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Structural and Functional Studies of the Rap1 C-Terminus Reveal Novel Separation-of-Function Mutants

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The yeast Rap1 protein plays an important role in transcriptional silencing and in telomere length homeostasis. Rap1 mediates silencing at the HM loci and at telomeres by recruiting the Sir3 and Sir4 proteins to chromatin via a Rap1 C-terminal domain, which also recruits the telomere length regulators, Rif1 and Rif2. We report the 1.85 Å resolution crystal structure of the Rap1 C-terminus, which adopts an all-helical fold with no structural homologues. The structure was used to engineer surface mutations in Rap1, and the effects of these mutations on silencing and telomere length regulation were assayed *in vivo*. Our surprising finding was that there is no overlap between mutations affecting mating-type and telomeric silencing, suggesting that Rap1 plays distinct roles in silencing at the silent mating-type loci and telomeres. We also found novel Rap1 phenotypes and new separation-of-function mutants, which provide new tools for studying Rap1 function. Yeast two-hybrid studies were used to determine how specific mutations affect recruitment of Sir3, Rif1, and Rif2. A comparison of the yeast two-hybrid and functional data reveals patterns of protein interactions that correlate with each Rap1 phenotype. We find that Sir3 interactions are important for telomeric silencing, but not mating type silencing, and that Rif1 and Rif2 interactions are important in different subsets of telomeric length mutants. Our results show that the role of Rap1 in silencing differs between the HM loci and the telomeres and offer insight into the interplay between HM silencing, telomeric silencing, and telomere length regulation. These findings suggest a model in which competition and multiple recruitment events modulate silencing and telomere length regulation.

Keywords: Rap1; Sir complex; Rif proteins; transcriptional silencing; telomere length

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Introduction

The *Saccharomyces cerevisiae* protein Rap1 plays multiple roles in transcription regulation and genomic stability. Rap1 is a DNA-binding protein that activates transcription at promoters, represses transcription at the silent mating-type loci and telomeres, and negatively regulates telomere length.[1] Deletion of RAP1 is lethal,[2] most likely because of its role in activating some essential genes. Mutations in RAP1 have a variety of consequences that include loss of silencing at the HM loci[3] and at telomeres,[4] telomere lengthening,[5] telomere fusion,[6] and chromosome loss.[7]

Rap1 mediates mating-type and telomeric silencing, as well as telomere length regulation, by binding DNA and recruiting other proteins to chromatin. Rap1 binds specifically to the E sites in the silent mating-type (HMR and HML) loci and to the telomeric $(C_{1-3}A)_n$ repeats.[8] At each of these regions, Rap1 recruits a complex of silent information regulator (Sir) proteins, which includes Sir2, Sir3, and Sir4,[1] via direct interactions with Sir3 and Sir4.[9] Sir2, which is recruited indirectly through Sir4, deacetylates histones and thereby establishes silent chromatin.[1] At telomeres, Rap1 also recruits the Rif1 and Rif2 proteins, which are negative regulators of telomere

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Abbreviations used: 5-FOA, 5-fluoroorotic acid; GAD, GAL4-activation domain; ORC, origin recognition complex; TEV, tobacco etch virus; MPD, 2-methyl-2,4-pentanediol; SC-Trp, synthetic complete medium lacking tryptophan; SC-His, synthetic complete medium lacking histidine; Sir, silent information regulator.

length.[10] Rif1 and Rif2 are thought to mediate a telomere repeat counting mechanism in which the number of Rap1 and associated Rif1 and Rif2 molecules bound to the telomeres controls telomere elongation.[11]

Rap1 mediates mating-type and telomeric silencing as well as telomere length regulation through a C-terminal domain spanning residues 679–827. Deletion of this domain causes a loss of mating-type and telomeric silencing similar to that observed when the silencing proteins Sir2, Sir3, or Sir4 are deleted,[9] as well as an increase in telomere length[7] similar to that observed when the telomere length regulation proteins Rif1 and Rif2 are deleted.[10] Tethering the Rap1 C-terminus to DNA restores silencing in a Rap1 mutant strain[12] and causes a decrease in telomere length.[13] Yeast two-hybrid studies have demonstrated that the Rap1 C-terminal domain is sufficient for interaction with the silencing proteins Sir3 and Sir4[14] as well as with the telomere length regulators Rif1[9] and Rif2.[10] Because of these interactions with numerous proteins, we term residues 679–827 the Rap1 protein-interaction domain.

