

Regulation of Telomere Length Requires a Conserved N-Terminal Domain of Rif2 in *Saccharomyces 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 **B**locks **A**ddition of **T**elomeres. 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.

KEYWORDS telomeres; telomerase; *Rif2*; length regulation

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; Ijima 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 includes 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 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,

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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, 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, Rad50, 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 rif1 Δ rif2 Δ 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*, that limits 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 recombination (Di Virgilio *et al.* 2013; Escribano-Diaz *et al.* 2013; Zimmermann *et al.* 2013).

Like RIF1, the RIF2 gene in yeast also plays a role in protecting chromosome ends from resection. rif2 Δ 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 rif2 Δ 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 rif2 Δ , 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 polymerase chain reaction (PCR) and for construction of plasmids used in this study are listed in Supporting Information, Table S1, Table S2, Table S3, Table S4, Table S5, and Table 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 1.67-kb product was subcloned into pCR2.1-TOPO (Life Technologies) according to the manufacturer’s instructions. RIF2 was then subcloned into pRS406 (Sikorski and Hieter 1989) using KpnI and EcoRI. This plasmid was used to generate 66 rif2NAAIRS mutants using a three-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 2–7 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 2–7. 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 *rif2*–NAAIRS2 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 six 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 W303-1 α 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*/*rif2* Δ ::*kanMX4* diploid (CVy245) with the NAAIRS mutant plasmids cut with *NcoI* 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*/*rif2* Δ ::*kanMX4*, *ura3-1*/*ura3-1*::(*rif2*NAAIRS–*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 *rif2*NAAIRS 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 *rif2*–NAAIRS 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 245 bp 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 was 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. 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 *RIF2* locus in OAY1002. Proper integration of the *rif2* mutants at the endogenous *RIF2* locus was confirmed by PCR and verified by sequencing.

***RAP1*–*RIF2*₆₀ fusion constructs integrated at the *RAP1* locus**

We used Gibson assembly (Gibson 2011) to generate the integrating plasmid pHK35, containing the *RAP1*–*RIF2*₆₀ fusion gene. The final construct, based in the pRS405 vector, contains the following elements stitched together: the C-terminal region of *RAP1* amplified from genomic DNA, fused to a flexible glycine 10 (G10) linker (HK89, HK98); the N-terminal 60 aa of *Rif2*, including a stop codon, from p406-Rif2 (HK97, HK94); the 250-bp *CYC1* terminator from p414-GALS (ATCC87344) (HK117, HK118); the *URA3* cassette from p406-Rif2 (HK93, HK96); and 176 bp of genomic DNA 3' of *RAP1* to target to the construct to the *RAP1* locus (HK95, HK90). The construct was sequence verified and then digested

with *SacI/NotI* and integrated at the *RAP1* locus in CVy245 or HKy639. Ura⁺ 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-Rif2₃₆*, was created from pHK35 using the mutagenesis method described above for the generation of single amino acid changes removing codons 37–60 (HK121, HK123) to create pHK68. The plasmid was sequence verified, digested with *SacI/NotI*, and then integrated at the *RAP1* genomic locus in HKy639. Ura⁺ integrants were verified by PCR and diploids were sporulated to generate haploid cells of the specific genotype.

The mutant *RAP1-RIF2₆₀ F8A, F8Y, and F8W* fusion genes were created using the site-directed mutagenesis method described above for the generation of single amino acid 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 *SacI/NotI* and integrated at the *RAP1* genomic locus. Proper integration of the *RAP1* fusion genes was verified by colony PCR and sequencing.

The *RAP1ΔC-RIF2₆₀* fusion gene was created using Gibson assembly of two fragments: the region amplified from genomic DNA, which removes 498 bp from the end of *RAP1* at amino acid 662 (HK128, HK129), and the region from pHK35, which contains the glycine10 linker-RIF2₆₀-CYC1 components (HK130, HK131) that were reassembled into pHK35 cut with *NotI/BglII*, creating pHK72. After sequence verification, the fusion construct was digested with *NotI/SacI* and introduced into the *RAP1* genomic locus in HKy639 selecting Ura⁺ integrants (HKy768, HKy769). As a control, a construct containing only *rap1ΔC* (HK128, HK132) that truncated the *RAP1* gene at codon 662 was engineered in a similar fashion, creating pHK71. This plasmid was sequence verified and integrated at the *RAP1* genomic locus in HKy639 selecting Ura⁺ integrants (HKy754, HKy755).

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 *tlc1-Δ::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° in liquid medium yeast extract-peptone-dextrose (YPD). Five 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 min of vigorous shaking (Eppendorf mixer 5432) in equal volumes of 0.5 mm glass beads (Biospec Products), phenol-chloroform (50:50), and lysis buffer [1% sodium dodecyl sulfate (SDS), 2% Triton X-100, 100 mM sodium chloride (NaCl), 10 mM Tris, pH 8.0, 1 mM ethylenediamine tetracetic acid (EDTA)]. DNA was precipitated in ethanol and resuspended at 37° in TE (10 mM Tris, pH 8.0, 1 mM EDTA) and RNaseA (10 μg/ml). Samples were digested with *XhoI* and separated by electrophoresis on a 1% agarose gel in 1× TTE buffer (20× = 1.78 M Tris base, 0.57 M taurine, 0.01 M EDTA). On each gel, 250 ng of 2-log DNA ladder (NEB N3200) was included as a reference. After electrophoresis was complete, the gel was denatured for 30 min [0.2 M sodium hydroxide (NaOH), 0.34M NaCl] and neutralized (1.5 M NaCl, 0.5 M Tris, pH 7.0) for 30 min before vacuum transfer (Boeckel Appligene vacuum blotter) at 50 mbar onto Amersham Hybond-N+ membrane (GE Healthcare) in 10× SSC (1.5 M NaCl, 0.17 M sodium citrate) for 1 hr. After UV-crosslinking (UV Stratalinker 2400, Stratagene), the membrane was prehybridized for 1–2 hr in Church buffer (0.5 M Tris, pH 7.2, 7% SDS, 1% bovine serum albumin, 1 mM EDTA) and hybridized with a radiolabeled subtelomeric Y' fragment (750-bp fragment generated by PCR from yeast genomic DNA using primers YPrimeFWD and YPrimeREV) and radiolabeled 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 kilobases. The numbers on the y-axis represent the lane numbers in the agarose gel.

Western blot analysis

Yeast strains were grown at 30° in YPD until OD₆₀₀ reached 0.4–0.6. Whole cell protein lysates were prepared by trichloroacetic acid (TCA) extraction as follows: three OD₆₀₀ of cells were collected and resuspended in 10 ml of 10% TCA for 30 min. After centrifugation, the TCA-treated cells were resuspended in 1 ml 1 M HEPES buffer, pH 7.5 and transferred into a microcentrifuge tube. Cells were pelleted and resuspended in 50 μl 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer [125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.2 M dithiothreitol (DTT), 0.012% bromophenol blue dye (BPB)] then ruptured with an equal amount of 0.5 mm glass beads for 3 min on a high-speed vortex mixer. An additional 50 μl of 2× SDS-PAGE buffer was added to each sample, followed by 5 min at 100° and 15 sec of vigorous shaking. The samples were centrifuged for

10 min at top speed in a microcentrifuge and the supernatant was collected and stored at -20° .

Whole cell lysate (3 μ l) was loaded per lane on a 10% TGX SDS-PAGE gel (Bio-Rad) along with molecular weight protein standards (Bio-Rad no. 161-0373) and resolved by electrophoresis. The proteins were transferred to 0.45 μ m Immobilon-FL membrane (Millipore) according to recommended protocol (Bio-Rad). All Blue, Precision Plus protein standards (Bio-Rad no. 161-0373) were used as molecular weight markers. All transferred membranes were blocked in Odyssey blocking buffer (LI-COR) for 1 hr at room temperature (RT). The membranes were washed three times in 1 \times wash buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.01% IGEPAL CA-630; Sigma) for 15 min, 5 min, and 5 min at RT. When using LI-COR detection, the final wash contained no detergents (1 \times TBS: 10 mM Tris, pH 8.0, 150 mM NaCl). The 13xMyc epitope was detected with a 1:10,000 dilution of anti-Myc 9E10 antibody (National Cell Culture Center, Minneapolis). Anti-actin (1:500 dilution of Anti-actin; Sigma A2066) and anti-PGK (1:10,000 dilution of anti-PGK; Invitrogen 459250) 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 hr 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 no. 460705) for 1 hr at RT. Anti-mouse immunoglobulin G horseradish peroxidase (IgG-HRP)-linked secondary antibody (Cell Signaling Technologies no. 7076) was diluted 1:10,000 in 5% milk (Bio-Rad), 0.05% Triton X-100, and incubated for 45 min at RT. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific no. 34095) was used to detect the chemiluminescent signal on an ImageQuant LAS4000 mini (GE Healthcare). Since the loading control comigrated with the V5-tagged *Rif2*, the blots were stripped with Restore Western Blot Stripping Buffer for 25 min at room temperature (Thermo Scientific no. 21059), washed three times in 1 \times TBS as described above, and verified that no signal was present. After treating 30 min to 1 hr in Odyssey blocking buffer, the membranes were then incubated for 1 hr 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.

Data availability

All strains and plasmids described here and in the [Supporting Information](#) are available upon request.

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 NAAIRS (Loneragan *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 codons 2–7, and NAAIRS8 substitutes codons 8–13. Each mutant was integrated into the yeast genome at the *URA3* locus in a *RIF2/rif2 Δ* heterozygous diploid and verified by sequencing. Wild-type *RIF2* integrated at *URA3*, as a control, was able to complement a *rif2 Δ* (data not shown). Telomere length was examined in *rif2 Δ ::kanMX4 ura3-1::rif2NAAIRS-URA3* haploid segregants by Southern blot and classified as wild type, long (similar to *rif2 Δ*), or medium (between *rif2 Δ* and wild type). There were a number of regions in *RIF2* where NAAIRS mutations resulted in a long telomere phenotype similar to *rif2 Δ* (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 (NAAIRS2–NAAIRS44) was located in this unstructured N-terminal domain of *Rif2*.

Mutant	Phenotype	Protein expression	Mutant	Phenotype	Protein expression
NAAIRS2	long	medium (0.52)	NAAIRS194	medium	low (0.33±0.03)
NAAIRS8	long	WT (1.49±0.12)	NAAIRS200	wt	
NAAIRS14	long	WT (0.74±0.03)	NAAIRS206	long	low (0.00±0)
NAAIRS20	long	medium (0.53±0.01)	NAAIRS212	long	low (0.37±0)
NAAIRS26	medium	WT (0.78±0.14)	NAAIRS218	wt	
NAAIRS32	long	low (0.44±0.03)	NAAIRS224	wt	
NAAIRS38	medium	WT (2.12±0.52)	NAAIRS230	long	low (0.16±0.02)
NAAIRS44	medium	WT (0.95±0.13)	NAAIRS236	long	low (0.19±0.04)
NAAIRS50	wt		NAAIRS242	long	low (0.23±0.09)
NAAIRS56	wt		NAAIRS248	long	low (0.11±0.02)
NAAIRS62	wt		NAAIRS254	wt	
NAAIRS68	wt		NAAIRS260	wt	
NAAIRS74	long	low (0.34±0.11)	NAAIRS266	wt	
NAAIRS80	medium	medium (0.55±0.44)	NAAIRS272	long	low (0.00±0)
NAAIRS86	long	medium (0.52±0.03)	NAAIRS278	wt	
NAAIRS92	wt		NAAIRS284	wt	
NAAIRS98	long	low (0.29±0.04)	NAAIRS290	long	low (0.00±0)
NAAIRS104	long	low (0.29±0.04)	NAAIRS296	long	low (0.22±0.04)
NAAIRS110	long	low (0.15±0.06)	NAAIRS302	wt	WT (0.81±0.12)
NAAIRS116	long	low (0.00±0)	NAAIRS308	long	low (0.00±0)
NAAIRS122	medium	WT (1.00±0.01)	NAAIRS314	wt	
NAAIRS128	medium	low (0.19±0.04)	NAAIRS320	long	low (0.38±0)
NAAIRS134	wt		NAAIRS326	medium	low (0.32±0.01)
NAAIRS140	medium	low (0.33±0.04)	NAAIRS332	medium	low (0.22±0.06)
NAAIRS146	wt		NAAIRS338	medium	low (0.26±0.04)
NAAIRS152	wt		NAAIRS344	long	low (0.38±0.06)
NAAIRS158	wt		NAAIRS350	medium	low (0.18±0.03)
NAAIRS164	wt		NAAIRS356	long	low (0.02±0.01)
NAAIRS170	wt		NAAIRS362	long	low (0.20±0.02)
NAAIRS176	wt		NAAIRS368	medium	low (0.50±0.03)
NAAIRS182	wt		NAAIRS374	long	low (0.00±0)
NAAIRS188	wt		NAAIRS380	wt	
			NAAIRS386	medium	WT (0.83±0.04)
			NAAIRS392	wt	

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 Southern and Westerns for these mutants are shown in Figure S1.

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*, *rif2*Δ, 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 six-codon change in *rif2*-NAAIRS8 and *rif2*Δ (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 six codons were mutated 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

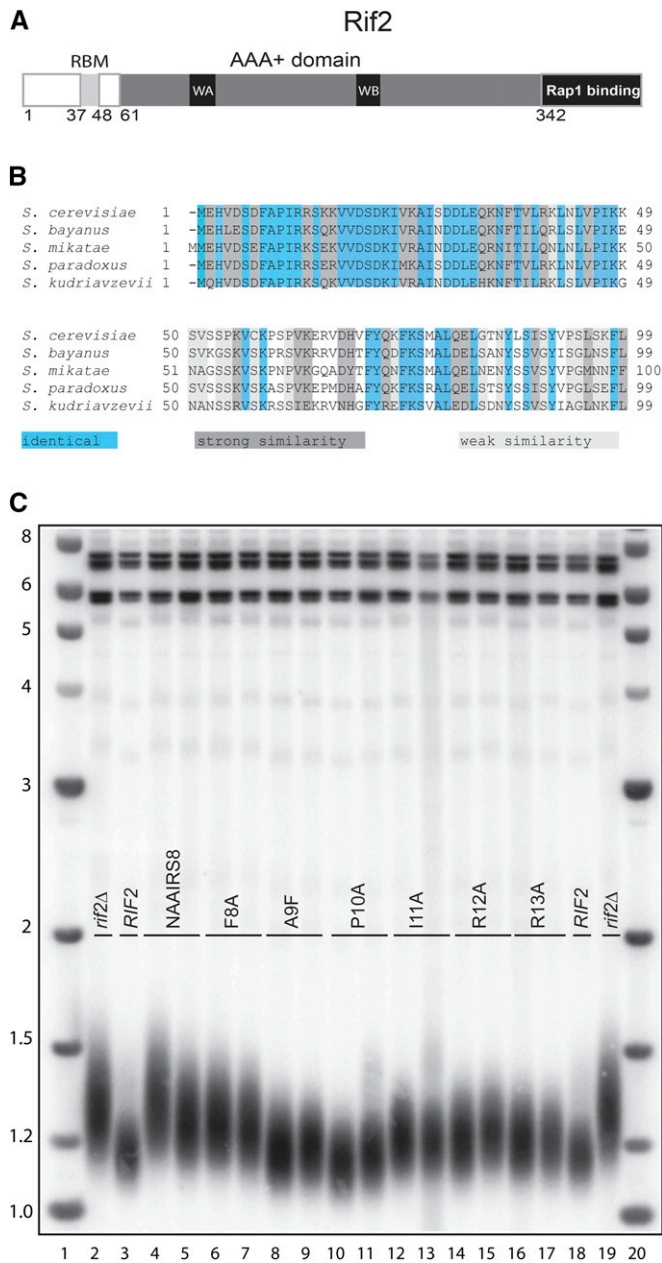


Figure 2 Point mutants in *RIF2* disrupt protein function. (A) The domain structure of Rif2 protein is shown. The C-terminal region binds Rap1. There is an AAA+ domain that contains Walker A and B motifs (designated WA and WB). The N-terminal domain is not well structured but contains a short helix (RBM) 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 kilobases. Two independent haploid transformants for each single point mutant are shown.

