative element (NE) is shown as a pink square downstream from *EPA1* and the Sil2126 is
813 represented as an orange arrow. Note the discontinuity from the NE close to the 3' UTR of *EPA1* up
814 to the native Sil2126 element near the telomere.

815 (B) Assessment of the level of silencing of *URA3* reporter in strains with deletions of *cis*-acting
816 elements (Sil2126 and NE) using a growth plate assay on the indicated media. The genomic
817 structure at the subtelomeric region of telomere E_R for each strain tested is shown to the left of each
818 line. Note that insertion of the Sil@-32kb generates a duplication of Sil2126 in this region. Lines 1 -
819 4 show the silencing activity of the Sil@-32kb in the presence or absence of the NE. Lines 5 - 7
820 show the silencing activity of derivatives of these strains in which the native copy of Sil2126 has
821 been deleted. Strains were grown to stationary phase in YPD, diluted and spotted on the media
822 indicated as described in Figure 1B.

823

824 **Figure 3.** The *EPA2-EPA3* intergenic region is required for Sil@-32kb activity.

825 (A, top) Schematic representation of the Sil@-32kb and the *URA3* reporter integrated in the right
826 telomere of the chromosome E (Chr E_R) between *ISC1* and *HYR1* genes in the *silΔ* strain. (A,
827 bottom) Silencing activity of the Sil@-32kb in strains with a replacement of the *EPA1-EPA2* and
828 *EPA2-EPA3* intergenic regions and in the absence of the native Sil2126 element. Schematic
829 representation of the genetic structure at telomere E_R in each strain evaluated is shown on the left
830 side. The wavy line represents the replacement of the indicated intergenic region by vector
831 sequences. The distance between genes was maintained. Each strain contains a different
832 combination of the intergenic region replacements. Note the discontinuity from the *EPA1* promoter
833 up to the -32kb region where Sil@-32kb is inserted. (B, top) Schematic representation of the Sil@-
834 32kb and the *URA3* reporter integrated in the telomere E_R between *ISC1* and *HYR1* genes in the
835 parental strain (note that this strain contains a duplication of Sil2126). (B, bottom) Silencing activity

836 of the Sil@-32kb in strains with a replacement of the intergenic regions between *EPA* genes as in
837 (A, bottom). The level of silencing in each strain is shown on the right side as assessed by growth
838 on 5-FOA plates as described in Fig. 1B.

839

840 **Figure 4.** The binding sites for Rap1 and Abf1 are required for Sil@-32kb activity.

841 (A) Schematic representation of the Sil@-32kb and the *URA3* reporter integrated in the right
842 telomere of the chromosome E (Chr E_R), between *ISC1* and *HYR1* genes in the absence of the
843 original copy of Sil2126 between *EPA3* and the telomere (*silΔ*).

844 (B) Level of silencing of several Sil2126 deletions of Rap1 and Abf1 putative binding sites. The
845 control strains (Sil@-32kb-*URA3* reporter and only the *URA3* reporter integrated at -32 kb from
846 telomere E_R) are shown in lines 1 and 2. The orange rectangles represent the different deletions of
847 Sil2126. Numbers on the rectangles indicate the end nucleotide position of each version of Sil2126
848 deletions. All constructs were integrated at -32 kb from telomere E_R. Rap1 and Abf1 binding sites
849 are represented by red and green rectangles respectively. Equal numbers of cells of each strain
850 were spotted on each media to assess the level of silencing as described in Fig. 1B.

851

852 **Figure 5.** Rap1 and Abf1 are recruited to Sil2126 and at several positions throughout the
853 subtelomeric region of Chr E_R.

854 (A, top, middle and bottom right) Map of the right telomere of chromosome E_R showing Rap1 (red
855 vertical lines) and Abf1 (green vertical lines) putative binding sites. Lines are drawn above or under
856 the map to indicate the DNA strand on which the putative binding sites are localized. We used the
857 indicated *S. cerevisiae* consensus binding sites for Rap1 and Abf1, to predict the putative binding
858 sites in *C. glabrata* using JASPAR 2016 server (A, bottom right). (A, Bottom left) Schematic
859 representation of the tagged versions of Rap1 and Abf1 used for ChIP experiments. Rap1 was
860 fused with the Flag epitope at the C-terminal end and the wild-type allele was replaced by the
861 tagged version in its original chromosomal location. Abf1 construct is provided on a replicative

862 plasmid in which Abf1 is fused to the c-Myc epitope at the N-terminal end. The fusion is driven by
863 the inducible promoter P_{MT1} , which is induced in the presence of copper. **(B, C)** Rap1 is recruited by
864 Sil2126 at its native position and/or propagated from the telomere. **(Top)** Schematic representation
865 of Chr E_R indicating the regions tested in the ChIP assay. Each amplified fragment with the
866 corresponding primer set is numbered and the numbers correspond to each bar in the graphs the
867 arrows indicate the position where the qPCR primers anneal. The distance from Sil2126 to telomere
868 is indicated. **(Bottom)** Rap1-Flag and cMyc-Abf1 enrichment is represented as percentage of input
869 relative to binding at *ISC1* for Rap1 or at the telomere repeats for Abf1. Each column corresponds to
870 the regions amplified by qPCR, represented in the Chr E_R map as numbered rectangles. The
871 number of each primer set indicates the same region amplified across the different strains. The
872 percentage of input was calculated by percent input method using the formula $100 \cdot 2^{(\text{Adjusted}$
873 $\text{input to } 100\% - \text{Ct (IP)})}$. **(B)** ChIP assay in the parental strain (Sil2126 in its original position). **(C)**
874 ChIP assay in a *silΔ* strain.

875

876 **Figure 6. Sil2126 can recruit Rap1 and Abf1 when inserted 32 kb away from the telomere.**

877 **(A)** Sil@-32kb can recruit Rap1 and Abf1. **(Top)** Schematic representation of the subtelomeric
878 region of Chr E_R in the strain where the original copy of Sil2126 is deleted and the Sil@-32kb-*URA3*
879 reporter is inserted at Chr E_R . The position of the fragments amplified with the indicated primer sets
880 for the ChIP assays is indicated below the map. Each amplified fragment with the corresponding
881 primer set is numbered and the numbers correspond to each bar in the graphs in all panels and to
882 Fig. 5. **(Bottom)** Rap1-Flag and cMyc-Abf1 enrichment represented as percentage of input relative
883 to binding at *ISC1* for Rap1 or at the telomere repeats for Abf1, which was calculated as described
884 in Fig. 5B. **(B)** Rap1-Flag and cMyc-Abf1 are recruited at -32 kb in the absence of the *EPA2-EPA3*
885 intergenic region. **(Top)** Schematic representation of the Chr E_R in the absence of the original copy
886 of Sil2126 and with a replacement of the *EPA2-EPA3* intergenic region by vector sequences
887 (represented by the wavy line). The regions tested are indicated as described for Fig. 6A and
888 correspond to the bars in the graph. **(Bottom)** Rap1-Flag and cMyc-Abf1 enrichment represented as

889 percentage of input as in Fig. 5B. **(C)** There is a higher enrichment of Rap1-Flag and cMyc-Abf1
890 when a 5' fragment of Sil2126 (334 bp) containing the putative Abf1 and Rap1 binding sites is
891 integrated at -32 kb. **(Top)** Schematic representation of the subtelomeric region of Chr E_R in the
892 strain in which a 334 bp fragment from the 5' end of Sil2126 was inserted at -32 kb, followed by the
893 *URA3* reporter. The regions tested by qPCR are indicated as described for Fig. 6A and correspond
894 to the bars in the graph. **(Bottom)** Rap1-Flag and cMyc-Abf1 enrichment is represented as
895 percentage of input as described in Fig. 5B.

896 **Figure 7.** Sil2126 placed at -32 kb interacts with a fragment downstream *EPA1* to propagate
897 silencing. And Sil2126 in its native position interacts with the *EPA2-EPA3* intergenic region.

898 **(7A, top)** 3C analysis represented by crosslinking frequencies throughout the Chr E_R in derivatives
899 of the *silΔ* strain. Each point in the graph represents the crosslinking frequency of each *HindIII*
900 fragment tested in the different locations across the subtelomeric region. The crosslinking
901 frequencies in a strain with Sil@-32kb is represented by the purple line, the strain with the deletion
902 construct [*sil(1-262)Δ*] inserted at -32 kb is represented by the green line and the *rap1-21* strain is
903 represented by the red line. The silencing activity of these constructs is indicated. **(7A, bottom)**
904 Schematic representation of the telomere E_R with the Sil@-32kb and the *URA3* reporter inserted at
905 -32 kb. The arrowheads above the map represent the primers used in combination with the anchor
906 H and the TaqMan probe H located in Sil2126 (also indicated as blue and pink arrowheads
907 respectively). The digestion sites of restriction enzyme (*HindIII*) are indicated (H1-H9). **(7B top)**. 3C
908 analysis shown as crosslinking frequencies throughout the Chr E_R in the parental strain with
909 Sil2126 at its native locus. Each point in the graph represents the crosslinking frequency of each
910 *HindIII* fragment tested in the different locations across the subtelomeric region. The crosslinking
911 frequencies in the parental strain with Sil2126 at its native locus is represented by the orange line.
912 **(7B, bottom)** Schematic representation of the telomere E_R in the parental strain. The arrowheads
913 above the map represent the primers used in combination with the anchor H and the TaqMan probe
914 H located in Sil2126 (also indicated as blue and pink arrowheads respectively). The location and
915 numbers of the primers correspond to the primers in Fig. 7A bottom. Note that the Y-axis is
916 discontinuous.