Previous studies have indicated that there is cross-talk and competition between mating-type and telomeric silencing, as well as between silencing and telomere length homeostasis. One rap1 missense mutant (*rap1–12*) gives a loss of mating-type silencing and an increase in telomeric silencing.[15] Long telomeres, which contain an increased number of Rap1 binding sites, also cause a decrease in mating-type silencing and an increase in telomeric silencing,[4] suggesting that the HM loci and telomeres compete for a limited pool of Sir proteins.[12] Deletion of Sir3 or Sir4 causes a decrease in telomere length,[16] while deletion of Rif1 and Rif2 causes a decrease in mating-type silencing and an increase in telomeric silencing.[10] These findings suggest that Sir proteins compete with Rif1 and Rif2 for binding to Rap1; such competition has been demonstrated for Sir3 and Rif1.[9] Unraveling the cross-talk and competition between the multiple Rap1-mediated processes requires an elucidation of Rap1 interactions at the molecular level.

To better understand how Rap1 mediates the diverse interactions necessary for transcriptional silencing and telomere length homeostasis, we have determined the crystal structure of the Rap1 protein-interaction domain at 1.85 Å resolution and probed the functional importance of surface residues through an extensive mutational analysis. Our results suggest that Rap1 plays distinct roles in HM silencing, telomeric silencing, and telomere length regulation and that each of these processes requires a unique set of protein–protein interactions. These findings suggest a model in which competition and multiple recruitment events modulate silencing and telomere length regulation.

Results

Structure of the Rap1 protein-interaction domain

The crystal structure of the Rap1 protein-interaction domain was determined at 1.85 Å resolution using single-wavelength anomalous dispersion phasing with selenomethionine-substituted protein. The crystallized Rap1 fragment includes residues 672–827 of Rap1, along with a 3-residue N-terminal extension and an 11-residue C-terminal extension that includes a hexahistidine tag. The crystals contained two Rap1 molecules in the asymmetric unit, although the crystallographic dimer is unlikely to be physiologically relevant, since Rap1 migrates as a monomer on a gel filtration column (data not shown). The final model reported here contains two molecules of Rap1: molecule A, containing residues 674–822, and molecule B, containing residues 674–824. With the exception of the two missing residues at the C-terminus of molecule A, the two Rap1 molecules are very similar in structure and superimpose with a root mean square deviation (r.m.s.d.) of 0.7 Å for C^{α} atoms. We focus on molecule B in the discussion here.

The Rap1 protein-interaction domain is entirely helical, containing seven α-helices and one turn of 3_{10} helix (Fig. 1). A search of known structures using the Dali server[18] found no structures with significant homology (Z score > 3.2) to the Rap1 C-terminus. Even the highest-scoring hit (2CQ8, Z score = 3.2) had only 72 residues within four helices that could be aligned with the Rap1 structure, with an r.m.s.d. of 5.0 Å for C^{α} atoms. The absence of structural homologues indicates that, to our knowledge, the Rap1 protein-interaction domain adopts a previously unrecognized fold.