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-RIF2₆₀* and to the resulting fusion

protein as *Rap1-Rif2₆₀* (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 glycine linker sequence between *Rap1* and the *Rif2₆₀* N-terminal domain. The *RAP1-RIF2₆₀* 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 *rif1Δ rif2Δ* mutants.

We noted that there was a slight difference in telomere length in *rif1Δ rif2Δ* cells compared to *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 *rif1Δ* or *rif2Δ* mutants expressing the *RAP1-RIF2₆₀* 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 the *RAP1-RIF2₆₀* construct was working through the telomerase pathway we deleted the telomerase RNA component, *TLC1*, in a *RIF2/rif2Δ*, *RAP1/RAP1-RIF2₆₀* diploid. Telomere elongation in *rif2Δ* mutants was blocked by the loss of telomerase (Figure S5), as shown previously (Teng *et al.* 2000). Expression of the *RAP1-RIF2₆₀* fusion in *tlc1Δ* had little effect on telomere length, while *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

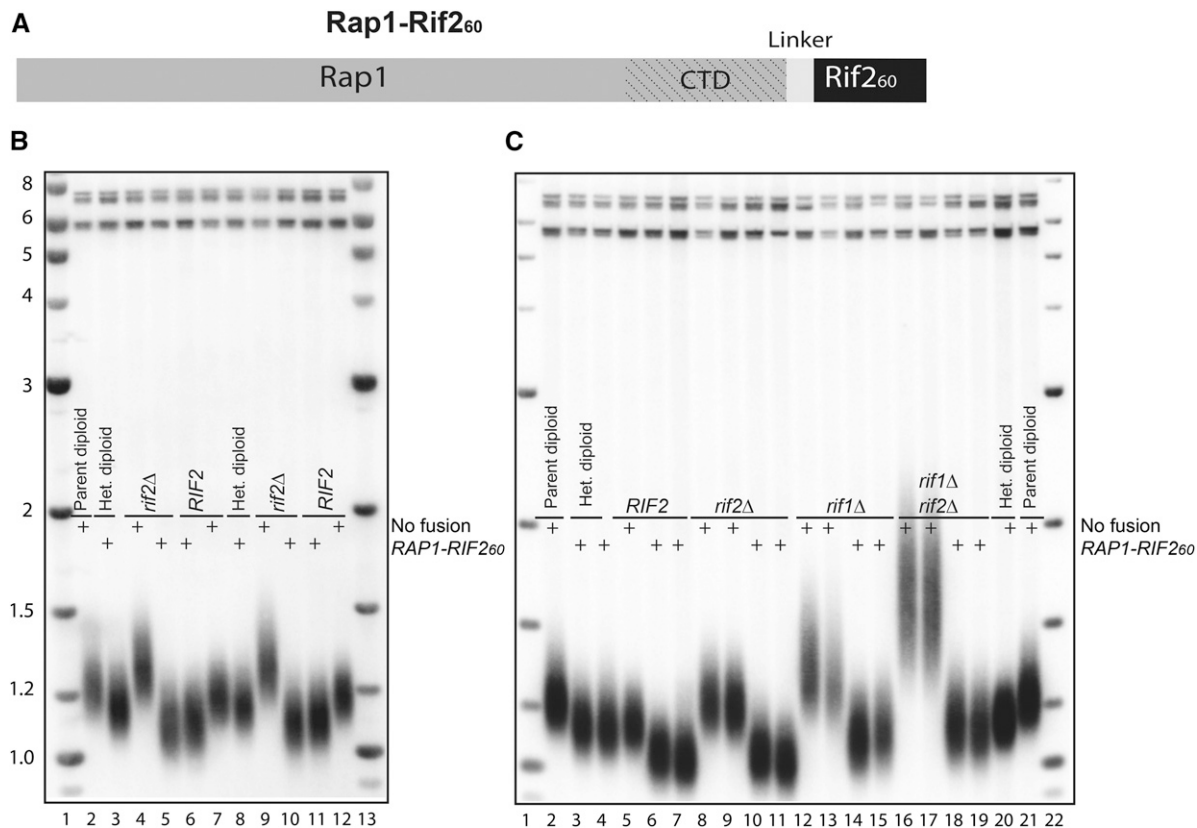


Figure 3 Rap1-Rif2₆₀ fusion protein blocks telomere elongation. (A) Schematic of Rap1-Rif2₆₀ fusion protein. The full-length *RAP1* coding region was fused in frame to a glycine 10 linker followed by the first 60 codons of *RIF2*. (B) *rif2Δ* mutants expressing the *RAP1-RIF2₆₀* construct. The genotype 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 shown with a + sign. The size markers on the side represent kilobases.

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 *tlc1Δ rif2Δ* cells. The loss of *Rif2* in a telomerase mutant has previously been shown to promote recombination and accelerate survivor formation (Teng *et al.* 2000; Chang *et al.* 2011; Ballew and Lundblad 2013; Hu *et al.* 2013). We found that the rapid appearance of survivors in *tlc1Δ rif2Δ* cells led to slightly longer bulk 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-Rif2₆₀ fusion protein and the requirement for telomerase indicate that this small domain of *Rif2* can block telomere overelongation 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 the *RAP1* C-terminal domain

The N-terminal 60 amino acids of *Rif2* include a small helical domain termed RBM between positions T37 and K48 that makes crystal contacts with the C-terminal domain of *Rap1*

(Shi *et al.* 2013). To determine whether the binding to the *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-RIF2₆₀*) 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-RIF2₆₀* 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 *rif1Δ*, *rif2Δ*, or *rif1Δ rif2Δ* (Figure S6).

Next we examined a fusion with a shortened BAT domain, *rap1ΔC-RIF2₃₆*, which lacks the RBM residues (Figure 2). Expression of *Rap1-Rif2₃₆* fully blocked telomere elongation in *rif1Δ*, *rif2Δ*, and *rif1Δ rif2Δ* mutants (Figure 4, C and D). While this *RAP1-RIF2₃₆* construct restored wild-type telomere

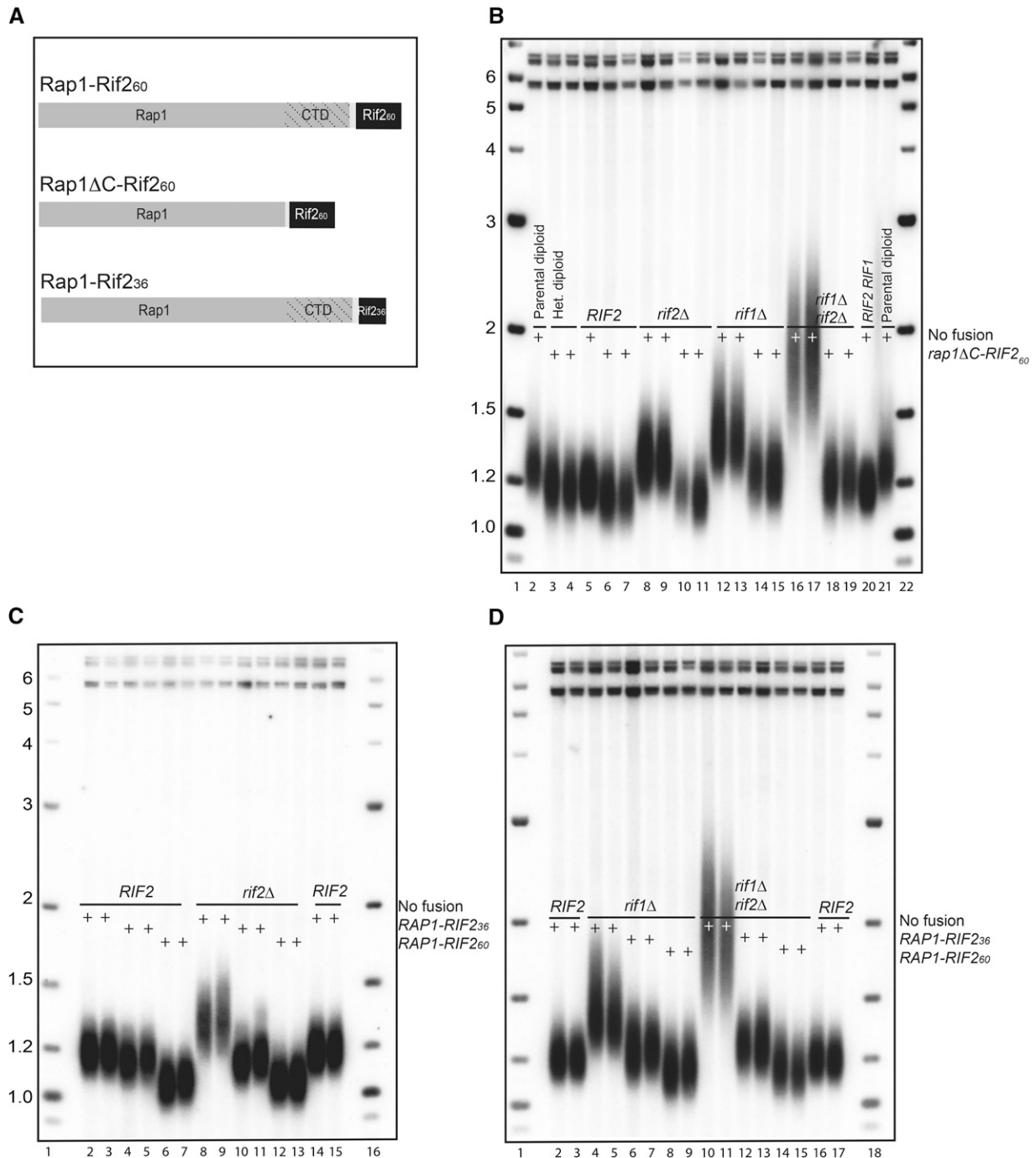


Figure 4 The C-terminal domain of Rap1 is not required for BAT domain function. (A) Schematic of three fusion protein constructs. (B) *rif1Δ*, *rif2Δ*, and *rif1Δ rif2Δ* double mutants expressing *rap1ΔC-RIF2₆₀* construct were analyzed by Southern blot. The genotype of the strain is indicated above each 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-RIF2₃₆*. The genotype of the strain is indicated above each lane and the presence or absence of either the *RAP1-RIF2₃₆* construct or *RAP1-RIF2₆₀* construct is shown with a + sign. (D) *rif1Δ* and *rif1Δ rif2Δ* double mutants expressing the shortened Rif2 N terminus, *RAP1-RIF2₃₆*. The genotype of the strain is indicated above each lane and the presence or absence of either the *RAP1-RIF2₃₆* construct or the *RAP1-RIF2₆₀* construct is shown with a + sign. The size markers on the side represent kilobases.

length, it did not cause shortening below wild-type length like the *RAP1-RIF2₆₀* construct (Figure 4, C and D). This lesser degree of telomere shortening by the *RAP1-RIF2₃₆* compared to the *RAP1-RIF2₆₀* 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 *RAP1-RIF2₃₆* 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.

***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 *et al.* 2009). To determine whether the C-terminal domain of *XRS2* is required for the ability of the BAT domain to limit telomere length, we introduced the *RAP1-RIF2₆₀* construct into a doubly heterozygous strain *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-Rif2₆₀* fusion protein resulted in significant shortening in the *rif2Δ xrs2ΔCt* cells (Figure 5, lanes 16 and 17). These results suggest that the *Rif2* BAT domain can regulate telomere length independent of the *Xrs2* C-terminal domain.

Mutations at F8 abolish the shortening effect of the *Rap1-Rif2₆₀* 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-Rif2₆₀* protein, we made a fusion construct containing this F8A substitution. When this mutant *RAP1-rif2₆₀ [F8A]* fusion construct was expressed in a *rif2Δ* background, telomere shortening did not occur (Figure 6A, lanes 14 and 15), indicating the *RAP1-rif2₆₀ [F8A]* mutation renders the BAT domain nonfunctional. Curiously, long telomeres were seen when the *RAP1-rif2₆₀ [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-Rif2₆₀ [F8A]* protein and thus may interfere with the function of the wild-type *Rif2*.

Aromatic amino acid tryptophan can mimic phenylalanine at F8

The functional importance of the *F8A* mutation in both the *RIF2* gene and in the *RAP1-rif2₆₀ [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 aromatic amino acids, tryptophan or tyrosine. Remarkably, the *RAP1-rif2₆₀ [F8W]* restored telomere shortening in *rif2Δ*, *rif1Δ*, and *rif1Δ rif2Δ* (Figure 6, C and D), indicating an aromatic amino acid can at least partially restore the function of the BAT domain. The *RAP1-rif2₆₀ [F8Y]* mutation more closely resembled the *RAP1-rif2₆₀ [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.

