Telomere length regulation by Rif1 protein from *Hansenula polymorpha*

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ABSTRACT

Rif1 is a large multifaceted protein involved in various processes of DNA metabolism – from telomere length regulation and replication to double-strand break repair. The mechanistic details of its action, however, are often poorly understood. Here, we report functional characterization of the Rif1 homologue from methylotrophic thermotolerant budding yeast *Hansenula polymorpha* DL-1. We show that, similar to other yeast species, *H. polymorpha* Rif1 suppresses telomerase-dependent telomere elongation. We uncover two novel modes of Rif1 recruitment at *H. polymorpha* telomeres: via direct DNA binding and through the association with the Ku heterodimer. Both of these modes (at least partially) require the intrinsically disordered N-terminal extension – a region of the protein present exclusively in yeast species. We also demonstrate that Rif1 binds Stn1 and promotes its accumulation at telomeres in *H. polymorpha*.

29 INTRODUCTION

30

31 Chromosomes of most eukaryotic organisms end in distinctive nucleoprotein structures, called
 32 telomeres, which are essential for protection of chromosomes from degradation and fusions by the DNA
 33 repair machinery (de Lange, 2018). Short G/C-rich telomeric DNA repeats provide a platform for
 34 loading of a specific set of telomeric proteins forming complex dynamic assemblies at the ends of the
 35 chromosomes. Dividing cells are constantly losing telomeric repeats due to incomplete replication unless
 36 counteracted by either recombination or activity of the ribonucleoprotein complex telomerase.
 37 Replenishing of telomeric DNA by telomerase is crucial for long-term proliferation of the majority of
 38 eukaryotic cell types. This process is highly coordinated, and plenty of positive and negative regulators
 39 of telomerase have been identified to date, many of which are components of the telomeric chromatin
 40 (Lee et al., 2020; Lim and Cech, 2021; Shay and Wright, 2019).

41 In budding yeast *Saccharomyces cerevisiae*, the double-stranded portion of telomeres is bound by Rap1
 42 through its duplicated MYB domain (Henry et al., 1990; Shore and Nasmyth, 1987). Two additional
 43 telomeric proteins – Rif1 and Rif2 – are recruited to telomeres via interaction with Rap1 C-terminus
 44 (RCT) (Hardy et al., 1992; Wotton and Shore, 1997). Both of them restrict telomerase action and their
 45 double deletion (as well as deletion of the RCT) leads to telomere hyperelongation (Teng et al., 2000;
 46 Wotton and Shore, 1997). Rap1 also recruits the histone deacetylase complex Sir2/Sir3/Sir4 (through an
 47 interaction between Sir4 and RCT) providing the basis for the silencing of telomere-proximal genes
 48 (Moretti et al., 1994). The C-terminal portion of Rif1 contains a Rap1-binding motif (Rif1_{RBM}) and a
 49 tetramerization module (Rif1_{CTD}), while Rif2 contains two Rap1-binding sites, therefore Rap1/Rif1/Rif2
 50 can form complex DNA-protein structures at the chromosome ends, inhibiting both telomerase
 51 recruitment and telomere silencing (Shi et al., 2013). In addition, Rif1 can localize at telomeres in a
 52 Rap1-independent fashion through its large HEAT repeats containing N-terminal domain (Rif1_{NTD}) that
 53 exhibits strong affinity to DNA with some preference towards 3'-overhang containing ss-ds junctions
 54 (Mattarocci et al., 2017). This binding mode is important for telomerase regulation and attenuation of

55 end resection at telomeres. Recently, the ScRif1_{NTD} was proposed to contain an interaction site for a yet
 56 unidentified protein partner at telomeres (Shubin et al., 2021).

57 The single-stranded region of telomeres (3'-overhang) is bound and protected by the Cdc13 protein
 58 (Garvik et al., 1995; Lin and Zakian, 1996; Nugent et al., 1996) together with its partners Stn1 and Ten1
 59 (the CST complex) (Grandin, 2001; Grandin et al., 1997; Mersaoui and Wellinger, 2019; Wellinger and
 60 Zakian, 2012). Cdc13 is a central hub for telomeric DNA synthesis as it mediates the main pathway of
 61 telomerase recruitment (synthesis of the G-strand), as well as assists in loading of the Pol α (synthesis of
 62 the C-strand) (Qi and Zakian, 2000; Wellinger and Zakian, 2012). Telomerase recruitment in *S.*
 63 *cerevisiae* also depends on another telomeric complex – the Ku70/Ku80 heterodimer (Ku): it binds a
 64 stem-loop structure within telomerase RNA and the Sir4 protein at telomeres (Hass and Zappulla, 2015;
 65 Peterson et al., 2001; Stellwagen, 2003). In addition, Ku can bind DNA directly and apparently does so
 66 at sub-telomeric regions and, perhaps, at the ds-ss junction of the telomere (Larcher et al., 2016; Lopez
 67 et al., 2011). Ku loss leads to telomere shortening and Exo1-dependent accumulation of single-stranded
 68 telomeric DNA (Bonetti et al., 2010; Boulton, 1996; Gravel, 1998; Polotnianka et al., 1998; Porter et
 69 al., 1996).

70 Almost every telomeric chromatin component plays important roles outside telomeres. Rap1 protein is
 71 a central transcription factor, controlling expression of hundreds of genes in budding yeast (Azad and
 72 Tomar, 2016). A regulatory role during RNA PolII transcription was recently described for ScCST
 73 complex (Calvo et al., 2019). Ku heterodimer is a key component of the non-homologous end joining
 74 (NHEJ) machinery (Frit et al., 2019). Rif1 protein binds Glc7 (PP1) phosphatase and this interaction is
 75 important for regulation of replication initiation at several origins (Davé et al., 2014; Hiraga et al., 2014;
 76 Mattarocci et al., 2014), but not for telomere length control (Shubin and Greider, 2020). Interestingly,
 77 this function in replication, but not the telomeric role, is conserved among eukaryotic Rif1 homologues
 78 (see (Alavi et al., 2021) for a recent review). The only known species outside budding yeast clade which
 79 contains Rif1 as a component of “normal” telomeric chromatin is fission yeast *Schizosaccharomyces*
 80 *pombe*; although SpRif1 is recruited through an interaction with Taz1 protein rather than Rap1 (Kano

81 and Ishikawa, 2001). Mammalian Rif1 associates with dysfunctional telomeres (and other types of DSB)
 82 together with 53BP1, limiting accumulation of ssDNA through a mechanism involving Rev7, the
 83 Shieldin complex, CST and Pol α , and driving the DSB repair towards NHEJ (Barazas et al., 2018;
 84 Chapman et al., 2013; Dev et al., 2018; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Gupta et
 85 al., 2018; Mirman et al., 2018; Noordermeer et al., 2018; Tomida et al., 2018; Zimmermann et al., 2013).
 86 Interestingly, *S. cerevisiae* Rif1 also localizes to non-telomeric DSBs and promotes NHEJ through its
 87 DNA binding NTD, suggesting that Rif1 may be a conserved NHEJ factor (Mattarocci et al., 2017).
 88 The thermotolerant methylotrophic budding yeast species *Hansenula polymorpha* DL-1 is distantly
 89 related to *S. cerevisiae*. Several interesting differences in telomere biology between the two species have
 90 already been documented. Rap1 has two paralogues in *H. polymorpha* (Rap1A and Rap1B) with distinct
 91 DNA recognition properties (Malyavko et al., 2019). HpRap1A is located at the subtelomeric regions
 92 with no reported telomeric role. HpRap1B is the major telomeric dsDNA binder with the ability to
 93 control telomere length, although the primary target of its inhibition appears to be recombination rather
 94 than telomerase (Malyavko et al., 2019). The *RIF2* gene is absent from *H. polymorpha* genome (and
 95 other yeasts outside *Saccharomycetaceae* family). A shorter version of Cdc13 protein is present in *H.*
 96 *polymorpha*, which possesses strong affinity for telomeric G-strand *in vitro* and binds Stn1 protein
 97 (Malyavko and Dontsova, 2020). HpCdc13 also interacts HpTERT in the yeast-two-hybrid (Y2H) assay.
 98 Here, we explored the conservation of the telomeric roles of Rif1 protein within budding yeast clade by
 99 investigating Rif1 function in the telomere maintenance of *Hansenula polymorpha*. We found that Rif1
 100 restricts telomerase action at *H. polymorpha* telomeres. We demonstrate that the Rif1 N-terminal
 101 extension (Rif1_{NTE}) – a portion of Rif1 present exclusively in yeast homologues of the protein – is an
 102 intrinsically disordered domain with the ability to bind DNA *in vitro*. We show that this DNA-binding
 103 domain helps to recruit Rif1 to telomeres. Moreover, we found that the interaction with the Ku
 104 heterodimer is required for Rif1 telomeric localization in *H. polymorpha*. Finally, we found that the
 105 conserved N-terminal domain of HpRif1 binds to HpStn1 protein, and this interaction is crucial for
 106 HpStn1 recruitment at telomeres.

