Regulation of telomere length requires a conserved N-terminal Domain of Rif2 in *S. cerevisiae*

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Abstract
The regulation of telomere length equilibrium is essential for cell growth and survival since critically short telomeres signal DNA damage and cell cycle arrest. While the broad principles of length regulation are well established, the molecular mechanism of how these steps occur is not fully understood. We mutagenized the RIF2 gene in Saccharomyces cerevisiae to understand how this protein blocks excess telomere elongation. We identified an N-terminal domain in Rif2 that is essential for length regulation, which we have termed BAT domain for Blocks Addition of Telomeres. Tethering this BAT domain to Rap1 blocked telomere elongation not only in rif2Δ mutants but also in rif1Δ and rap1C-terminal deletion mutants. Mutation of a single amino acid in the BAT domain, phenylalanine at position 8 to alanine, recapitulated the rif2Δ mutant phenotype. Substitution of F8 with tryptophan mimicked the wild-type phenylalanine, suggesting the aromatic amino acid represents a protein interaction site that is essential for telomere length regulation.

Introduction
The establishment and maintenance of telomere length equilibrium is essential for cell survival. Yeast cells that fail to maintain telomeres undergo senescence (Lundblad and Szostak 1989) which is mediated by the DNA damage response to short telomeres (Enomoto et al. 2002; Ijpma and Greider 2003). In primary human cell cultures short telomeres initiate replicative senescence (Harley et al. 1990;
Bodnar et al. (1998) by signaling DNA damage (D’adda Di Fagagna et al. 2003). This cellular response to short telomeres underlies a spectrum of human diseases that include bone marrow failure, pulmonary fibrosis, and immune senescence, collectively called the telomere syndromes (Armanios and Blackburn 2012). Conversely, inappropriate telomere maintenance allows the survival of cancer cells (Kim et al. 1994), and mutations that increase telomerase expression predispose people to familial melanoma (Horn et al. 2013; Huang et al. 2013) and other cancers (Heidenreich et al. 2014). To fully address the role of telomeres in disease, a detailed mechanistic understanding of telomere length maintenance is critical.

Telomere sequence DNA repeats are bound by a set of proteins that mediate two essential functions; first to protect the ends from degradation, recombination, and initiation of a damage response, and second to regulate telomere elongation by telomerase. Telomerase adds telomere repeats to chromosome ends to counterbalance the shortening that occurs during replication (Greider and Blackburn 1985), but this addition must be regulated. While the telomere-binding proteins in Saccharomyces cerevisiae and mammalian cells are not conserved in sequence, the function of telomere binding proteins limiting telomere elongation is conserved across eukaryotes (Smogorzewska and De Lange 2004). In yeast, deletion of the genes encoding the telomere binding proteins Rif1 and Rif2 leads to telomere elongation (Hardy et al. 1992; Wotton and Shore 1997). In mammals, removal of telomere binding proteins from the telomere including TRF1, TRF2, and POT1, also results in telomere elongation (Van Steensel and De Lange 1997; Smith and De...
Lange 2000; Colgin et al. 2003; Veldman et al. 2004; Palm and De Lange 2008). The conservation of this negative length regulation pathway highlights the importance of understanding the mechanism that limits telomere elongation.

In yeast, the Rap1/Rif1/Rif2 protein complex binds to the double-stranded telomere repeats (Longtine et al. 1989; Hardy et al. 1992; Wotton and Shore 1997) and the Cdc13 complex (Lin and Zakian 1996; Nugent et al. 1996) binds to the single-stranded G-rich 3’ overhang. Cdc13 interacts with Stn1 and Ten1 (Grandin et al. 1997; Grandin et al. 2001) to form a RPA (Replication Protein A)-like trimeric complex (Gao et al. 2007) that regulates telomere elongation. In addition to these DNA binding complexes, there are other proteins that associate with telomeres and help regulate both end-protection and telomere length. These include a number of proteins that are involved in DNA break repair and checkpoint signaling such as the MRX (Mre11, Rad 50, Xrs2) complex, the Ku70/80 heterodimer, and the Tel1/ATM protein kinase (Shore and Bianchi 2009; Wellinger and Zakian 2012).

In yeast, Rap1 recruits the Rif1 and Rif2 proteins that limit telomere elongation (Hardy et al. 1992; Marcand et al. 1997; Wotton and Shore 1997; Levy and Blackburn 2004). Deletion of either RIF1 or RIF2 results in long telomeres and deletion of the two together has an additive effect, resulting in very long telomeres. Rif1 and Rif2 bind to the C-terminal domain of Rap1, and deletion of this domain results in very long telomeres, similar to the rif1Δ rif2Δ double mutant (Wotton and
Shore 1997). This additive effect suggests these two proteins might use different mechanisms to limit telomere elongation.

A protein-counting model for negative regulation of telomere elongation was first proposed in yeast (Marcand et al. 1997). This model suggests the more Rap1/Rif1/Rif2 complexes that are bound along the telomere, the larger the repressive effect of telomere elongation. Thus, short telomeres have fewer repressive proteins bound and are more frequently elongated, whereas on longer telomeres the Rap1/Rif1/Rif2 exerts a strong repressive effect so these telomeres are elongated less frequently. Recruiting Rif1 and Rif2 to the telomere by fusion to other DNA binding domains also limits telomere elongation, indicating it is Rif1 and Rif2 function, rather than Rap1 per se, which limit telomere elongation (Levy and Blackburn 2004). The interplay of the preferential elongation of short telomeres (Bianchi and Shore 2008) and limiting the extent of elongation at long telomeres is thought to mediate the telomere length equilibrium.

The molecular mechanism by which Rif1 and Rif2 limit telomere elongation remains unclear. In mammalian cells Rif1 was shown to play a role in replication fork progression (Buonomo et al. 2009) and in the timing of replication origin firing (Cornacchia et al. 2012; Yamazaki et al. 2012). This role in regulating origin firing is conserved in yeast (Lian et al. 2011; Mattarocci et al. 2014; Peace et al. 2014) where Rif1 also functions in regulating resection of double-strand breaks and DNA.

Like RIF1, the RIF2 gene in yeast also plays a role in protecting chromosome ends from resection. rif2Δ mutants show increased single-stranded DNA at telomeres (Bonetti et al. 2010a) that requires the MRX complex and its regulator, the Tel1 kinase (Bonetti et al. 2010b). Moreover, in vitro experiments have suggested that Rif2 binds directly to the C-terminal region of Xrs2 and regulates telomere length through the Tel1 pathway (Hirano et al. 2009). Rif2 also protects telomeres from chromosome fusion events (Dubois et al. 2002), and the increased rate of senescence of telomerase mutants in the absence of RIF2 further supports a role for Rif2 in end protection (Chang et al. 2011; Ballew and Lundblad 2013; Hu et al. 2013). Whether this end-protection role of Rif2 is related to the telomere elongation phenotype of the rif2Δ is not known.