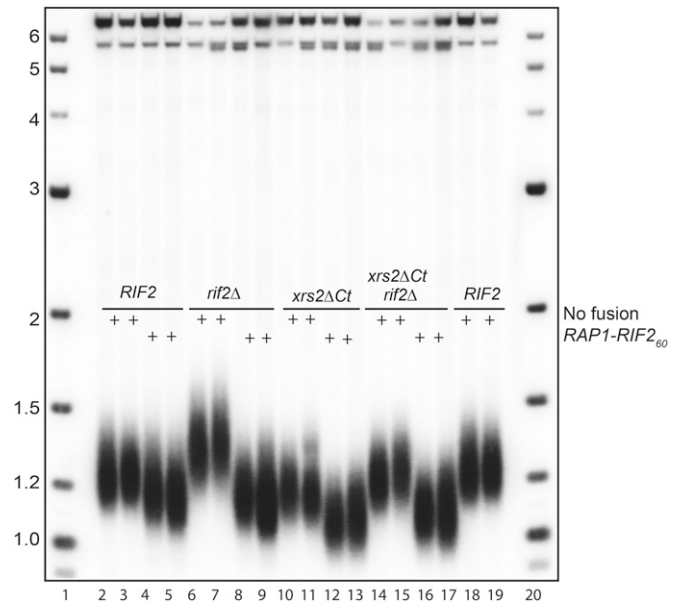


Figure 5 *Xrs2* C terminus is not required for the *Rif2* BAT domain to shorten telomeres. Southern blot telomere analysis of *xrs2ΔCt* and *rif2Δ xrs2ΔCt* cells expressing *RAP1-RIF2₆₀* construct. The genotype of the strain is indicated above each lane and the presence or absence of the *RAP1-RIF2₆₀* construct is shown with a + sign. The size markers on the side represent kilobases.

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 blocking excess elongation, as the *RAP1-RIF2₆₀ [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.

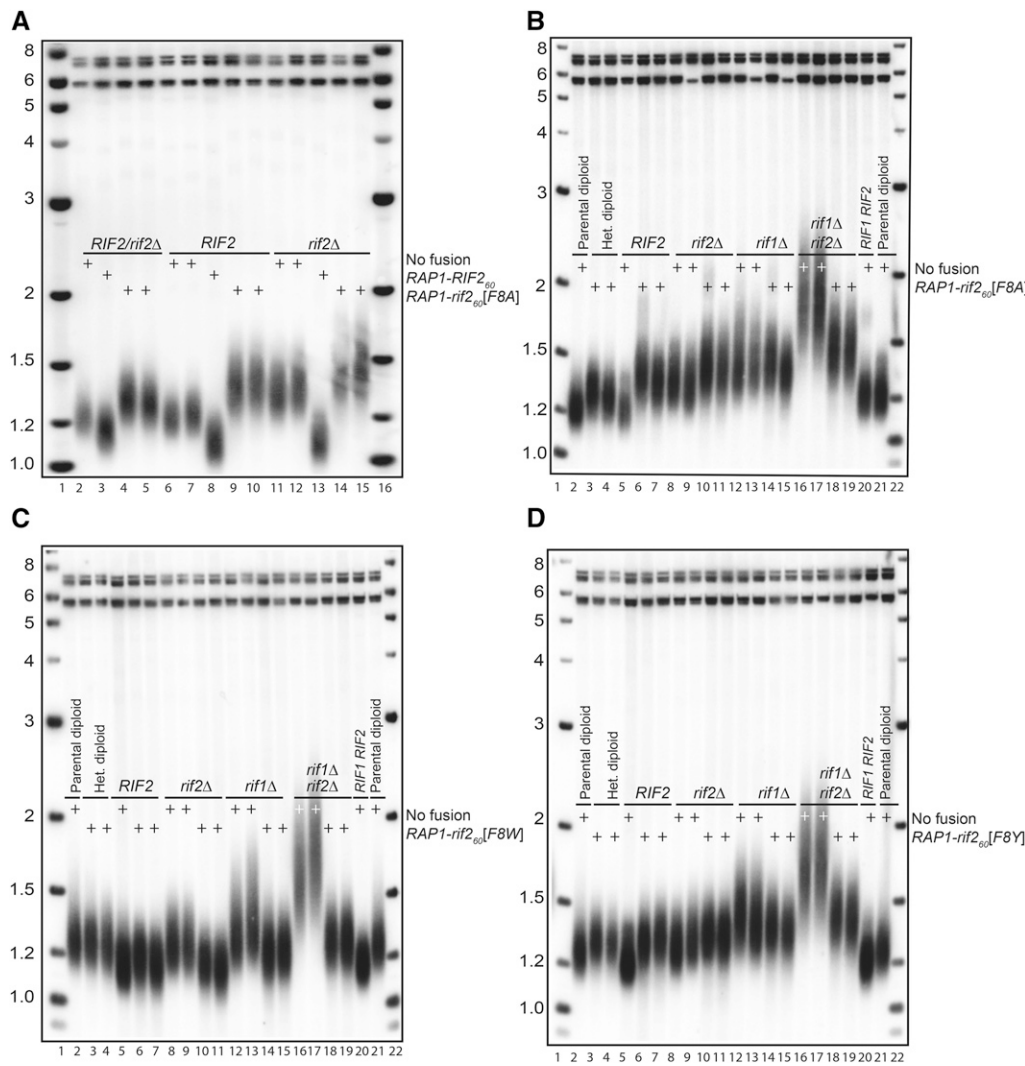


Figure 6 Aromatic residue at position 8 is important for Rif2 BAT domain function. (A) Southern blot telomere analysis of WT and *rif2Δ* cells expressing either *RAP1-RIF2₆₀* or *RAP1-rif2₆₀ [F8A]* mutant fusion construct. The genotype of the strain is indicated above each lane and the presence or absence 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-rif2₆₀ [F8A]* fusion or no fusion. The genotype of the strain is indicated above each lane and the presence or absence of the *RAP1-rif2₆₀ [F8A]* is shown with a + sign. The parental diploid was transformed with the fusion construct to yield the heterozygous diploid (Het. diploid) and dissected for the haploid segregants. (C) Southern blot telomere analysis of WT, *rif2Δ*, *rif1Δ*, and *rif1Δ rif2Δ* mutants expressing *RAP1-rif2₆₀ [F8W]* fusion. The genotype of the strain is indicated above each lane and the presence of the *RAP1-rif2₆₀ [F8W]* is shown with a + sign. (D) Southern blot telomere analysis of WT, *rif2Δ*, *rif1Δ*, and *rif1Δ rif2Δ* mutants expressing *RAP1-rif2₆₀ [F8Y]* fusion. The genotype of the strain is indicated above each lane and the presence of *RAP1-rif2₆₀ [F8Y]* fusion protein is shown with a + sign. The size markers on the side represent kilobases.

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 groups 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 role (Hirano *et al.* 2009) since Xrs2 C-terminal truncations still showed telomere shortening with the Rap1-Rif2₆₀ fusion protein.

The Rap1-Rif2₆₀ fusion causes telomere shortening

The dominant effect of the Rap1-Rif2₆₀ 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 associations 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–Rif2₆₀ fusion also shortened telomeres in a *rif1Δ* mutant. Rif1 regulates telomere elongation through a mechanism independent of Rif2, thus the ability of the Rap1–Rif2₆₀ to block telomerase elongation after the loss of Rif1 suggests that the mechanism by which BAT blocks telomere elongation is not pathway specific. Experiments by Levy and Blackburn (2004) 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. This further suggests 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 that binds to the F8 residue in the BAT domain and blocks telomere elongation as depicted in Figure 7A. When Rif2 is missing, telomerase can overextend 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 *S. 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

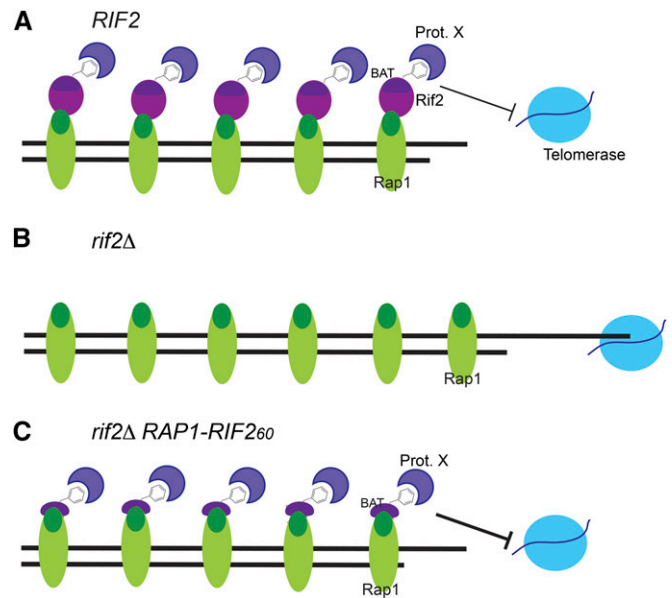


Figure 7 Model for separation of function of Rif2 and the BAT domain. In *RIF2* cells, the Rap1 protein, shown in green, binds to the telomeric double-stranded DNA and the C-terminal domain (dark green) recruits Rif1 (not shown for simplicity) and Rif2 (purple). Rif2 binds the Rap1 C-terminal domain 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-RIF2₆₀* fusion construct, even in a *rif2Δ* mutant, there is strong blocking of telomerase.

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, which limits telomere elongation, may be a conserved feature of telomere length regulation; however 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 may contribute to conservation of the telomere length equilibrium mechanism across species.

Acknowledgments

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GENETICS

Supporting Information

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Regulation of Telomere Length Requires a Conserved N-Terminal Domain of Rif2 in *Saccharomyces cerevisiae*

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Supporting Information

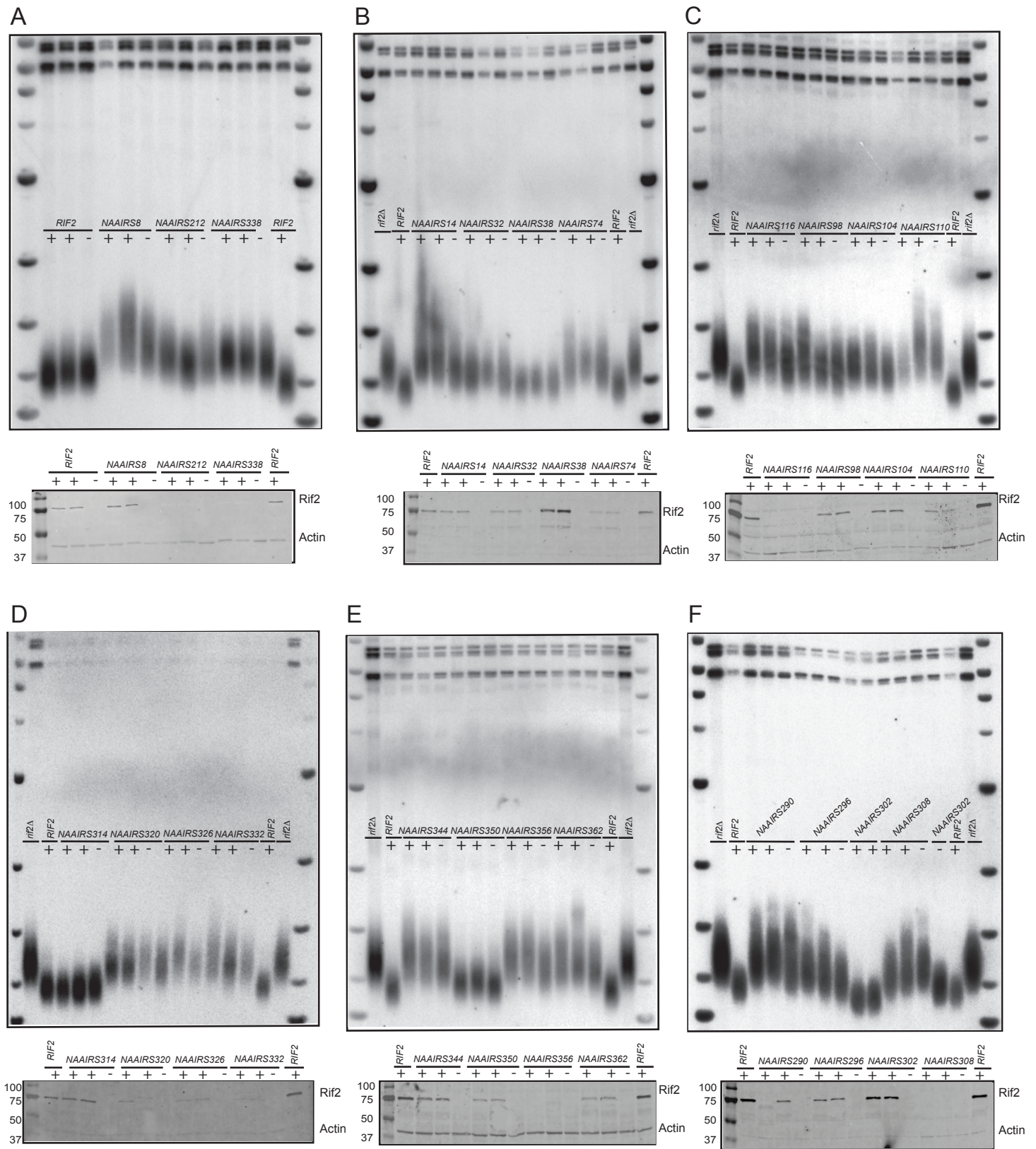


Figure S1. NAAIRS scanning mutagenesis identifies functional regions of RIF2. (A)-(F) The original Southern blots (Top) and western (bottom) for the NAAIRS screen are shown. In each gel two independent 13xMyc tagged haploids are shown (+) and one untagged control of the same mutant (-). The Rif2-13xMyc tagged protein level was normalized to the actin loading control as described in the materials and methods. When discrepancies were found between independent transformants additional independent transformants were examined to determine the phenotype. The mutants were assayed as they were sequence verified, and thus the mutants are not in numerical order on these preliminary gels.