917 **Figure 8.** *EPA1* expression is not induced when Sil@-32kb and *URA3* reporter are placed at -32 kb
918 from the telomere E_R.

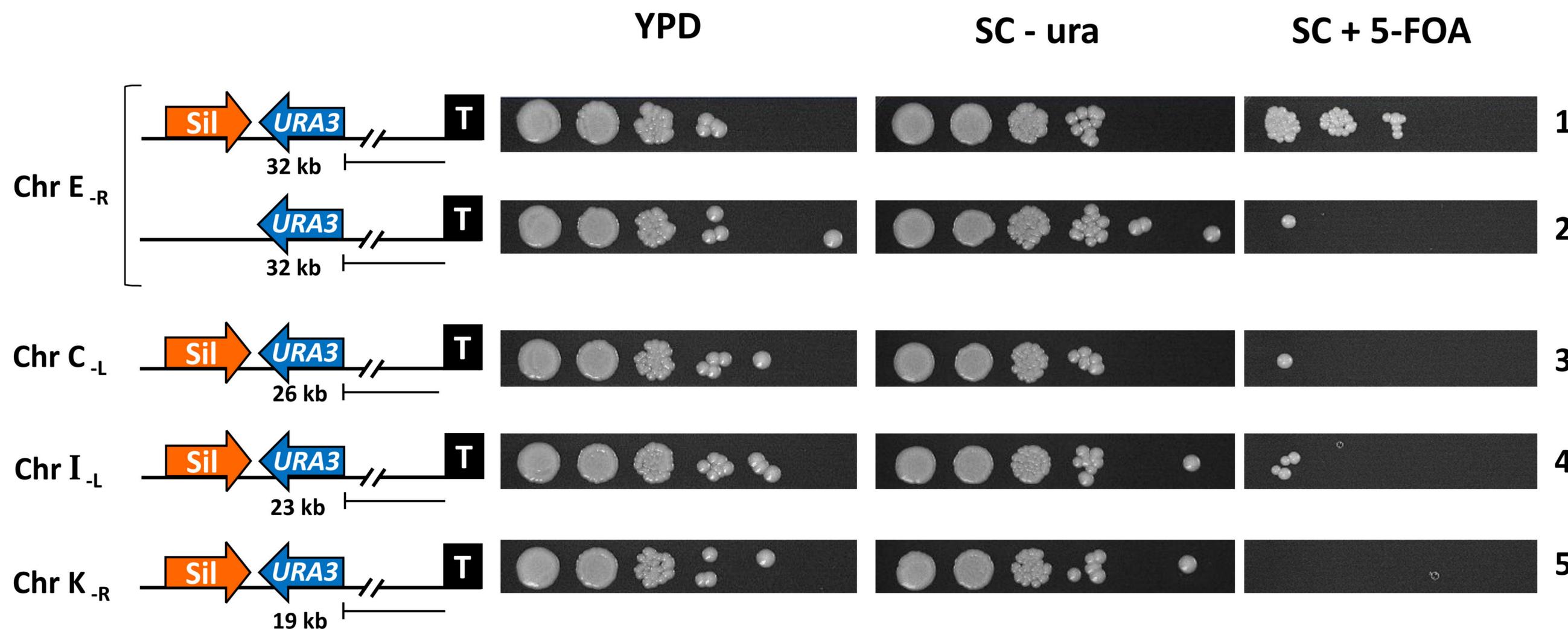
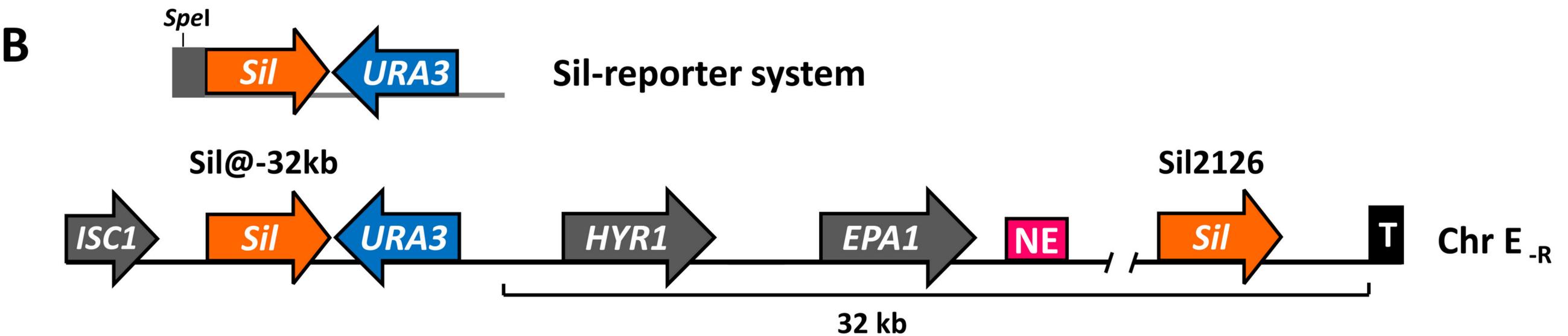
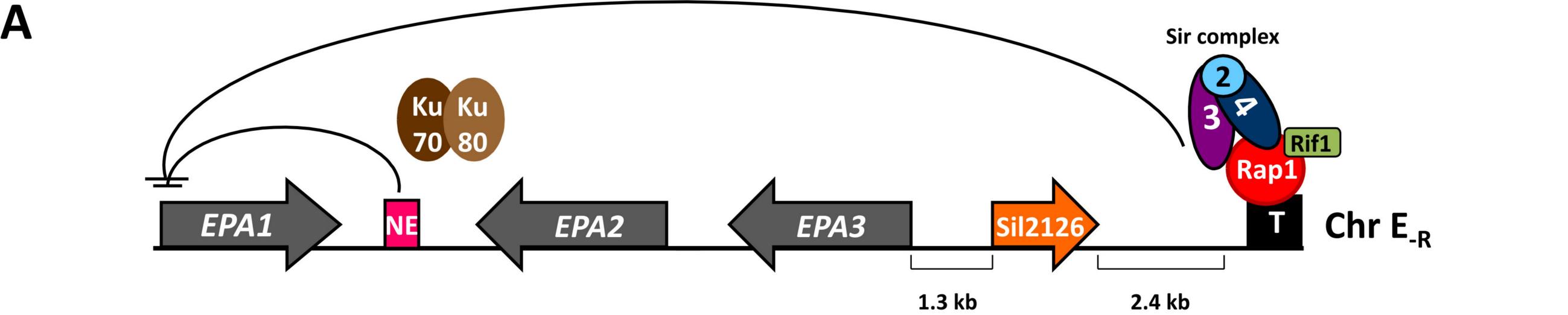
919 Activity of the *EPA1* promoter as measured by FACS. Strains were grown in SC medium
920 supplemented with 25 mg/L uracil for 48 hr at 30°. Cells were diluted into fresh medium and
921 samples were taken every 2 hr. Schematic representation of the genetic structure at telomere E_R in
922 each strain evaluated is shown on the right side. Fig. S9 shows the histograms corresponding to the
923 last strain in the graph.