The Rif2 protein structure was recently determined both alone and in complex with the C-terminal region of Rap1 (Shi et al. 2013). This structure revealed two different areas of contact between Rif2 and Rap1. The authors propose a Velcro model of interlocking protein interactions between Rap1, Rif1, and Rif2, which generate a chromatin scaffold that limits telomere elongation.

To more clearly define the molecular functions of Rif2, we carried out a mutagenesis screen across the entire RIF2 coding region. We describe here the identification of a
domain in the N-terminus that blocks telomere elongation by telomerase. Furthermore, mutation of a single amino acid in this domain mimics telomere lengthening seen in deletion of rif2Δ suggesting that it is a critical residue for RIF2 function.

**Materials and methods**

**Construction of Plasmids and Yeast Strains**

All of the *S. cerevisiae* strains (termed yeast strains) and oligonucleotides (termed primers) for PCR (Polymerase Chain Reaction) and for construction of plasmids used in this study are listed in the supplementary tables S1-S6. All restriction enzymes used in these experiments were from New England Biolabs.

**NAAIRS mutagenesis**

We scanned the entire RIF2 gene substituting every six codons with the sequence specifying the amino acid sequence asparagine-alanine-alanine-isoleucine-arginine-serine (NAAIRS) in continuous blocks. RIF2 was amplified from yeast genomic DNA using primers Rif2-up and Rif2-down by PCR and the 1.67kb product was subcloned into pCR2.1-TOPO (Life Technologies) according to manufacturer’s instructions. RIF2 was then subcloned into pRS406 (Sikorski and Hieter 1989) using *Kpnl* and *EcoRI*. This plasmid was used to generate 66 rif2NAAIRS mutants using a 3-step PCR strategy (Mosher et al. 2006). Using the construction of NAAIRS2 as an example, the first PCR incorporated the sequence encoding the NAAIRS amino acids at positions 2 through 7 by amplifying the 5’ end of RIF2 using NAAIRS2 reverse primer, Rif2
forward primer, and the template p406-Rif2. In the second PCR, the anti-NAAIRS2 forward primer, Rif2 reverse primer, and the template p406-Rif2 were used to amplify the remaining 3’ end of RIF2, also incorporating the NAAIRS substitution at amino acids 2 through 7. The PCR products from reactions 1 and 2 were diluted 1:100 and used as templates for amplification in a third PCR using Rif2 forward primer and Rif2 reverse primer. This generated a full-length rif2NAAIRS2 product that was subcloned into pCR2.1-TOPO, and then inserted into pRS406 using KpnI and EcoRI to generate pNAAIRS2. Using this approach, 66 plasmids with the NAAIRS amino acid sequences substituted for continuous blocks of 6 amino acids within RIF2 were constructed. All plasmids were confirmed by sequencing and are available upon request.

**Integration of the RIF2-NAAIRS mutants at the URA3 locus**

The parental diploid strain for the rif2 mutagenesis was derived from W303-1a and W303-1α strains provided by O. Aparicio (OAy1002, OAy1003) (Viggiani and Aparicio 2006). RIF2 was deleted by PCR-based methods as described (Brachmann et al. 1998). Yeast strains containing the RIF2-NAAIRS mutations were constructed by transforming a RIF2/rif2Δ: kanMX4 diploid (CVy245) with the NAAIRS mutant plasmids cut with Ncol within the plasmid-borne URA3, which directs integration of the plasmid into the *ura3-1* locus. Transformants were selected for Ura+. Proper integration into the yeast chromosome was confirmed by PCR. The resulting *RIF2/rif2Δ::kanMX4, ura3-1/ura3-1::(rif2NAAIRS-URA3)* strains were sporulated,
dissected, and haploids of the desired genotype were grown for Southern blot analysis of telomere length. Following telomere length analysis the rif2NAAIRS integrant was PCR amplified from genomic DNA and sequenced to reconfirm the expected NAAIRS mutation. *RIF2* was integrated at the *URA3* locus to generate the wild-type control strain. Yeast culture conditions, transformations, and dissections were performed as described (Green and Sambrook 2012).

**Epitope tagged *RIF2***

We used two different epitope tags to determine the expression level of *RIF2* mutants by western analysis. We used a 13xMyc epitope to tag the NAAIRS mutants at the *URA3* locus and actin as a loading control. For the single amino acid mutants integrated at the *RIF2* locus we used a V5 epitope tag and phosphoglycerol kinase (PGK) as a loading control. We tagged *RIF2* and specific *rif2NAAIRS* mutants integrated at the *URA3* locus by a one-step PCR-based method using pFA6a-13xMyc-*His3MX6* (Longtine *et al.* 1998) using the Myc-tag forward primer and Myc-tag reverse primer. His\(^+\) integrants were verified by colony PCR. The *RIF2* single amino acid mutants at the endogenous *RIF2* locus were tagged with V5 epitope and constructed in the *RIF2*-V5 epitope tagged plasmid pHK70. The plasmid was assembled according to the protocol: Creating Insertions or Deletions Using Overlap Extension PCR Mutagenesis (Green and Sambrook 2012). This construct was made in two steps using plasmid p406-Rif2 described above that contains the *RIF2* coding region flanked by 245bp of genomic sequences. The primers used for each of the steps are given in parentheses. The first step amplified the C-terminus of *RIF2*
together with a glycine 8 (G8) linker (HK3, HK4), a unique NotI site (HK7, HK9), and V5 tag from pLenti6/UbC/V5-DEST Gateway Vector (Life Technologies) (HK5, HK6) which was cloned into p406-Rif2 cut with BspEI/NotI, creating pHK1. The second step used overlap extension to amplify the genomic region downstream of RIF2 (HK18, HK19), adjacent to the upstream region of RIF2 (HK20, HK15), and p406-Rif2 backbone which included a KpnI site (HK16, HK17). This step removed some of the upstream region of RIF2 retaining a unique BsrGI site. After assembly, this product was cloned into pHK1 cut with restriction enzymes BsrGI and KpnI creating pHK70. The product was sequence verified. Proper chromosomal integration of epitope tagged RIF2 and NAAIRS mutants were confirmed by PCR.

**Generation of single amino acid rif2 mutants**

Single amino acid changes in the N-terminal region at positions 2-49 were generated in RIF2 in the plasmid pHK70 according to Protocol 3: *In Vitro* Mutagenesis Using Double-Stranded DNA Templates: Selection Of Mutants With DpnI (Green and Sambrook 2012). To expedite this mutagenesis some of the point mutants (pHK28-34, pHK37-64) were made by GENEWIZ, Inc. The region of each construct containing a rif2 mutation was PCR amplified and sequenced to confirm the presence of the desired mutation. Plasmids containing the rif2 mutants were cut using AfeI (except where indicated otherwise) and targeted to the RIF2 endogenous locus in OAy1002 by recombination. In cases where the mutation created an AfeI site (pHK3, pHK5-27, pHK51) plasmids were digested using BsrGI and integrated at
the \textit{RIF2} locus in OAy1002. Proper integration of the \textit{rif2} mutants at the endogenous \textit{RIF2} locus was confirmed by PCR and verified by sequencing.

