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Fob1p recruits DNA topoisomerase I to ribosomal genes locus and contributes to its transcriptional silencing maintenance

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ABSTRACT

S. cerevisiae ribosomal DNA (rDNA) locus hosts a series of highly complex regulatory machineries for RNA polymerase I, II and III transcription, DNA replication and units recombination, all acting in the Non Transcribed Spacers (NTSs) interposed between the repeated units by which it is composed. DNA topoisomerase I (Top1p) contributes, recruiting Sir2p, to the maintenance of transcriptional silencing occurring at the RNA Polymerase II cryptic promoters, located in the NTS region. In this paper we found that Fob1p presence is crucial for Top1p recruitment at NTS, allowing transcriptional silencing to be established and maintained. We also showed the role of Nsr1p in Top1p recruitment to rDNA locus. Our work allows to hypothesize that Nsr1p targets Top1p into the nucleolus while Fob1p is responsible for its preferential distribution at RFB.

1. Introduction

S. cerevisiae rDNA consists of 100–200 tandem repeated units organized in a single cluster on chromosome XII and interrupted by a NTS (Petes, 1979). Each NTS region is subdivided into NTS1 and NTS2 by the 5S RNA coding sequence, transcribed by RNA polymerase III (Nomura, 2001). The 35S RNA is instead transcribed by RNA polymerase I. Furthermore, NTS1 and NTS2 host two RNA polymerase II cryptic promoters (E-PRO and C-PRO respectively) subjected to transcriptional silencing and producing the so called non coding ribosomal RNAs (ncRNAs) (Li et al., 2006; Ganley et al., 2005). Each rDNA unit also contains regulatory elements related to DNA replication such as the rDNA replication origin (rARS) and the Replication Fork Barrier (RFB), whose activity ensures a rDNA unidirectional replication (Brewer and Fangman, 1988) only when is bound by Fob1p. This prevents collisions between replication forks and the 35S transcription bubbles moving in the opposite direction (Kobayashi and Horiuchi, 1996). In the accomplishment of this task, Fob1p is assisted by Tof1p and Csm3p. These two factors act on the stalled forks and protect them from Rrm3p helicase action whose activity can release the fork block *via* the Fob1p temporary dislocation (Mohanty et al., 2006). While the RNA polymerase I and III activities are regulated by specific transcription factors, E-PRO and C-PRO transcription activity depends mainly on the presence/absence of nucleosomes occupying these sequences. The positioning of these nucleosomes depends on histone modifications (Li et al., 2006), particularly acetylation and methylation. Furthermore, rDNA histone acetylation state is mainly controlled by the histone deacetylase Sir2p (Vaquero et al., 2007). Recombination events occurring at the rDNA are necessary for the maintenance of units identity, but also act to maintain the physiological units copy number in a Sir2p dependent manner (Kobayashi et al., 2004). Therefore, within no more than 2500 bp the regulation of three extremely complex processes (DNA replication, transcription and recombination) takes place. The absence or the mutation of even only one among Sir2p, Fob1p or Upstream Activating Factor (UAF) (the ancillary factor for RNA polymerase I transcription) affects not only rDNA replication, but also its recombination and ncRNA transcription (Bryk et al., 1997; Smith and Boeke, 1997; Huang, 2003; Cioci et al., 2003). In this context Top1p, releasing DNA torsional stress accumulated during transcription and replication events, plays a pivotal role also in maintaining rDNA silencing (Bryk et al., 1997; Smith et al., 1999). Its cleavage activity at rDNA is sequence specific, since produces two high defined cleavage sites at RFB region [at -2143bp and -2236bp from RNA Pol I Transcriptional Start Site (TSS)], and a single one in the 35S RNA promoter region (-171 bp from the TSS) (Di Felice et al., 2005). Moreover Top1p acts as a scaffold protein contributing, regardless of its catalytic activity, to Sir2p recruitment at RFB (D'Alfonso et al., 2016). In S. cerevisiae, a physical interaction between Top1p and the product of the gene encoding Nsr1p, the yeast

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Table 1
Yeast strains used in this work.

Strains	Description
GCY99 (WT)	*Matα; ura 3-52, trp 1-289, his 3-Δ1, leu 2-3,112, gal 2, gal 10* (kindly provided by M.E. Bianchi)
nsr1Δ	Same as GCY99 but *nsr1::KAN* (this study)
W303-1a	MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100
NOY1064	Same as W303-1a, but fob1Δ::HIS3; rDNA copy number ~190 (Shou et al., 2001)
AMR51	Same as W303-1a but *top1::LEU2*
tof1Δ	Same as W303-1a but *tof1::G418* (Mohanty et al., 2006) (kindly provided by D. Bastia)
UKY403	*Mata; ade 2-101, his-Δ200, leu 2-3,112, lys 2-801, ura 3-52, trp 1-Δ419, Δhhf1::HIS3, Δhhf2::LEU2/Pmh3110 (trp1 cen3 ars1 UASgal-hhf2)* [30]
UKY/fob1Δ	Same as UKY403 but *fob1::KAN* (this study)

Table 2
Oligonucleotides used in this work.