107 RESULTS

108

109 **Rif1 protein regulates telomere length in *H. polymorpha***

110 Iterative PSI-BLAST search against NCBI protein database yielded a single plausible *H. polymorpha*
 111 Rif1 homologue: the open reading frame HPODL_04218 (UniProt accession number W1QFB8). Amino
 112 acid sequence similarity between ScRif1 and HpRif1 is very low (16% identity), however alignment of
 113 multiple budding yeast homologues revealed several conserved elements within HpRif1 (Figure 1A,
 114 Supplementary file 1): the RVxF/SILK motif (residues 277-306), the N-terminal HEAT repeats
 115 containing domain (Rif1_{NTD}, residues ~310-1000) and the C-terminal domain (Rif1_{CTD}, residues ~1475-
 116 1521). HpRif1 also contains large potentially disordered regions (residues ~1000-1475 and 1-277). The
 117 residues located N-terminally to the RVxF/SILK motif we term N-terminal extension or NTE (residues
 118 1-277).

119 To test whether the identified HpRif1 homologue is involved in telomere maintenance, we deleted the
 120 *RIF1* gene and measured telomere length in the mutant strain. We observed markedly elongated
 121 telomeres in the Δ *rif1* strain (Figure 1B). Concomitant deletion of the *RAD52* gene did not influence
 122 telomere overelongation in the Δ *rif1* strain, whereas deletion of telomerase RNA gene (*TER*) led to
 123 telomere shortening (Figure 1B). The Δ *rif1* Δ *ter* double knock-out strain senesced upon continuous
 124 propagation, suggesting that Rif1 inhibits telomerase activity at *H. polymorpha* telomeres (Figure 1C).
 125 Next, we investigated telomeric localization of HpRif1. We created the *RIF1-HA* strain expressing
 126 HpRif1 protein tagged with hemagglutinin epitope (HA) at the C-terminus. Chromatin
 127 immunoprecipitated from this strain via anti-HA antibodies was enriched with telomere proximal
 128 fragment “TEL” compared to the internal fragment “ALA1”, suggesting that Rif1 localizes at *H.*
 129 *polymorpha* telomeres *in vivo* (Figure 1D). Thus, Rif1 is a telomeric protein inhibiting telomerase action
 130 in *H. polymorpha* similar to its *S. cerevisiae* homologue.

Figure 1. Rif1 regulates telomere length in *H. polymorpha*. (A) Schematic illustration of the domain organization of the full length HpRif1 and HA-tagged Rif1 fragments expressed in *H. polymorpha*. (B) Southern blot analysis of terminal restriction fragments from the indicated mutant strains. Genomic DNA was isolated from the strains after the Nth streak (where N is a number under a lane; each streak is ~20 generations). “0” streak – gDNA was isolated from the colonies on the transformation plate, without additional restreaks. “M” – telomeric DNA containing fragments that served as markers of length (their sizes are indicated on the left of each blot). (C) viability of the strains with the indicated genotypes was monitored during three serial restreaks on YPD agar plates, the plates were photographed after 2 days growth at 37 °C. (D) ChIP analysis. Chromatin from the indicated strains was immunoprecipitated on anti-HA magnetic beads. DNA was analyzed by qPCR with primers targeting either subtelomere region of the right end of chromosome VII (“TEL”) or ALA1 gene locus (negative control, “ALA1”). The amount of DNA fragments in the IP samples as a percentage of the input DNA was calculated, the % of input of the “TEL” Rif1-HA sample was set to 1. Error bars indicate SD, n = 3. “Rif1*-HA” – the strain expressing the Rif1-HA protein but in a slightly different background (see METHODS). (E) same as (B) but with different strains. (F) Western blot analysis of the total proteins isolated from the indicated strains using antibodies targeting HA epitope (upper panel). Ponceau S-stained membrane (lower panel) served as a loading control.

147 **Involvement of Rap1 in recruitment of Rif1 to telomeres**

148 Telomeric localization of ScRif1 is largely dependent on the interaction with ScRap1, and mutation in
 149 the Rif1_{RBM}, located within the C-terminal part of the protein, greatly reduce the amount of telomere-
 150 bound ScRif1 (Shi et al., 2013). We could not find a similar Rap1-binding motif in the HpRif1 sequence
 151 (Supplemental file 1), suggesting that HpRif1 may be recruited at telomeres in a Rap1-independent
 152 fashion, or such RBM is located in a different region of the protein. Phenotypic consequences of the
 153 ScRif1 loss or removal of the C-terminal domain of ScRap1 are similar, as they both result in the
 154 telomerase-dependent telomere overelongation. Telomere elongation in the $\Delta rif1$ strain of *H.*
 155 *polymorpha* appear to rely exclusively on telomerase action (Figure 1B, C). However, we have
 156 previously found that elongation of telomeres in the B¹⁻⁵²⁶ strain (expressing C-terminally truncated
 157 Rap1B) is largely Rad52-dependent, providing further indication for a Rap1-independent Rif1 function.
 158 If Rap1B is not involved in Rif1 recruitment – then Rif1 should not bind to double stranded telomeric
 159 DNA. To test this, we constructed the intTEL18 strain of *H. polymorpha* by inserting 18 telomeric
 160 repeats upstream of the WSC3 gene on the chromosome I (Figure 1 – figure supplement 1A). As
 161 expected, Rap1B binds telomeric repeats with similar efficiency at the internal locus or at the
 162 chromosome end (only ~2-fold difference between WSC3 and TEL signals for the intTEL18 strain,
 163 Figure 1 – figure supplement 1B). On the contrary, Rif1 association with the internal telomeric repeats
 164 is weak and close to the background levels (ChIP WSC3 signal is ~8-fold lower than TEL, Figure 1 –
 165 figure supplement 1C). Therefore, we conclude that interaction with Rap1 is not a major determinant of
 166 telomeric localization of Rif1 in *H. polymorpha*.

167

168 **NTE aids in recruitment of HpRif1 at telomeres**

169 In an attempt to identify portions of HpRif1 important for its telomeric localization, we substituted the
 170 *RIF1* locus with several HA-tagged C-terminal truncation constructs of Rif1. However, the only variant
 171 showing robust expression was the 1-264 fragment containing residues from the NTE region of HpRif1.
 172 Quite unexpectedly, we found that this Rif1_{NTE}¹⁻²⁶⁴ fragment can be efficiently recruited to telomeres,

according to our ChIP experiments (Figure 1D). Moreover, removal of residues 1-264 from HpRif1 (Rif1^{ΔNTE}) strongly reduced its ability to associate with telomeric DNA (Figure 1D), and led to telomere elongation (Figure 1E), while having no effect on the accumulation of the protein (Figure 1F). Thus, Rif1_{NTE}¹⁻²⁶⁴ is an important determinant of Rif1 telomeric localization in *H. polymorpha*.

Rif1_{NTE}¹⁻²⁶⁴ is intrinsically disordered in *H. polymorpha*

Multiple protein sequence alignment of Rif1 homologues from distantly related budding yeasts revealed that N-terminal extensions have no significant similarity (Supplemental file 1), suggesting that Rif1 NTE may not have any conserved folding. Moreover, MetaDisorder prediction (Kozlowski and Bujnicki, 2012) for 1-264 fragment of HpRif1 suggests that it lacks any secondary structure (Figure 2A), i.e. HpRif1_{NTE} may be unstructured or intrinsically disordered. It is worth noting that during gel-filtration and denaturing gel electrophoresis 28 kDa Rif1_{NTE}¹⁻²⁶⁴ behaves like a protein with higher molecular weight (Figure 2B, C), which is common for many intrinsically disordered proteins (IDPs) (Tomba, 2002). To directly examine the Rif1_{NTE}¹⁻²⁶⁴ structure we applied biophysical methods. Circular dichroism spectra of Rif1_{NTE}¹⁻²⁶⁴ recorded at different temperatures demonstrated that it contains no significant α -helical or β -strand secondary structural elements over this temperature range, as judged by the absence of well-defined peaks in the 215–230 nm region (Figure 2D). The isoelliptical point near 210 nm is indicative of the equilibrium between left-handed PPII and truly unordered conformation, which has been often observed in CD spectra of IDPs (Bienkiewicz et al., 2000). We also recorded two-dimensional ¹H-¹⁵N HSQC NMR spectrum of ¹⁵N-labeled Rif1_{NTE}¹⁻²⁶⁴. Narrow chemical shift dispersion of amide protons (7,8-8,6 ppm) in the spectrum confirms that Rif1_{NTE}¹⁻²⁶⁴ is intrinsically disordered (Figure 2E).