\textbf{\textit{RAP1-RIF2}_{60} fusion constructs integrated at the \textit{RAP1} locus}

We used Gibson assembly (Gibson 2011) to generate the integrating plasmid pHK35, containing the \textit{RAP1-RIF2}_{60} fusion gene. The final construct, based in the pRS405 vector, contains the following elements stitched together: the C-terminal region of \textit{RAP1} amplified from genomic DNA, fused to a flexible glycine 10 (G10) linker (HK89, HK98), the N-terminal 60aa of Rif2, including a stop codon, from p406-Rif2 (HK97, HK94), the 250bp \textit{CYC1} terminator from p414-GALS (ATCC87344) (HK117, HK118), the \textit{URA3} cassette from p406-Rif2 (HK93, HK96), and 176bp of genomic DNA 3' of \textit{RAP1} to target to the construct to the \textit{RAP1} locus (HK95, HK90). The construct was sequence verified and then digested with \textit{SacI}/\textit{NotI} and integrated at the \textit{RAP1} locus in CVy245 or HKy639. Ura\textsuperscript{+} integrants were verified by colony PCR, sporulated, and tetrads were analyzed. Strains with the desired genotypes were selected for Southern and western analysis.

The shorter version of the fusion protein, Rap1-Rif2\textsubscript{36}, was created from pHK35 using the mutagenesis method described above for the generation of single amino acids changes removing codons 37-60 (HK121, HK123) to create pHK68. The plasmid was sequence verified, digested with \textit{SacI}/\textit{NotI}, and then integrated at the \textit{RAP1} genomic locus in HKy639. Ura\textsuperscript{+} integrants were verified by PCR and diploids were sporulated to generate haploid cells of the specific genotype.
The mutant \textit{RAP1-RIF2}_{60} F8A, F8Y, and F8W fusion genes were created using the site-directed mutagenesis method described above for the generation of single amino acids changes using pHK35 as a template. The resulting plasmids (pHK65, pHK74, and pHK73, respectively) were sequenced, and those containing the desired mutations were digested with \textit{SacI}/\textit{NotI} and integrated at the \textit{RAP1} genomic locus. Proper integration of the \textit{RAP1} fusion genes was verified by colony PCR and sequencing.

The \textit{RAP1ΔC-RIF2}_{60} fusion gene was created using Gibson assembly of two fragments: the region amplified from genomic DNA which removes 498bp from the end of \textit{RAP1} at amino acid 662 (HK128, HK129) and the region from pHK35 which contains the Glycine 10 linker-\textit{Rif2}_{60}-\textit{Cyc1} components (HK130, HK131) that were re-assembled into pHK35 cut with \textit{NotI}/\textit{BglII}, creating pHK72. After sequence verification, the fusion construct was digested with \textit{NotI}/\textit{SacI} and introduced into the \textit{RAP1} genomic locus in HKy639 selecting \textit{Ura}^+ integrants (HKy768, 769). As a control, a construct containing only \textit{rap1ΔC} (HK128, HK132) that truncated the \textit{RAP1} gene at codon 662 was engineered in a similar fashion creating pHK71. This plasmid was sequence verified and integrated at the \textit{RAP1} genomic locus in HKy639 selecting \textit{Ura}^+ integrants (HKy754, 755).
Generation of RIF1, XRS2 and TLC1 deletion strains

To generate the RIF1 deletion the LEU2 cassette was amplified from pRS405 (OCC85, OCC86) (Brachmann et al. 1998) and integrated into the RIF1 locus of CVy245, yielding HKy639. Leu+ integrants were verified by PCR before tetrad analysis. A diploid yeast strain containing XRS2/xrs2ΔCt (yYM311), previously generated in our laboratory (Ma and Greider 2009), was transformed to delete the RIF2 locus using a PCR product containing the LEU2 cassette from pRS405 (OCC122, OCC123) (Brachmann et al. 1998) to generate JHUy912. The Rap1-Rif260 fusion was introduced into these strains by transformation to replace the RAP1 locus as described above.

A deletion of TLC1 was introduced into RIF2/rif2Δ, RAP1/rap1::(Rap1-Rif260 –URA3) (HKy551) by transformation of a PCR product from yeast strain YCC115 containing a tlc1Δ::LEU2 cassette (OCC168, OCC171) to generate two independent diploids HKy668 and HKy669. The Leu+ integrants were verified by PCR.

Southern analysis and telomere length measurement

Strains for telomere length analysis were grown overnight at 30°C in liquid medium yeast extract-peptone-dextrose (YPD). 5 OD600 of cells were collected per sample and washed with water. Genomic DNA was prepared from each strain as follows: Cell pellets were ruptured by 8 minutes of vigorous shaking (Eppendorf Mixer 5432) in equal volumes of 0.5mm glass beads (Biospec Products), phenol-chloroform (50:50), and lysis buffer (1% sodium dodecyl sulfate (SDS), 2%Triton X-
100, 100mM sodium chloride (NaCl), 10mM Tris,pH8.0, 1mM ethylenediamine tetracetic acid (EDTA)). DNA was precipitated in ethanol and resuspended at 37°C in TE (10mM Tris, pH 8.0, 1mM EDTA) and RNaseA (10µg/ml). Samples were digested with Xhol and separated by electrophoresis on a 1% agarose gel in 1X TTE buffer (20X=1.78M Tris Base, 0.57M Taurine, 0.01M EDTA). On each gel, 250ng of 2-log DNA ladder (NEB N3200) was included as a reference. After electrophoresis was complete, the gel was denatured for 30 minutes (0.2M sodium hydroxide (NaOH), 0.34M NaCl) and neutralized (1.5M NaCl, 0.5M Tris, pH 7.0) for 30 minutes before vacuum transfer (Boekel Appligene Vacuum Blotter) at 50mbar onto Amersham Hybond-N+ membrane (GEHealthcare) in 10X SSC (1.5M NaCl, 0.17M sodium citrate) for 1 hour. After UV-crosslinking (UV Stratalinker 2400, Stratagene), the membrane was pre-hybridized for 1-2 hours in Church buffer (0.5M Tris, pH7.2, 7% SDS, 1% bovine serum albumin, 1mM EDTA) and hybridized with a radiolabelled subtelomeric Y' fragment (750bp fragment generated by PCR from yeast genomic DNA using primers YPrimeFWD and YPrimeREV) and radiolabelled 2-log DNA ladder probe. Hybridized nylon membranes were exposed to Storage Phosphor Screens (GE Healthcare) and scanned on a Storm 825 imager (GE Healthcare). The images were converted using Adobe Photoshop CS6 and adjusted for contrast using the curves feature within the software. In the represented Southern blots, the numbers on the X-axis indicate the sizes of the 2-log ladder in kb. The numbers on the Y-axis represent the lane numbers in the agarose gel.
**Western blot analysis**

Yeast strains were grown at 30°C in YPD until OD$_{600}$ reached 0.4-0.6. Whole cell protein lysates were prepared by trichloroacetic acid (TCA) extraction as follows: 3 OD$_{600}$ of cells were collected and resuspended in 10mL of 10% TCA for 30 minutes. After centrifugation the TCA treated cells were resuspended in 1mL 1M HEPES buffer, pH7.5 and transferred into a microcentrifuge tube. Cells were pelleted and resuspended in 50µl 2X SDS- polyacrylamide gel electrophoresis (PAGE) sample buffer (125mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.2M dithiothreitol (DTT), 0.012% bromophenol blue dye (BPB)) then ruptured with an equal amount of 0.5mm glass beads for 3 minutes on a high-speed vortex mixer. An additional 50µL of 2X SDS-PAGE buffer was added to each sample, followed by 5 minutes at 100°C and 15 seconds of vigorous shaking. The samples were centrifuged for 10 minutes at top speed in a microcentrifuge and the supernatant collected and stored at -20°C.