Oligonucleotide	Sequence
RFB f	5'-CGATGAGGATGATAGTGTGTAAGA-3'
RFB r	5'-ACCCATCTTTGCAACGAAAA-3'
PROM-171 f	5'-TTCCGTATTTTCCGCTTCC-3'
PROM-171 r	5'-TCGCCGAGAAAAACTTCAAT-3'
NTS1F1 f	5'-TGTTAGTGCAGGAAAGCGGGAAGGA-3'
NTS1R1 r	5'-GCACTATCCAGCTGCACTCTTCTTC-3'
ACT INTRA f	5'-ACGTTCCAGCCTTCTACGTTTCCA-3'
ACT INTRA r	5'-AGTCAGTCAAATCTCTACCGGCCA-3'
R32	5'-GGGGCCTAGTTTAGAGAGAAGTAG-3'
R3	5'-CGCGTTTCCGTATTTTCCGC-3'

orthologue of mammalian nucleolin, has been described (Edwards et al., 2000). This protein is involved in pre-RNA processing, specifically binds nuclear localization sequences and, according to Edwards' and coworkers' experiments, also plays a role in the cellular localization of yeast Top1p, whose distribution results to be altered in its absence (Edwards et al., 2000). We previously showed that Top1p cleavage sites at the RFB are absent when Fob1p is missing (Di Felice et al., 2005), while its ability to recognize and cleave the C-PRO region remains unaltered. Here we wanted to deeper detail the molecular basis responsible for this behavior.