Mapping published loss-of-function mutations onto the Rap1 structure

To examine the spatial distribution of previously identified Rap1 loss-of-function mutants[3,4,15] (Table S1), we mapped these mutations onto the structure (Fig. S1). Only two mutations, D727A and R747S, map to the surface of the domain, while the remainder map to buried residues or have the potential to disrupt the global fold of the domain. The two surface mutations, D727A and R747S,[3] give rise to loss of mating-type silencing and increased telomere length. Because these residues are solvent accessible and functionally important, they suggest potential interaction sites for the Sir and Rif proteins. However, D727 and R747 are on opposite sides of the structure, indicating that multiple Rap1 surfaces are required for interactions with the multiple proteins involved in mating-type silencing and telomere length regulation. We also found that, unlike the D727 and R747 mutations, other published loss-of-function mutations have the potential to disrupt the global fold of the domain and thus offer little insight into how Rap1 recruits other proteins. For example, a series of Rap 1 C-terminal deletion mutants[15] lack at least one helix that may be required for proper folding of other parts of the domain. Several reported mutations—L736M, K809A, H810Y, and R818A[15]—alter buried residues and are likely to affect the tertiary structure of the protein rather than directly disrupting protein–protein interactions. Although

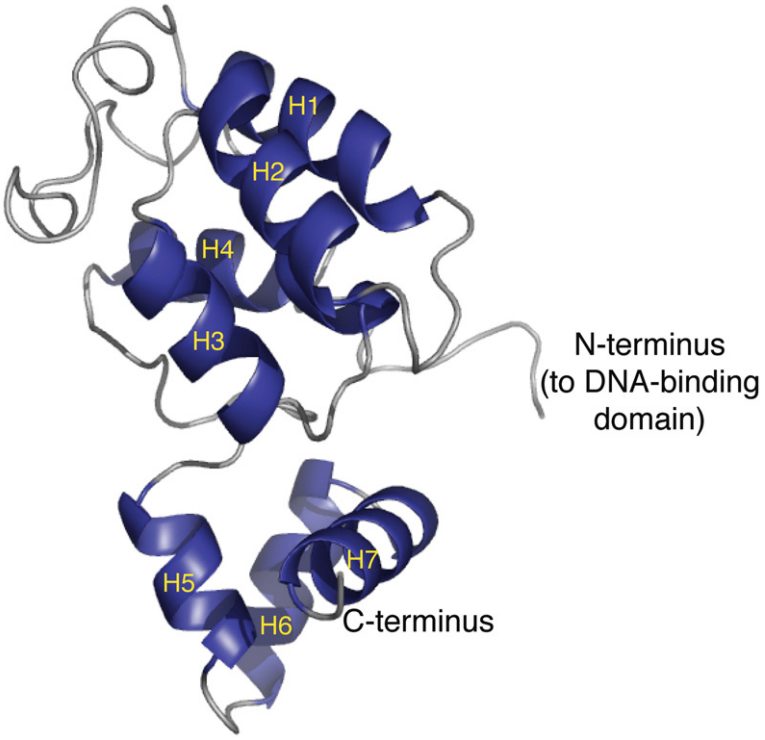

Fig. 1. Crystal structure of the Rap1 protein-interaction domain. Helices H1 (713–725), H2 (733–744), H3 (748–757), H5 (789–796), H6 (800–810), and H7 (812–822) are entirely α-helical; helix H4 (762–775) includes one turn with 3_{10} geometry followed by three turns with α geometry. All structure graphics were made using PyMOL.[17]

these mutations do not seem to completely destabilize the domain, since the mutants function as well as wild type in a subset of functional assays, they offer little insight into the role these mutated residues play in protein–protein interactions. Mutation of pairs of residues near the C-terminus, F821 F822 and E823 D825, to glycine[15] introduces backbone flexibility that could have a global effect on tertiary structure. Similarly, mutation of a glycine, G726, to aspartic acid[3] has the potential to restrict backbone flexibility. In the structure, G726 adopts Ramachandran angles that do not preclude substitution with an aspartic acid. However, the helix and loop that flank residue 726 seem to be flexible, since they adopt slightly different orientations in each of the two Rap1 molecules of the asymmetric unit. Restricting the flexibility of G726 could, therefore, have a more global effect on interactions if this local flexibility is important for interaction with the Sir or Rif proteins. Another previously identified mutation, P694L,[19] is also likely to have structural effects, since mutation of a proline introduces backbone flexibility. This mutant also has a DNA-binding defect,[19] suggesting that the P694 mutation causes a conformational change that allows the protein-interaction domain to interfere with the structure or function of the DNA-binding domain. Of the 25 previously described loss-of-function mutants, just two—D727A and R747S[3]—offer insight into the local effects of Rap1 surface substitutions on function and interactions.