Whole cell lysate (3µL) was loaded per lane on a 10% TGX SDS-PAGE gel (BIO-RAD) along with molecular weight protein standards (BIO-RAD #161-0373) and resolved by electrophoresis. The proteins were transferred to 0.45µm Immobilon FL membrane (Millipore) according to recommended protocol (BIO-RAD). All Blue, Precision Plus Protein Standards (BIO-RAD #161-0373) were used as molecular weight markers. All transferred membranes were blocked in Odyssey Blocking Buffer (LI-COR) for 1 hour at room temperature (RT). The membranes were washed three times in 1X wash buffer (10mM Tris, pH8.0, 150mM NaCl, 0.1% TritonX-100,
0.01% IGEPAL CA-630 (SIGMA)) for 15 minutes, 5 minutes, and 5 minutes at RT. When using LI-COR detection, the final wash contained no detergents (1X TBS: 10mM Tris, pH8.0, 150mM NaCl). The 13XMyC epitope was detected with a 1:10,000 dilution of Anti-Myc 9E10 antibody (National Cell Culture Center, Minneapolis, MN). Anti-actin (1:500 dilution of anti-actin, SigmaA2066) and anti-phosphoglycerate kinase (PGK) (1:10,000 dilution of Anti-PGK, Invitrogen459250) antibodies served as loading controls. The appropriate species IRDye secondary antibodies (LI-COR) were diluted at 1:15,000 in Odyssey Blocking Buffer and incubated for 2 hours at RT. Immunoblots were analyzed on an Odyssey Infrared Imaging System (LI-COR Biosystems) using the quantification software provided. The Rif2 protein levels of the samples were compared to the actin or the PGK loading control; this ratio in the wild type (WT) was set to 1 and the other samples were normalized to this value. The Rif2 protein level from two independent haploids was determined, and the average of these two values is reported in Figure 1.

The V5-epitope was detected with a 1:2000 dilution of Anti-V5 antibody (Invitrogen #460705) for one hour at RT. Anti-mouse Immunoglobulin G horseradish peroxidase (IgG-HRP)-linked secondary antibody (Cell Signaling Technologies #7076) was diluted 1:10,000 in 5% milk (BIO-RAD), 0.05% TritonX-100 and incubated for 45 minutes at RT. SuperSignal West Femto Maximum Sensitivity Substrate (Thermoscientific #34095) was used to detect the chemiluminescent signal on an ImageQuant LAS4000mini (GE Healthcare). Since the loading control co-migrated with the V5-tagged Rif2, the blots were stripped with Restore Western Blot
Stripping Buffer for 25 minutes at room temperature (Thermoscientific #21059), washed three times in 1X TBS as described above, and verified that no signal was present. After treating 30 minutes – 1 hour in Odyssey Blocking Buffer, the membranes were then incubated for one hour at RT with 1:10,000 of Anti-PGK using the same IgG-HRP-secondary antibody as described. The Rif2 protein levels of the samples and loading controls were quantitated using ImageJ (Schneider et al. 2012) and normalized relative to WT levels.

Results

Scanning mutagenesis of RIF2

To better understand the role of Rif2 in telomere length maintenance, we scanned the entire coding region of RIF2 substituting every 6 contiguous codons with a sequence encoding asparagine-alanine-alanine-isoleucine-arginine-serine (NAAIRS) (Lonergan et al. 1998); generating a set of 66 rif2-NAAIRS mutants. The mutations were named for the first codon where the substitution begins. For example, NAAIRS2 substitutes codon 2-7, and NAAIRS8 substitutes codons 8-13. Each mutant was integrated into the yeast genome at the URA3 locus in a RIF2/rif2Δ heterozygous diploid and verified by sequencing. Wild-type RIF2 integrated at URA3, as a control, was able to complement a rif2Δ (data not shown). Telomere length was examined in rif2Δ::kanMX4 ura3-1::rif2NAAIRS-URA3 haploid segregants by Southern blot and classified as wild-type, long (similar to rif2Δ), or medium (between rif2Δ and wild-type). There were a number of regions in RIF2
where *NAAIRS* mutations resulted in a long telomere phenotype similar to *rif2Δ* (Figure 1 and Figure S1).

To determine whether the mutant proteins were expressed at wild-type levels, we tagged each of the mutants that showed long telomeres with a 13xMyc tag and quantified Rif2 protein levels by western analysis. As a control, we tagged a wild-type copy of *RIF2* integrated at the *URA3* locus and showed the 13xMyc tag did not affect telomere length (Figure S2A). A number of the NAAIRS substitutions resulted in significantly reduced protein expression, for example the mutations NAAIRS212 and NAAIRS338 (Figure S1A), disrupted protein stability suggesting the long telomeres in these mutants were likely due to low Rif2 levels. However, there was a distinct subset of mutants in which wild-type or near wild-type protein levels were present and yet the NAAIRS substitution resulted in longer than wild-type length telomeres (Figure 1). Strikingly, 7 of 11 of the long or medium length mutants that expressed at least 50% protein levels were in the N-terminal domain of Rif2. We thus focused our attention on this N-terminal region.

While this work was underway, the Thoma lab reported the crystal structure of Rif2 protein and Rif2 complexed with the C-terminal domain of Rap1 (Shi *et al.* 2013). Rif2 contains a large central AAA+ domain with Walker A and Walker B motifs. The crystal structure revealed a C-terminal domain in Rif2 that interacts with Rap1 (Figure 2A). The N-terminal domain of the Rif2 protein from amino acid (aa) 1-60 was mostly unstructured but contained a short helix from T37-K48 called the Rap1
Binding Motif (RBM). A peptide spanning Rif2 residues 30-49 bound to the Rap1 C-terminal domain in solution. This peptide has two residues, L42 and L44, which have crystal contacts with the Rap1 C-terminal domain, while residues 49-60 were unstructured (Shi et al. 2013). As mentioned above, the block of NAAIRS mutations that affected protein function (NAAIRS 2- NAAIRS44) was located in this unstructured N-terminal domain of Rif2.