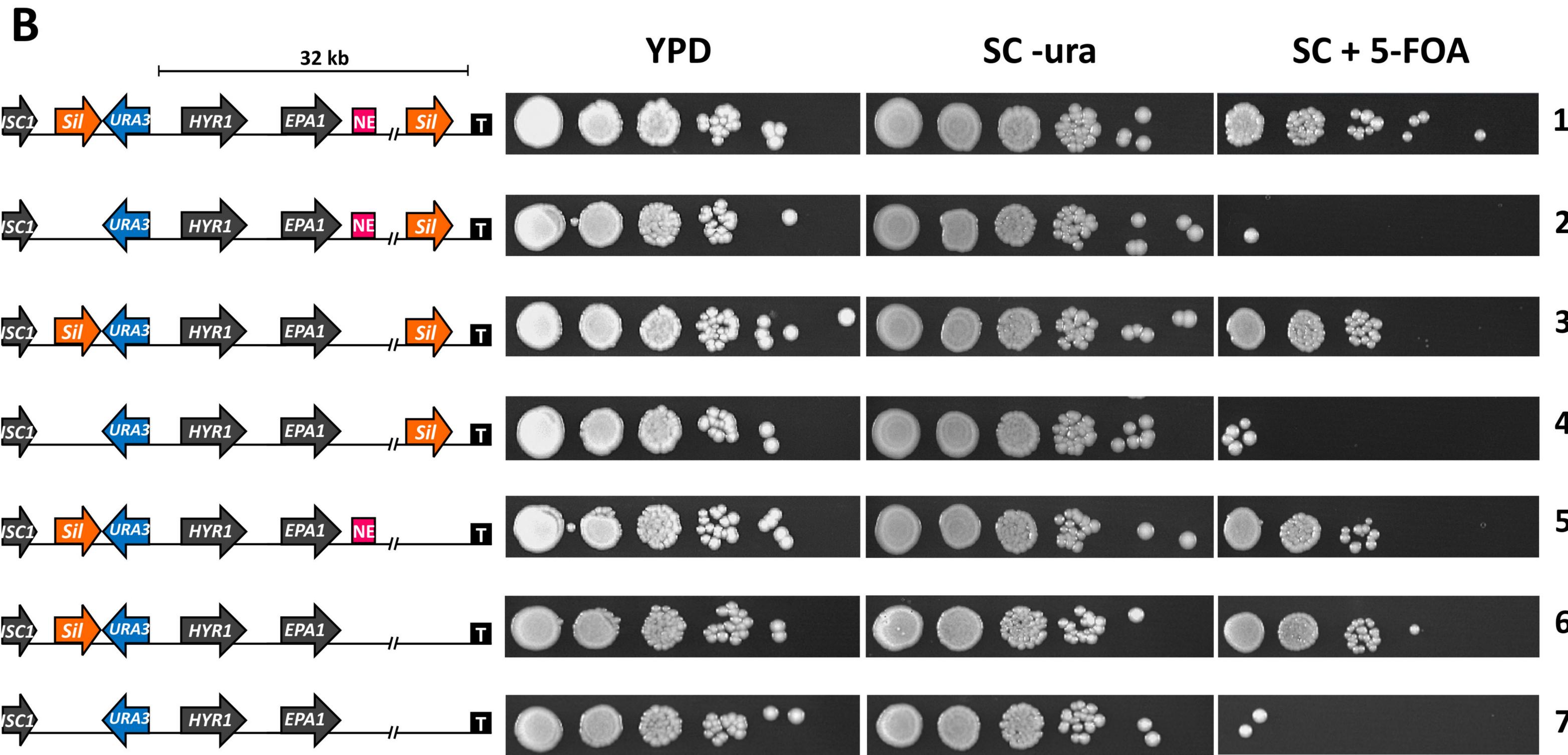
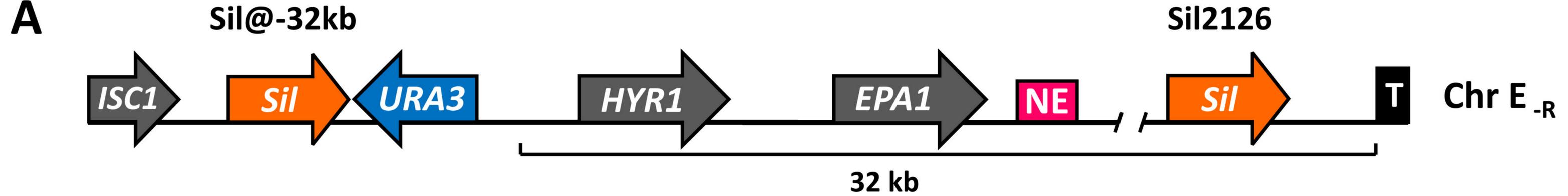
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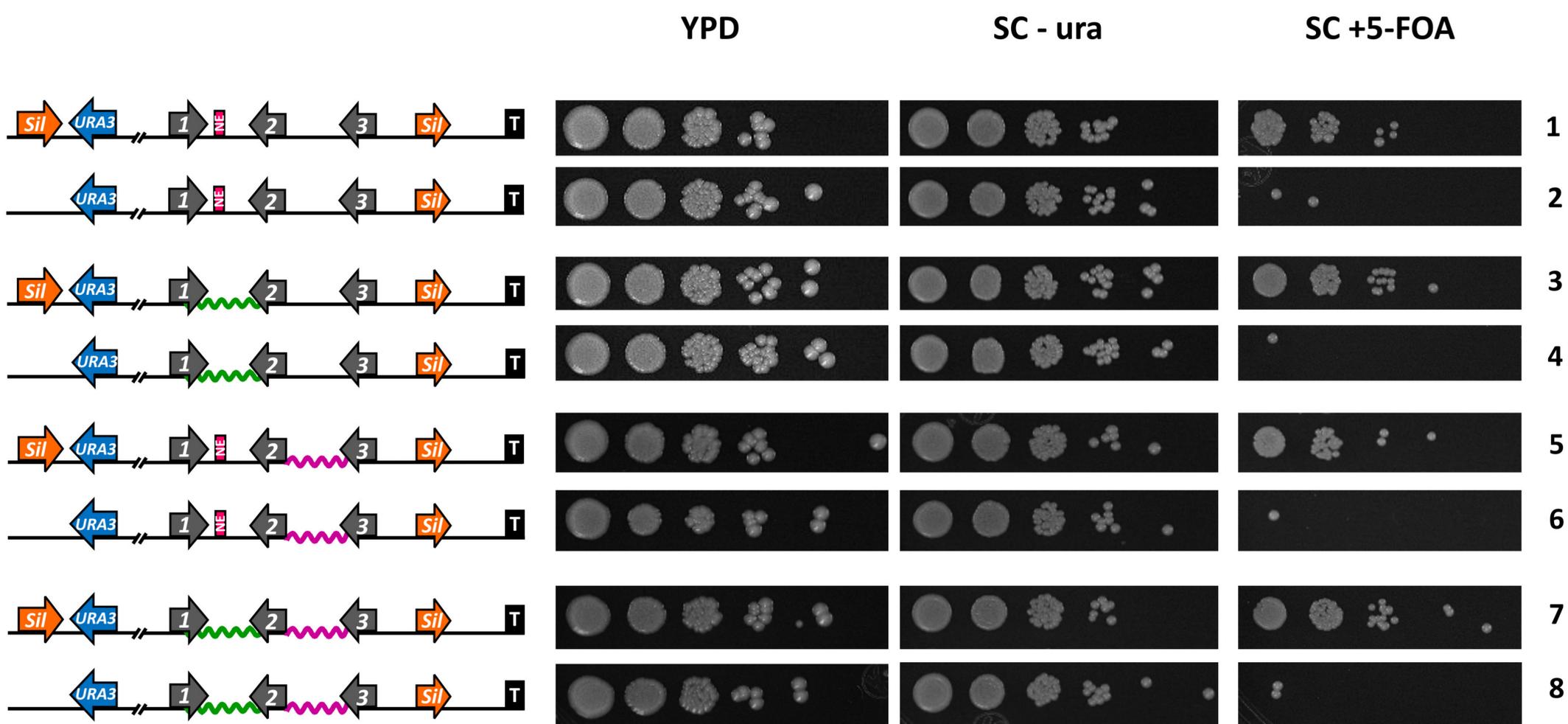
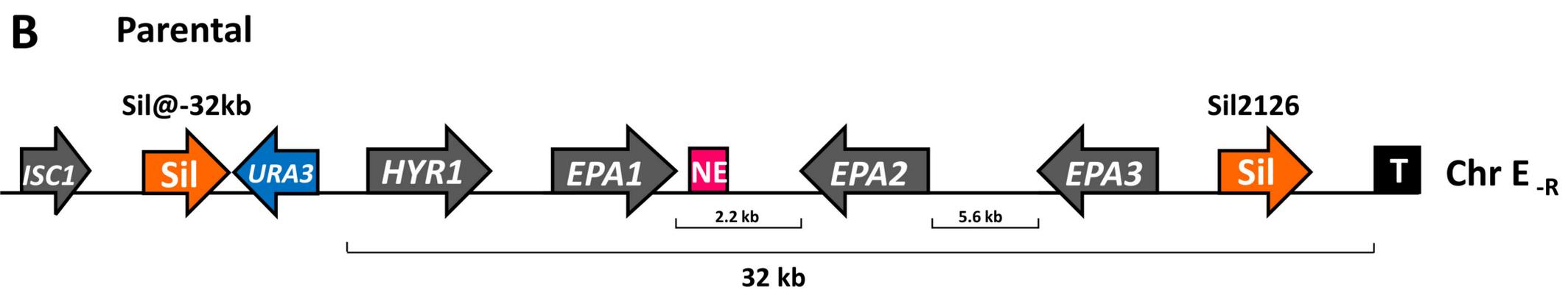
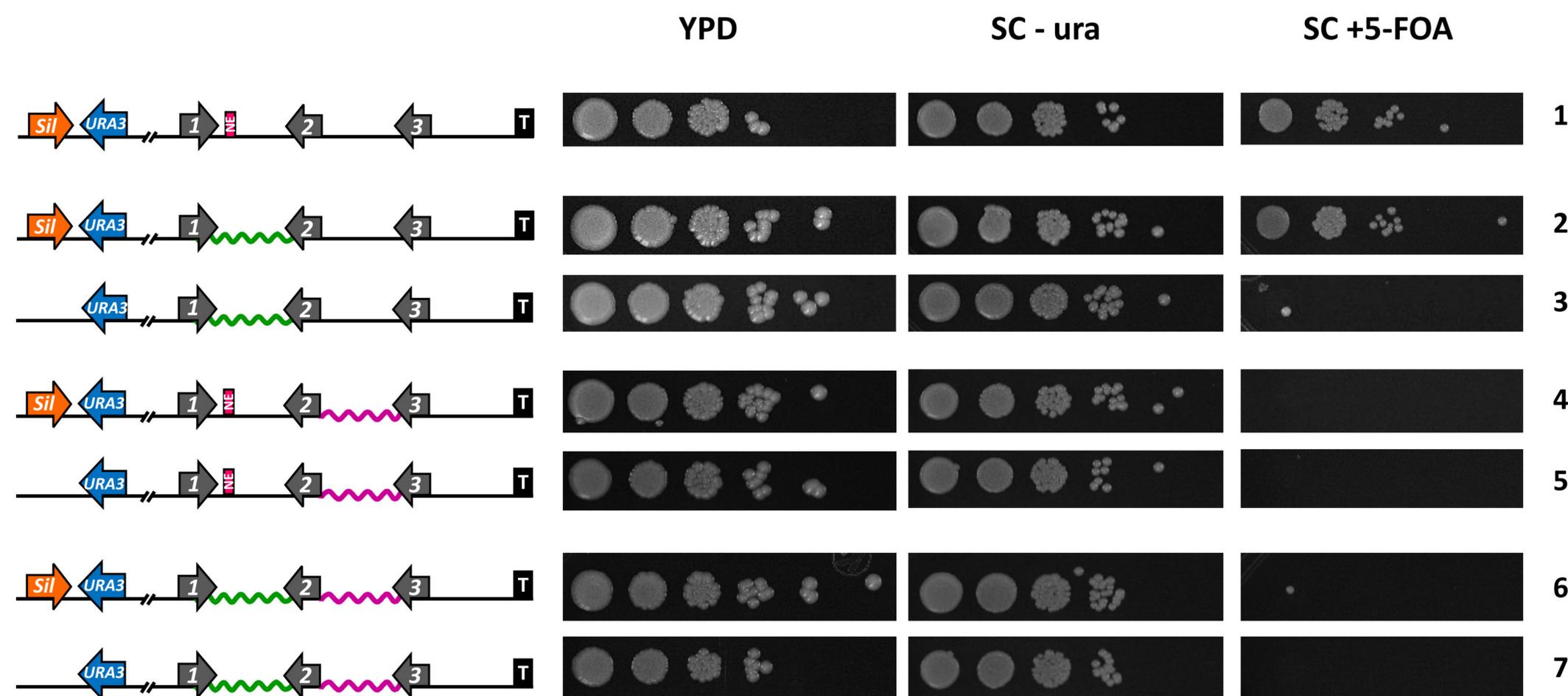
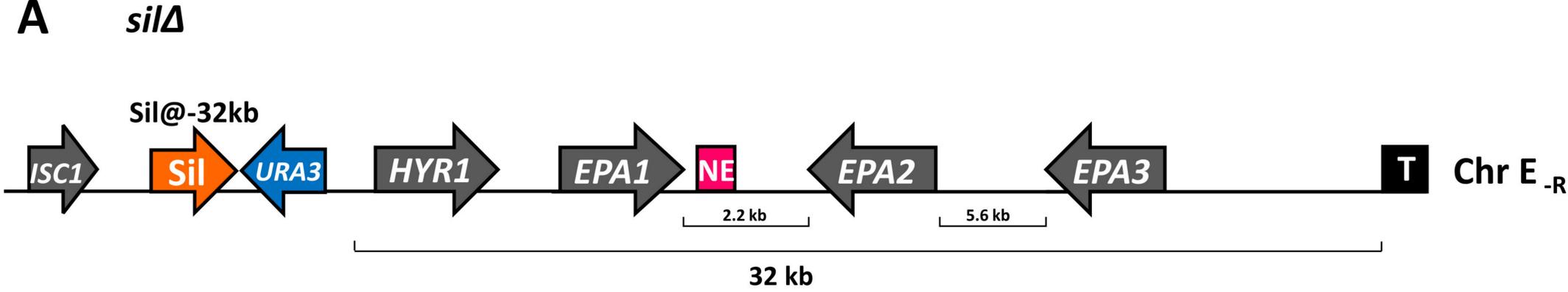
925 **Figure 9. Models for alternative silenced superstructures formed in two strains: a *silΔ* strain with a**
926 ***cis*-acting element Sil2126 inserted at -32 kb (Sil@-32) and in the parental strain with Sil2126 is at**
927 **is native position.**