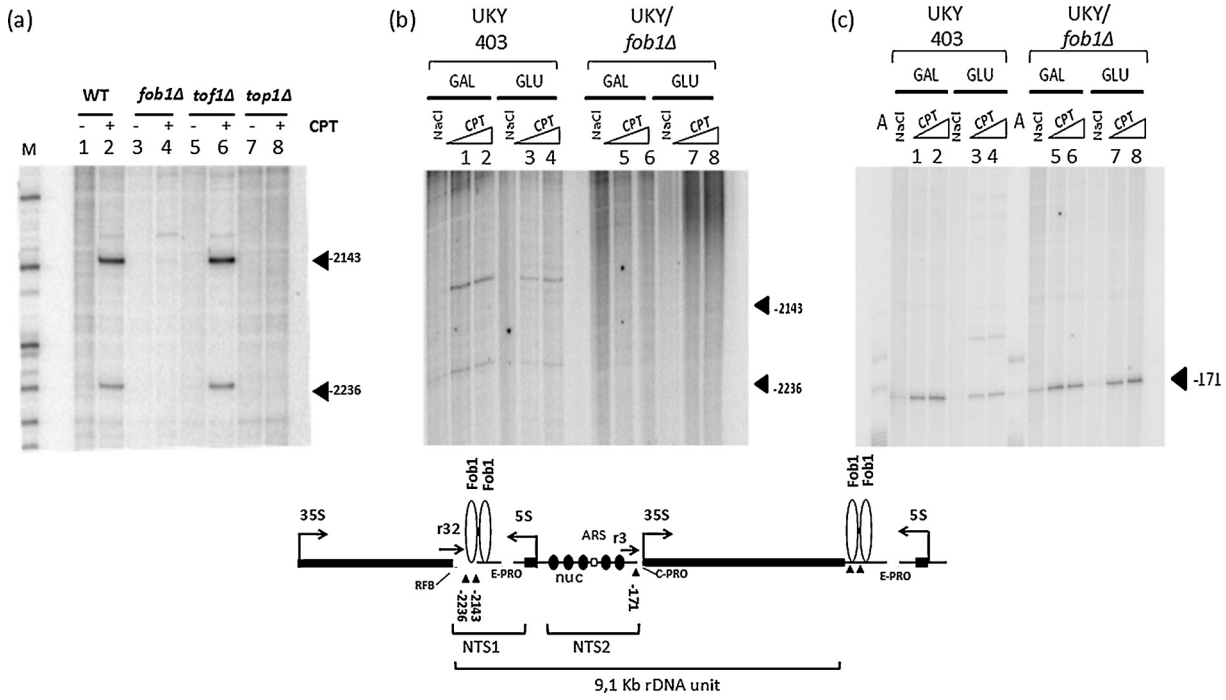

Fig. 1. *Fork movement, nucleosomes presence and Top1p cleavage activity at rDNA*. a) High resolution analysis of *in vivo* Top1p cleavage activity at RFB region in WT, *fob1*Δ, *tof1*Δ and *top1*Δ cells in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of CPT treatment (200 μM). All samples were primer extended by Vent (exo$^-$) DNA polymerase in PCR conditions, starting from 5′ labeled oligo r32 (see map below). M: size marker (pBR322/MspI); b) High resolution analysis of *in vivo* Top1p cleavage activity at RFB and C-PRO regions in UKY403 and UKY/*fob1*Δ cells. Lanes 1 and 2, 5 and 6: spheroplasts from UKY403 or UKY/*fob1*Δ cells growing in galactose medium (H4 synthesis ON) and treated *in vivo* with increasing amounts (empty triangles) of CPT (100 and 200 μM, respectively). Lanes 3 and 4, 7 and 8: spheroplasts from UKY403 or UKY/*fob1*Δ cells growing in glucose medium (H4 synthesis OFF) and treated *in vivo* with increasing amounts (empty triangles) of CPT (100 and 200 μM, respectively). Lanes NaCl: spheroplasts treated with 0.8 M NaCl. (c) High resolution analysis of *in vivo* Top1p cleavage activity at C-PRO region. Lanes 1 and 2, 5 and 6: spheroplasts from UKY403 or UKY/*fob1*Δ cells growing in galactose medium (H4 synthesis ON) and treated *in vivo* with increasing amounts (empty triangles) of CPT (100 and 200 μM, respectively). Lanes 3 and 4, 7 and 8: spheroplasts from UKY403 or UKY/*fob1*Δ cells growing in glucose medium (H4 synthesis OFF) and treated *in vivo* with increasing amounts (empty triangles) of CPT (100 and 200 μM, respectively). Lane NaCl: spheroplasts treated with 0.8 M NaCl. Lane A: sequencing lane.
The schematic map on the bottom of the figure represents the investigated regions: positions of the Top1p cleavage sites at RFB region (-2236bp and -2143bp from TSS) and C-PRO region (-171bp from TSS) are indicated by black arrowheads.

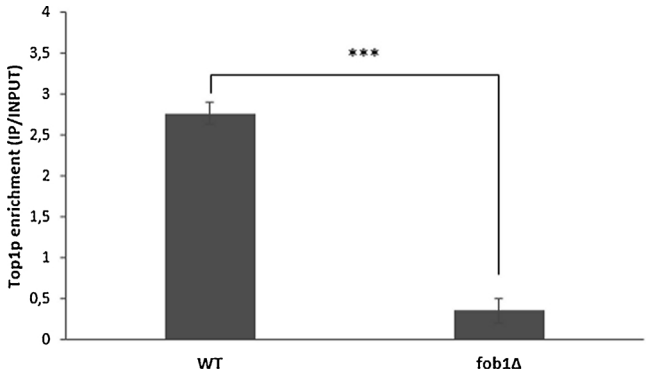

Fig. 2. *Fob1p recruits Top1p at RFB region.*
ChIP analysis of Top1p enrichment at RFB region, in *fob1*Δ and WT cells using α-Topo1 antibody. Results are presented as the mean ± s.d.; ***p < 0.001.

2. Material and methods

2.1. Yeast strains

Yeast strains used in these study are listed in Table 1.

2.2. Culture media and conditions

Yeast cells were grown and manipulated according to standard protocols (Sherman et al., 1983). YPD medium (1% bacto yeast extract, 2% bacto peptone, 2% glucose) was used for all strains; YPGAL medium (1% bacto yeast extract, 2% bacto peptone, 2% galactose) was used for UKY403 or UKY/fob1Δ when specified.

2.3. Oligonucleotides sequences

Oligonucleotide sequences are listed in Table 2

2.4. ChIP analysis

ChIP assays were performed as indicated in (D'Alfonso et al., 2016). Briefly: exponential growing cells (0.5 OD_{600}/ml) were formaldehyde-crosslinked, treated with glycine, harvested, resuspended in lysis buffer (140 mM NaCl, 50 mM Hepes/KOH pH 7.5, 1 mM EDTA, 1% TritonX-100, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors) and then subjected to lysis with glass beads. Lysates were sonicated and 300 µg of chromatin extracts were treated as Input (genomic sample), IP (Immunoprecipitated DNA) or beads only (BO, no antibody).