*N-terminal rif2 point mutants have long telomeres*

The N-terminal region of Rif2 is highly conserved among *Saccharomyces* (Figure 2B). To probe this region more closely, we substituted each individual residue from aa 2-37 with alanine (or a different residue if alanine was the wild-type residue). These point mutants were tagged with a V5 epitope and integrated at the *RIF2* genomic locus in haploid cells. The telomere length of two independent transformants of each mutant was compared to four controls: wild type, *RIF2, rif2Δ*, and the original NAAIRS mutant haploid strain (Figure 2C and Figure S3). Control experiments showed that the V5 tag did not affect telomere length at the wild-type *RIF2* locus (Figure S2B). If discordant results were obtained with two independent transformants, additional transformants were analyzed to determine the effect of that mutant.

Mutations in six residues in the N-terminal region resulted in telomere elongation: D5, F8, I11, R12, R13, and D29 (Figure 2C and Figure S2C, also see Figure S3). Mutants in F8, I11, R12, and R13 were expressed at or just above wild-type levels,
while D5 and D29 were slightly reduced (Figure S2D). All of these residues fall within the unstructured region in the crystal structure (Shi et al. 2013). Remarkably, the single amino acid change F8A showed significant telomere lengthening comparable to both the 6-codon change in rif2-NAAIRS8 and rif2Δ (Figure 2C), suggesting this is a key residue in Rif2 that is critical for telomere length regulation. In addition to residues with a major effect, there were also residues that had smaller effects. For some NAAIRS mutants, such as rif2-NAAIRS14 and rif2-NAAIRS20, none of the single mutants affected telomere length, however when all 6 codons were mutatated in combination, telomere lengthening was observed (Figure S3). These results are consistent with this region being a binding site for some protein, in which docking of the F8 residue is the most critical and neighboring residues contribute to the interaction.

The N-terminal domain of Rif2 mediates telomere length regulation

Because the mutants in the N-terminus of Rif2 mimic loss-of-function of the Rif2 protein, we next asked whether this N-terminal domain would function alone if tethered at the telomere. We generated a fusion gene encoding the N-terminal 60 codons of RIF2 fused to the C-terminus of RAP1. We will refer to this construct as RAP1-RIF2_{60} and to the resulting fusion protein as Rap1-Rif2_{60} (Figure 3A). We chose the N-terminal 60 aa of Rif2 because in the crystal structure the well-structured protein begins at residue 61. To promote flexibility of the fusion domain, we added a flexible glycine linker sequence between Rap1 and the Rif2_{60} N-terminal domain. The RAP1-RIF2_{60} construct was transformed into a RIF2/rif2Δ heterozygous
diploid and integrated at the RAP1 genomic locus. RIF2/rif2Δ, RAP1/RAP1-RIF2₆₀ double heterozygotes were sporulated and telomere lengths were examined in two independent spores as well as in the diploids. Remarkably, the RAP1-RIF2₆₀ construct fully suppressed the telomere lengthening in a rif2Δ mutant (Figure 3B lanes 4 and 5). Moreover, this domain dominantly shortened telomeres since telomeres were shorter in the heterozygous diploid RIF2/rif2Δ containing the RAP1-RIF2₆₀ construct than in the parental diploid (Figure 3B lanes 2 and 3). This shortening effect was also observed in haploid cells. Wild-type cells expressing the fusion had telomeres shorter than wild-type cells without the fusion (Figure 3B lanes 6 and 7). This gain-of-function effect may result from alteration of the regulated cell cycle dissociation of Rif2 from the telomere (Smith et al. 2003), as discussed below. The suppression of the rif2Δ phenotype by the RAP1-RIF2₆₀ construct suggests that tethering this functional domain of Rif2 at the telomere blocks excessive telomere elongation.

Rif1 and Rif2 act through different pathways to limit telomere extension (Wotton and Shore 1997). To test whether the RAP1-RIF2₆₀ construct would also block rif1Δ telomere elongation, we generated a triple heterozygous diploid: RIF1/ rif1Δ, RIF2/ rif2Δ, RAP1/RAP1-RIF2₆₀. Telomere length was measured in two independent haploids of each genotype (Figure 3C). The Rap1-Rif2₆₀ fusion protein fully suppressed the long telomeres in both rif1Δ and in rif1Δ rif2Δ mutants.
We noted that there was a slight difference in telomere length in \textit{rif1Δ rif2Δ} cells compared to \textit{rif2Δ} cells expressing the fusion construct (Figure 3C compare lanes 10 and 11 to lanes 18 and 19). To test whether this difference was due to insufficient cell divisions needed to reach steady state, we passaged cells four times in subcultures. The telomere length of each mutant expressing the fusion protein was stable over the successive passages (Figure S4). The slight difference in final telomere length in \textit{rif1Δ} or \textit{rif2Δ} mutants expressing the \textit{RAP1-RIF2}_{60} construct might reflect the fact that Rif1 affects telomere length through a different pathway than Rif2 (Wotton and Shore 1997).

To determine whether telomere shortening in cells expressing \textit{RAP1-RIF2}_{60} construct was working through the telomerase pathway we deleted the telomerase RNA component, \textit{TLC1}, in a \textit{RIF2/rif2Δ, RAP1/RAP1-RIF2}_{60} diploid. Telomere elongation in \textit{rif2Δ} mutants was blocked by the loss of telomerase (Figure S5), as shown previously (Teng \textit{et al.} 2000). Expression of the \textit{RAP1-RIF2}_{60} fusion in \textit{tlc1Δ} had little affect on telomere length, while \textit{tlc1Δ rif2Δ} cells not expressing the fusion construct had slightly longer telomeres than those expressing the construct (Figure S5A compare lanes 10 and 11 to lanes 12 and 13). While this slight difference could be due to effects of the fusion protein on other telomere maintenance pathways, we suspect it is due to increased telomere recombination in the \textit{tlc1Δ rif2Δ} cells. The loss of Rif2 in a telomerase mutant has previously been shown to promote recombination and accelerate survivor formation (Teng \textit{et al.} 2000; Chang \textit{et al.} 2011; Ballew and Lundblad 2013; Hu \textit{et al.} 2013). We found that
the rapid appearance of survivors in tlc1Δ rif2Δ cells led to slightly longer terminal
Xhol telomere bands than tlc1Δ (Figure S5B compare lanes 4 and 5 to lanes 6 and
7). Expression of the fusion construct may delay telomere recombination. Taken
together, the telomere shortening caused by the Rap1-Rif2Δ fusion protein and the
requirement for telomerase indicate that this small domain of Rif2 can block
telomere over-elongation by telomerase in both rif1Δ and rif2Δ mutants. We will
refer to this functional N-terminal domain as the BAT domain for _Blocks Addition of_Telomeres.