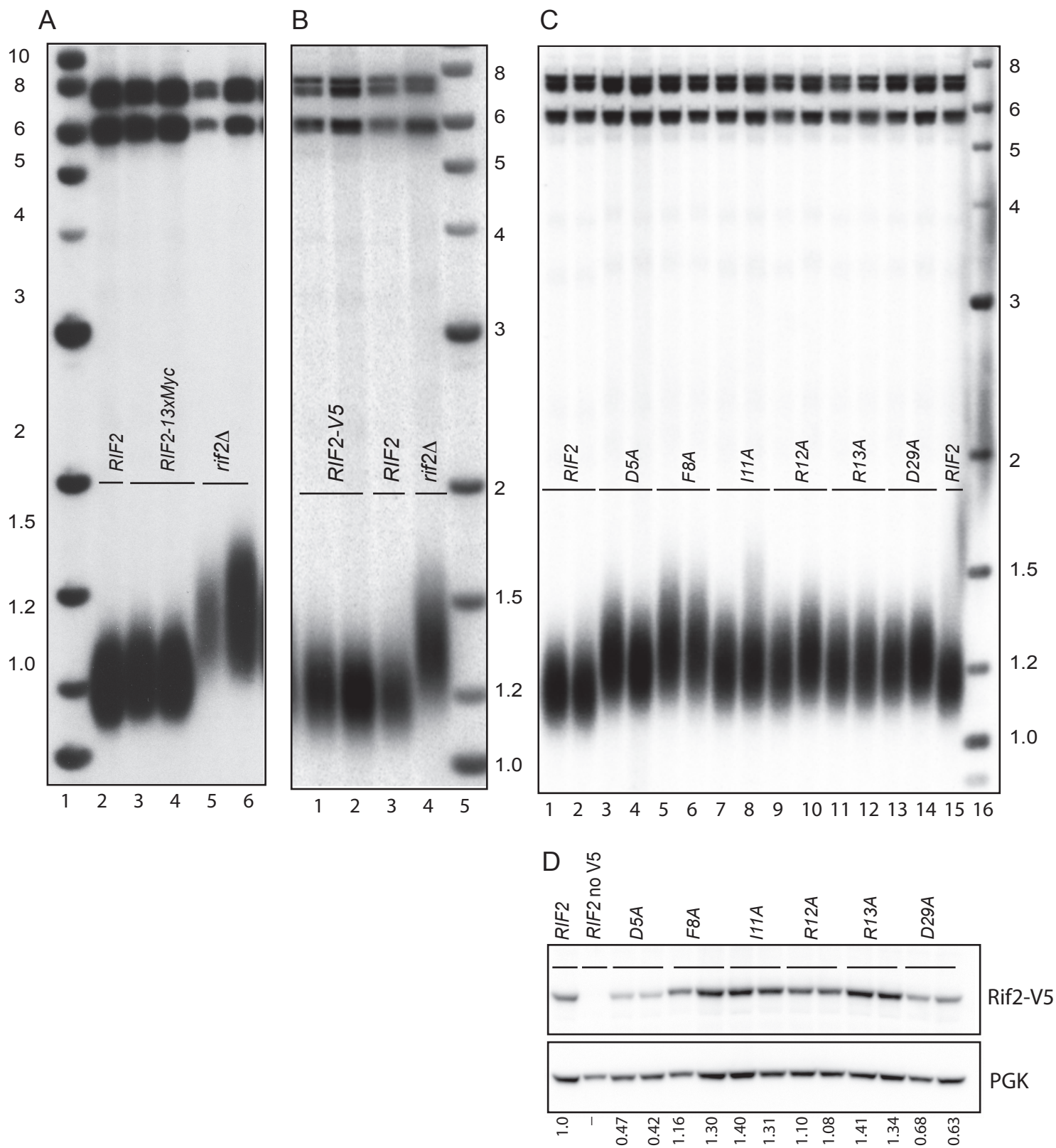


Figure S2. Tagging of *RIF2* does not disrupt function. (A) The telomere length in the wild type 13xMyc tagged *RIF2* strains are similar to the untagged wild type strain. (B) The telomere length in the V5 tagged *RIF2* integrated at the endogenous *RIF2* locus is similar to wild type. (C) Single amino acid mutations that show long telomeres were retested for protein telomere length and (D) for Rif2 protein expression level. The numbers below represent quantification of Rif2 levels, normalized to the PGK loading control, and then to the wild type *RIF2* (See Materials and Methods).

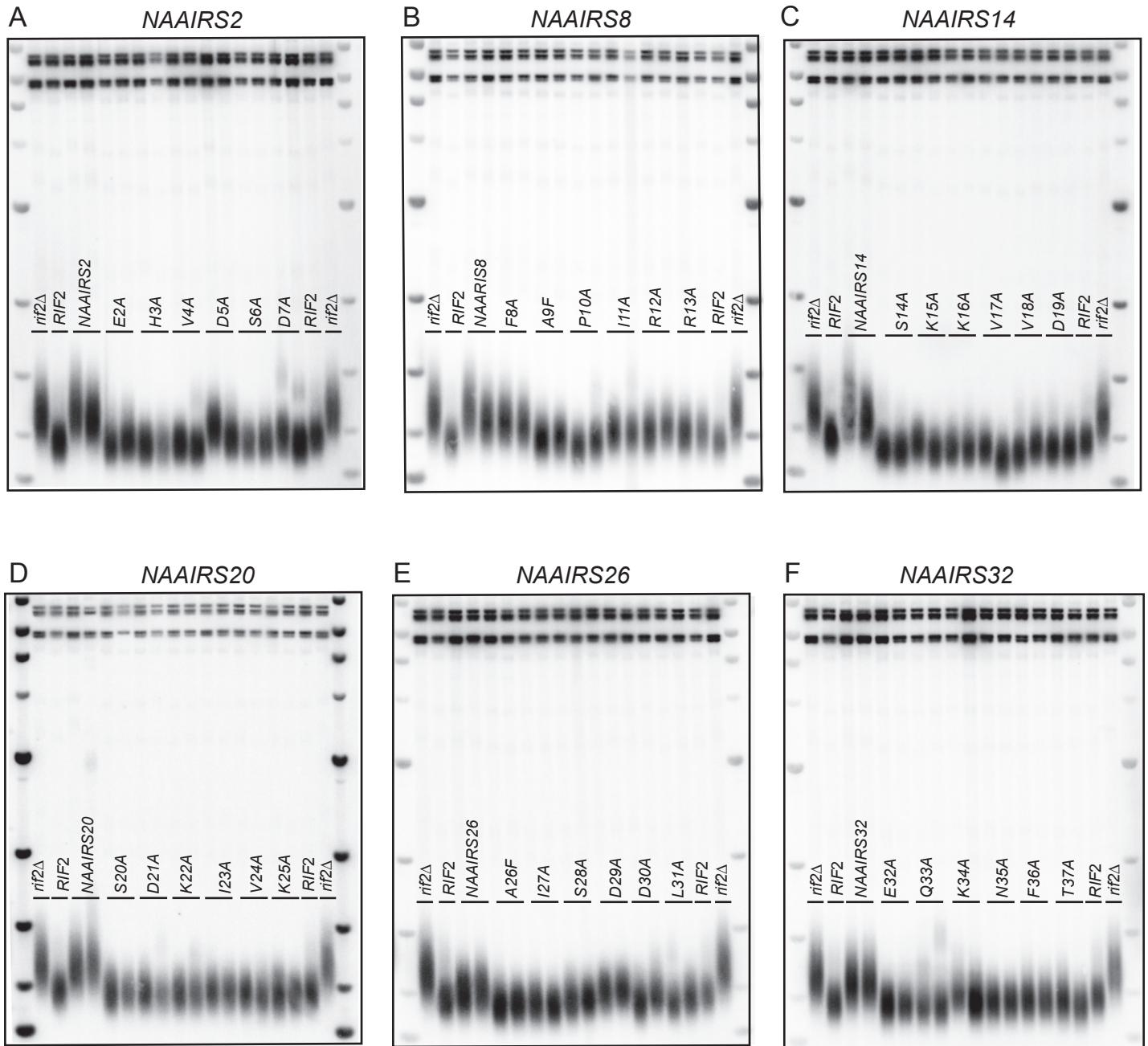


Figure S3. Individual single amino acid changes across Rif2 N-terminus. (A)-(F). Each amino acid across the six N-terminal NAAIRS mutants: *rif2*-NAAIRS2, *rif2*-NAAIRS8, *rif2*-NAAIRS14, *rif2*-NAAIRS20, *rif2*-NAAIRS26, and *rif2*-NAAIRS32 were individually mutated and telomere length was measured and compared to *rif2* Δ and RIF2 telomere length on the same gel. For each amino acid change two independent transformants were measured.

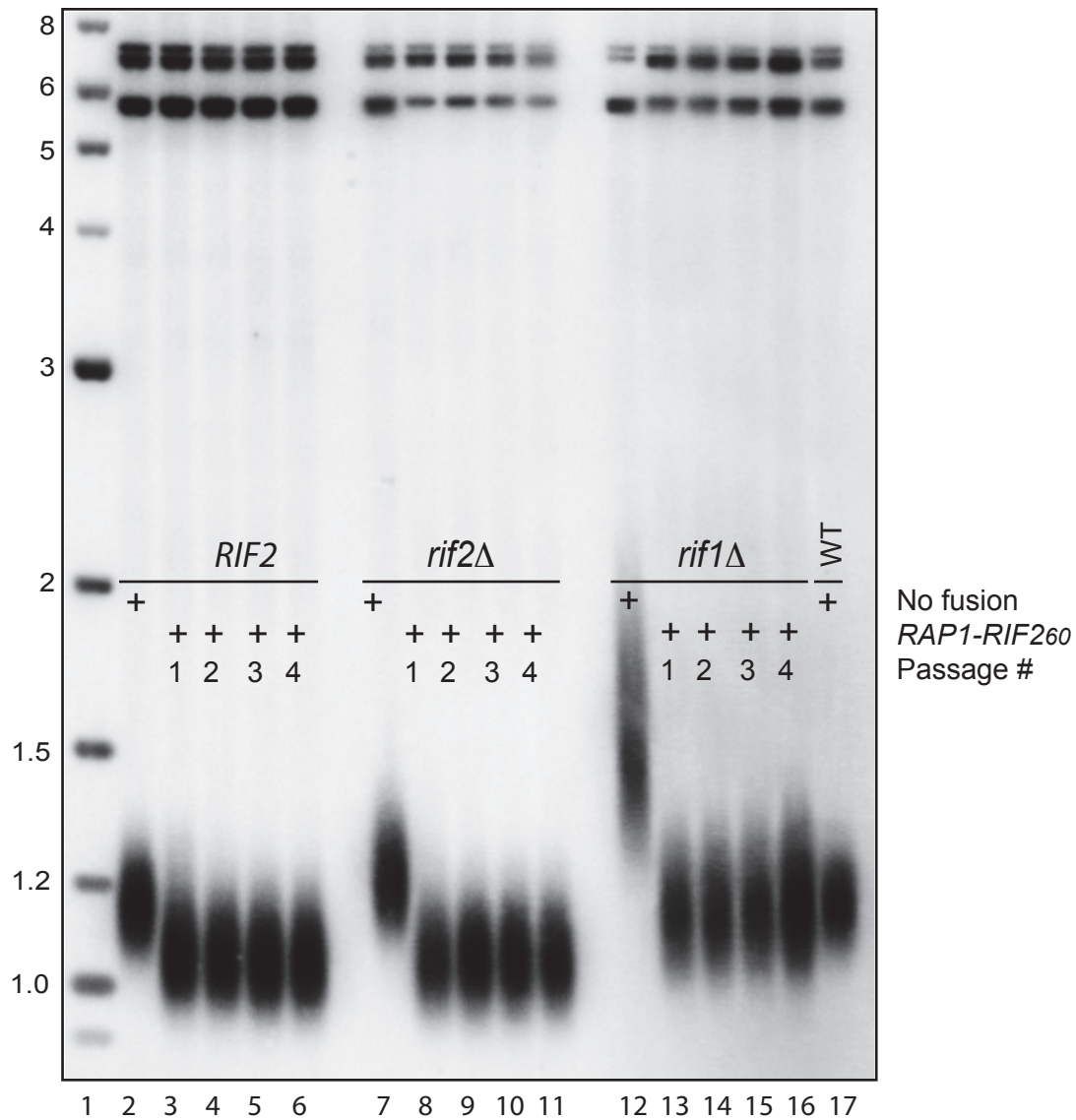


Figure S4. Telomere length during passaging of cells expressing of *RAP1-RIF260* fusion. Haploid segregants of *RIF2*, *rif2Δ*, or *rif1Δ* mutants containing *RAP1-RIF260* were grown for 4 consecutive passages in YPD broth. Telomere length at each passage was analyzed. The size markers on the side represent kb.

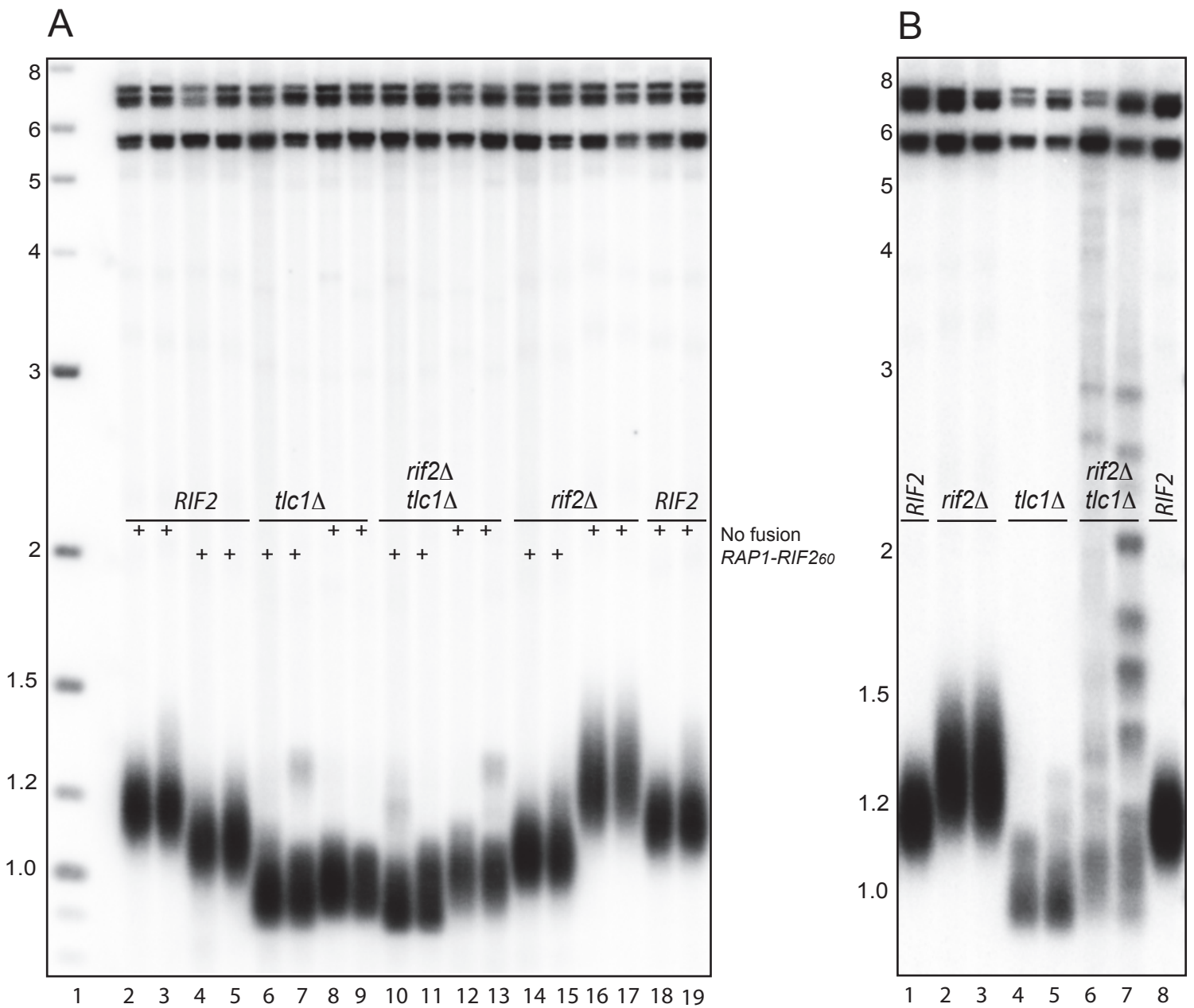


Figure S5. Telomerase is epistatic to *RAP1-RIF260* fusion (A) Southern blot telomere analysis of *RIF2*, *rif2Δ tlc1Δ*, and *rif2Δ tlc1Δ* cells expressing the *RAP1-RIF260* or no fusion protein. The genotype of the strain is indicated above each lane and the presence or absence of the fusion protein is shown with a + sign. (B) Southern blot telomere analysis of *RIF2 RIF1*, *rif2Δ*, *tlc1Δ*, and *rif2Δ tlc1Δ* cells grown for additional generations. The higher bands that appear between 1-3 kb in the *rif2Δ tlc1Δ* cells represent the early emergence of telomerase null survivors (Chang et al. 2011; Ballew and Lundblad 2013; Hu et al. 2013). The shortest telomeres are longer in these *rif2Δ tlc1Δ* survivors. The size markers on the side represent kb