Figure 2. N-terminal extension of HpRif1 is intrinsically disordered. (A) Disorder prediction by Metadisorder (Kozlowski and Bujnicki, 2012) for 1-264 fragment of HpRif1 using three different combinations of algorithms: MetaDisorder (black), MetaDisorderMD (purple), MetaDisorderMD2 (blue). All residues with the disorder probability over 0.5 are considered to be disordered. (B) An aliquot of the purified recombinant Rif1_{NTE}¹⁻²⁶⁴ was analyzed by SDS-PAGE and Coomassie staining. (C) Profiles of size-exclusion chromatography of the mixture of the standard proteins (upper profile) and the recombinant Rif1_{NTE}¹⁻²⁶⁴ (lower profile). (D) Circular dichroism spectra of the Rif1_{NTE}¹⁻²⁶⁴ recorded at 5 °C (purple), 15 °C (blue), 25 °C (green), 35 °C (yellow) and 50 °C (red). (E) Two-dimensional ¹H-¹⁵N HSQC NMR spectrum of ¹⁵N-labeled Rif1_{NTE}¹⁻²⁶⁴ recorded at 25 °C.

204 Two “clusters” within Rifl_{NTE} are important for DNA binding *in vitro* and promote telomeric 205 localization *in vivo*

206 DNA binding by Rifl has been demonstrated in several species (including mammals, fission and
207 budding yeasts) (Kano et al., 2015; Mattarocci et al., 2017; Moriyama et al., 2018; Sukackaite et al.,
208 2014; Xu et al., 2010). We decided to test whether Rifl-DNA interaction is important for its telomeric
209 localization in *H. polymorpha*. We divided HpRifl into several parts (Figure 3A) and expressed them as
210 6His-S-tagged recombinant proteins in *E. coli*. After affinity chromatography on Ni-NTA resin we
211 obtained protein preparations significantly enriched with the HpRifl fragments (Figure 3B). We
212 analyzed the ability of these fragments to interact with ss- and ds-DNA oligonucleotides (FAM-Tel4(G)
213 and FAM-Tel4(GC), respectively) comprised of four telomeric repeats using EMSA. We found that only
214 two fragments (both containing amino acids 1-264 of HpRifl) could bind telomeric DNA (both single-
215 and double-stranded, Figure 3C). Thus, HpRifl contains a DNA-binding domain at its N-terminus.
216 The titration experiments revealed that affinities for telomeric ssDNA and dsDNA are very close (K_d^{app}
217 ~ 240 nM for FAM-Tel4(G), and $K_d^{app} \sim 430$ nM for FAM-Tel4(GC)) in our experimental conditions
218 (Figure 3D, Figure 3 – figure supplement 1A). The competition EMSA confirmed that Rifl_{NTE}¹⁻²⁶⁴
219 poorly differentiates between Tel4(G), Tel4(C) (C-rich telomeric strand) and Tel4(GC) oligonucleotides
220 (Figure 3E, Figure 3 – figure supplement 1B). However, it also revealed that Rifl_{NTE}¹⁻²⁶⁴ has some
221 preference towards telomeric substrates compared with the G/C-poor DNA oligonucleotides (Figure 3E,
222 Figure 3 – figure supplement 1B, Table 1).

Figure 3. Rifl^{NTE}¹⁻²⁶⁴ binds DNA *in vitro*. (A) Schematic illustration of the 6His-S-tagged Rifl fragments expressed in *E. coli* and used in EMSA assays. (B) Aliquots of the Ni-NTA purified fragments were analyzed by SDS-PAGE and Coomassie staining. "M" – protein weight marker. (C) 5 μ M of the indicated 6His-S-tagged HpRifl fragments were subjected to the electrophoretic mobility shift assay, using 0.5 μ M of either ss- (Tel4(G)) or ds- (Tel4(GC)) DNA oligonucleotide comprising four telomeric repeats as a probe. "–" – no protein control. Positions of the free DNA and protein/DNA complexes are indicated by arrows. (D) Quantification of the titration EMSA experiment (two replicates) with increasing concentration of recombinant (tag-free) Rifl^{NTE}¹⁻²⁶⁴ (0, 1, 3, 10, 30, 100, 300, 1000, 3000, 10000 nM) and 30 nM FAM-Tel4(G) (black circles) or FAM-Tel4(GC) (black squares) as probes (gels are shown in Figure 3 – figure supplement 1A). The fits into the "Specific binding with Hill slope" model are shown in dark red (FAM-Tel4(G) – solid curves; FAM-Tel4(GC) – dashed curves). The best-fit values for K_d apparent and Hill coefficient are shown. (E) Quantification of the competition EMSA experiment (the correspondent gels are shown in Figure 3 – figure supplement 1B) with 30 nM FAM-Tel4(G), 1 μ M (tag-free) Rifl^{NTE}¹⁻²⁶⁴ and increasing concentration of competitor DNA oligonucleotides (0.1, 0.3, 1, 3, 10, 30 μ M). The sequences of the competitors are in Table 1. We note that competition with the Tel4(C) oligo may be difficult to interpret since it may first titrate out the FAM-Tel4(G) probe.

239 **Table 1.** Oligonucleotides used in the EMSA experiments.

Oligo name	Sequence (5'-3')
Tel4 (G)	GGGTGGCGGGGTGGCGGGGTGGCGGGGTGGCG
Tel4 (C)	CGCCACCCCGCCACCCCGCCACCCCGCCACCC
Tel4 (GC)	annealed from Tel4 (G) and Tel4 (C)
ssrnd1	ACGACTCACTGTAGATACGACTCACTGTAGAT
ssrnd2	ATCTACAGTGAGTCGTATCTACAGTGAGTCGT
ssrnd3	AAATCTAGACATGAAAAAAAAAATGTTAGTAATCGAAATCTC
dsrnd1	annealed from ssrnd1 and ssrnd2
dsrnd2antisense	GAGATTTTCGATTACTAACATTTTTTTTTTTCATGTCTAGATTT
dsrnd2	annealed from ssrnd3 and dsrnd2antisense

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241

Next, we sought to identify residues within the N-terminal extension of HpRif1 which are involved in DNA binding, in order to provide evidence for direct interaction between HpRif1 and telomeric DNA in living cells. Lack of conservation and intrinsic disorder of Rif1_{NTE}¹⁻²⁶⁴ precluded us from predicting DNA-contacting residues by comparison with other known DNA-binding proteins. We tested several NTE truncation constructs for their ability to shift telomeric DNA *in vitro* and found amino acids 101-264 to be dispensable for DNA-binding activity (Figure 4 – figure supplement 1). Then we noticed that region 1-100 of HpRif1 contains two similar clusters enriched in positively charged residues (³⁸KRNNRSR⁴⁴ and ⁷⁹KRSTNNKSK⁸⁷, Figure 4A). Lysines and arginines often mediate DNA-protein contacts, and we presumed that these two clusters might be responsible for the observed Rif1_{NTE}¹⁻²⁶⁴-DNA interaction. We expressed and purified three mutant 6His-S-Rif1_{NTE}¹⁻²⁶⁴ proteins (Figure 4A, B): two with four alanine substitutions in either cluster 1 or cluster 2 (³⁸AANNASA⁴⁴ or 4A¹, ⁷⁹AASTNNASA⁸⁷ or 4A²) and one with eight alanine substitutions (³⁸AANNASA⁴⁴/⁷⁹AASTNNASA⁸⁷ or 8A). According to the results of the EMSA experiments, 8ARif1_{NTE}¹⁻²⁶⁴ retained little (if any) ability to bind DNA *in vitro* (Figure 4C). Thus, we conclude that regions 38-44 and 79-87 are crucial elements of DNA-binding activity of Rif1_{NTE}¹⁻²⁶⁴ *in vitro*.