Identification of new Rap1 loss-of-function mutants

To identify additional regions of the Rap1 protein surface important for silencing and telomere length regulation, we generated 49 Rap1 mutants containing substitutions at one or more surface side chains (Table S2) and tested their effect on mating-type silencing, telomeric silencing, and telomere length. Using our structure to determine the solvent accessibility of all residues, we identified 37 surface side chains where substitutions would not be expected to have an effect on protein folding (Fig. S2a). A total of 49 Rap1 mutants were generated, each containing one or more substitutions at these solvent-exposed residues. These included 32 single mutants, 8 double mutants, and 9 "patch" mutants with three or more substitutions at spatially adjacent residues (Fig. S2b). In addition, a mutant with a complete deletion of the C-terminal Rap1 fragment used for crystallization, Δ672–827, exhibited a loss-of-function phenotype in all assays, as expected from previous studies of similar C-terminal deletion mutants.[15] Western blot analysis was performed to verify that impairment of Rap1 function was not due to decreased protein expression (Figs. S3 and S4b). We focus here on mutants that exhibited partial or complete loss of function; mutants with wild-type phenotypes are listed in Supplementary Data (Table S2).

Mating-type silencing by Rap1 mutants was assayed with a TRP1 marker at an hmrΔA locus[3]; growth on synthetic complete medium lacking tryptophan (SC-Trp) indicates a loss of Rap1 function. The weakened hmrΔA was used because the HMR locus has redundant sites for Sir complex recruitment and only requires two of its three silencers to completely silence the HMR locus. Mutants that inactivate Rap1, therefore, show no phenotype unless one of the silencers is deleted. Two mutants gave a decrease or loss of HMR silencing in our assay (Fig. 2a). The previously reported D727A single mutant[3]