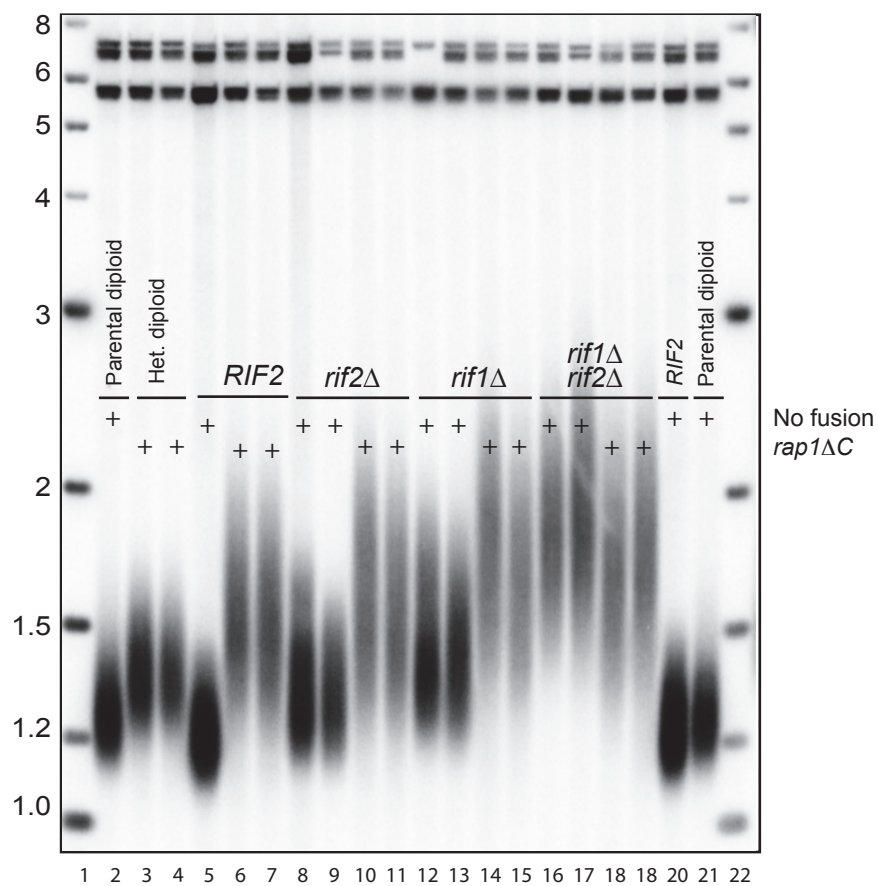


Figure S6. Expression of *Rap1ΔC* without the BAT domain does not rescue telomere length. A *rap1ΔC* truncation without the Rif260 BAT domain was expressed in in *rif1Δ*, *rif2Δ*, and *rif1Δ rif2Δ* cells and Southern blot telomere analysis was carried out. The genotype of the strain is indicated above each lane and the presence or absence of *rap1ΔC* truncation 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. The size markers on the side represent kb.

Table S1 Yeast Strains 1: *rif2NAAIRS* mutants integrated at the *URA3* locus

Strain	Genotype	Source
OAY1002	<i>MATa ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 RAD5</i>	Viggiani and Aparicio (2006)
OAY1003	<i>MATα ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 RAD5</i>	Viggiani and Aparicio (2006)
CVy242	<i>MATa/MATα ade2-1/ade2-1 trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 RAD5/RAD5</i>	This study
CVy245	<i>MATa/MATα ade2-1/ade2-1 trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 RAD5/RAD5 RIF2/rif2Δ::kanMX4</i>	This study
CVy275	CVy245 <i>ura3-1/ura3-1::(RIF2-URA3)</i>	This study
HKy295, 296	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS2-URA3)</i>	This study
HKy297, 298	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS8-URA3)</i>	This study
HKy299, 300	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS14-URA3)</i>	This study
HKy342, 343	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS20-URA3)</i>	This study
HKy344, 345	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS26-URA3)</i>	This study
HKy301, 302	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS32-URA3)</i>	This study
HKy303, 304	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS38-URA3)</i>	This study
HKy346, 347	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS44-URA3)</i>	This study
KGy155 A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS50-URA3)</i>	This study
KGy107 A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS56-URA3)</i>	This study
KGy108 A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS62-URA3)</i>	This study
KGy109 A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS68-URA3)</i>	This study
HKy305, 306	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS74-URA3)</i>	This study
HKy348, 349	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS80-URA3)</i>	This study
HKy350, 351	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS86-URA3)</i>	This study
HKy352, 353	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS92-URA3)</i>	This study
HKy354, 355	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS98-URA3)</i>	This study
HKy356, 357	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS104-URA3)</i>	This study
HKy358, 359	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS110-URA3)</i>	This study
HKy307, 308	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS116-URA3)</i>	This study
HKy309, 310	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS122-URA3)</i>	This study
HKy311, 312	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS128-URA3)</i>	This study
KGy120A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS134-URA3)</i>	This study
HKy314, 315	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS140-URA3)</i>	This study
KGy122A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS146-URA3)</i>	This study
KGy123A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS152-URA3)</i>	This study
KGy156A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS158-URA3)</i>	This study
KGy124A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS164-URA3)</i>	This study
KGy125A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS170-URA3)</i>	This study
KGy126A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS182-URA3)</i>	This study
KGy127A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS188-URA3)</i>	This study
HKy316, 317	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS194-URA3)</i>	This study
HKy318, 319	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS200-URA3)</i>	This study

Table S1, *continued*

Strain	Genotype	Source
HKy320, 321	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS206-URA3)</i>	This study
HKy322, 323	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS212-URA3)</i>	This study
KGy132A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS218-URA3)</i>	This study
HKy324, 325	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS230-URA3)</i>	This study
HKy326, 327	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS236-URA3)</i>	This study
HKy328, 329	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS242-URA3)</i>	This study
HKy330, 331	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS248-URA3)</i>	This study
KGy137A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS260-URA3)</i>	This study
KGy138A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS266-URA3)</i>	This study
HKy360, 361	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS272-URA3)</i>	This study
KGy139A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS278-URA3)</i>	This study
KGy140A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS284-URA3)</i>	This study
HKy362, 363	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS290-URA3)</i>	This study
HKy364, 365	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS296-URA3)</i>	This study
HKy366, 367	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS302-URA3)</i>	This study
HKy368, 369	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS308-URA3)</i>	This study
HKy370, 371	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS314-URA3)</i>	This study
HKy372, 373	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS320-URA3)</i>	This study
HKy332, 333	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS326-URA3)</i>	This study
HKy334, 335	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS332-URA3)</i>	This study
HKy336, 337	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS338-URA3)</i>	This study
HKy338, 339	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS344-URA3)</i>	This study
HKy340, 341	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS350-URA3)</i>	This study
HKy374, 375	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS356-URA3)</i>	This study
HKy376, 377	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS362-URA3)</i>	This study
HKy378, 379	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS368-URA3)</i>	This study
HKy382, 383	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS374-URA3)</i>	This study
KGy147A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS380-URA3)</i>	This study
HKy380-381	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS386-URA3)</i>	This study
KGy148A, B	CVy245 <i>ura3-1/ura3-1::(rif2NAAIRS392-URA3)</i>	This study

Table S1 Yeast strain 1: *rif2* NAAIRS mutants integrated at the *URA3* locus

The diploid yeast strains for NAAIRS176, 224, and 254 integrated at the *URA3* locus were inadvertently not saved, however, all the other strains and the haploid segregants for all of the NAAIRS mutants are available upon request.

Table S2 Yeast Strains 2: Epitope-tagged *rif2* mutants

Strain	Genotype	Source
KGy149, 151	<i>MATα</i> <i>ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 RAD5 ura3-1::(RIF2-URA3)</i>	This study
HKy385, 386	KGy149 <i>ura3-1::(RIF2-13Myc-URA3)</i>	This study
HKy393, 394	KGy149 <i>ura3-1::(rif2NAAIRS2-13Myc-URA3)</i>	This study
HKy387, 388	KGy149 <i>ura3-1::(rif2NAAIRS8-13Myc-URA3)</i>	This study
HKy401, 402	KGy149 <i>ura3-1::(rif2NAAIRS14-13Myc-URA3)</i>	This study
HKy405, 406	KGy149 <i>ura3-1::(rif2NAAIRS20-13Myc-URA3)</i>	This study
HKy407, 408	KGy149 <i>ura3-1::(rif2NAAIRS26-13Myc-URA3)</i>	This study
HKy395, 396	KGy149 <i>ura3-1::(rif2NAAIRS32-13Myc-URA3)</i>	This study
HKy409, 410	KGy149 <i>ura3-1::(rif2NAAIRS44-13Myc-URA3)</i>	This study
HKy403, 404	KGy149 <i>ura3-1::(rif2NAAIRS74-13Myc-URA3)</i>	This study
HKy435, 436	KGy149 <i>ura3-1::(rif2NAAIRS80-13Myc-URA3)</i>	This study
HKy437, 438	KGy149 <i>ura3-1::(rif2NAAIRS86-13Myc-URA3)</i>	This study
HKy411, 412	KGy149 <i>ura3-1::(rif2NAAIRS98-13Myc-URA3)</i>	This study
HKy413, 414	KGy149 <i>ura3-1::(rif2NAAIRS104-13Myc-URA3)</i>	This study
HKy415, 416	KGy149 <i>ura3-1::(rif2NAAIRS110-13Myc-URA3)</i>	This study
HKy397, 398	KGy149 <i>ura3-1::(rif2NAAIRS116-13Myc-URA3)</i>	This study
HKy439, 440	KGy149 <i>ura3-1::(rif2NAAIRS122-13Myc-URA3)</i>	This study
HKy441, 442	KGy149 <i>ura3-1::(rif2NAAIRS128-13Myc-URA3)</i>	This study
HKy451, 452	KGy149 <i>ura3-1::(rif2NAAIRS140-13Myc-URA3)</i>	This study
HKy453, 454	KGy149 <i>ura3-1::(rif2NAAIRS194-13Myc-URA3)</i>	This study
HKy399, 400	KGy149 <i>ura3-1::(rif2NAAIRS206-13Myc-URA3)</i>	This study
HKy389, 390	KGy149 <i>ura3-1::(rif2NAAIRS212-13Myc-URA3)</i>	This study
HKy417, 418	KGy149 <i>ura3-1::(rif2NAAIRS230-13Myc-URA3)</i>	This study
HKy419, 420	KGy149 <i>ura3-1::(rif2NAAIRS236-13Myc-URA3)</i>	This study
HKy421, 422	KGy149 <i>ura3-1::(rif2NAAIRS242-13Myc-URA3)</i>	This study
HKy423, 424	KGy149 <i>ura3-1::(rif2NAAIRS248-13Myc-URA3)</i>	This study
HKy462, 463	KGy149 <i>ura3-1::(rif2NAAIRS290-13Myc-URA3)</i>	This study
HKy425, 426	KGy149 <i>ura3-1::(rif2NAAIRS296-13Myc-URA3)</i>	This study
Hky427, 428	KGy149 <i>ura3-1::(rif2NAAIRS302-13Myc-URA3)</i>	This study
HKy464, 465	KGy149 <i>ura3-1::(rif2NAAIRS308-13Myc-URA3)</i>	This study
HKy443, 444	KGy149 <i>ura3-1::(rif2NAAIRS314-13Myc-URA3)</i>	This study
HKy445, 446	KGy149 <i>ura3-1::(rif2NAAIRS320-13Myc-URA3)</i>	This study
HKy447, 448	KGy149 <i>ura3-1::(rif2NAAIRS326-13Myc-URA3)</i>	This study
HKy449, 450	KGy149 <i>ura3-1::(rif2NAAIRS332-13Myc-URA3)</i>	This study
HKy391, 392	KGy149 <i>ura3-1::(rif2NAAIRS338-13Myc-URA3)</i>	This study
HKy429, 430	KGy149 <i>ura3-1::(rif2NAAIRS344-13Myc-URA3)</i>	This study
HKy431, 432	KGy149 <i>ura3-1::(rif2NAAIRS350-13Myc-URA3)</i>	This study
HKy458, 459	KGy149 <i>ura3-1::(rif2NAAIRS356-13Myc-URA3)</i>	This study
HKy433, 434	KGy149 <i>ura3-1::(rif2NAAIRS362-13Myc-URA3)</i>	This study
HKy460, 461	KGy149 <i>ura3-1::(rif2NAAIRS368-13Myc-URA3)</i>	This study