IP samples were incubated overnight at 4 °C with 3 µg of α-Top1p (Santa Cruz Biotechnology, sc-26167), α-Sir2p (Santa Cruz Biotechnology, sc-25753), α-Rpb1 RNA polymerase II subunit (Santa Cruz Biotechnology, sc-25758), α-H3 C-Term (Abcam, ab1791), or α-H3 Ac (Millipore, 06–599) where appropriate. These samples and BO samples were then incubated with Dynabeads protein A or G (Invitrogen) according to antibodies manifacturer's indications, to isolate chromatin-antibody complexes. After extensive washing, samples were eluted with 100 µl of elution buffer (50 mM Tris−HCl pH 8, 10 mM EDTA, and 1% SDS) and incubated overnight at 65 °C in order to reverse crosslink. All samples were treated with proteinase K and RNase A. DNA was then extracted and purified in standard conditions.

Input samples were dissolved in 500 µl of water, while IP and BO samples in 50 µl of water; all samples were then amplified by q-PCR using specific primers.

The relative fold increase was calculated respect to Actin, for both IP and Input samples according the formula: Fold increase = $2^{-\Delta Ct}$,

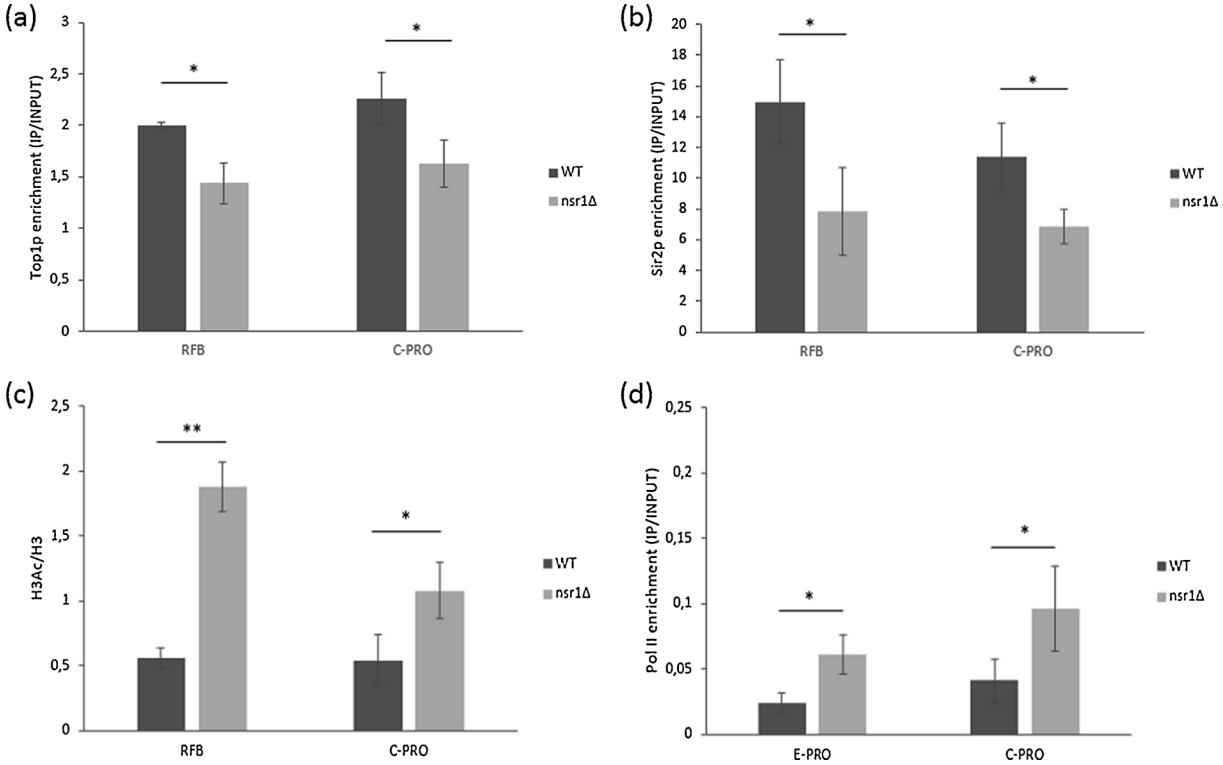

Fig. 3. *Fob1p absence leads to Sir2p amount reduction, H3 hyperacetylation and loss of transcriptional silencing only at RFB region.*
(a) Sir2p enrichment at RFB and C-PRO regions was measured by ChIP using a α-Sir2 antibody; values obtained from *fob1*Δ cells (grey bar) were compared with those obtained from WT (black bar). (b) Histone H3 acetylation at rDNA. These data were obtained by ChIP using α-H3 Ac or α-H3 C-term; histone H3 acetylation data were normalized to total histone H3 and then the values obtained from *fob1*Δ cells (grey bar) were compared to those obtained from WT (black bar). (c) RNA polymerase II enrichment at E-PRO and C-PRO measured by ChIP using a α-Rpb1 RNA polymerase II subunit antibody. Gray bar refers to *fob1*Δ cells while black bar refers to WT cells. Results are presented as the mean ± s.d.; *p < 0.05, **p < 0.01.