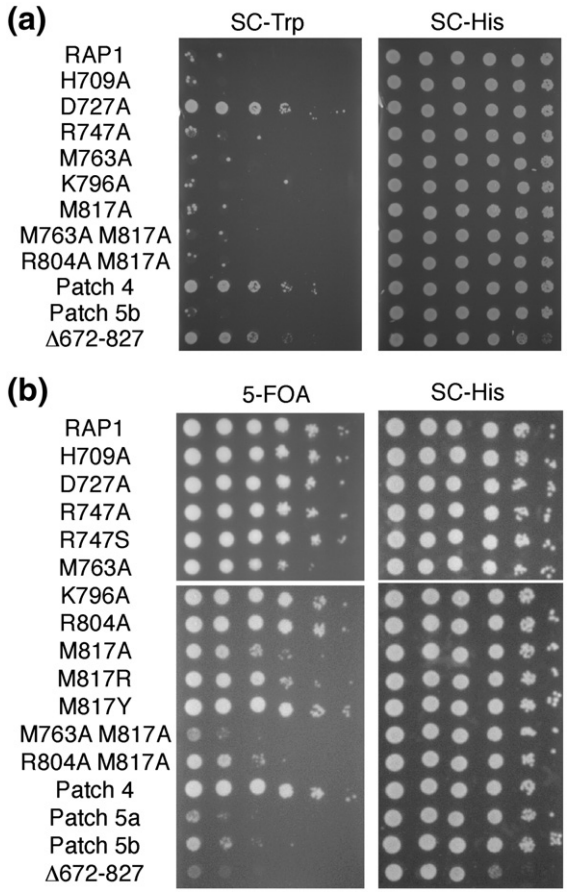

Fig. 2. Silencing phenotypes of Rap1 mutants. (a) Silencing of the mating-type loci was tested in an HMR silencing assay with YEF strains. Serial dilutions of each mutant strain were plated on medium lacking tryptophan (SC-Trp) or histidine (SC-His). Growth on SC-Trp indicates a loss of mating-type locus silencing. SC-His was used as a growth control. (b) Silencing of the telomeres was tested in a telomeric silencing assay with CLY strains. Serial dilutions of each mutant strain were plated on medium containing 5-FOA, which is lethal to cells expressing URA3, or medium lacking histidine (SC-His). Inability to grow on 5-FOA indicates a loss of telomeric silencing. SC-His was used as a growth control. Patch mutants are as follows: Patch 4, S725A D727A E729A; Patch 5a, D761A M763A M817A; Patch 5b, D761A M763A R814A M817A.

showed a decrease in silencing in our assay, as expected. Combining the D727A mutation with S725A and E729A mutations (Patch 4) gave the same loss-of-silencing phenotype as the D727A single mutant. Because an R747S mutation had been reported to cause a decrease in silencing,[3] an R747A mutation was tested. Unlike the serine mutation, the alanine mutation at R747 caused no decrease in silencing.

Telomeric silencing by Rap1 mutants was assayed with a URA3 marker at the VII_L telomere;[15] inability to grow on medium containing 5-fluoroorotic acid (5-FOA)[20] indicates a loss of Rap1 function. Eight mutants, all of which included substitutions at either M763 or M817, exhibited a decrease or loss of telomeric silencing in our assay (Fig. 2b). A M763A single mutant gave a slight decrease in silencing. Phenotypes of M817 mutants depended on the nature of the side chain; a tyrosine mutant showed no decrease in silencing, an arginine mutant showed a slight decrease, and an alanine mutant showed a more significant decrease. The M763A and M817A had additive effects when combined in a double mutant, giving a decrease in silencing more severe than with either single mutant. Two mutants, R804A M817A and D761A M763A M817A (Patch 5a), gave a loss of silencing almost as severe as that observed with the C-terminal deletion mutant Δ672–827. Aside from D761A and R804A, additional mutations on surfaces near M763 or M817 did not enhance the telomeric silencing defect. The D727A and Patch 4 mutants, which caused a loss of mating-type silencing, showed no telomeric silencing defect. We also tested an R747S mutant, which had previously been shown to cause mating-type silencing and telomere length defects,[3] in the telomeric silencing assay (Fig. 2b). Like the other mating-type silencing mutants, R747S showed no telomeric silencing defect.

The effect of Rap1 mutations on telomere length was measured by Southern blot analysis (Fig. 3). Many mutations gave rise to an increase in telomere length, although none of the effects was as severe as that of a C-terminal deletion mutant. Consistent with published results,[3] a D727A single mutant exhibited an increase in telomere length, as did the single mutations H709A, R747A, K796A, M817A, and M817R. Mutating additional residues around D727 (Patch 4) gave rise to increased heterogeneity in telomere length. None of the other multiple mutants exhibited greater telomere lengthening than the corresponding single substitution. Interestingly, the M763A mutation caused telomere shortening (Fig. S4a), while combining this M763A mutation with the M817A mutation restored wild-type telomere length. To our knowledge, this is the first report of a Rap1 mutant that causes a decrease in telomere length at physiological temperature.

Effects of loss-of-function mutations on Rap1 interactions

Loss of Rap1 function presumably results from the effect of mutations on the ability of Rap1 to interact with other proteins. To probe more specifically which interactions are affected by Rap1 mutations, we selected 10 Rap1 loss-of-function mutants and used quantitative yeast two-hybrid assays to study their interactions with Sir3, Rif1, and Rif2 (Fig. 4 and Table S3). We also used yeast two-hybrid assays to examine Rap1 interaction with Sir4, but found that a GAL4-activation domain (GAD)/Sir4 fusion causes comparable levels of β-galactosidase activation with LexA alone as with a LexA/Rap1 fusion (data not shown). As previously reported,[12,14] the β-galactosidase activation observed with the GAD/Sir4 fusions was significantly greater than that with GAD alone, suggesting that Sir4 interacts weakly with both LexA and LexA/Rap1. Because of this weak yeast two-hybrid signal, we were unable to assess the effect of our Rap1 mutants on Sir4 interactions.