Table S2, continued

Strain	Genotype	Source
HKy374	KGy149 <i>ura3-1::(rif2NAAIRS374-13Myc-URA3)</i>	This study
HKy455, 456	KGy149 <i>ura3-1::(rif2NAAIRS386-13Myc-URA3)</i>	This study
HKy466, 467	<i>MATα ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 RAD5</i>	Dissection CVy245
HKy468, 469	<i>MATα ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 RAD5 rif2Δ::kanMX4</i>	Dissection CVy245
HKy470, 471	OAY1002 <i>RIF2-V5-URA3</i>	This study
HKy472, 473	OAY1002 <i>rif2(S28A)-V5-URA3</i>	This study
HKy474, 475	OAY1002 <i>rif2(Y122A)-V5-URA3</i>	This study
HKy476, 477	OAY1002 <i>rif2(G124A)-V5-URA3</i>	This study
HKy478, 479	OAY1002 <i>rif2(I125A)-V5-URA3</i>	This study
HKy480, 481	OAY1002 <i>rif2(R127A)-V5-URA3</i>	This study
HKy482, 483	OAY1002 <i>rif2(D126A)-V5-URA3</i>	This study
HKy498, 499	OAY1002 <i>rif2(L39A)-V5-URA3</i>	This study
HKy500, 501	OAY1002 <i>rif2(R40A)-V5-URA3</i>	This study
HKy502, 503	OAY1002 <i>rif2(K41A)-V5-URA3</i>	This study
HKy504, 505	OAY1002 <i>rif2(V38A)-V5-URA3</i>	This study
HKy506, 507	OAY1002 <i>rif2(L47A)-V5-URA3</i>	This study
HKy508, 509	OAY1002 <i>rif2(K269A)-V5-URA3</i>	This study
HKy510, 511	OAY1002 <i>rif2(N43A)-V5-URA3</i>	This study
HKy512, 513	OAY1002 <i>rif2(I233A)-V5-URA3</i>	This study
HKy514, 515	OAY1002 <i>rif2(L44A)-V5-URA3</i>	This study
HKy516, 517	OAY1002 <i>rif2(H120A)-V5-URA3</i>	This study
HKy518, 519	OAY1002 <i>rif2(I233C)-V5-URA3</i>	This study
HKy520, 521	OAY1002 <i>rif2(F8A)-V5-URA3</i>	This study
HKy522, 523	OAY1002 <i>rif2(A9F)-V5-URA3</i>	This study
HKy524, 525	OAY1002 <i>rif2(P10A)-V5-URA3</i>	This study
HKy526, 527	OAY1002 <i>rif2(I11A)-V5-URA3</i>	This study
HKy528, 529	OAY1002 <i>rif2(R12A)-V5-URA3</i>	This study
HKy531, 532	OAY1002 <i>rif2(R13A)-V5-URA3</i>	This study
HKy538, 539	OAY1002 <i>rif2(S14A)-V5-URA3</i>	This study
HKy540, 541	OAY1002 <i>rif2(K15A)-V5-URA3</i>	This study
HKy542, 543	OAY1002 <i>rif2(K16A)-V5-URA3</i>	This study
HKy549, 550	OAY1002 <i>rif2(V17A)-V5-URA3</i>	This study
HKy544, 545	OAY1002 <i>rif2(V18A)-V5-URA3</i>	This study
HKy546, 547	OAY1002 <i>rif2(D19A)-V5-URA3</i>	This study
HKy555, 556	OAY1002 <i>rif2(S20A)-V5-URA3</i>	This study
HKy557, 558	OAY1002 <i>rif2(D21A)-V5-URA3</i>	This study
HKy559, 560	OAY1002 <i>rif2(K22A)-V5-URA3</i>	This study
HKy561, 562	OAY1002 <i>rif2(I23A)-V5-URA3</i>	This study
HKy563, 564	OAY1002 <i>rif2(V24A)-V5-URA3</i>	This study
HKy565, 566	OAY1002 <i>rif2(K25A)-V5-URA3</i>	This study
HKy567, 568	OAY1002 <i>rif2(V45A)-V5-URA3</i>	This study
HKy659, 570	OAY1002 <i>rif2(P46A)-V5-URA3</i>	This study

Table S2, *continued*

Strain	Genotype	Source
HKy571, 572	OAY1002 <i>rif2(I47A)</i> -V5-URA3	This study
HKy573, 574	OAY1002 <i>rif2(K48A)</i> -V5-URA3	This study
HKy575, 576	OAY1002 <i>rif2(K49A)</i> -V5-URA3	This study
HKy577, 578	OAY1002 <i>rif2(E2A)</i> -V5-URA3	This study
HKy579, 580	OAY1002 <i>rif2(H3A)</i> -V5-URA3	This study
HKy581, 582	OAY1002 <i>rif2(V4A)</i> -V5-URA3	This study
HKy583, 584	OAY1002 <i>rif2(D5A)</i> -V5-URA3	This study
HKy585, 586	OAY1002 <i>rif2(S6A)</i> -V5-URA3	This study
HKy587, 588	OAY1002 <i>rif2(D7A)</i> -V5-URA3	This study
HKy589, 590	OAY1002 <i>rif2(A26F)</i> -V5-URA3	This study
HKy591, 592	OAY1002 <i>rif2(I27A)</i> -V5-URA3	This study
HKy593, 594	OAY1002 <i>rif2(D29A)</i> -V5-URA3	This study
HKy595, 596	OAY1002 <i>rif2(D30A)</i> -V5-URA3	This study
HKy597, 598	OAY1002 <i>rif2(L31A)</i> -V5-URA3	This study
HKy599, 600	OAY1002 <i>rif2(E32A)</i> -V5-URA3	This study
HKy601, 602	OAY1002 <i>rif2(Q33A)</i> -V5-URA3	This study
HKy603, 604	OAY1002 <i>rif2(K34A)</i> -V5-URA3	This study
HKy605, 606	OAY1002 <i>rif2(N35A)</i> -V5-URA3	This study
HKy607, 608	OAY1002 <i>rif2(F36A)</i> -V5-URA3	This study
HKy609, 610	OAY1002 <i>rif2(T37A)</i> -V5-URA3	This study
HKy548	OAY1002 <i>rif2Δ::KanMX4</i>	This study

Table S3 Yeast Strains 3: *RAP1-RIF2* fusions integrated at the *RAP1* locus

Strain	Genotype	Source
HKy551, 552	CVy242 <i>RIF2/rif2Δ::kanMX4 RAP1/rap1::(RAP1-RIF2₆₀-URA3)</i>	This study
HKy621, 622	CVy242 <i>RIF2/rif2Δ::kanMX4 RIF1/rif1Δ::LEU2 RAP1/rap1::(RAP1-RIF2₆₀-URA3)</i>	This study
HKy639	CVy242 <i>RIF2/rif2Δ::kanMX4 RIF1/rif1Δ::LEU2</i>	This study
HKy662, 663	HKy639 <i>RAP1/rap1::(RAP1-rif2₆₀ [F8A]-URA3)</i>	This study
HKy782, 783	HKy639 <i>RAP1/rap1::(RAP1-rif2₆₀ [F8W]-URA3)</i>	This study
HKy796, 797	HKy639 <i>RAP1/rap1::(RAP1-rif2₆₀ [F8Y]-URA3)</i>	This study
HKy768, 769	HKy639 <i>RAP1/rap1::(rap1ΔC-RIF2₆₀-URA3)</i>	This study
HKy754, 755	HKy639 <i>RAP1/rap1::(rap1ΔC-URA3)</i>	This study
HKy736, 737	HKy639 <i>RAP1/rap1::(RAP1-RIF2₃₆-URA3)</i>	This study
YCC115	<i>MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 TLC1/tlc1Δ::LEU2</i>	This study
HKy668, 669	CVy242 <i>RIF2/rif2Δ::kanMX4 RAP1/rap1::(RAP1-RIF2₆₀-URA3) TLC1/tlc1Δ::LEU2</i>	This study
JHUy912	<i>MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3Δ0/ura3Δ0 XRS2/xrs2::xrs2ΔCt-13myc-kanMX6 RIF2/rif2Δ::LEU2</i>	This study
HKy688, 689	JHUy912 <i>RAP1/rap1::(RAP1-RIF2₆₀-URA3)</i>	This study

Table S4: Primers used in the construction of *rif2* NAAIRS mutants

<i>rif2</i> mutant	Primer name	Sequence (5' to 3')
WT	Rif2-up	CTGACATGGTTTTCATACAC
WT	Rif2-down	CAGCAACCAAATCCAAGTCCTAG
WT	Rif2-forward	ATATAGATATAAATACGAACGTGGTTAGTATATAGAGACACGGATCCCCGG GTTAATTAA
WT	Rif2-reverse	TCTTTGTATTGTTCGAACTCTTTCAAAGACCTTGGTAATGAATTCGAGCTC GTTTAAAC
NAAIRS 2	NAAIRS 2 reverse	CCTTATAGGTGCAAACGATCGTATAGCATCATTATTGTCTCTATATA
	Anti-NAAIRS 2 forward	TATATAGAGACAAATGAATGCTGCTATACGATCGTTTTGCACCTATAAGG
NAAIRS 8	NAAIRS 8 reverse	AAACAACCTTTTTCGACGATCGTATAGCATCATTATCGGAATCTACATG
	Anti-NAAIRS 8 forward	CATGTAGATTCCGATAATGCTGCTATACGATCGTCGAAAAAGGTTGTT
NAAIRS 14	NAAIRS 14 reverse	CACAATCTTGTCACTCGATCGTATAGCATCATTTCTCCTTATAGGTGC
	Anti-NAAIRS 14 forward	GCACCTATAAGGAGAAATGCTGCTATACGATCGAGTGACAAGATTGTG
NAAIRS 20	NAAIRS 20 reverse	ATCATCGCTTATTGCCGATCGTATAGCATCATTGTCAACAACCTTTTT
	Anti-NAAIRS 20 forward	AAAAAGGTTGTTGACAATGCTGCTATACGATCGGCAATAAGCGATGAT
NAAIRS 26	NAAIRS 26 reverse	AAAATTTTTTGTCTCCGATCGTATAGCATCATTTTTTACAATCTTGTC
	Anti-NAAIRS 26 forward	GACAAGATTGTGAAAAATGCTGCTATACGATCGGAGCAAAAAAATTTT
NAAIRS 32	NAAIRS 32 reverse	CAACTTTCTCAGTACCGATCGTATAGCATCATTCAAATCATCGCTTAT
	Anti-NAAIRS 32 forward	ATAAGCGATGATTTGAATGCTGCTATACGATCGGTAAGTACTGAGAAAGTTG
NAAIRS 38	NAAIRS 38 reverse	TTTAATTGGAACAAGCGATCGTATAGCATCATTAGTAAAATTTTTTTG
	Anti-NAAIRS 38 forward	CAAAAAAATTTTACTAATGCTGCTATACGATCGCTTGTTCCAATTTAA
NAAIRS 44	NAAIRS 44 reverse	TGGGCTGCTAACACTCGATCGTATAGCATCATTGTTCAACTTTCTCAG
	Anti-NAAIRS 44 forward	CTGAGAAAGTTGAACAATGCTGCTATACGATCGAGTGTTAGCAGCCCA
NAAIRS 50	NAAIRS 50 reverse	ACTCGGCTTACACACCGATCGTATAGCATCATTTTTTTAATTGGAAC
	Anti-NAAIRS 50 forward	GTTCCAATTAAAAAAATGCTGCTATACGATCGGTGTGTAAGCCGAGT
NAAIRS 56	NAAIRS 56 reverse	CACTCGCTCCTTAACCGATCGTATAGCATCATTCTTTGGGCTGCTAAC
	Anti-NAAIRS 56 forward	GTTAGCAGCCCAAAGAATGCTGCTATACGATCGGTTAAGGAGCGAGTG
NAAIRS 62	NAAIRS 62 reverse	CTGGTAGAAAACATGCGATCGTATAGCATCATTTGGACTCGGCTTACA
	Anti-NAAIRS 62 forward	TGTAAGCCGAGTCCAAATGCTGCTATACGATCGCATGTTTTCTACCAG
NAAIRS 68	NAAIRS 68 reverse	GGCCATTGATTTGAACGATCGTATAGCATCATTGTCCACTCGCTCCT
	Anti-NAAIRS 68 forward	AAGGAGCGAGTGGACAATGCTGCTATACGATCGTTCAAATCAATGGCC
NAAIRS 74	NAAIRS 74 reverse	GGTGCCTAGCTCTTGCGATCGTATAGCATCATTCTTCTGGTAGAAAAC
	Anti-NAAIRS 47 forward	GTTTTCTACCAGAAGAATGCTGCTATACGATCGCAAGAGCTAGGCACC
NAAIRS 80	NAAIRS 80 reverse	GCTTATTGACAAATACGATCGTATAGCATCATTCAAGGCCATTGATTT
	Anti-NAAIRS 80 forward	AAATCAATGGCCTTGAATGCTGCTATACGATCGTATTTGTCAATAAGC
NAAIRS 86	NAAIRS 86 reverse	ACTTAAGCTCGGAACCGATCGTATAGCATCATTATTGGTGCCTAGCTC
	Anti-NAAIRS 86 forward	GAGCTAGGCACCAATAATGCTGCTATACGATCGGTTCCGAGCTTAAGT
NAAIRS 92	NAAIRS 92 reverse	ATTTTTTGAAGAAACGATCGTATAGCATCATTGTAGCTTATTGACAA
	Anti-NAAIRS 92 forward	TTGTCAATAAGCTACAATGCTGCTATACGATCGTTTTCTTTCAAAAAAT
NAAIRS 98	NAAIRS 98 reverse	ATTTTTTCACTCCTCGATCGTATAGCATCATTCTTACTTAAGCTCGG
	Anti-NAAIRS 98 forward	CCGAGCTTAAGTAAGAATGCTGCTATACGATCGAGGAGTATGAAAAAT
NAAIRS 104	NAAIRS 104 reverse	GTCGAAGAAAACGATCGATCGTATAGCATCATTAAGATTTTTTTGAAAG
	Anti-NAAIRS 104 forward	CTTTCAAAAAATCTTAATGCTGCTATACGATCGATCGTTTTCTTCGAC
NAAIRS 110	NAAIRS 110 reverse	GTGTATATGTTCAACCGATCGTATAGCATCATTACAATTTTTCTACT
	Anti-NAAIRS 110 forward	AGTATGAAAAATTGTAATGCTGCTATACGATCGGTTGAACATATACAC
NAAIRS 116	NAAIRS 116 reverse	GTCGATACCAGCATACGATCGTATAGCATCATTGTTGTCGAAGAAAAC
	Anti-NAAIRS 116 forward	GTTTTCTTCGACAAAAATGCTGCTATACGATCGTATGCTGGTATCGAC
NAAIRS 122	NAAIRS 122 reverse	TGTTTTCTGAAACTGCCGATCGTATAGCATCATTTTGGTGTATATGTTT
	Anti-NAAIRS 122 forward	GAACATATACACAAAAATGCTGCTATACGATCGGCAGTTTCAGAAACA
NAAIRS 128	NAAIRS 128 reverse	TATATCGACTAAAAGACGATCGTATAGCATCATTACGGTCGATACCAGC
	Anti-NAAIRS 128 forward	GCTGGTATCGACCGTAATGCTGCTATACGATCGTCTTTAGTCGATATA
NAAIRS 134	NAAIRS 134 reverse	TTCTATAATTACGACCGATCGTATAGCATCATTCAAGTGTCTGAAAC
	Anti-NAAIRS 134 forward	GTTTCAGAAACACTGAATGCTGCTATACGATCGGTCGTAATTATAGAA
NAAIRS 140	NAAIRS 140 reverse	CATTAAGTAGTCATTTCGATCGTATAGCATCATTATTTATATCGACTAA
	Anti-NAAIRS 140 forward	TTAGTCGATATAAATAATGCTGCTATACGATCGAATGACTACTTAATG