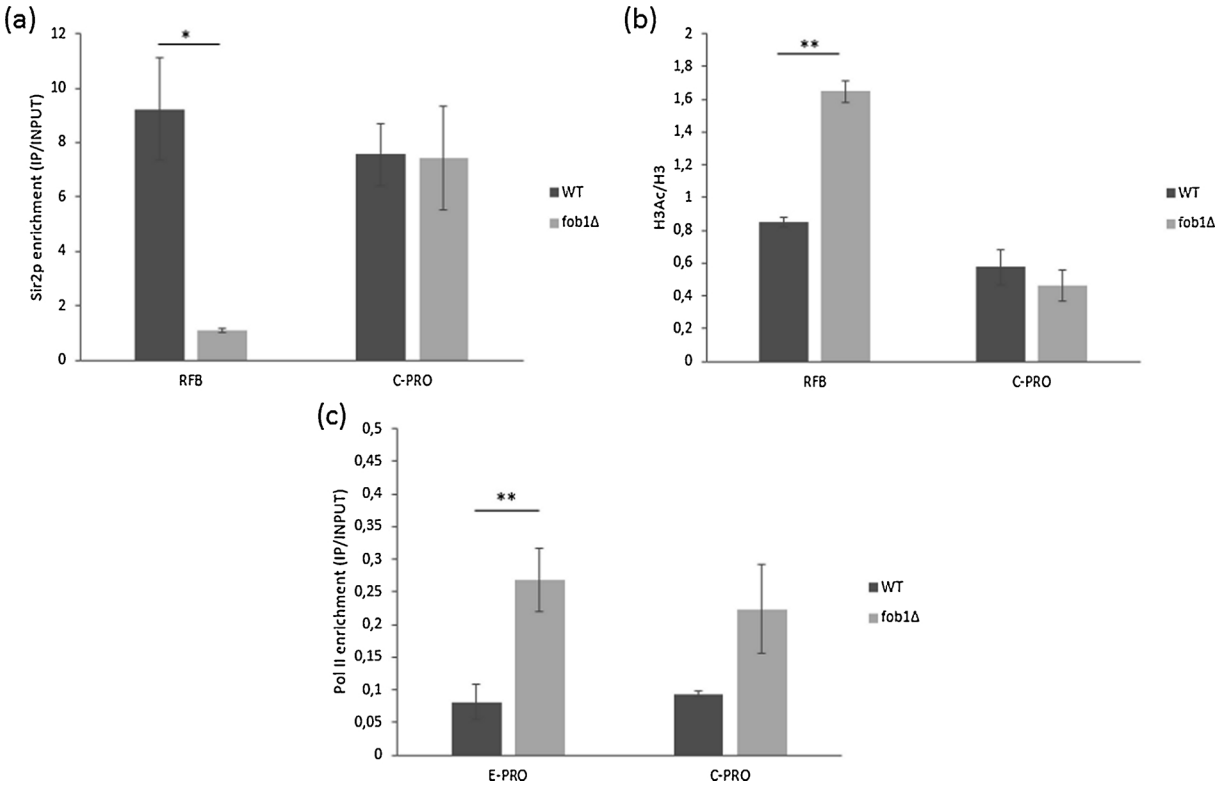

Fig. 4. *Nsr1p recruits Top1p at NTS and its absence leads to loss of transcriptional silencing* (a) Top1p enrichment at RFB and C-PRO regions measured by ChIP using α-Topo1 antibody; Values obtained from *nsr1*Δ (grey bar) were compared to those obtained from WT (black bar); (b) Sir2p enrichment at RFB and C-PRO regions measured by ChIP using an α-Sir2 antibody. Values obtained from *nsr1*Δ (grey bar) were compared to those obtained from WT (black bar); (c) Histone H3 acetylation at RFB and C-PRO regions measured by ChIP using α-H3Ac or α-H3 C-term; histone H3 acetylation data were normalized to total histone H3 and then the values obtained from *nsr1*Δ (grey bar) were compared to those obtained from WT (black bar); (d) RNA polymerase II enrichment at E-PRO and C-PRO measured by ChIP using an α-Rpb1 RNA polymerase II subunit antibody. Results are presented as the mean ± s.d.; **p < 0.05, *p < 0.01.

with $\Delta Ct = Ct_{Gene} - Ct_{Actin}$.