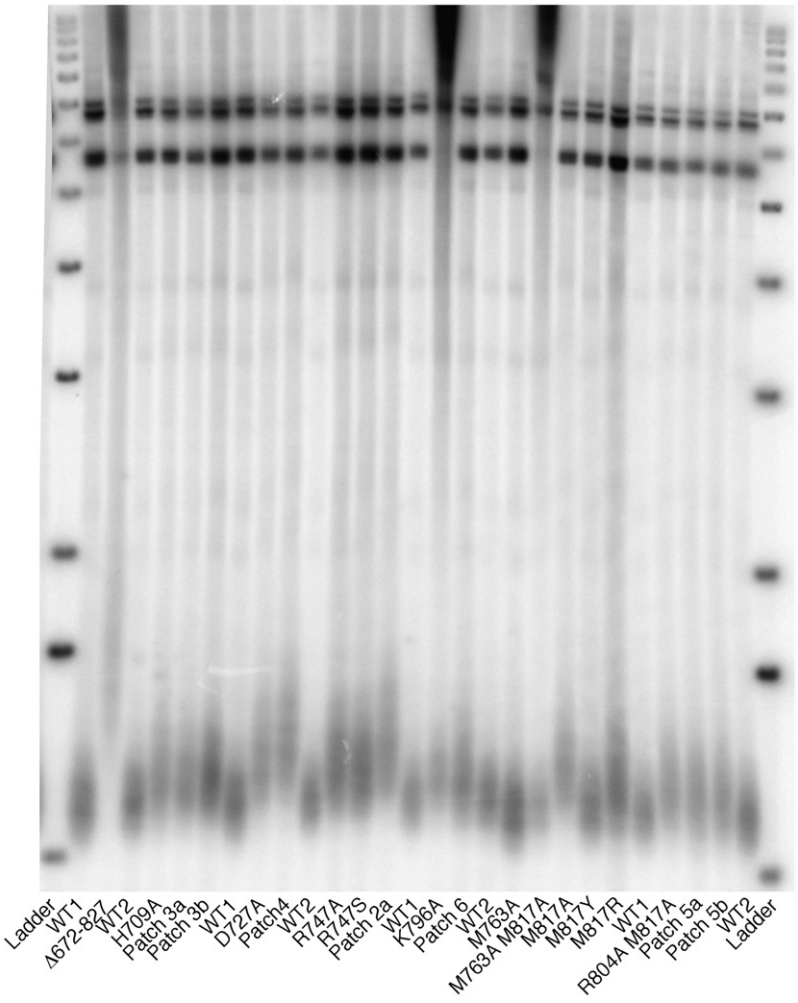

Fig. 3. Telomere length phenotypes of Rap1 mutants. Genomic DNA from strains containing each Rap1 mutant was digested with XhoI and analyzed by Southern blot with a ^{32}P-labeled poly(dC-dA) probe. Reduced migration on the gel indicates an increase in telomere length. Patch mutants are as follows: Patch 2a, T700A D701A R747A K748A N749A; Patch 3a, H709A D742A E743A; Patch 3b, L706A H709A D742A E743A; Patch 4, S725A D727A E729A; Patch 5a, D761A M763A M817A; Patch 5b, D761A M763A R814A, M817A; Patch 6, K796A R804A T812A. Ladder is a 1-kb DNA ladder (Invitrogen) with bands at 1-, 1.6-, 2-, and 1-kb intervals thereafter.