Then effect of the mutations in N-terminal extension of HpRif1 on its telomeric functions *in vivo* was tested. Even four alanine substitutions (in either motif) reduce HpRif1 association with telomere VII to the background levels (Figure 4D), while having little effect on the protein abundance (Figure 4E). Telomeres in all three mutant strains were elongated, and comparable to the Rif1^{ΔNTE} strain (Figure 4F). Collectively, these data demonstrate an important role of regions 38-44 and 79-87 for HpRif1 function and argue that DNA-binding activity of the N-terminal extension helps to recruit HpRif1 to telomeres. ScRif1 binds DNA *in vitro* via its crook-shaped structural NTD, however we did not observe DNA binding activity of the HpRif1 fragments corresponding to this region of the protein (Figure 3). This may be explained by the difficulties to correctly fold large protein fragments in a heterologous system, and we tried to find whether DNA-contacting residues from ScRif1 NTD are conserved in HpRif1. According to the published ScRif1_{NTD}/DNA structure, 19 charged residues have the potential to contact

268 the DNA backbone (Mattarocci et al., 2017). Of these 19, only four are conserved in *H. polymorpha*
 269 Rif1 (Figure 4 – figure supplement 2A, Supplementary file 1). Double mutant HpRif1_{K658E/K666E} is
 270 expressed at considerably lower levels than WT HpRif1, indicating that residues K658 and K666 are
 271 important for protein stability (Figure 4 – figure supplement 2B). Substitution of the other two conserved
 272 amino acids (K504 and R539) for glutamines does not lead to detectable changes in Rif1 telomere
 273 occupancy or telomere length (Figure 4 – figure supplement 2C, D). Thus, HpRif1 may lack the DNA-
 274 binding mode described for ScRif1 or it utilizes different amino acids for such interaction.

Figure 4. Two K/R clusters within Rif1 are important for its telomeric localization. (A) Sequences of the two clusters enriched in positively charged residues in the wild type and mutant versions of HpRif1. (B) Aliquots of the Ni-NTA purified wild type (“WT”) and mutant 1-264 fragments of HpRif1 were analyzed by SDS-PAGE and Coomassie staining. “M” – protein weight marker. (C) 10 μ M of the indicated 6His-S-tagged proteins were subjected to EMSA, using 1 μ M of ss-oligonucleotide comprising four telomeric repeats (FAM-Tel4(G)) as a probe. “–” – no protein control. “30x” – 30 μ M of the 8A mutant protein. Positions of the free DNA and protein/DNA complexes are indicated by arrows. (D) ChIP analysis of the indicated strains, same as in Figure 1D; the % of input of the “TEL” Rif1*-HA WT sample was set to 1. Error bars indicate SD, n = 3. (E) Western blot analysis of the total proteins isolated from the indicated strains using antibodies targeting HA epitope (upper panel). Ponceau S-stained membrane (lower panel) served as a loading control. (F) Southern blot analysis as in Figure 1B but with different strains.

287 **Rif1 telomere localization depends on the Ku heterodimer**

288 To further investigate the mechanism of Rif1 recruitment, we sought to identify protein-protein
 289 interactions in which Rif1 may be involved at *H. polymorpha* telomeres. Rif1 and several other telomeric
 290 proteins were tested in yeast-two-hybrid (Y2H) assay, which revealed two Rif1-interacting partners:
 291 Ku80 and Stn1 (Figure 5A). Interestingly, the interaction with Ku80 (but not with Stn1) appears to be
 292 mediated through the Rif1_{NTE}¹⁻²⁶⁴ fragment, according to the Y2H analysis (Figure 5A). By testing
 293 several deletion constructs of the Rif1_{NTE}¹⁻²⁶⁴, we found that the residues required for Ku80 binding are
 294 localized within the 220-240 region of Rif1 (hereafter referred to as Rif1 Ku80-binding motif, or
 295 Rif1_{KBM}) (Figure 5 – figure supplement 1). This region is fairly conserved in five species closely related
 296 to *H. polymorpha* (Figure 5B, Supplementary file 2), and substitution of either F225 or R230 with a
 297 negatively charged glutamic acid residue abolish the Rif1_{NTE}¹⁵⁰⁻²⁶⁴-Ku80 interaction in the Y2H system
 298 (Figure 5C).

299 Rif1-Ku interaction can also be observed *in vivo* by Co-IP experiments (Figure 5D). Deletion of either
 300 *KU80* or *KU70* led to a ~4-fold reduction in Rif1-HA telomere occupancy (Figure 5E), consistent with
 301 the idea that binding to Ku is important for Rif1 telomere association. In agreement with the Y2H data,
 302 Rif1_{NTE}¹⁻²⁶⁴ fragments with either F225E or R230E mutation were unable to localize to telomeres (Figure
 303 5F, G), confirming that Rif1_{NTE}-Ku80 interaction does occur *in vivo*. However, F225E and R230E
 304 substitutions did not affect the ability of the full-length Rif1 to bind telomeric DNA (Figure 5H). Double
 305 mutation F225E/R230E and substitution of amino acids 225-230 with alanines (the 6A mutant) also did
 306 not perturb Rif1 telomere binding (Figure 5H). Moreover, neither mutation led to a noticeable telomere
 307 length increase (Figure 5I). Thus, Rif1 contains other sites for Ku binding which are more important *in*
 308 *vivo* than Rif1_{NTE}-Ku80 interaction.

309 The idea that Rif1 is recruited at telomeres by Ku relies on the assumption that Ku itself binds telomeric
 310 DNA. To verify this, we performed ChIP experiments with the Ku70-HA and Ku80-HA strains, which
 311 showed that telomeric DNA is co-immunoprecipitated with Ku (Figure 5 – figure supplement 2A).
 312 Moreover, deletion of either Ku component perturbs telomere length maintenance (Figure 5 – figure

313 supplement 2B), confirming that Ku heterodimer is indeed a part of telomeric chromatin in *H.*
 314 *polymorpha*. Deletion of *RIF1* leads to only ~2-fold reduction in Ku80 occupancy (Figure 5 – figure
 315 supplement 2C), in agreement with the idea that Rif1 is more reliant on the Ku's presence at telomeres
 316 than Ku on Rif1's. However, Ku loss leads to telomere shortening (~25-50% reduction in telomere
 317 length, Figure 5 – figure supplement 2B, Figure 5J), indicating to a positive role of Ku in telomere
 318 lengthening. To verify that Rif1 telomere localization defect upon Ku loss is not simply a consequence
 319 of the telomere shortening, we measured Rif1 telomere binding in a strain lacking telomerase RNA
 320 (Figure 5 – figure supplement 2D, E). TER knock-out resulted in ~40% reduction in telomere length,
 321 whereas Rif1-HA telomere occupancy diminished only ~2-fold (Figure 5 – figure supplement 2D, E);
 322 contrasting to ~4-fold Rif1 ChIP signal drop in case of Ku mutants (Figure 5E).

323 Interestingly, *RIF1* deletion in a $\Delta ku80$ background does not lead to strong telomere elongation
 324 compared to the parental $\Delta ku80$ strain, suggesting that inhibition of telomerase by Rif1 is attenuated in
 325 the absence of Ku (Figure 5J). Moreover, inhibition by the Rif1_{NTE}¹⁻²⁶⁴ fragment is lost completely: no
 326 telomere lengthening is observed in the Rif1^{ΔNTE} $\Delta ku80$ strain compared to the $\Delta ku80$ strain (Figure 5J).
 327 These results suggest that Ku potentially recruits some telomere addition factor, while the Rif1 fragment
 328 may compete with this putative factor for Ku80 binding. In *S. cerevisiae*, Ku is one of the accessory
 329 subunits of telomerase stably bound to its RNA component. We found that HpTER cannot be efficiently
 330 co-purified with Ku80-HA, while it robustly binds TERT-HA, Est1-HA and Est3-HA in the same
 331 experimental conditions (Figure 5 – figure supplement 2F, (Shepelev et al., 2020)), suggesting that
 332 HpKu is not a stable telomerase component. This does not exclude, however, the possibility of weak or
 333 transient association between Ku and telomerase in *H. polymorpha*. Consistent with this idea, we observe
 334 weak but noticeable interaction between HpTERT and HpKu80 in the Y2H assay ((Figure 5 – figure
 335 supplement 2G).