_The Function BAT domain does not require interaction with RAP1 C-terminal domain_

The N-terminal 60 amino acids of Rif2 include a small helical domain termed RBM
between positions T37-K48 that makes crystal contacts with the C-terminal domain
of Rap1 (Shi et al. 2013). To determine whether the binding to Rap1 C-terminus is
required for the function of the BAT domain we took two approaches: first we
removed the C-terminus of Rap1, and second we created a fusion protein with a
shorter version of the BAT domain that lacks the RBM region (Figure 4A).

We generated a new fusion construct (termed _RAP1ΔC -RIF2_60) in which the Rap1 C-
terminus is truncated at amino acid 662 with the Rif2 BAT domain fused to the Rap1
DNA binding domain. The expression of the _RAP1ΔC -RIF2_60 fusion completely
suppressed the long telomere phenotype in rif1Δ, rif2Δ, and rif1Δ rif2Δ mutants
(Figure 4B). This result indicates the BAT domain does not need the Rap1 C-
terminus to block telomere elongation. As a control, in the same strain we generated
a Rap1ΔC-truncated at amino acid 662 that lacks Rif2 BAT domain. As expected expression of the Rap1ΔC-truncation showed long telomeres and did not rescue telomere length in \textit{rif1Δ, rif2Δ, or rif1Δ rif2Δ} (Figure S6).

Next we examined a fusion with a shortened BAT domain, \textit{RAP1ΔC-RIF2}_{36}, which lacks the RMB residues (Figure 2). Expression of Rap1-Rif2_{36} fully blocked telomere elongation in \textit{rif1Δ, rif2Δ, and rif1Δ rif2Δ} mutants (Figure 4C and Figure 4D). While the \textit{RAP1-RIF2}_{36} construct restored wild-type telomere length, it did not cause shortening below wild-type length like the \textit{RAP1-RIF2}_{60} construct (Figure 4C and Figure 4D). This lesser degree of telomere shortening by the \textit{RAP1-RIF2}_{36} compared to the \textit{RAP1-RIF2}_{60} could be due to a slightly reduced accessibility or flexibility of this shorter domain. The lack of requirement for the Rap1 C-terminal domain, together with the ability of the \textit{RAP1-RIF2}_{36} to fully restore wild-type telomere length, suggest that the BAT domain of Rif2 does not require the presence of the Rap1 C-terminus to affect telomere shortening.

\textit{XRS2 C-terminus does not mediate the function of the Rif2 BAT domain}

Recent studies have suggested that Rif2 binds to the C-terminal region of Xrs2 and that this blocks Tel1 telomere association, thereby limiting telomere elongation (Hirano \textit{et al.} 2009). To determine whether the C-terminal domain of \textit{XRS2} is required for the ability of the BAT domain to limit telomere length, we introduced the \textit{RAP1-RIF2}_{60} construct into a doubly heterozygous strain \textit{RIF2Δ/rif2Δ XRS2/xrs2ΔCt} expressing an Xrs2 C-terminal truncation protein that fails to bind
Tel1 (Ma and Greider 2009), and examined telomeres in the single and double mutant haploid segregants. Expression of the Rap1-Rif2<sub>60</sub> fusion protein resulted in significant shortening in the rif<sub>2</sub>Δ xrs2ΔCt cells (Figure 5 lanes 16 and 17). These results suggest that Rif2 BAT domain can regulate telomere length independent of the Xrs2 C-terminal domain.

**Mutations at F8 abolish the shortening effect of Rap1-Rif2<sub>60</sub> fusion protein**

In the NAAIRS scanning mutagenesis experiments, we identified the F8 residue as playing a major role in Rif2 function. To determine if this amino acid was also important in the effect of the Rap1-Rif2<sub>60</sub> protein, we made a fusion construct containing this F8A substitution. When this mutant RAP1-rif2<sub>60</sub> [F8A] fusion construct was expressed in a rif<sub>2</sub>Δ background telomere shortening did not occur (Figure 6A lanes 14 and 15), indicating the RAP1-rif2<sub>60</sub> [F8A] mutation renders the BAT domain non-functional. Curiously, long telomeres were seen when the RAP1-rif2<sub>60</sub> [F8A] fusion construct was expressed in wild-type haploids (Figure 6A lanes 9 and 10) suggesting that tethering the mutant BAT domain to Rap1 dominantly interferes with normal length regulation. Since this construct is the only copy of RAP1 in the cell, all of the telomeres should be bound by the Rap1-Rif2<sub>60</sub> [F8A] protein and thus may interfere with the function of the wild-type Rif2.

**Aromatic amino acids tryptophan and tyrosine can mimic phenylalanine at F8.**

The functional importance of the F8A mutation in both the RIF2 gene and in the RAP1-rif2<sub>60</sub> [F8A] fusion suggests this region may be a protein-protein interaction
site. To test whether aromatic phenylalanine may be specifically recognized, we substituted this amino acid with either of the similar aromatic amino acids, tryptophan or tyrosine. Remarkably, the RAP1-rif2Δ [F8W] restored telomere shortening in rif2Δ, rif1Δ, and rif1Δ rif2Δ (Figure 6C and Figure 6D), indicating an aromatic amino acid can at least partially restore the function of the BAT domain. The RAP1-rif2Δ [F8Y] mutation more closely resembled the RAP1-rif2Δ [F8A] mutant, suggesting this residue may interfere with function. These experiments support the model that the F8 aromatic residue in the BAT domain is an important binding determinant for an as yet unknown protein that limits telomere elongation.

**Discussion**

To probe the mechanism of telomere length regulation, we carried out scanning mutagenesis of RIF2 and identified an N-terminal domain that is essential for blocking telomere elongation. The substitution of a single amino acid, F8A, within the BAT domain mimicked the long telomeres in a rif2Δ mutant. Further, tethering this 60 amino acid domain to Rap1 fully blocked telomere elongation. The F8 residue was essential for this blocking excess elongation, as the RAP1-RIF2Δ [F8A] fusion did not block telomere elongation in rif2Δ mutants. These results imply the BAT domain regulates telomere length by a similar mechanism in the context of either full length RIF2 or as an isolated domain tethered to RAP1.

The Rap1/Rif1/Rif2 scaffold is not essential to block telomere elongation
Our results suggest the recently proposed molecular Velcro model for telomere length regulation may be incomplete. In this Velcro model Rif1, Rif2, and Rap1 are suggested to generate an interlocking molecular scaffold that limits telomerase access to the telomere (Shi et al. 2013). Specifically Rif2 is proposed to contribute to the scaffold by bridging two Rap1 molecules, binding one through the RBM and the other through the AAA+ domain. Rif1 is likewise proposed to make contact with two Rap1 molecules to further support the scaffold. While our data do not address whether this scaffold forms in wild-type cells, they do suggest that such a structure is not required to block the elongation of telomeres by telomerase.