A comparison of the yeast two-hybrid data and genetic phenotypes (Table 1) reveals patterns of protein interactions that correlate with each Rap1 function. These patterns are most consistent for mutations that reduce the yeast two-hybrid assay to less than 30% of wild-type values. We found that all mutants with telomeric silencing defects have decreased interactions with Sir3. Interestingly, those mutants that affect telomeric silencing without increasing telomere length regulation had the most dramatic effect on Sir3 recruitment, reducing activation levels to that of the negative control. Mutants with both telomere length and silencing defects were distinguished by marked decreases in interactions with Rif1. In contrast, three of the four mutants tested that were impaired only in telomere length regulation had dramatically decreased interactions with Rif2 only.

The general correlation of phenotype with the yeast two-hybrid data is, however, imperfect. The M763A mutant, which confers a decrease in both telomeric silencing and telomere length, has decreased interactions with both Sir3 and Rif2, suggesting that an impaired Sir3 interaction can reverse the effect of the impaired Rif2 interaction. A K796A mutant has a moderate defect in telomere length regulation but no significant decrease in interaction with Rif1 or Rif2. It should also be noted that the correlation between telomeric silencing and Sir3 interactions is only qualitative. For example, the M763A mutant shows no Sir3 interaction but has only a mild decrease in telomeric silencing, while the R804A M817A mutant retains some Sir3 interaction but has an almost complete loss of telomeric silencing. This lack of quantitative correlation is most likely due to the compounded effects of mutations that alter Rap1 interactions with more than one binding partner. Despite these caveats, this pattern of functional and interaction defects reveals protein interactions essential for each Rap1 function.

Functionally important surfaces of Rap1

Mapping our loss-of-functions mutants onto the Rap1 structure reveals a common surface important for Rap1 function and interactions (Fig. 5). This surface includes residues S725, D727, E729, D761, M763, and M817; we term these residues the M817 cluster. Mutation of residues in this cluster causes defects in mating-type silencing, telomeric silencing, or one form of silencing plus telomere length regulation. Residues in this cluster are also required for interactions with Sir3, Rif1, and Rif2.

Although the majority of the loss-of-function mutations map to the M817 cluster, other regions of the