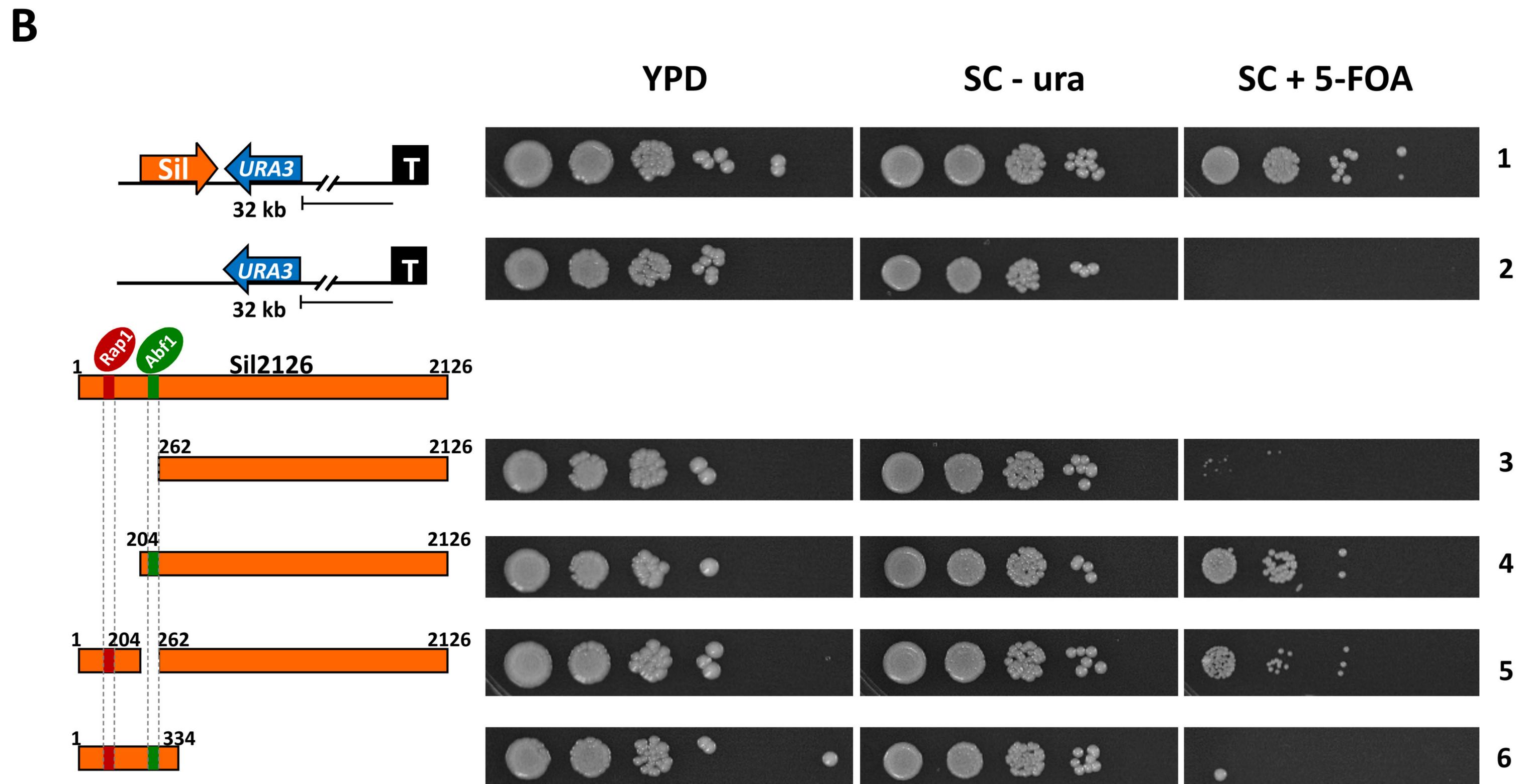
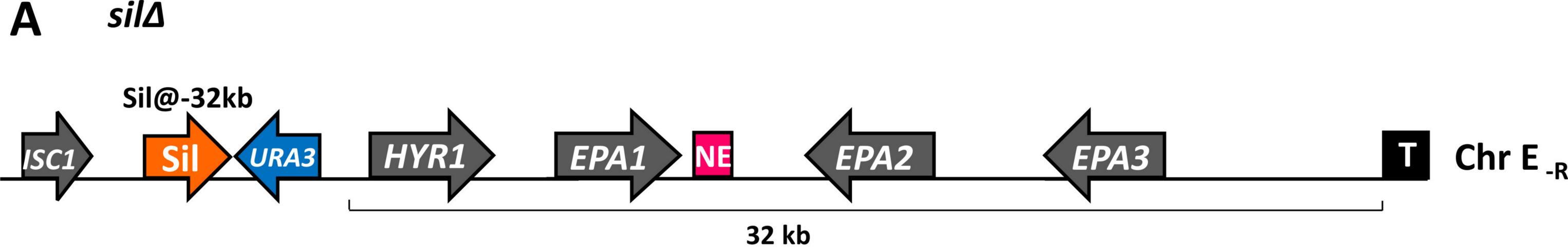
928 **(9A) Proposed DNA loops formed in the *silΔ* strain with Sil@-32kb.** The protosilencer Sil@-32kb
929 and the *EPA1-EPA2* intergenic region between *EPA1* and *EPA2* interact to form a loop. This
930 structure is probably formed through the interaction between different silencing proteins, Rap1, Abf1
931 and SIR complex to maintain a silenced superstructure. The silencing can propagate up to 32 kb
932 due to the presence of Sil2126 at this position, which presumably would act by recruiting Rap1 and
933 Abf1 proteins. SIR complex is represented by: light blue circles (Sir2), purple ovals (Sir3) and dark
934 blue ovals (Sir4). Rap1 is represented as red ovals, the Ku proteins (yKu70 and yKu80) are
935 represented as a yellow circle and Abf1 as green ovals. *EPA* genes are represented as gray arrows
936 and Sil2126 as an orange arrow. The model shows another proposed loop formed between the
937 telomere and the NE in this strain. This loop is inferred from genetic data showing that silencing
938 from the telomere directly affects *EPA1* expression (Gallegos-Garcia *et al.*, 2012). **(9B)** Proposed
939 chromatin loop formed between Sil2126 at its native position and the *cis*-acting elements in the
940 *EPA2-EPA3* intergenic region in the parental strain. Silencing proteins are represented in the same
941 way as Fig. 9A.