Figure 5. Rif1 interacts with Ku80 in *H. polymorpha* (A) Y2H analysis. AH109 colonies expressing pairs of the indicated proteins (fused to either Gal4-BD (GBD) or Gal4-AD (GAD)); cultures with $A_{600} \sim 0.5$ and four 10-fold serial dilutions were plated on the SC medium lacking amino acids as indicated, and incubated at 30 °C for four days. “T” – SV40 large T antigen. (B) A fragment of the alignment of the NTE regions from *H. polymorpha* DL-1 (HANPO) and five of its closest relatives. Full alignment is in Supplemental file 2. (C) Y2H analysis as in (A), “NTE^{R230E}” and “NTE^{F225E}” – 150-264 fragments of Rif1 with the R230E and F225E mutations, respectively. (D) Co-IP analysis. IP on the anti-Flag resin. The amount of tagged proteins in whole cell extracts (WCE) and the IP samples (IP) was monitored by Western blot (WB) using anti-Flag and anti-HA antibodies. The IP experiment was performed in the presence of benzonase nuclease. (E, F) ChIP analysis of the indicated strains, same as in Figure 1D; the % of input of the “TEL” Rif1-HA WT sample was set to 1 (E); the % of input of the “TEL” Rif1¹⁻²⁶⁴-HA WT sample was set to 1 (F). (G) Western blot analysis. Same as in Figure 1F, but with different strains. (H) ChIP analysis of the indicated strains, same as in Figure 1D; Error bars indicate SD, n = 3. “6A” mutation: ²²⁵FQAVVR^{230/225}AAAAAA²³⁰. (I, J) Southern blot analysis as in Figure 1B (J) Mean lengths of the brightest TFR bands are: *RIF1KU80* 476 bp, *RIF1Δku80* 399 bp. WT telomere length reported to be ~160 bp (~20 telomeric repeats, (Sohn et al., 1999)), therefore telomere length is reduced by ~50% in the knockout strain.

352 **Rif1 is important for recruitment of Stn1 protein to telomeres**

353 Next, we decided to study telomeric role of the interaction between Rif1 and Stn1. First, we confirmed
 354 the ability of the proteins to form a complex *in vivo* by the Co-IP experiment. Stn1-HA co-elutes with
 355 Rif1-HA following immunoprecipitation on the anti-Flag resin (Figure 6A). Benzonase treatment of the
 356 extracts prior to the IP does not diminish the amount of co-precipitated Stn1 (Figure 6A), suggesting
 357 that the Rif1-Stn1 interaction is DNA-independent. We were unable to test the effect of the *STN1*
 358 knockout on the Rif1 telomere binding, since Stn1 is essential for cell viability (as two other components
 359 of the CST complex – Cdc13 and Ten1, Figure 6 – figure supplement 1A). However, we found that
 360 removal of either N- or C-terminal Stn1 domains is sufficient to completely disrupt binding to Rif1 in
 361 the Y2H assay (Figure 6B, C). C-terminal truncations in Stn1 are well tolerated by cells, although the
 362 Stn1^{ΔC381}-Flag and Stn1^{ΔC221}-Flag strains have greatly elongated telomeres (Figure 6D), similar to the
 363 Stn1 truncation mutants in *S. cerevisiae* (Petreaca et al., 2007, 2006; Puglisi et al., 2008). Rif1 telomere
 364 association is reduced in the strains with these *stn1* mutations, although the reduction is relatively modest
 365 (~2.5-fold, Figure 6E). Interestingly, we found that Stn1 telomere binding is completely abolished upon
 366 *RIF1* deletion, suggesting that it is actually Rif1 that recruits Stn1 to telomeres, and not vice versa
 367 (Figure 6F). Stn1 telomere occupancy is also diminished (~2-fold) in the $\Delta ku80$ strain, which has smaller
 368 amount of the telomere-bound Rif1 (Figure 6F). Finally, we found that Rif1 bind Stn1 in Y2H through
 369 its conserved NTD, more precisely – the 265-897 region (Figure 6G).

Figure 6. Rif1 recruits Stn1 at *H. polymorpha* telomeres. (A) Co-IP analysis. Same as in Figure 5I, but with different strains; the IP experiment was performed either with or without benzonase treatment as indicated. (B) Y2H analysis. Same as in in Figure 5A, but with different protein pairs. (C) Schematic illustration of the domain organization of the full length HpStn1 and its truncation variants used in this study. (D) Southern blot analysis as in Figure 1B but with different strains. (E, F) ChIP analysis of the indicated strains, same as in Figure 1D (IP on the anti-HA beads); the % of input of the “TEL” Rif1-HA/Stn1-Flag sample was set to 1. Error bars indicate SD, n = 3. (E) or the % of input of the “TEL” Stn1-HA sample was set to 1 (F). (G) Y2H analysis. Same as in in Figure 5A, but with different protein pairs.

379 DISCUSSION

380

381 We found that Rif1 from the methylotrophic yeast *H. polymorpha* prevents hyperelongation of
 382 telomeres, similar to its other yeast counterparts. In *S. cerevisiae*, Rif1's function strongly depends on
 383 the recruitment by Rap1 protein (specifically, by the Rap1^{RCT} domain). Hence, telomerase-dependent
 384 telomere overelongation can be observed in the strains with either deletion of the *RIF1* gene, or
 385 mutations that disrupt Rap1-telomere association (Hardy et al., 1992; Shi et al., 2013; Teng et al., 2000).
 386 Long telomeres in the $\Delta rif1$ strain of *H. polymorpha* do appear to be solely maintained by telomerase
 387 (Figure 1B, C). *H. polymorpha* has two Rap1 paralogues, however, HpRap1A does not recognize
 388 telomeric DNA and RCT removal from Rap1A has no effect on telomere length (Malyavko et al., 2019).
 389 HpRap1B associates with double-stranded telomeric DNA and the 50 a.a. C-terminal truncation
 390 (Rap1B¹⁻⁵²⁶) leads to more than 10-fold drop in Rap1B expression and strong telomere elongation. In
 391 contrast to the $\Delta rif1$ strain, longer telomeres in this B¹⁻⁵²⁶ strain were found to depend primarily on
 392 recombination (Malyavko et al., 2019). This phenotypic discrepancy indicates that functions of Rap1
 393 and Rif1 in *H. polymorpha* may not be as intimately linked as in *S. cerevisiae*. In addition, we could not
 394 identify the Rap1-binding motif within HpRif1. We failed to detect a direct interaction between HpRif1
 395 and either of the two HpRap1 paralogues in the Y2H experiment (Figure 5A). Finally, Rap1B can
 396 efficiently localize to internal as well as terminal telomeric dsDNA (as expected from a sequence specific
 397 telomeric factor), whereas Rif1 has a clear preference for telomeric repeats located at the end of the
 398 chromosome (Figure 1 – figure supplement 1). Thus, although we cannot exclude the possibility of an
 399 interaction between HpRap1 and HpRif1, we believe that it is highly unlikely that Rif1 binding to Rap1
 400 is the major mechanism of Rif1 telomeric recruitment in *H. polymorpha* in a manner it is described for
 401 its *S. cerevisiae* homologues.

402 Rif1 homologues from many species contain a DBD, and DNA binding by the NTD of *S. cerevisiae*
 403 Rif1 promotes its accumulation at telomeres in a Rap1-independent fashion (Kano et al., 2015;
 404 Mattarocci et al., 2017; Moriyama et al., 2018; Sukackaite et al., 2014; Xu et al., 2010). This prompted

us to test whether HpRif1 might be recruited at telomeres by means of direct interaction with telomeric DNA. We found that HpRif1 fragments containing amino acids 1-264 possess DNA-binding activity *in vitro*. The Rif1_{NTE}¹⁻²⁶⁴ fragment can be detected at telomeres by ChIP, while its removal (the Rif1^{ΔNTE} strain) strongly diminishes the telomere binding of Rif1. Moreover, mutations that abolish DNA-binding *in vitro* lead to the substantial (if not complete) loss of HpRif1 from telomeres and telomere elongation comparable to the Rif1^{ΔNTE} strain (Figure 4). These results are in favor of the “recruitment via direct telomeric DNA binding” hypothesis. The identified DNA-binding mode by the intrinsically disordered portion of HpRif1, however, is clearly different from the one utilized by the conserved structured NTD of ScRif1. We could not detect a DNA binding activity of the HpRif1_{NTD} (residues ~310-1000, Figure 3A-C), although this may be due to improper folding of the large polypeptides in the *E. coli* expression system or suboptimal conditions of the EMSA reactions. Despite the fact that the NTD is a relatively well conserved region of Rif1, we found that of 19 charged amino acids constituting the DBD of ScRif1 only 4 can be found in HpRif1 and their mutation has no apparent effect on Rif1-telomere association (Figure 4 – figure supplement 2). Given the high evolvability of telomeric proteins, we do not, however, dismiss the possibility that HpRif1 may contact DNA through different amino acids within its NTD domain.

Interestingly, there is a discrepancy between our ChIP experiments, which suggest that Rif1_{NTE} is necessary and sufficient for the telomeric localization of Rif1, and the telomere length analysis, which points to the relatively minor contribution of the Rif1_{NTE} to the telomere length control (Figure 4D, F). This points to the existence of another mode(s) of HpRif1 recruitment, which may evade detection by ChIP, perhaps, because it is restricted to the short window of the cell cycle or due to its transient nature. This second mode could be attributed to the DNA binding by the NTD (similar to the ScRif1) and/or to the interaction of HpRif1 with another telomeric protein.