The fold enrichment values were calculated as [IP/ Input]. Moreover, regarding acetylation levels, the final values were obtained normalizing H3 acetylation values to total H3 values.

2.5. Statistical analysis

All quantified data are shown as mean and standard deviation (s.d.) of relative enrichments calculated from at least three different biological replicates.

p values were obtained using Student's T-Test.

2.6. Top1p cleavage sites

Top1p cleavage sites were produced as described in (Vogelauer and Camilloni, 1999). Briefly:

exponential growing cells were harvested and resuspended in 10 ml of a buffer containing 1 M sorbitol, 50 mM Tris − HCl (pH 7.5), 10 mM β-mercaptoethanol and 0.05 mg/3×10^7 cells of Zymolyase 100 T (Seygakaku). Cells were incubated at 30 °C for 10 min to obtain spheroplasts that were then resuspended in cleavage buffer (3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris − HCl (pH 8.0), 1 M sorbitol and nystatin 100 µg/ml). Aliquots were incubated with different camptothecin (CPT) concentrations (100 µM or 200 µM) for 2 min at room temperature. The reaction was stopped with 1% SDS and 5 mM EDTA. Samples were treated with Proteinase K, DNA extracted by phenol/chloroform and then purified by ethanol precipitation followed by RNAse A treatment. NaCl samples, in which CPT treatment was replaced by 0.8 M NaCl treatment, provided information on DNA integrity.

2.7. Multiple-round primer extension and high-resolution analysis of Top1p cleavage sites

Genomic DNA (1–2 µg) obtained as reported above, was reacted with Vent(exo$^-$) polymerase as described previously 15] and end-labeled oligonucleotide. The extension products were phenol extracted, ethanol precipitated, dissolved in formamide buffer and analyzed by 6% denaturing PAGE. The Top1p cleavage sites were detected by autoradiography.

2.8. Oligonucleotide labeling procedure

The r3 and r32 oligonucleotides were end-labeled using [γ −32 P]ATP; T4 polynucleotide kinase reaction was performed according to (Vogelauer et al., 1998).

3. Results

3.1. Top1p specific cleavages at RFB are fork block-independent

The Fob1p main role at rDNA is to efficiently block replication fork at RFB sequences (Kobayashi and Horiuchi, 1996) together with other elements such as Tof1p and Csm3p (Mohanty et al., 2006) as previously reported in the introduction section. We asked whether the absence of the fork block at RFB could influence Top1p cleavage activity at this region. Thus, we studied the cleavage sites production in WT cells (active fork block given the presence of Fob1p bound to RFB), in *fob1*Δ cells (inactive fork block) or in a *tof1*Δ strain (inactive fork block). In absence of Tof1p also its action conteracting Rrm3p is lost. As a consequence, Fob1p is temporary displaced from RFB and the fork block is

released even if Fob1p is present). (Bairwa et al., 2010)). These comparisons would help to distinguish whether the role of Fob1p in determining Top1p cleavages at RFB, is specific or indirect.

Top1p cleavage sites were obtained as in (Di Felice et al., 2005) using the DNA topoisomerase I inhibitor Camptothecin (CPT). As reported in Fig. 1A, cleavage sites at positions -2236 and -2143 from the 35S RNA TSS are observed in WT (active fork block) and in *tof1*Δ strain (inactive fork block) at the same positions (compare WT lane 2 with *tof1*Δ, lane 6). Conversely, no cleavage sites are observed in *top1*Δ or *fob1*Δ strains (compare WT lane 2 with *fob1*Δ, lane 4 and *top1*Δ lane 8) as previously reported in (Di Felice et al., 2005). Cleavage sites occurrence is observed only when Fob1p is present (in WT or *tof1*Δ cells) indicating that Top1p cleavage activity at RFB is independent on an active block of the replication forks.

3.2. Top1p doesn't cleave RFB sequence regardless nucleosome presence in Fob1Δ cells

Since Top1p cleavage activity is affected by nucleosome presence (Di Felice et al., 2008) we wanted to verify whether, in absence of Fob1p, chromatin rearrangements could occur at RFB locus involving nucleosomes and preventing Top1p cleavage activity. We used two yeast strains (UKY403 and UKY/*fob1*Δ) in which histone H4 is under the *GAL1* promoter control (Kim et al., 1988). Cells stop H4 synthesis and completely loose chromatin organization within few hours when grown in glucose, while maintain a regular chromatin asset in galactose containing medium, due to a standard H4 synthesis. Thus, Top1p cleavage activity has been evaluated at RFB and 35S Promoter regions, with or without a regularly organized chromatin.