942







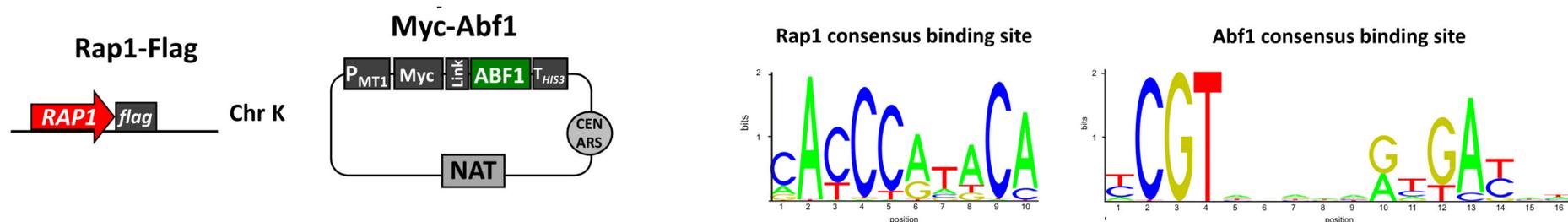


A

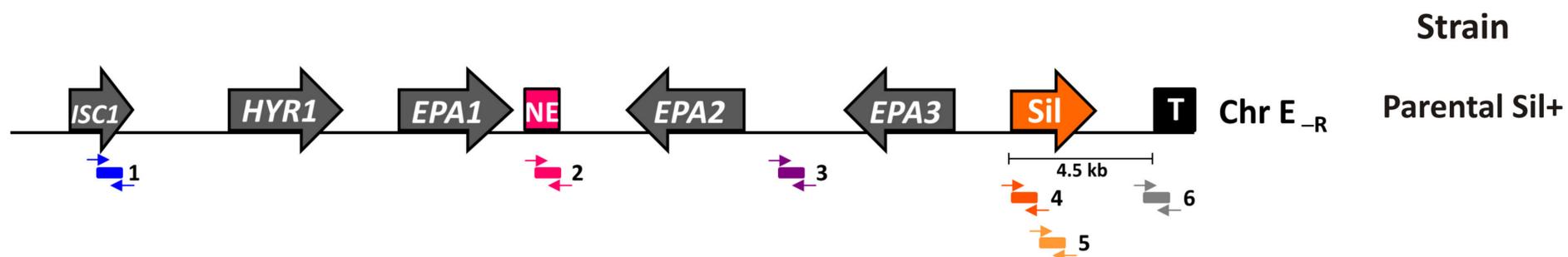
Rap1 binding sites



Abf1 binding sites

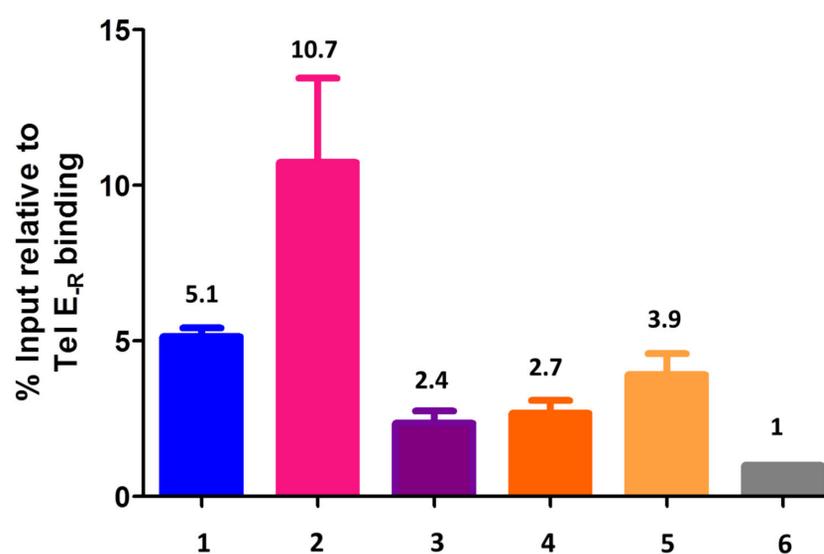
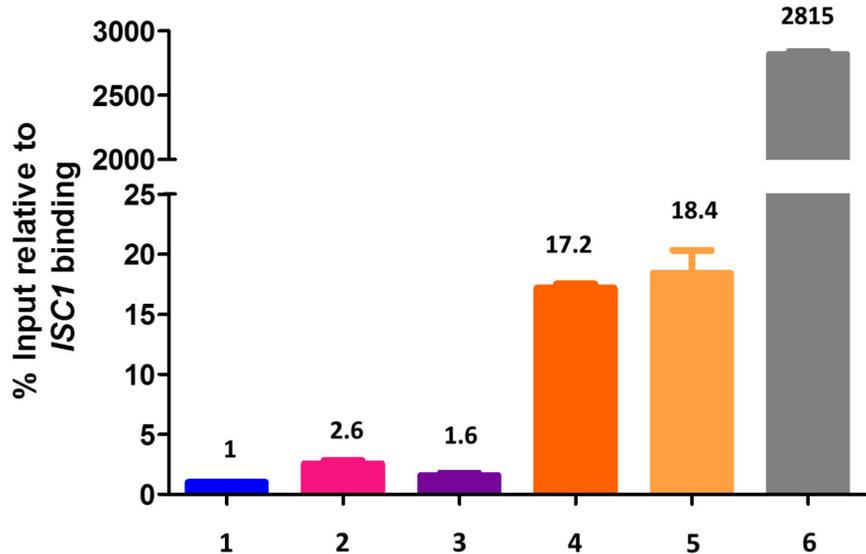


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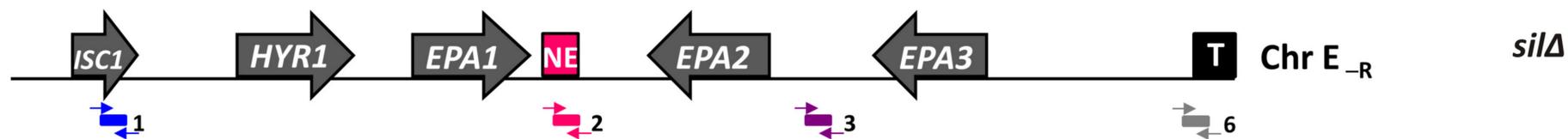