Our search for Rif1’s protein partners at telomeres revealed two hits – Ku80 and Stn1 – and deletion of the *KU80* gene does lead to strong reduction in Rif1 telomere occupancy, suggesting that this interaction plays an important role in localizing Rif1 to telomeres (Figure 5). Although in the Y2H assay the Ku80-

431 Rif1 interaction depends primarily on the short motif within Rif1_{NTE}, our experiments indicate that in *H.*
 432 *polymorpha* cells the robust Ku80-Rif1 association requires other Rif1 domains. The Rif1_{KBM} may thus
 433 serve only as an auxiliary module, which is consistent with the fact that it is conserved only in five
 434 species closest to *H. polymorpha*. The interaction of NTE-less Rif1 with Ku could therefore represent
 435 the second mode of telomeric recruitment of HpRif1 responsible for the telomerase inhibition in the
 436 Rif1^{ΔNTE} strain.

437 In *S. cerevisiae*, telomere tract length determines the probability of the elongation of a chromosomal
 438 end: shorter telomeres are preferred substrates for telomerase (Marcand, 1999; Teixeira et al., 2004). At
 439 the basis of this regulation is “counting” of telomeric repeats by Rap1 that defines the number of
 440 (telomerase-inhibitory) Rif1 and Rif2 molecules present at any given telomere (Levy and Blackburn,
 441 2004; Marcand et al., 1997). *H. polymorpha* lacks Rif2 entirely, and at this point it is not obvious how
 442 mechanistically Rif1 recruitment could be linked to telomere length. In fact, HpRif1 appears to have
 443 only limited ability to bind double-stranded telomeric DNA *in vivo* (Figure 1 – figure supplement 1). It
 444 should be noted, that Rif1 is a large protein with many functions and, even in *S. cerevisiae*, it is not fully
 445 involved in the “counting” process. The Rif1^{RBM} mutant lacking the ability to contact Rap1 is almost
 446 completely lost from telomeres as judged by ChIP, yet retains a significant portion of its telomerase
 447 inhibitory potential (Mattarocci et al., 2017; Shi et al., 2013). Thus, budding yeast Rif1 homologues may
 448 operate (at least partially) in a telomere length-independent way.

449 Similarly to *S. cerevisiae* (Boulton, 1996; Porter et al., 1996), deletion of the subunits of the HpKu
 450 heterodimer leads to pronounced telomere shortening (Figure 5 – figure supplement 1B). ScKu
 451 recognizes a specific stem-loop structure within telomerase RNA, mediating one of the two pathways of
 452 telomerase recruitment (Chen et al., 2018; Peterson et al., 2001; Stellwagen, 2003). Our previous
 453 analysis of the *H. polymorpha* telomerase RNA structure did not reveal a similar Ku-binding hairpin
 454 (Smekalova et al., 2013) and the lack of a stable interaction between Ku and HpTER was confirmed
 455 experimentally in this study (Figure 5 – figure supplement 2F). Shorter telomeres in the HpKu knock-
 456 out strains could be explained, however, by a transient association with telomerase via another

telomerase subunit (for example like in human cells, where an interaction between Ku and TERT has been detected (Chai et al., 2002)). Indeed, we found that HpTERT binds HpKu80 in the Y2H experiment (Figure 5 – figure supplement 2G). Interestingly, we observed only minor telomere elongation upon HpRif1 loss in the $\Delta ku80$ background, suggesting that Rif1 counteracts the positive effect of the Ku at telomeres (Figure 5J). We propose that Rif1's inhibitory function in *H. polymorpha* may involve a direct competition with telomerase for Ku binding at telomeres (Figure 6 – figure supplement 2).

The second pathway of telomerase recruitment in *S. cerevisiae* relies on the binding of the Est1 telomerase subunit to the RD (recruitment domain) of the Cdc13 protein (Chen et al., 2018; Evans and Lundblad, 1999; Pennock et al., 2001). Association with Stn1 blocks this interaction, thereby limiting the amount of telomerase at telomeres (Chandra, 2001; Li et al., 2009; Pennock et al., 2001). Although HpCdc13 is shorter than ScCdc13 and lacks the RD domain (and, apparently, the ability to bind Est1), we have demonstrated recently, that it can bind both telomerase (via the TERT subunit) and Stn1 in a Y2H assay, indicating that a similar mechanism of telomerase regulation may be operating in *H. polymorpha* (Malyavko and Dontsova, 2020). Alternatively, Stn1 may influence telomerase binding indirectly, by controlling the amount of ssDNA (and therefore Cdc13, which is a ssDNA binding protein) at telomeres (Grandin et al., 1997). In any case, we found that Stn1 accumulation at telomeres depends strongly on Rif1, and that mutations disrupting Stn1-Rif1 (and Stn1-Cdc13) interaction lead to highly elongated telomeres (Figure 6). Thus, we speculate that the negative effect of Rif1 on telomere length may also be explained by its ability to promote Stn1 telomere localization, thereby attenuating the telomerase recruitment via Cdc13 (Figure 6 – figure supplement 2).

Remarkably, the existence of a physical interaction between Rif1 and the CST complex was proposed (but not demonstrated experimentally) in a study by Anbalagan et al (Anbalagan et al., 2011) to explain the specific requirement of Rif1 for viability of *S. cerevisiae* cells with hypomorphic mutations in Cdc13 and Stn1. Our Y2H experiments show that Stn1 binds the NTD of Rif1 (Figure 6G). Rif1_{NTD} contains the most conserved portion of the protein (Supplementary file 1) that is present in all Rif1 orthologues

482 (Mattarocci et al., 2017; Sreesankar et al., 2012). Hence it is plausible that the Stn1-Rif1 direct
483 interaction observed in our study may be present in other species.

484 In summary, in the present study we have identified several novel aspects of Rif1 function in budding
485 yeasts. We demonstrated that the N-terminal extension (NTE) of Rif1 is an accessory domain partially
486 promoting Rif1 telomeric localization in *H. polymorpha*. Given that DNA binding by Rif1_{NTE} is weak
487 and lacks clearly defined specificity towards a particular telomeric substrate, we believe that it may
488 simply stabilize Rif1 at telomeres, while the preference for the telomeric chromatin is provided by the
489 ability to contact Ku heterodimer. Consistently, Rif1_{NTE}^{1-264(F225E)} and Rif1_{NTE}^{1-264(R230E)} mutants (unable
490 to bind Ku80, but with intact DBD) fail to localize to telomeres (Figure 5G). Our results also indirectly
491 point to the possibility of the DNA recognition by the HpRif1_{NTD} in a manner akin to ScRif1, which
492 recognizes the 3'-overhang containing ss-ds junctions (Mattarocci et al., 2017). This may also be a factor
493 driving HpRif1 to the chromosomal ends. Finally, we uncovered a HpRif1's role in loading Stn1 protein
494 onto telomeres. Curiously, the mammalian Rif1 homologue is important for the recruitment of CST
495 complex to promote NHEJ at several types of DSBs (although the Rif1-CST interaction is not direct,
496 but mediated via the Shieldin complex) (Barazas et al., 2018; Chapman et al., 2013; Dev et al., 2018; Di
497 Virgilio et al., 2013; Escribano-Díaz et al., 2013; Gupta et al., 2018; Mirman et al., 2018; Noordermeer
498 et al., 2018; Tomida et al., 2018; Zimmermann et al., 2013). It is tempting to speculate that Rif1's role
499 in recruiting Stn1 may be conserved from yeasts to human.

500

501 MATERIALS AND METHODS

502 Yeast strains and constructs

503 The strains used in this study are listed in Supplemental Table S1. The DL1-L strain (Kang et al., 2005)
504 was used as a wild type (no tag) control in all experiments. Gene replacements (detailed below) and
505 random spore analysis was performed as described (Malyavko et al., 2019). The gene replacements were
506 verified by PCR, mutagenesis was verified by sequencing.

507 1-1001 bp region of the *RAD52* gene, 190-480 bp region of the *HpTER* gene and 196-2076 bp region of
508 the *KU80* gene were replaced with pKAM555 plasmid (linearized with *Sma*I) (Agaphonov et al., 2009).

509 For the *HpRIF1* knockout, -33-4430 bp region of the *RIF1* gene was replaced with *HpLEU2* gene from
510 pCHLX vector (Sohn et al., 1996). For the *HpKU70* knockout, 214-1698 bp region of the *KU70* gene
511 was replaced with either *HpLEU2* gene from pCHLX, or pKAM555 (linearized with *Sma*I).