Fig. 1B shows Top1p cleavages at RFB, both when H4 synthesis is defective (GLU) or efficient (GAL). Conversely cleavage sites are lost in UKY/*fob1*Δ strain both in presence (GAL) or absence (GLU) of nucleosomes. The presence of the canonical cleavage site at -171 bp from the 35S RNA TSS (Fig. 1C) indicates that neither nucleosome presence/absence, nor Fob1p presence/absence, affect Top1p cleavage activity at the C-PRO region.

3.3. Top1p is not recruited at RFB sequence in fob1Δ

Results reported in Fig. 1 indicate that the presence of Fob1p is crucial for Top1p site-specific activity at RFB regardless of fork block activity or nucleosome presence or positioning. Thus, we asked whether the loss of Top1p cleavage activity at RFB is a consequence of its inhibition or is due to its absence. To this purpose we performed a ChIP assay using α-Top1 antibody and evaluated, by qPCR, the Top1p enrichment at RFB region in WT and *fob1*Δ cells. As shown in Fig. 2, a clear and significant difference is observed, with the Top1p enrichment at RFB almost 10-fold reduced in *fob1*Δ cells compared to WT. Further analysis of C-PRO region has not been performed since the Top1p cleavage site detected at this region (Fig. 1C) proves the presence of Top1p.

This result indicates that loss of Top1p cleavage activity reported in Fig. 1, is due to its absence and does not depend on something preventing its ability to reach, recognize and cleave the RFB sequence.

3.4. Functional Top1p-mediated consequences at rDNA in fob1Δ cells

A defective Top1p recruitment at RFB determines a Sir2p reduction at the same locus that, in turn, is associated with increased H3 acetylation levels and loss of transcriptional silencing (D'Alfonso et al., 2016). Thus, we evaluated by ChIP assay: the presence of Sir2p, histone acetylation levels (at RFB and C-PRO) and RNA polymerase II recruitment at cryptic promoters (to measure transcriptional silencing) in WT and *fob1*Δ cells.

Fig. 3A shows a sharp decrease of Sir2p at RFB in *fob1*Δ strain (grey bar) respect to WT (black bar), while Sir2p levels remain those of the WT at the C-PRO region. To further detail the consequences of Fob1p absence, we measured the enrichment of H3 Ac relative to H3 C-term. Fig. 3B shows increased H3 acetylation level in *fob1*Δ cells (grey bar) respect to the WT (black bar) only at RFB, coherently with the previously observed Sir2p reduced recruitment in the same region. Sir2p decrease and rDNA chromatin hyperacetylation have been associated with an increased ncRNA transcription from E-PRO and C-PRO cryptic promoters (Cesarini et al., 2012) and with an increased RNA Pol II binding (Mayan and Aragon, 2010). Thus, we evaluated the RNA polymerase II enrichment at cryptic promoters instead of directly quantifying ncRNAs, to avoid errors due to the annealing positions of specific oligonucleotides used in the Reverse Transcription - PCR reaction (Sasano et al., 2017). In addition, overlapping among the different ncRNAs deriving from both cryptic promoters (Li et al., 2006) and their intrinsic instability, make their study by RT-qPCR less reliable. Fig. 3C, in agreement with our results concerning Sir2p recruitment and H3 Ac abundance, shows a significant increase of RNA polymerase II enrichment particularly at E-PRO region in the *fob1*Δ strain (grey bar) compared to WT (black bar).

Data reported in Fig. 3 indicate that lack of Fob1p leads to a less efficient Sir2p recruitment at RFB, associated to increased H3 Ac in the same region, while no significant differences are observed at the C-PRO; furthermore RNA polymerase II is recruited at E-PRO with higher efficiency.

3.5. Effects of missing NSR1 on Top1p recruitment at rDNA

Edwards and collegues (Edwards et al., 2000) previously suggested that Top1p is targeted in the nucleus by Nsr1p, the yeast orthologue of human nucleolin.

We wanted to evaluate the consequences of a *nsr1* null mutation on Top1p mediated transcriptional silencing. Thus, we performed a ChIP analysis in WT and *nsr1*Δ cells to measure Top1p at the RFB and C-PRO regions where Top1p specific cleavage activity occurs (Vogelauer and Camilloni, 1999). Fig. 4A, shows a significant reduction of Top1p in *nsr1*Δ cells (grey bar) compared to WT(black bar), both at RFB and C-PRO.

Since previous data indicated that Top1p contributes to Sir2p recruitment at rDNA locus (D'Alfonso et al., 2016), we also evaluated the Sir2p enrichment at RFB and C-PRO regions of *nsr1*Δ cells. Fig. 4B shows a statistically significant reduction of Sir2p at both RFB and C-PRO regions in *nsr1*Δ cells (grey bar) compared to WT cells (black bar). Since a decrease of Sir2p at the rDNA leads to histone hyperacetylation of the same locus (Li et al., 2006), to support this observation we also measured by ChIP the H3 acetylation amount in the same two target regions.