Table S4, continued

<i>rif2</i> mutant	Primer name	Sequence (5' to 3')
NAAIRS 146	NAAIRS 146 reverse	TGATTGAATACCCTCCGATCGTATAGCATCATTCAATTTCTATAATTAC
	Anti-NAAIRS 146 forward	GTAATTATAGAAATGAATGCTGCTATACGATCGGAGGGTATTCAATCA
NAAIRS 152	NAAIRS 152 reverse	ACATTCTTTTGATTTGATCGTATAGCATCATTTCATTAAGTAGTC
	Anti-NAAIRS 152 forward	GACTACTTAATGAAAAATGCTGCTATACGATCGAAATCAAAAAGAAATGT
NAAIRS 158	NAAIRS 158 reverse	CTGCCCCATTGACTCCGATCGTATAGCATCATTGCTTGATTGAATACC
	Anti-NAAIRS 158 forward	GGTATTCAATCAAGCAATGCTGCTATACGATCGGAGTCAATGGGGCAG
NAAIRS 164	NAAIRS 164 reverse	TTGTCCGCTATATGACGATCGTATAGCATCATTGATACATTCTTTTGA
	Anti-NAAIRS 164 forward	TCAAAAAGAAATGTATCAATGCTGCTATACGATCGTCATATAGCGGACAA
NAAIRS 170	NAAIRS 170 reverse	ACTAGCTTCGAAATCCGATCGTATAGCATCATTAGCCTGCCCCATTGA
	Anti-NAAIRS 170 forward	TCAATGGGGCAGGCTAATGCTGCTATACGATCGGATTTTGAAGCTAGT
NAAIRS 176	NAAIRS 176 reverse	GTGATTTGAAGGTTTCGATCGTATAGCATCATTTAGTTGTCCGCTATA
	Anti-NAAIRS 176 forward	TATAGCGGACAATAAATGCTGCTATACGATCGAAACCTTCAAATCAC
NAAIRS 182	NAAIRS 182 reverse	CATCATTAGGTCAGACGATCGTATAGCATCATTTCCTAGCTTCGAA
	Anti-NAAIRS 182 forward	TTCGAAGCTAGTGAATAATGCTGCTATACGATCGTCTGACCTAATGATG
NAAIRS 188	NAAIRS 188 reverse	TATTTTCTCATAACCGATCGTATAGCATCATTCTGTGATTTGAAGG
	Anti-NAAIRS 188 forward	CCTTCAAATCACACGAATGCTGCTATACGATCGGTTATGAGGAAAATA
NAAIRS 194	NAAIRS 194 reverse	GATACTTTTCGTCATTTCGATCGTATAGCATCATTATCATCATTAGGTC
	Anti-NAAIRS 194 forward	GACCTAATGATGATGAATGCTGCTATACGATCGAATGACGAAAGTATC
NAAIRS 200	NAAIRS 200 reverse	GAAGTAGACAATATGCGATCGTATAGCATCATTATTTATTTTCTCAT
	Anti-NAAIRS 200 forward	ATGAGGAAAATAAATAATGCTGCTATACGATCGCATATTGTCTACTTC
NAAIRS 206	NAAIRS 206 reverse	ATCTAATTGTTTGAACGATCGTATAGCATCATTATCGATACTTTTCGTC
	Anti-NAAIRS 206 forward	GACGAAAGTATCGATAATGCTGCTATACGATCGTTCGAACAATTAGAT
NAAIRS 212	NAAIRS 212 reverse	AGTTGAAGTAGATAACGATCGTATAGCATCATTTCGAAAGTAGACAAT
	Anti-NAAIRS 212 forward	ATTGTCTACTTCAAAAATGCTGCTATACGATCGTTATCTACTTCAACT
NAAIRS 218	NAAIRS 218 reverse	CTTCGAAGGTTCTATCGATCGTATAGCATCATTTCATCTAATTGTTTC
	Anti-NAAIRS 218 forward	GAACAATTAGATAAAAAATGCTGCTATACGATCGATAGAACCCTTCGAAG
NAAIRS 224	NAAIRS 224 reverse	ATTGATAAATTCGGTTCGATCGTATAGCATCATTATAGTTGAAGTAGA
	Anti-NAAIRS 224 forward	TCTACTTCAACTATAAATGCTGCTATACGATCGACCGAATTTATCAAT
NAAIRS 230	NAAIRS 230 reverse	TTCAAGTACCGATAACGATCGTATAGCATCATTAAGCTTCGAAGGTTTC
	Anti-NAAIRS 230 forward	GAACCTTCGAAGCTTAATGCTGCTATACGATCGTTATTCGGTACTTGAA
NAAIRS 236	NAAIRS 236 reverse	TGCAATGTTTACTCGATCGTATAGCATCATTAACATTGATAAATTC
	Anti-NAAIRS 236 forward	GAATTTATCAATGTTAATGCTGCTATACGATCGAGTAATAACATTGCA
NAAIRS 242	NAAIRS 242 reverse	ATAAATGAGGACCTTCGATCGTATAGCATCATTTCGAAAGTACCGA
	Anti-NAAIRS 242 forward	TCGGTACTTGAAAAAATGCTGCTATACGATCGAAGGTCCTCATTTAT
NAAIRS 248	NAAIRS 248 reverse	AATGCTAACGTTATTCGATCGTATAGCATCATTAATGCAATGTTATT
	Anti-NAAIRS 248 forward	AATAACATTGCATTTAATGCTGCTATACGATCGAATAACGTTAGCATT
NAAIRS 254	NAAIRS 254 reverse	TGTCGATAGGAGAGACGATCGTATAGCATCATTTCGAAATAATGAGGAC
	Anti-NAAIRS 254 forward	GTCCTCATTTATTCAAATGCTGCTATACGATCGTCTCTCTATCGACA
NAAIRS 260	NAAIRS 260 reverse	GAGTTTCTTTTTCGATCGTATAGCATCATTTCGAAATGCTAACGTT
	Anti-NAAIRS 260 forward	AACGTTAGCATTTTGAATGCTGCTATACGATCGCTCAAAAAGAAACTC
NAAIRS 266	NAAIRS 266 reverse	CACAGTATATTTTTCGATCGTATAGCATCATTGGATGTGATAGGAG
	Anti-NAAIRS 266 forward	CTCCTATCGACATCCAATGCTGCTATACGATCGACAAAATATACTGTG
NAAIRS 272	NAAIRS 272 reverse	TAATATCGGCATCTCCGATCGTATAGCATCATTGTTGAGTTTCTTTTT
	Anti-NAAIRS 272 forward	AAAAAGAACTCAACAATGCTGCTATACGATCGGAGATGCCGATATTA
NAAIRS 278	NAAIRS 278 reverse	TTGTTCTTGAGCGCACGATCGTATAGCATCATTAACACAGTATATTT
	Anti-NAAIRS 278 forward	AAATATACTGTGTTAATGCTGCTATACGATCGTGCCTCAAGAACAA
NAAIRS 284	NAAIRS 284 reverse	CATTTTTTTTCAAATACGATCGTATAGCATCATTTGTTAATATCGGCAT
	Anti-NAAIRS 284 forward	ATGCCGATATTAACAAATGCTGCTATACGATCGTATTTGAAAAAATG
NAAIRS 290	NAAIRS 290 reverse	ATCAAAGGTAAACTTCGATCGTATAGCATCATTTCCTGTTCTTGAGC
	Anti-NAAIRS 290 forward	GCTCAAGAACAAGAAAATGCTGCTATACGATCGAAGTTTACCTTTGAT
NAAIRS 296	NAAIRS 296 reverse	TAATAACTTGCTTCCGATCGTATAGCATCATTTATCATTTTTTTTCAA
	Anti-NAAIRS 296 forward	TTGAAAAAATGATAAATGCTGCTATACGATCGGGAAGCAAGTTATTA
NAAIRS 302	NAAIRS 302 reverse	AAGCGAGTTGTAAGACGATCGTATAGCATCATTGGAATCAAAGGTAAA
	Anti-NAAIRS 302 forward	TTTACCTTTGATTCCAATGCTGCTATACGATCGTCTTACAACCTCGCTT

Table S4, continued

<i>rif2</i> mutant	Primer name	Sequence (5' to 3')
NAAIRS 308	NAAIRS 308 reverse	ATTCAACTGGCATGTCGATCGTATAGCATCATTCTGTAATAACTTGCT
	Anti-NAAIRS 308 forward	AGCAAGTTATTACAGAATGCTGCTATACGATCGACATGCCAGTTGAAT
NAAIRS 314	NAAIRS 314 reverse	TAAGTTGGATTCTTTTCGATCGTATAGCATCATTGACAAGCGAGTTGTA
	Anti-NAAIRS 314 forward	TACAACTCGCTTGTCAATGCTGCTATACGATCGAAAAGATCCAACCTTA
NAAIRS 320	NAAIRS 320 reverse	AAATTCGAAAAGATCGATCGTATAGCATCATTATTATTCAACTGGCA
	Anti-NAAIRS 320 forward	TGCCAGTTGAATAATAATGCTGCTATACGATCGATCTTTTTTCGAATTT
NAAIRS 326	NAAIRS 326 reverse	GTGCGGAAAGACCTTCGATCGTATAGCATCATTGCTAAGTTGGATTC
	Anti-NAAIRS 326 forward	GAATCCAACCTTAGCAAATGCTGCTATACGATCGAAGGTCTTCCGCAC
NAAIRS 332	NAAIRS 332 reverse	AAACAAATAGGTAACGATCGTATAGCATCATTCAAAAATTCGAAAAA
	Anti-NAAIRS 332 forward	TTTTTCGAATTTTTGAATGCTGCTATACGATCGTTTACCTATTTGTTT
NAAIRS 338	NAAIRS 338 reverse	AATCTCAGTGTAAGCCGATCGTATAGCATCATTAGGGTGCAGAAAGAC
	Anti-NAAIRS 338 forward	GTCTTTCCGCACCCTAATGCTGCTATACGATCGGCTTACACTGAGATT
NAAIRS 344	NAAIRS 344 reverse	AGTTCTACTCTGGACCGATCGTATAGCATCATTGTTAAACAAATAGGT
	Anti-NAAIRS 344 forward	ACCTATTTGTTTAAACAATGCTGCTATACGATCGGTCCAGAGTAGAACT
NAAIRS 350	NAAIRS 350 reverse	ATCCAACAATTCATCCGATCGTATAGCATCATTATAATCTCAGTGTA
	Anti-NAAIRS 350 forward	TACACTGAGATTATAAATGCTGCTATACGATCGGATGAATTGTTGGAT
NAAIRS 356	NAAIRS 356 reverse	CAGTCTGTTTCTGATCGATCGTATAGCATCATTAAAAGTTCTACTCTG
	Anti-NAAIRS 356 forward	CAGAGTAGAACTTTTAAATGCTGCTATACGATCGATCAGAAACAGACTG
NAAIRS 362	NAAIRS 362 reverse	TGGGTAATTTTTTATCGATCGTATAGCATCATTCTTATCCAACAATTC
	Anti-NAAIRS 362 forward	GAATTGTTGGATAAGAATGCTGCTATACGATCGATAAAAAATTACCCA
NAAIRS 368	NAAIRS 368 reverse	AAAGTTATAAGCACTCGATCGTATAGCATCATTGTGCTGTTTCT
	Anti-NAAIRS 368 forward	AGAAACAGACTGACAAATGCTGCTATACGATCGAGTGCTTATAACTTT
NAAIRS 374	NAAIRS 374 reverse	AAGACGCTGGTTTTTTCGATCGTATAGCATCATTATGTGGGTAATTTTT
	Anti-NAAIRS 374 forward	AAAAATTACCCACATAATGCTGCTATACGATCGAAAAACCAGCGTCTT
NAAIRS 380	NAAIRS 380 reverse	TCGAGTTAACTTAAGCGATCGTATAGCATCATTCTTAAAGTTATAAGC
	Anti-NAAIRS 380 forward	GCTTATAACTTTAAGAATGCTGCTATACGATCGCTTAAAGTTAACTCGA
NAAIRS 386	NAAIRS 386 reverse	TTATCTATCATGTACCGATCGTATAGCATCATTGGAAGACGCTGGTT
	Anti-NAAIRS 386 forward	AACCAGCGTCTTCCAAATGCTGCTATACGATCGGTACATGATAGATAA
NAAIRS 392	NAAIRS 392 reverse	TCAAAAGACCTTGGTTTACGATCGTATAGCAGCATTTTTTTCGAGTTAACTT
	Anti-NAAIRS 392 forward	AAGTTAACTCGAAAAAATGCTGCTATACGATCGTAAACCAAGGTCTTTTGA