512 For C-terminal 3HA tagging, stop codon (or a relevant portion of the gene, in case of a concomitant C-
513 terminal truncation) was replaced with *Sma*I-*Pme*I fragment of the pFA6a-3HA-*HpURA3* plasmid
514 (Malyavko et al., 2019). The pFA6a-*Rif1*-3HA-*HpLEU2* vector was constructed by replacing the
515 *Bgl*II/*Pme*I fragment of the pFA6a-*Rif1*-3HA-*HpURA3* with the *HpLEU2* marker gene (generated by
516 PCR from the pCHLX vector with primers F: 5'-aaaagatctccaccgcggtggc-3' and R: 5'-
517 cgggggatcctactttttttctcc-3'); then the 3HA sequence was replaced with 3Flag by PCR and self-ligation

518 of the pFA6a-*Rif1*-3HA-*HpLEU2* (primers F: 5'-
519 caccgtcatggtctttgtagtctccaccccgctcccccgcgtcttttttcaaacacgtc-3' and R: 5'-
520 attataaagatcatgacatcgactacaaggatgacgatgacaagtagggcgccgacttctaataag-3'), generating the pFA6a-
521 *Rif1*-3Flag-*HpLEU2* vector. For C-terminal 3Flag tagging, stop codon (or a relevant portion of the gene,
522 in case of a concomitant C-terminal truncation) was replaced with the 3FlagLEU2 fragment (primers F:
523 5'-gggggaggcgggggtggagactac-3' and R: 5'-cgggggatcctactttttttctcc-3') of the pFA6a-*Rif1*-3Flag-
524 *HpLEU2*.

525

526 For mutagenesis of the NTE of HpRifI we generated the p5rCHLXpr plasmid by cloning upstream *RIF1*
527 flanking region (primers F: 5'-aacgcggaccaggtcatctacagagacgag-3'; R: 5'-
528 aaatctagacgggtgtgtgattctgcaaacc-3') and *RIF1* promoter (primers F: 5'-
529 aagatccaaaaacaaaaaaatgccagcttgaaaaaattg-3'; R: 5'-aagaattcggcttctgggttgaaaatacag-3') at
530 SacII/XbaI sites and BamHI/EcoRI sites, respectively, of the pCHLX vector. Then *RIF1* fragments (F:
531 5'-aaagatatcatgagtctaatgacaacgacag-3'; R: 5'-aaagtcgacaggcggactcactttcaagattg-3') and (F: 5'-
532 aaagatatcatgcgggacgcggccggcaac-3' R: 5'-aaagtcgactccacggcgtgcaagctca-3') were cloned at
533 SalI/EcoRV sites to generate templates for RifI*-HA and RifI^{ΔNTE}-HA cassettes, respectively.
534 Templates for integration cassettes with mutations in NTE were introduced by PCR amplification of the
535 plasmid with the template for RifI*-HA cassette and subsequent self-ligation. All resulting cassettes
536 were PCR amplified and transformed into RifI-HA strain to generate RifI*-HA, RifI^{ΔNTE}-HA and the
537 mutant RifI*-HA strains.

538 In the heterozygous *H. polymorpha* CBS4732 strains the portions of the genes (-17-951 bp of *CDC13*;
539 22-1618 bp of *STN1*; 1-365 bp of *TEN1*; 78-1582 bp of *KU70*) were replaced with *HpLEU2* gene from
540 pCHLX.

541 For the intTEL18 strain creation we generated the pUC19LGPH36 plasmid by cloning *leu2* gene (from
542 DL1-L genome) with flanking regions (primers F: 5'-gcgcgtgtctcagcatgaac-3'; R: 5'-
543 ggtgtgggaggtagaagagg-3') at the pUC19 SmaI site. The *leu2* ORF was replaced (primers F: 5'-
544 aaaaagatctaattatactgttgcgcgaagtagtcccatggttaggatctcgaataattcctaaataatcc-3'; R: 5'-
545 gattgcaaatgatggaactattttgc-3') by the PstI(blunted)-BclI fragment of the HARS36 sequence (Sohn et
546 al., 1999) from the AMIpSL1 vector (Agaphonov et al., 1999). Then the G418^R gene (primers F: 5'-
547 aaaaaagacaggaatgagtaaataagatcctttgatcttttctacgg-3'; R: 5'-
548 ccgggaaaaactgaaaaccattggcagcagaggtttcccgac-3' from the pKAM555 vector) was inserted at the ClaI
549 site using HiFi assembly (NEB). For the intTEL0 strain creation the pUC19LG plasmid (lacking the
550 PstI(blunted)-BclI fragment of the HARS36 sequence) was used.

551

552 **Yeast growth conditions**

553 Normally, single colonies from the transformation plates were restreaked on YPD plates and grown for
554 2 days at 37 °C. After 10 restreaks (~230 cell divisions) cells were grown in 10 ml of YPD at 37 °C
555 overnight and used for Southern blot analysis.

556 For the Δter strains viability assay (Figure 1B, C) several colonies from the transformation plate were
557 resuspended in 100 μ l of water: 50 μ l were used for yeast colony PCR, 2 μ l were plated onto a fresh
558 YPD plate and grown for 2 days at 37 °C (“streak 1” plate), 48 μ l were added to 10 ml of YPD and
559 grown overnight at 37 °C (used for gDNA isolation and Southern blot, “streak 0”). Single colonies from
560 the “streak 1” plate were resuspended in 10 μ l of water, 3 μ l were plated onto a fresh YPD plate and
561 grown for 2 days at 37 °C (“streak 2” plate), 7 μ l were added to 10 ml of YPD and grown overnight at
562 37 °C (used for gDNA isolation and Southern blot, “streak 1”). Single colonies from the “streak 2”
563 (smears of colonies in case of the Δter strains) plate were restreaked onto a fresh YPD plate and grown
564 for 2 days at 37 °C (“streak 3” plate). The *TER* strains were restreaked seven additional times, single
565 colonies from the “streak 10” were grown in 10 ml of YPD overnight at 37 °C and used for Southern
566 blot. Two colonies of each genotype were analyzed in this experiment (biological replicates).

567

568 **Telomere Southern blots**

569 Southern blot experiments were carried out as previously described (Shepelev et al., 2020). TRF length
570 were calculated using the ImageQuant TL 1D software version 7.0.

571

572 **Chromatin Immunoprecipitation (ChIP)**

573 ChIP experiments were performed as previously described (Malyavko et al., 2019) with the following
574 modifications. Cells were grown in 100 ml of YPD at 37 °C and fixed with 1% formaldehyde for 30 min
575 at 25 °C. Lysates were incubated with anti-HA magnetic beads for 2 hours. High-salt lysis buffer
576 contained 0.5 M NaCl, and wash buffer did not contain any SDS. We used the standard deviation

577 between at least three replicates (cultures from three colonies of the same strain) as a value of the
578 experimental error.

579

580 **Co-immunoprecipitation (Co-IP) and Western blots**

581 Co-IP experiments were performed as previously described (Shyian et al., 2020) with the following
582 modifications. OD₆₀₀ ~0.9 cell cultures were used to prepare 1 ml of lysate. Cells were broken with glass
583 beads in a Precellys Evolution homogenizer. 20 µl of anti-Flag M2 gel (Sigma) per 1 ml of lysate were
584 used. Proteins were eluted by boiling at 95 °C for 10 min in 30 µl of 2x SDS-PAGE buffer (100 mM
585 Tris pH 8, 4% SDS, 10% glycerol, 0.2% bromophenol blue). Total proteins were isolated as previously
586 described (Kushnirov, 2000). Proteins were separated on 8% PAGE gels, transferred onto Hybond P 0.22
587 PVDF membrane (GE Healthcare), stained with Ponceau S (Amresco), and blocked in 5% BSA. Anti-
588 HA-HRP antibodies (clone 3F10, Sigma) at 1:5000, anti-FLAG M2 antibodies (Sigma) at 1:5000, goat
589 anti-mouse IgG-HRP (62-6520, Thermo Fisher Scientific) at 1:5000 and SuperSignal™ West Femto
590 Maximum Sensitivity Substrate (Thermo Fisher Scientific) were used for protein detection.