**BAT domain as a functional protein-binding site**

The modular nature of the BAT domain, and the critical F8 residue, suggests that this region of Rif2 may be a protein-protein interaction domain. The high conservation of the BAT domain in *Saccharomyces* (Figure 2B) further supports a role in protein binding. We propose that the F8 residue is the critical determinant in a binding site and that surrounding amino acids also contribute to important protein contacts. When residues near F8 were singly mutated there was less effect on telomere length than when they were mutated together as group of six residues (Figure 2). This additive effect was also seen in the NAAIRS2, NAAIRS14, and NAAIRS20 mutants, in which the single amino acid changes did not have as strong an effect as the group of 6 mutations (Figure S3). This finding suggests that there is a protein interaction interface over a region surrounding F8, and this aromatic residue is the key player in a protein interaction. The BAT domain may recruit an
unknown protein as described below or it may interact directly with known proteins such as Cdc13, Stn1, Ten1, or telomerase to block elongation. We ruled out the Xrs2 C-terminal domain as playing a major a role (Hirano et al. 2009) since Xrs2 C-terminal truncations still showed telomere shortening with the Rap1-Rif2$_{60}$ fusion protein.

**The Rap1-Rif2$_{60}$ fusion causes telomere shortening**

The dominant effect of the Rap1-Rif2$_{60}$ fusion in diploid cells and its ability to shorten telomeres in haploids may be due to altered cell cycle regulation, altered affinity, loss of end protection, or a combination of these factors. The cell cycle regulated association of Rap1 and Rif2 with the telomere differ; Rap1 telomere association increases while Rif2 decreases in late S-phase (Smith et al. 2003). It may be this dissociation of Rif2 in late S-phase that allows telomere elongation. In our experiment, by tethering the functional BAT domain of Rif2 to Rap1 this dissociation at late S-phase cannot occur and thus telomere elongation may be more efficiently blocked.

The Rap1-Rif2$_{60}$ fusion also shortened telomeres in a rif1Δ mutant. Rif1 regulates telomere elongation through a mechanism independent of Rif2, thus the ability of the Rap1-Rif2$_{60}$ to block telomerase elongation after the loss of Rif1 suggest the mechanism by which BAT blocks telomere elongation is not pathway specific. Experiments by Levy and Blackburn also showed that both the rif1Δ and rif2Δ long telomere phenotypes could be counteracted by overexpression of a Rap1-PDZ
fusion protein that allows multimerization of Rap1 (Levy and Blackburn 2004). This further suggest that while Rif1 and Rif2 may normally act through different pathways, strengthening just one of those pathways may be sufficient to block telomere over-elongation.

**Putting the pieces together: a model for Rif2 BAT domain function**

Our data suggest that the BAT domain of Rif2 is a protein-binding domain that limits telomere elongation by telomerase. This domain may directly block telomere elongation or may recruit another protein which binds to the F8 residue in the BAT domain and block telomere elongation as depicted in Figure 7A. When Rif2 is missing, telomerase can over-extend the telomeres (Figure 7B). However tethering the BAT domain directly to Rap1 restores and even strengthens the block to telomerase (Figure 7C). The specific mechanism by which the BAT domain blocks telomerase elongation is not clear; it might directly interfere with the catalytic subunit Est2 recruitment or it could affect elongation indirectly by altering telomere processing or C-strand synthesis. In addition to blocking telomerase, Rif2 plays a role in end protection. Rif2 blocks telomere recombination (Teng et al. 2000), thereby delaying survivors (Chang et al. 2011; Ballew and Lundblad 2013; Hu et al. 2013). Rif2 also protects telomeres from fusion (Dubois et al. 2002) and from nuclease processing (Bonetti et al. 2010a). The increased rate of telomere sequence turnover in rif2Δ mutants (Krauskopf and Blackburn 1996) likely reflects the combined effects of increased telomere degradation, elongation, and recombination. It is not yet clear whether the functions of Rif2 in end-protection and in blocking
telomerase elongation are separable or are the result of one mechanism. Our data support the role of Rif2 in blocking telomere recombination in telomerase mutants (Figure S5) and suggest that the BAT domain plays a role in this function.

**Telomere length regulation throughout evolution**

The negative regulation of telomere elongation in length homeostasis is conserved throughout evolution. While Rif2 protein sequence is not conserved from yeast to humans, the loss of telomere binding proteins leads to telomere elongation in mammals as well (Palm and De Lange 2008). Rif2 in *Saccharomyces cerevisiae* is a paralog of the conserved Orc4 protein (Byrne and Wolfe 2005). Analysis of synteny indicates that Rif2 was generated by divergence after a whole genome duplication that occurred early in the *Saccharomyces* lineage (Barnett 2004). Interestingly, the sequence conservation of *RIF2* and *ORC4* (Marcand *et al.* 2008) does not include the BAT domain suggesting *RIF2* acquired this regulatory module after the duplication and divergence from *ORC4*. This functional BAT domain that limits telomere elongation may be a conserved feature of telomere length regulation, but it may be attached to different telomere proteins in different organisms. A regulatory motif with similar function to the BAT domain may be present on other proteins and contribute to conservation of the telomere length equilibrium mechanism across species.
Acknowledgments

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Figure Legends

Figure 1. Summary of telomere length and protein expression in rif2-NAAIRS mutants. The telomere length of each of the rif2-NAAIRS mutants was categorized as wild-type (WT), medium, or long. For mutants with medium or long telomeres, the rif2-NAAIRS construct was tagged with the 13xMyc epitope and the relative level of Rif2 protein was measured by western analysis. For each mutant, protein level in two independent haploids was measured and normalized to the loading control and to wild-type Rif2 protein levels (See Materials and Methods). Each mutant was characterized as having WT (>70%), medium (50-70%), or low expression level (0-50%). Highlighted mutants indicate those with either WT or medium Rif2 expression level and telomeres longer than WT. NAAIRS mutants that had WT telomere length were not retested to examine protein levels. Representative examples of Southerns and westerns for these mutants are shown in Figure S1.

Figure 2. Point mutants in RIF2 disrupt protein function. (A) The domain structure of Rif2 protein is shown. The C-terminal region binds Rap1. There is an AAA+ domain that contains Walker A and B motifs (designated WA and WB). The N-terminal domain is not well structured but contains a short helix (RBM) that interacts with Rap1. The regions in white have no known structure. (B) The alignment of residues in the Rif2 N-terminal region from five Saccharomyces species is shown. Identical residues are highlighted in blue, those residues with strong similarity are in dark gray, and those with weak similarity in light gray. (C) Southern blot analysis of telomeres from rif2-NAAIRS8 and the six individual mutations within the rif2-NAAIRS8 mutant. The size markers on the side represent kb. Two independent haploid transformants for each single point mutant are shown.

Figure 3. Rap1-Rif260 fusion protein blocks telomere elongation. (A) Schematic of Rap1-Rif260 fusion protein. The full length RAP1 coding region was fused in frame to a glycine 10 linker followed by the first 60 codons of RIF2. (B) rif2Δ mutants expressing the RAP1-RIF260 construct. The genotype of the strain is indicated in each lane and the presence or absence of the fusion protein is shown with a + sign in each lane. The parental diploid was transformed with the fusion construct to yield the heterozygous diploid (Het. diploid) and was dissected to generate the haploid segregants. (C) Expression of RAP1-RIF260 construct in rif1Δ mutants and rif1Δ rif2Δ mutants. The genotype of the strain is indicated in each lane and the presence or absence of the fusion protein is show with a + sign. The size markers on the side represent kb.