Rap1-Flag

Myc-Abf1

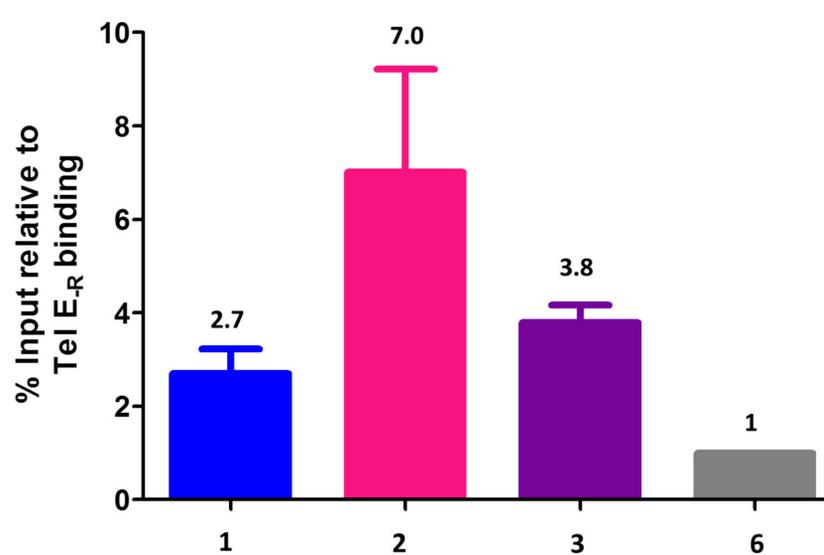
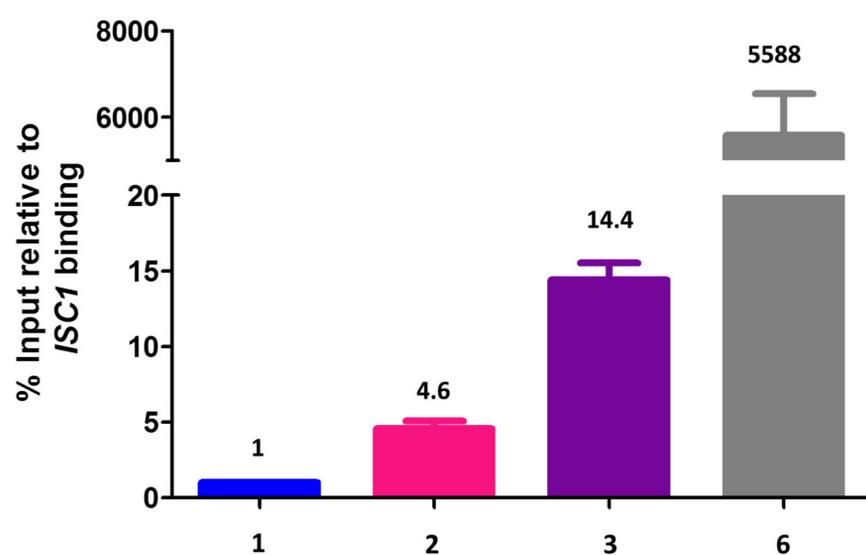


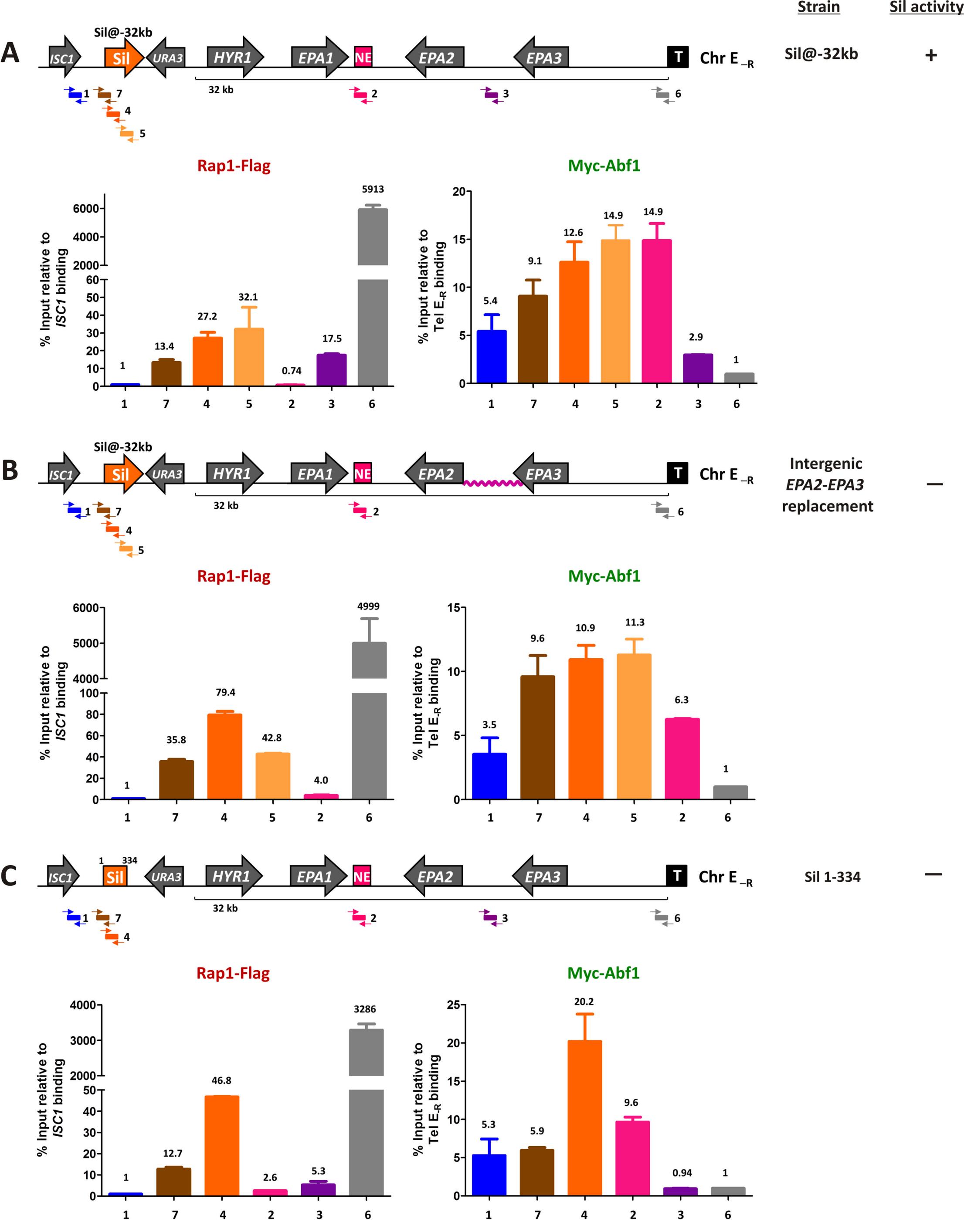
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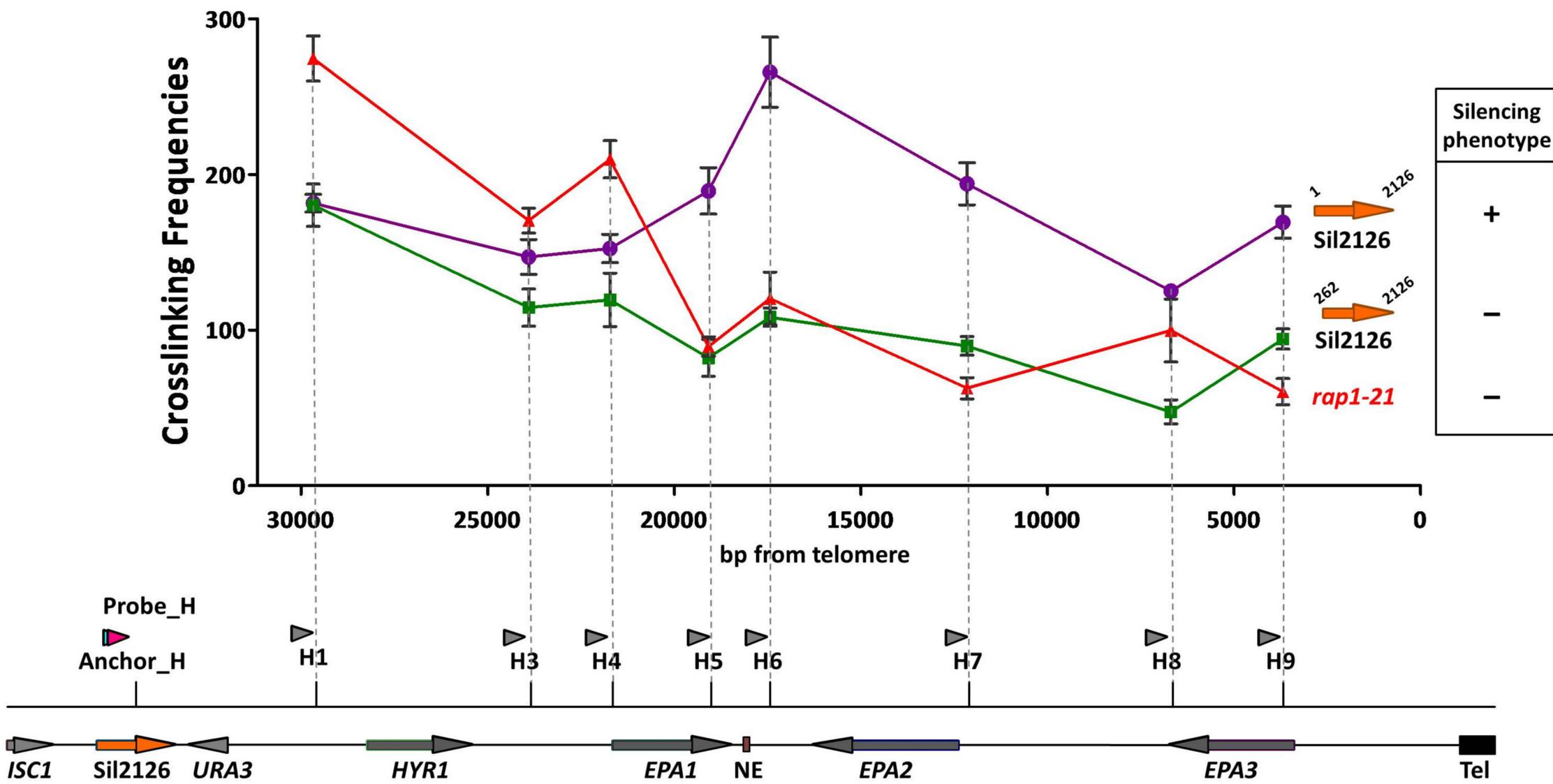
Rap1-Flag