Table S5: Primers for epitope-tagged *rif2* mutants

Primer Name	Sequence (5' to 3')	Primer Description
Myc F	GCGTCTTCCACTTAAGTTAACTCGAAAAGTACATGATAGACGGATCCCCGGGTT AATTAA	Tagging of NAAIRS mutants
Myc R	CGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGGAATTCGAGCTC GTTTAAAC	
HK3	TACCTTTGATTCCGGAAGCAAGTTATT	Used for inserting the V5 tag upstream of Rif2 via overlap extension method
HK4	AGGCTTACCACCACCACCACCACCACCACCTCTATCATGTACTTTTCG	
HK5	CATGATAGAGGTGGTGGTGGTGGTGGTGGTGGTAAAGCCTATCCCTAAC	
HK6	GGTGGCGGCCGCTCTAGAACGTAGAATCGAGACCGAG	
HK7	CTCGGTCTCGATTCTACGTTCTAGAGCGGCCGCCACCG	
HK9	TGGCACGACAGGTTTTCCCGACTGGAAA	
HK16	CTAATCAAGTTTTTTGGGGTTCGAGGTG	
HK17	ACGGAGGTGGGGTACCCAATTCGCCCTA	
HK18	TTGGGTACCCACCTCCGTTGTCTCTA	
HK19	TTCTGTACACCATTCCGGCGTTCTGCTG	
HK20	GCCGAATGGTGACAGAAGGGATCTTC	
HK15	GGTTCAACTTTCTCAGTACAGTAAAAT	
HK113	CCAAAAATTTTCAGCAGAACGCCGAATGGTGATCAATTGAATAAATTGGTAAACT TCAC	
HK114	GATGGTTAAGAAGATCCCTTCTGTACATATCTGTAAGCGCAATGTTTTCTTCATT G	
HK65	GCATGTAGATTCCGATGCCGCACCTATAAGGAGATC	
HK66	GATCTCCTTATAGGTGCGGCATCGGAATCTACATGC	Site-directed mutagenesis 3' primer for Rif2F8A
HK67	GAGCATGTAGATTCCGATTTTTTCCCTATAAGGAGATCGAAAAAG	Site-directed mutagenesis 5' primer for Rif2A9F
HK68	CTTTTTCGATCTCCTTATAGGGAAAAAATCGGAATCTACATGCTC	Site-directed mutagenesis 3' primer for Rif2A9F
HK69	GTAGATTCCGATTTTGCAGCCATAAGGAGATCGAAAAAG	Site-directed mutagenesis 5' primer for Rif2P10A
HK70	CTTTTTCGATCTCCTTATGGCTGCAAAAATCGGAATCTAC	Site-directed mutagenesis 3' primer for Rif2P10A
HK71	GATTCCGATTTTGCACCTGCAAGGAGATCGAAAAAGG	Site-directed mutagenesis 5' primer for Rif2I11A
HK72	CCTTTTTCGATCTCCTTGCAGGTGCAAAAATCGGAATC	Site-directed mutagenesis 3' primer for Rif2I11A
HK73	GATTCCGATTTTGCACCTATAGCGAGATCGAAAAAGGTTGTTG	Site-directed mutagenesis 5' primer for Rif2R12A
HK74	CAACAACCTTTTTCGATCTCGCTATAGGTGCAAAAATCGGAATC	Site-directed mutagenesis 3' primer for Rif2R12A
HK75	GATTTTGCACCTATAAGGGCATCGAAAAAGGTTGTTG	Site-directed mutagenesis 5' primer for Rif2R13A
HK76	CAACAACCTTTTTCGATGCCCTTATAGGTGCAAAAATC	Site-directed mutagenesis 3' primer for Rif2R13A
HK77	GATTTTGCACCTATAAGGAGAGCAAAAAGGTTGTTGACAGTGAC	Site-directed mutagenesis 5' primer for Rif2S14A
HK78	GTCACTGTCAACAACCTTTTTGCTCTCCTTATAGGTGCAAAAATC	Site-directed mutagenesis 3' primer for Rif2S14A
HK79	CACCTATAAGGAGATCGGCCAAAGGTTGTTGACAGTG	Site-directed mutagenesis 5' primer for Rif2K15A
HK80	CACTGTCAACAACCTTTGCCGATCTCCTTATAGGTG	Site-directed mutagenesis 3' primer for Rif2K15A
HK81	CTATAAGGAGATCGAAAGCGGTTGTTGACAGTGAC	Site-directed mutagenesis 5' primer for Rif2K16A
HK82	GTCACTGTCAACAACCGCTTTTCGATCTCCTTATAG	Site-directed mutagenesis 3' primer for Rif2K16A
HK83	CTATAAGGAGATCGAAAAAGGCTGTTGACAGTGACAAGATTG	Site-directed mutagenesis 5' primer for Rif2V17A

Table S5, *continued*

Primer Name	Sequence (5' to 3')	Primer Description
HK84	CAATCTTGCTCACTGTCAACAGCCTTTTTTCGATCTCCTTATAG	Site-directed mutagenesis 3' primer for Rif2V17A
HK85	GGAGATCGAAAAAGGTTGCTGACAGTGACAAGATTGTG	Site-directed mutagenesis 5' primer for Rif2V18A
HK86	CACAATCTTGCTCACTGTCAGCAACCTTTTTTCGATCTCC	Site-directed mutagenesis 3' primer for Rif2V18A
HK87	GAGATCGAAAAAGGTTGTTGCTAGTGACAAGATTGTGAAAGC	Site-directed mutagenesis 5' primer for Rif2D19A
HK88	GCTTTCACAATCTTGCTCACTAGCAACAACCTTTTTTCGATCTC	Site-directed mutagenesis 3' primer for Rif2D19A
HK21	GTGAAAGCAATAGCCGATGATTTGGAG	Site-directed mutagenesis 5' primer for Rif2AA28
HK22	CTCCAAATCATCGGCTATTGCTTTCAC	Site-directed mutagenesis 3' primer for Rif2AA28
HK33	CAAAAAAATTTACTGCCCTGAGAAAGTTGAACC	Site-directed mutagenesis 5' primer for Rif2V38A
HK34	GGTTCAACTTTCTCAGGGCAGTAAAATTTTTTTG	Site-directed mutagenesis 3' primer for Rif2V38A
HK35	GCAAAAAAATTTACTGTAGCCAGAAAGTTGAACCTTGTTC	Site-directed mutagenesis 5' primer for Rif2L39A
HK35	GGAACAAGGTTCAACTTTCTGGCTACAGTAAAATTTTTTTGC	Site-directed mutagenesis 3' primer for Rif2L39A
HK37	AAATTTTACTGTACTGGCAAAGTTGAACCTTGTTC	Site-directed mutagenesis 5' primer for Rif2R40A
HK38	GAACAAGGTTCAACTTTGCCAGTACAGTAAAATTT	Site-directed mutagenesis 3' primer for Rif2R40A
HK39	TTACTGTACTGAGAGCGTTGAACCTTGTTC	Site-directed mutagenesis 5' primer for Rif2K41A
HK40	GGAACAAGGTTCAACGCTCTCAGTACAGTAA	Site-directed mutagenesis 3' primer for Rif2K41A
HK41	CTGTACTGAGAAAGGCGAACCTTGTTC	Site-directed mutagenesis 5' primer for Rif2L42A
HK42	TGGAACAAGGTTTCGCTTTCTCAGTACAG	Site-directed mutagenesis 3' primer for Rif2L42A
HK43	GTAAGTACTGAGAAAGTTGGCCCTTGTTC	Site-directed mutagenesis 5' primer for Rif2N43A
HK44	TAATTGGAACAAGGGCCAACCTTTCTCAGTAC	Site-directed mutagenesis 3' primer for Rif2N43A
HK51	GTAAGTACTGAGAAAGTTGAACGCCGTTCCAATTA	Site-directed mutagenesis 5' primer for Rif2L44A
HK52	CTTTTTTAATTGGAACGGCGTTCAACTTTCTCAGTAC	Site-directed mutagenesis 3' primer for Rif2L44A
HK23	GTTGÁACATATACACCAAGCTGCTGGTATCGACCGTG	Site-directed mutagenesis 5' primer for Rif2AA122
HK24	CACGGTCGATACCAGCAGCTTGGTGTATATGTTCAAC	Site-directed mutagenesis 3' primer for Rif2AA122
HK25	CATATACACCAATATGCTGCTATCGACCGTGCAGTTTC	Site-directed mutagenesis 5' primer for Rif2AA124
HK26	GAAACTGCACGGTCGATAGCAGCATATTGGTGTATATG	Site-directed mutagenesis 3' primer for Rif2AA124
HK27	CATATACACCAATATGCTGGTGGCACCAGTTCAGAAAC	Site-directed mutagenesis 5' primer for Rif2AA125
HK28	GTTTCTGAAACTGCACGGTCGGCACCAGCATATTGGTGTATATG	Site-directed mutagenesis 3' primer for Rif2AA125
HK29	CCAATATGCTGGTATCGCCCGTGCAGTTTCAGAAAC	Site-directed mutagenesis 5' primer for Rif2AA126

Table S5, *continued*

Primer Name	Sequence (5' to 3')	Primer Description
HK30	GTTTCTGAAACTGCACGGGCGATACCAGCATATTGG	Site-directed mutagenesis 3' primer for Rif2AA126
HK31	CACCAATATGCTGGTATCGACGCTGCAGTTTCAGAAACACTGTC	Site-directed mutagenesis 5' primer for Rif2AA127
HK32	GACAGTGTTCGAAACTGCACGCTCGATACCAGCATATTGGTG	Site-directed mutagenesis 3' primer for Rif2AA127
HK53	CTTCGAAGCTTACCGAATTTGCCAATGTTTTATCGGTACTIONG	Site-directed mutagenesis 5' primer for Rif2I233A
HK54	CAAGTACCGATAAAACATTGGCAAATTCGGTAAGCTTCGAAG	Site-directed mutagenesis 3' primer for Rif2I233A
HK55	GAAGCTTACCGAATTTTGAATGTTTTATCGGTAC	Site-directed mutagenesis 5' primer for Rif2I233C
HK56	GTACCGATAAAACATTGCAAAATTCGGTAAGCTTC	Site-directed mutagenesis 3' primer for Rif2I233C
HK57	GACAAAGTTGAACATATAGCCCAATATGCTGGTATCG	Site-directed mutagenesis 5' primer for Rif2H120A
HK58	CGATACCAGCATATTGGGCTATATGTTCAACTTTGTC	Site-directed mutagenesis 3' primer for Rif2H120A
HK63	GATCATATTGTCTACTTCAAATTCACAATTAGATAAATTATCTACTTC	Site-directed mutagenesis 5' primer for Rif2E213Q
HK64	GAAGTAGATAATTTATCTAATTGTTGGAATTTGAAGTAGACAATATGATC	Site-directed mutagenesis 3' primer for Rif2E213Q
HK61	CGACATCCCTCAAAAAGGCACTCAACACAAAATATAC	Site-directed mutagenesis 5' primer for Rif2K269A
HK62	GTATATTTTGTGTTGAGTGCCTTTTTGAGGGATGTCCG	Site-directed mutagenesis 3' primer for Rif2K269A

Table S6: Primers used in the construction of *RAP1-RIF2* fusions at the *RAP1* locus

Primer Name	Sequence (5' to 3')	Primer Description
HK89	CGGGGATCCACTAGTTCTAGAGCGGCCGCGATCTGAT GGTCTTTCCTCGCTATTTCTTG	
HK90	CCTCACTAAAGGGAACAAAAGCTGGAGCTCTGCTAATGG GATTCTATAAACTGTTCCGC	Used to amplify the C-terminus of <i>RAP1</i> ; to be used in the construction of the <i>RAP1-RIF2₆₀</i>
HK97	GATTTTTTTGAGAAGGACCTGTTAGGTGGCGGAGGTGGC GGAGGTGGCGGAGGTATGGAGCATGTAGATTCCG	
HK98	CGGAATCTACATGCTCCATACCTCCGCCACCTCCGCCAC CTCCGCCACCTAACAGGTCCTTCTCAAAAAATC	
HK93	GCAGCCCAAAGGTGTGTAAGCCGAGTTTATTCAATTCAA TTCATCATTTTTTTTTTATTTC	
HK94	GAATAAAAAAAAAAATGATGAATTGAATTGAATAAACTCGG CTTACACACCTTTGGGCTGC	Used to construct the <i>URA</i> homology region for insertion of <i>RAP1-RIF2₆₀</i> construct into yeast chromosome
HK95	CATATTTGAGAAGATGCGGCCAGCAAACTAAGTAATTG AATTAAGTAACATCATTGTTTAACT	
HK96	GTAAACAATGATGTTACTTAATTCAATTACTTAGTTTTGC TGGCCGCATCTTCTCAAATATG	
HK115	ACAATGTTAATCCTCCTCCCAAC	Forward primer for lifting out <i>RAP1-RIF2₆₀</i>
HK116	GCGTGACATAACTAATTACATGAAGATCTCTAACTCGGCT TACACACCTTTG	Reverse primer for lifting out <i>RAP1-RIF2₆₀</i>
HK117	CAAAGGTGTGTAAGCCGAGTTAGAGATCTTCATGTAATTA GTTATGTCACGC	Forward primer for <i>CYC1</i> fragment
HK118	GAATAAAAAAAAAAATGATGAATTGAATTGCAAATTAAGC CTTCGAGCGTC	Reverse primer for <i>CYC1</i> fragment
HK119	GACGCTCGAAGGCTTTAATTTGCAATTCAATTCATCATT TTTTTTTATTTC	Forward primer for lifting out part of <i>URA3</i> promoter
HK120	TGCAGGTTTTTGTCTGTGCAGTTG	Reverse primer for lifting out part of <i>URA3</i> promoter
HK121	TAGAGATCTTCATGTAATTAGTTATG	Forward primer for making <i>RAP1-RIF2₃₆</i> construct from <i>RAP1-RIF2₆₀</i> construct
HK123	AAAATTTTTTTGCTCCAAATCATCGC	Reverse primer for making <i>RAP1-RIF2₃₆</i> construct from <i>RAP1-RIF2₆₀</i> construct
HK124	GCATGTAGATTCCGATTATGCACCTATAAGGAGATC	Forward primer for making F8Y construct
HK125	GATCTCCTTATAGGTGCATAATCGGAATCTACATGC	Reverse primer for making F8Y construct
HK126	CATGTAGATTCCGATTGGGCACCTATAAGGAGAT	Forward primer for making F8W construct
HK127	GATCTCCTTATAGGTGCCAATCGGAATCTACATG	Reverse primer for making F8W construct
HK128	CGGGGATCCACTAGTTCTAGAGCGGCCGCGATTGCAC GAGAATTTTTCAAGCATTTCG	Forward primer for making <i>rap1ΔC</i> fragment
HK129	ACCTCCGCCACCTCCGCCACCTCCGCCACCGGGCAAAC TATTTGAAATATTGGATAGATC	Reverse primer for making <i>rap1ΔC</i> fragment in fusion
HK130	GATCTATCCAATATTTCAAATAGTTTGCCCGGTGGCGGA GGTGGCGGAGGTGGCGGAGGT	Forward primer for making <i>RIF2</i> fragment for <i>rap1ΔC</i> Gibson
HK131	CCTTTTCGGTTAGAGCGGATGTGGGAGGAGGGC	Reverse primer for making <i>RIF2</i> fragment for <i>rap1ΔC</i> Gibson
HK132	GCGTGACATAACTAATTACATGAAGATCTCTAGGGCAA CTATTTGAAATATTGGATAG	Reverse primer for making <i>rap1ΔC</i> plasmid (forward primer is HK128)
HK133	GCGGTCAAGAAGCAGTTTTA	Forward primer for confirming <i>rap1ΔC</i> construct at <i>RAP1</i> locus
OCC85	ATTGCCATTGCAAAATCGTTTTTGTGGTCAATTTGCAATG AGATTGTAAGTACTGAGAGTGCAC	Deletion of <i>RIF1</i>
OCC86	ATTTATTGCCATTTTGTATCTATTCTACATACTAACAATCAC TGTGCGGTATTTACACCCG	Deletion of <i>RIF1</i>
OCC122	TAGATATAAATACGAACGTGGTTAGTATATAGAGACAATG AGATTGTAAGTACTGAGAGTGCAC	Deletion of <i>RIF2</i>
OCC123	TTGTATTGTTTCAACTCTTTCAAAGACCTTGGTAATTTA CTGTGCGGTATTTACACCCG	Deletion of <i>RIF2</i>
OCC168	GGTTCTGGTGGCATCTAT	Deletion of <i>TLC1</i>
OCC171	ATGCTTGCCGAAAGCTG	Deletion of <i>TLC1</i>
Yprime FWD	CGCGAATTCGCCCTACAGCACTTCTACATAGC	Y' fragment for Southern analysis
Yprime REV	CGAGAATTCAGCGTTTGCCTCCATGACG	Y' fragment for Southern analysis