Figure 4. The C-terminal domain of Rap1 is not required for BAT domain function. (A) Schematic of three fusion protein constructs. (B) rif1Δ, rif2Δ and rif1Δ rif2Δ double mutants expressing rap1ΔC-RIF260 construct were analyzed by Southern blot. The genotype of the strain is indicated above each lane and the presence or absence of the
fusion construct is shown with a + sign in each lane. (C) rif2Δ mutants expressing the fusion construct with a shortened Rif2 N-terminus, RAP1-RIF2_{36}. The genotype of the strain is indicated in above each lane and the presence or absence of either the RAP1-RIF2_{36} construct or RAP1-RIF2_{60} construct is shown with a + sign. (D) rif1Δ and rif2Δ double mutants expressing the shortened Rif2 N-terminus, RAP1-RIF2_{36}. The genotype of the strain is indicated in above each lane and the presence or absence of either the RAP1-RIF2_{36} construct or the RAP1-RIF2_{60} construct is shown with a + sign. The size markers on the side represent kb.

**Figure 5. Xrs2 C-terminus is not required for Rif2 BAT domain to shorten telomeres.** Southern blot telomere analysis of xrrs2ΔCt and rif2Δ xrrs2ΔCt cells expressing RAP1-RIF2_{60} construct. The genotype of the strain is indicated above each lane and the presence or absence of either the RAP1-RIF2_{36} construct or the RAP1-RIF2_{60} construct is shown with a + sign. The size markers on the side represent kb.

**Figure 6. Aromatic residue at position 8 is important for Rif2 BAT domain function.** (A) Southern blot telomere analysis of WT and rif2Δ cells expressing either RAP1-RIF2_{60} or RAP1-rif2_{60} [F8A] mutant fusion construct. The genotype of the strain is indicated above each lane and the presence or absence of the given fusion protein is shown with a + sign. (B) Southern blot telomere analysis of RIF1 RIF2, rif2Δ, rif1Δ, and rif1Δ rif2Δ mutants expressing RAP1-rif2_{60} [F8A] fusion or no fusion. The genotype of the strain is indicated above each lane and the presence or absence of RAP1-rif2_{60} [F8A] is shown with a + sign. The parental diploid was transformed with the fusion construct to yield the heterozygous diploid (Het. diploid) and was dissected for the haploid segregants. (C) Southern blot telomere analysis of WT, rif2Δ, rif1Δ, and rif1Δ rif2Δ mutants expressing RAP1-rif2_{60} [F8W] fusion. The genotype of the strain is indicated above each lane and the presence of RAP1-rif2_{60} [F8W] is shown with a + sign. (D) Southern blot telomere analysis of WT, rif2Δ, rif1Δ, and rif1Δ rif2Δ mutants expressing RAP1-rif2_{60} [F8Y] fusion. The genotype of the strain is indicated above each lane and the presence of RAP1-rif2_{60} [F8Y] fusion protein is shown with a + sign. The size markers on the side represent kb.

**Figure 7. Model for separation of function of Rif2 and the BAT domain.** (A) In RIF2 cells the Rap1 protein, shown in green, binds to the telomeric double-stranded DNA and the C-terminal domain (dark green) recruits Rif1 (not shown for simplicity) and Rif2 (purple). Rif2 binds the Rap1 C-terminal domain and blocks nuclease activity and telomerase elongation. The N-terminal BAT domain of Rif2 (dark purple) contains a critical phenylalanine residue. The F8 residue in the BAT domain serves as a protein recognition motif for a critical protein (Prot. X) that limits telomerase elongation of the telomere. (B) In rif2Δ telomerase elongation is not blocked by Rif2. (C) When the BAT domain is fused directly to the Rap1 C-terminal region in the RAP1-RIF2_{60} fusion construct, even in a rif2Δ mutant, there is strong blocking of telomerase.
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Figure 2

A

RBM
AAA+ domain

Rap1 binding

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B

S. cerevisiae

1  -MEHVDSDFAPIRRSKKVVDSDKIVKAISDDLEQKNFTVLRKLNLVPIKK 49

S. bayanus

1  -MEHLESDFAPIRKSQKVVDSDKIVRAINDDLEQKNFTILQRLSLVPIKK 49

S. mikatae

1  MMEHVDSEFAPIRSEKVVDSDKIVRAINDDLEQnitILQNLNLPLPNNK 50

S. paradoxus

1  -MEHVDSDFAPIRSEPPVDSDKIVAINDDLEQKNPTVLRKLNLVPIKK 49

S. kudriavzevii

1  -MQHVDSDFAPIRSEKVVDSDKIVRAINDDLEHNNPFLQRLSLVPIKK 49

S. cerevisiae

50 SVSSPKVCKPSPVKERVDHVFYQKFKSMALQELGTNYLSISYVPSLSKFL 99

S. bayanus

50 SVKGSKVSKPRSVKRRVDHTFYQDFKSMALDELSANYSSVGYISGLNSFL 99

S. mikatae

51 NAGSSKVSKPNPVKGQADYTFYQNFKSMALQELNENYSSVSYVPGMNNFF 100

S. paradoxus

50 SVSSSKVSKASPVKEPMDHAFYQKFKSRHALQELSTSYSSISYVPGLSEFL 99

S. kudriavzevii

50 NANSSRVSKRSSIEKRVNHGFYREFKSVALEDLSDNYSSVSYIAGLNKFL 99

Identical   strong similarity   weak similarity

C

1  2  3  5  6  8

rif2
rif2
NAA1RS8
F8A
A9F
P10A
17A
R12A
R13A
rif2
rif2

1.2
1.0
1.5
1.3
1.1
1.4
1.6
1.8
2.0

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Figure 3

A

Rap1-Rif260

Rap1
CTD
Rif260

Linker

B

C

B: Parent diploid
Het. diploid
rif2Δ
RIF2
Het. diploid
rif2Δ
RIF2

C: Parent diploid
Het. diploid
rif2Δ
RIF2
Het. diploid
rif2Δ
RIF2

No fusion
RAP1-RIF260

1 2 3 4 5 6 7 8 9 10 11 12 13

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

No fusion
RAP1-RIF260
Figure 4

A

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<td>Rap1ΔC-Rif260</td>
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<td>Rap1-Rif236</td>
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B

No fusion
rap1ΔC-RIF260

C

No fusion
RAP1-RIF236
RAP1-RIF260

D

No fusion
RAP1-RIF236
RAP1-RIF260
Figure 5

No fusion

$RAP1-RIF2_{60}$
Figure 7

A  \( RIF2 \)

B  \( rif2\Delta \)

C  \( rif2\Delta \ RAP1-RIF2_{60} \)