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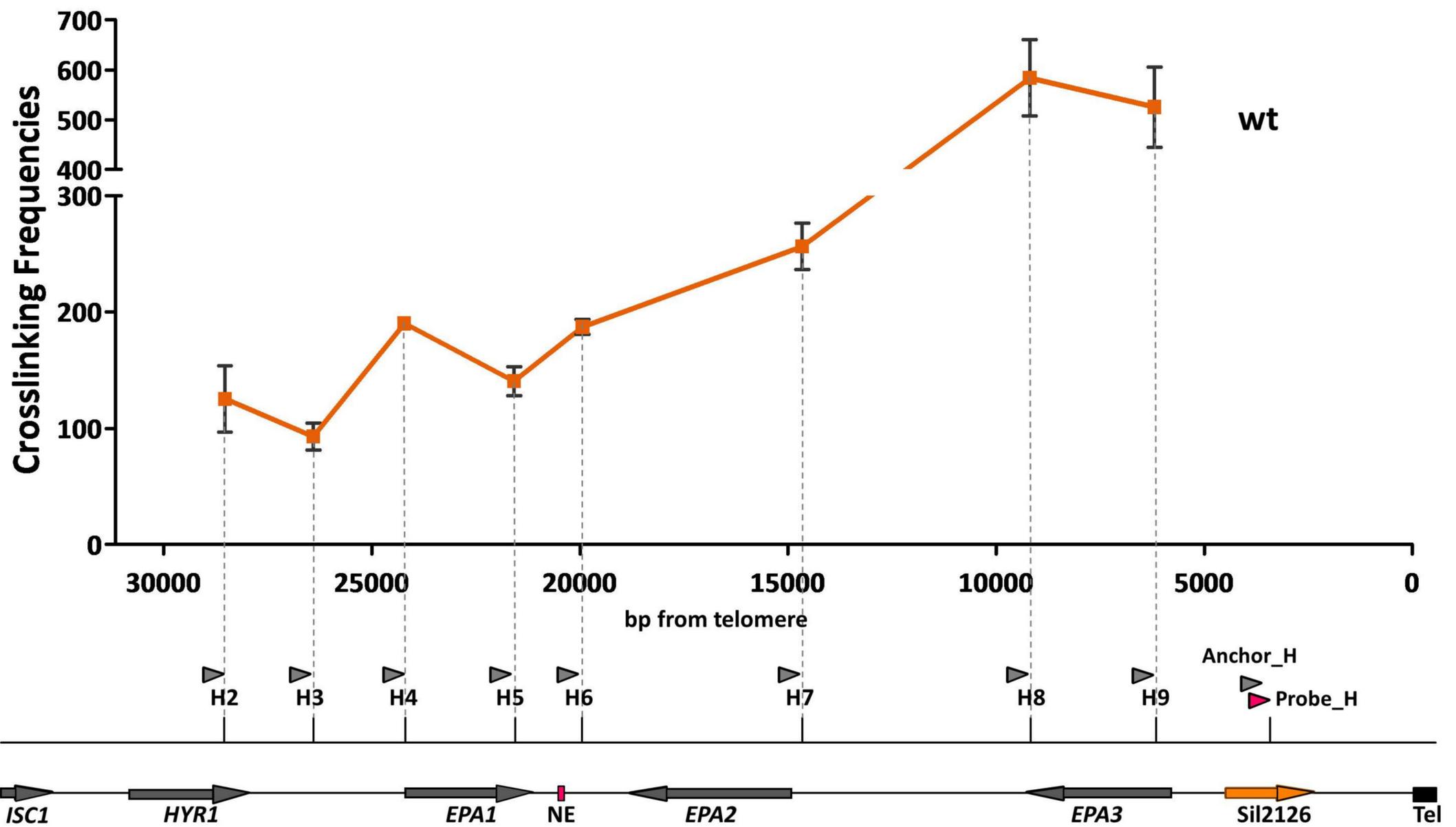