As shown in Fig. 4C, according to the observed decrease of Sir2p in the *nsr1*Δ strain, an increase of H3 acetylation is clearly visible at both RFB and C-PRO regions (grey bars compared to black black bars).

Furthermore we investigated whether the loss of Nsr1p interferes with transcriptional silencing maintenance by evaluating the RNA polymerase II recruitment to E-PRO and C-PRO cryptic promoters. Results of ChIP analysis are reported in Fig. 4D and show that RNA Pol II recruitment is increased in the *nsr1*Δ strain (grey bar) compared to WT (black bar), with the same efficiency on both promoters. Summarizing, results reported in Fig. 4 (A–D) indicate that the *nsr1*Δ strain is characterized by a reduced Top1p presence at the same extent both at RFB and C-PRO regions. This decrease is also associated with a Sir2p reduced amount at the same sequences, where histone H3 acetylation levels increase as a possible consequence. Furthermore, as expected and predicted by the E-PRO and C-PRO cryptic promoters hyperacetylation, they are bound by a greater amount of RNA polymerase II, indicating loss of transcriptional silencing.

4. Discussion

Recent experiments demonstrated that Top1p is responsible for Sir2p recruitment at NTS1 region of rDNA (D'Alfonso et al., 2016), where a complex series of DNA-protein interactions have been reported. In particular, a physical interaction between Fob1p and Top1p was deduced by MS analysis, together with several additional proteins (Huang et al., 2006). Moreover, the lack of Top1p site specific activity at the NTS1 region has been described in *fob1*Δ cells (Di Felice et al., 2005). Thus, we hypothesized that the absence of Top1p cleavage sites in *fob1*Δ strain would derive from a defective recruitment of this enzyme at the NTS1 region and we wanted to verify this hypothesis.

Previous data have shown that Top1p is recruited at nucleus by Nsr1p (Edwards et al., 2000). Here we observed that when Nsr1p is missing, the Top1p amount at rDNA is reduced without any preference between RFB and C-PRO regions; in addition, Sir2p presence is decreased and H3 acetylation increased with loss of transcriptional silencing. These phenotypes remind those observed in *top1*Δ mutant (Bryk et al., 1997; Smith et al., 1999).

The presence of Top1p at RFB is witnessed by its site specific cleavage activity strongly Fob1p-dependent only at RFB, as showed in Fig. 1 and in ref (Di Felice et al., 2005). Actually this behavior reminds the different Fob1p binding at NTS1 and NTS2, with a higher amount of the protein found at NTS1 (Huang et al., 2006). We then hypothesized a direct role of Fob1p in recruiting Top1p at RFB. Our hypothesis is confirmed by: i) Top1p-Fob1p physical interaction demonstrated by MS data from Huang and coworkers (Huang et al., 2006); ii) the preferential distribution of Fob1p at RFB associated with the reduction of Top1p in the *fob1*Δ strain at the same region (Fig. 2 and (Huang et al., 2006)); iii) loss of Top1p site specific cleavage activity at RFB in the *fob1*Δ strain (Di Felice et al., 2005) and shown in Fig. 1.

We thus confirmed that Top1p presence at RFB is Fob1p-dependent (Fig. 2), and observed a decreased Sir2p enrichment at the same locus, an increased H3 acetylation and loss of transcriptional silencing in the *fob1*Δ strain (Fig. 3).

Furthermore our data show that Fob1p absence affects Sir2p recruitment and H3 acetylation at RFB region. Previous studies clearly indicated that two different mechanisms control transcriptional silencing at E-PRO and C-PRO (Bairwa et al., 2010). Here we propose that both of them involve Sir2p, which recruitment is Fob1p-Top1p dependent at RFB (this work) and RNA Pol I-Net1p dependent at C-PRO (Shou et al., 2001; Buck et al., 2016). It is conceivable that transcriptional silencing would require the recruitment of different apparatuses at RFB and C-PRO respectively. In fact, *S. cerevisiae* NTS region at rDNA is the scenario for several mutually interfering processes, such as RNA Pol I, Pol II and Pol III transcription, DNA replication and recombination. So, Fob1p specifically binds at RFB, while RNA Pol I transcription complex specifically binds the RNA Pol I promoter.

In summary our findings show that the Fob1p-dependent Top1p recruitment at RFB is part of the silencing apparatus by which Top1p, *via* its scaffold activity, targets Sir2p at RFB. According to this model, Fob1p specificity for RFB determines Top1p specific cleavage activity (otherwise widespread) for this region, revealing mechanism evolved for Top1p specific localization.

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