591 RNA Co-IP experiments were performed as described in (Shepelev et al., 2020)

592

593 **Protein Expression and Purification**

594 Rif1 fragments for EMSA were expressed and purified as HpRap1B protein (Malyavko et al., 2019).
595 For CD and NMR studies 1-264 HpRif1 fragment was purified in 50 mM phosphate buffer, pH 7.5, 500
596 mM NaCl, 10 mM β-ME, 10% glycerol, 0,05% Tween 20, 30mM Imidazole, 0,3 mM PMSF followed
597 by ion-exchange chromatography on Heparine agarose (Sigma). Then 6His- and S-tags were excised by
598 overnight incubation with recombinant TEV protease at 4 °C; tags and TEV protease were removed by
599 an additional round of affinity chromatography on Ni-NTA-agarose (Sigma). As a final step gel filtration
600 on a Superdex 75 column in 20 mM phosphate buffer, pH 7.5, 50 mM NaCl, 5% glycerol was applied.
601 750 µg of ovalbumin, 400 µg of carbonic anhydrase and 400 µg of lactalbumin were injected separately

to serve as standards. For the ^{15}N isotope labeling cells were cultivated at 37 °C in M9 minimal medium with 1 g/L $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories, Inc.).

Biophysical Measurements

CD spectra were recorded at the following temperatures: 5, 15, 25, 35 and 50 °C. A protein concentration of 0.3 mg/ml was used in 20 mM phosphate buffer, pH 7.5, 50 mM NaCl, 5% glycerol. CD measurements were made on a Chirascan CD spectrometer (Applied Photophysics) using a 0.1-mm path length.

The NMR samples with concentration of 0.2 mM ^{15}N -labeled protein were prepared in 90 % H_2O /10 % D_2O , 50 mM NaCl, and 20 mM sodium phosphate buffer (pH 7.2). Spectra were acquired at 298 K on a Bruker Avance 600 MHz spectrometer equipped with a triple resonance (^1H , ^{13}C and ^{15}N) pulsed field z gradient probe. NMR spectra were processed and analyzed using the Mnova software (Mestrelab Research, Spain).

Electrophoretic Mobility Shift Assay (EMSA)

The sequences of the oligonucleotides used in EMSA are in Table 1. EMSA experiments were performed as described (Malyavko et al., 2019) with the following modification. Reaction buffer contained 10 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 0.5 mM DTT, 0.25 mg/ml bovine serum albumin, 5% glycerol. Band intensities (BI) were quantified in ImageQuant TL 7.0, values of “fraction DNA bound” (FDB) were calculated using formula $\text{FDB} = \text{BI}^*(\text{complex})/(\text{BI}^*(\text{complex}) + \text{BI}(\text{free DNA}))$, where $\text{BI}^*(\text{complex})$ is BI of the complex minus BI of the corresponding area in the “no protein control” lane. The fits into the “Specific binding with Hill slope” were done using GraphPad Prism version 7.00.

Yeast two-hybrid (Y2H) system

Y2H experiments were carried out as previously described (Malyavko and Dontsova, 2020).

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Figure 1 – figure supplement 1. (A) Schematic illustration of the *leu2* locus on the chromosome I in the WT, intTEL0 and intTEL18 strains. “TEL18” – 18 telomeric repeats, “Subtel” – 304 bp region of the subtelomeric DNA derived from HARS36 (see METHODS). Arrows correspond to qPCR primers targeting the WSC3 gene (located right next to the internal telomeric DNA). (B) ChIP analysis. Chromatin from the indicated strains was immunoprecipitated on anti-HA magnetic beads. DNA was analyzed by qPCR with primers targeting internal telomeric repeats/WSC3 gene locus (“WSC3”), subtelomere region of the right end of chromosome VII (“TEL”), ALA1 gene locus (negative control, “ALA1”) or RPS2 gene locus (negative control, “RPS2”). The amount of DNA fragments in the IP samples as a percentage of the input DNA was calculated (% of input). Error bars indicate SD, n = 3. (C) same as (B) but with Rif1-HA-containing strains.

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898
899 **Figure 3 – figure supplement 1.** (A) The titration EMSA experiment with increasing concentration of
900 recombinant (tag-free) Rif1_{NTE}¹⁻²⁶⁴ (0, 1, 3, 10, 30, 100, 300, 1000, 3000, 10000 nM) and 30 nM FAM-
901 Tel4(G) (upper gel) or FAM-Tel4(GC) (lower gel) as probes. (B) Competition EMSA with 30 nM
902 FAM-Tel4(G), 1 μM (tag-free) Rif1_{NTE}¹⁻²⁶⁴ and increasing concentration of the indicated competitor
903 DNA oligonucleotides (0.1, 0.3, 1, 3, 10, 30 μM).

904
905 **Figure 4 – figure supplement 1.** Rifl_{NTE} truncation constructs were purified from *E. coli* as 6His-S-
906 fusions. 10 μ M of the indicated fragments were subjected to the EMSA using 1 μ M of FAM-Tel4(G)
907 probe. “–” – no protein control. “100x” – 100 μ M of the protein used in the reaction.

908

909 **Figure 4 – figure supplement 2.** (A) List of the ScRif1_{NTD} residues potentially involved in the DNA
910 binding (“ScRif1”) and corresponding residues in the HpRif1 (“HpRif1”) (alignment in the
911 Supplementary file 1). ScRif1 residues which were confirmed to be important for telomeric function are
912 in bold. Conserved residues are marked by red. * – different HpRif1 residues correspond to ScRif1 K514
913 according to alignments with different algorithms. (B) Anti-HA western blot analysis (upper panel).
914 Ponceau S-stained membrane (lower panel) served as a loading control. (C) ChIP analysis of the
915 indicated strains, same as in Figure 1D. (D) Southern blot analysis as in Figure 1B but with different
916 strains.

917

918 **Figure 5 – figure supplement 1.** Y2H analysis. AH109 colonies expressing pairs of the indicated
919 proteins (fused to either Gal4-BD (GBD) or Gal4-AD (GAD)); cultures with A₆₀₀ ~ 0.5 and four 10-
920 fold serial dilutions were plated on the SC medium lacking amino acids as indicated, and incubated at
921 30 °C for four days. KBS – Ku80 binding site.

922

923 **Figure 5 – figure supplement 2.** (A) Anti-HA ChIP analysis of the indicated strains, same as in Figure 1D. The
924 amount of DNA fragments in the IP samples is represented as a percentage of the input DNA (mean $\pm$ SD, n = 3).
925 (B) Southern blot analysis as in Figure 1B but with different strains. Mean lengths of the brightest TFR bands are:
926 WT 474 bp, $\Delta ku70$ 430 bp, $\Delta ku80$ 433 bp. WT telomere length reported to be ~160 bp (~20 telomeric repeats,
927 (Sohn et al., 1999)), therefore telomere length is reduced by ~25% in each knockout strain. (C, D) Anti-HA ChIP
928 analysis same as (A) (E) Southern blot analysis (as in Figure 1B) of the strains analyzed by ChIP in (D). Mean
929 length of the brightest TFR bands are: TER 485 bp, Δter 420 bp (telomere length is reduced by ~40% in the Δter
930 strains compared to TER). (F) RNA co-precipitated from the indicated strains on anti-HA agarose was analyzed
931 by qRT-PCR targeting HpTER or snU1 RNA (HpU1). The amount of RNA fragments in the IP samples is
932 represented as a percentage of the input RNA (mean $\pm$ SD, n = 3). (G) Y2H analysis. cultures with $A_{600} \sim 0.5$ and
933 four 10-fold serial dilutions were plated on the SC medium lacking amino acids as indicated, and incubated at 30
934 °C for four days.

Figure 6 – figure supplement 1. (A) Results of the analysis of random spores derived from the indicated heterozygous strains. After ether treatment and growth on YPD plate, 24 colonies derived from each heterozygous strain were randomly picked, resuspended in water, plated onto the indicated selective plates, grown for 4 days at 37 °C and photographed. Each plate also contained the parental heterozygous strains (phenotype: Ade⁺Ura⁺Leu⁺). None of the spores from strains CDC13^{+/-}; STN1^{+/-} and TEN1^{+/-} displayed the Leu⁺ phenotype, whereas 50% (12/24) of the spores from the KU70^{+/-} had the Leu⁺ phenotype (as expected for the non-essential KU70 gene). (B) Y2H analysis. AH109 colonies expressing pairs of the indicated proteins (fused to either Gal4-BD (GBD) or Gal4-AD (GAD)); cultures with A₆₀₀ ~ 0.5 and four 10-fold serial dilutions were plated on the SC medium lacking amino acids as indicated, and incubated at 30 °C for four days.

948
949 **Figure 6 – figure supplement 2.** Schematic representation of the putative protein-protein interactions
950 at *H. polymorpha* telomeres. Double thick line represents subtelomeric (gray) and telomeric (black)
951 DNA. Thin lines denote interactions, blunt arrows – inhibitory effect.