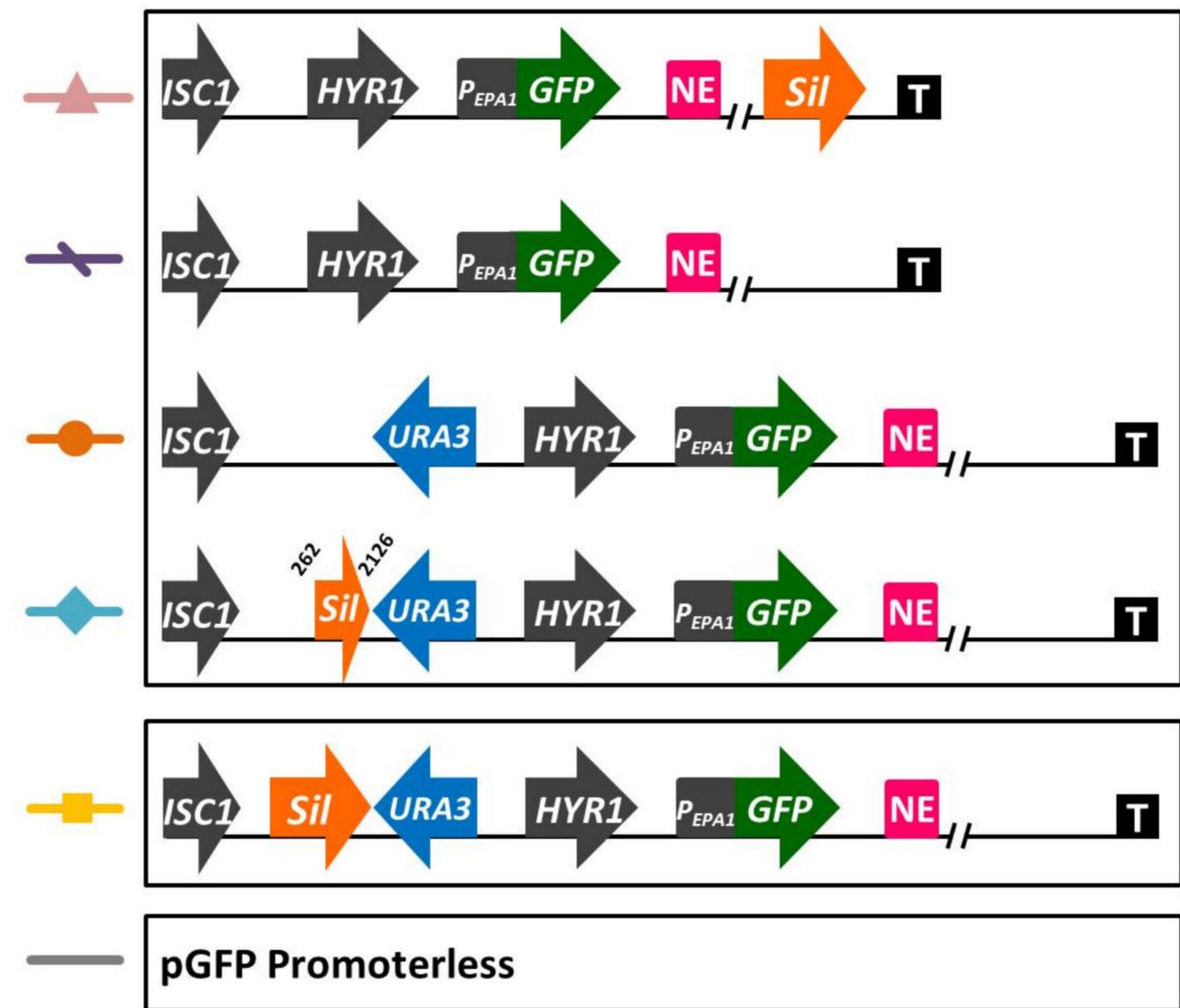
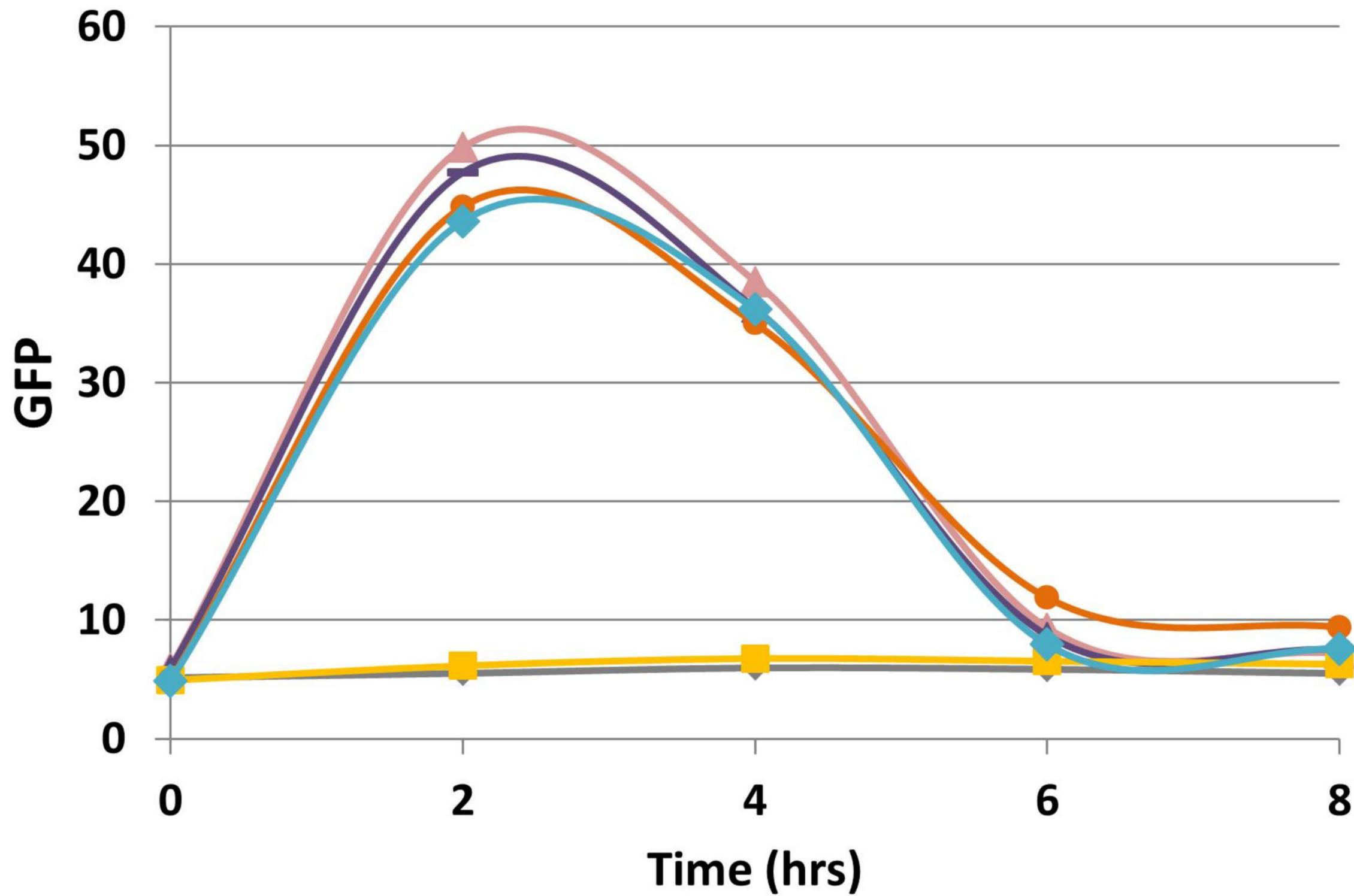


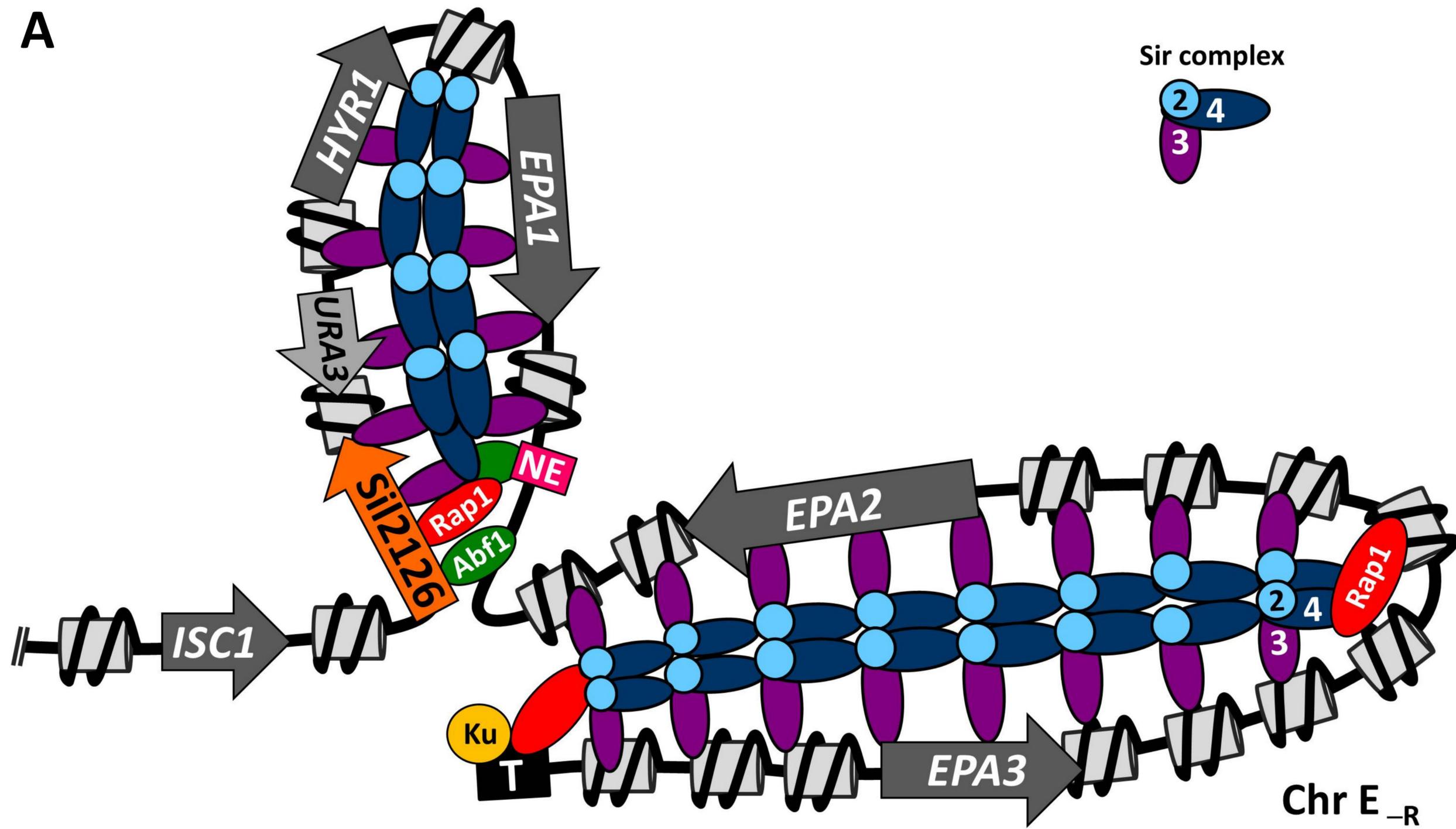
A



B





A**B**