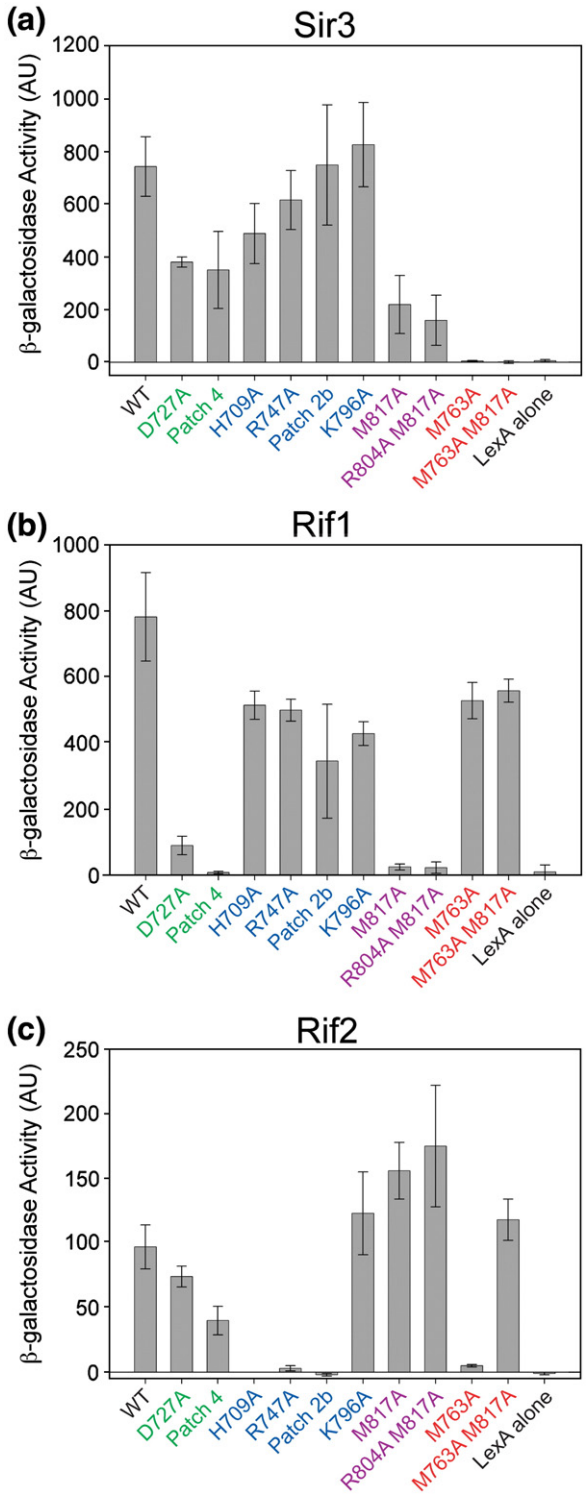

Fig. 4. Effects of Rap1 mutations on protein–protein interactions in a yeast two-hybrid assay. Interaction of LexA/Rap1(679–827) fusions with (a) GAD/Sir3(356–978), (b) GAD/Rif1(1614–1916), and (c) GAD/Rif2(14–395) was measured as β-galactosidase activity (see Materials and Methods). Error bars indicate one standard deviation. Colors indicate phenotypes as follows: green, mating-type silencing and telomere length defects; blue, telomere length defect only; purple, telomeric silencing and telomere length defects; red, telomeric silencing defect only. Patch mutants are as follows: Patch 2b, T700A D701A R747A K748A N749A S753A; Patch 4, S725A D727A E729A. AU, arbitrary units.

Rap1 protein-interaction domain are involved in function and interactions. Three residues required for telomere length regulation, H709, R747, and K796, form a ridge on a surface opposite the M817 cluster that we term the R747 ridge. This ridge likely functions in Rif2 interactions, since mutations at either H709 or R747 disrupt this interaction. Previous studies have also shown that R747 is important for mating-type silencing,[3] indicating that two distinct surfaces are required for HM silencing. One residue involved in telomeric silencing, R804, falls on a surface of the protein distant from both the M817 cluster and the R747 ridge and comprises a third functional surface. Thus, the M817 cluster is necessary for Rap1 function and interactions, but multiple regions of the protein-interaction domain play a role in each Rap1 function.

Discussion

The structure of the Rap1 protein-interaction domain and new mutagenesis experiments provide insight into Rap1 function and raise new questions about the diverse roles of Rap1. The crystal structure of the Rap1 C-terminus reveals an all-helical domain with no structural homologues. Using the structure to design a large set of surface mutations, we probed the role of the Rap1 protein surface in three Rap1 functions—mating-type silencing, telomeric silencing, and telomere length regulation—and in interactions with proteins essential for these functions—Sir3, Rif1, and Rif2. The results show a complex picture of how Rap1 mediates each of these functions with surprising separation-of-functions mutants, which reveal unexpected differences between mating-type and telomeric silencing.

Rap1 plays distinct roles in telomeric and mating type silencing

Despite a comprehensive examination of published and new Rap1 mutants, we found no missense mutants with defects in both mating-type and telomeric silencing (Fig. 6 and Table S2). Of the 49 mutants tested in our studies, two have defects in HM silencing, while eight have defects in telomeric silencing, yet there is no overlap between these two sets (Fig. 6). This result is surprising because both forms of silencing depend on the Sir complex and are thought to occur by the same general mechanism. Our yeast two-hybrid data also show that different sets of interactions correlate with each type of silencing (Table 1). Telomeric silencing mutants had a far greater effect on interactions with Sir3 than did HM silencing mutants. The lack of overlap between the two sets of silencing mutants suggests that Rap1 plays different, context-dependent roles in silencing at the HM loci and at telomeres.

Earlier studies[12,15] had noted differences between HM and telomeric silencing, but the complete lack of overlap between the two types of silencing was unexpected. Before the current study, only three

Table 1. Summary of functional and binding phenotypes

	TEL decrease	HM decrease	Length change	Sir3 (% WT)	Rif1 (% WT)	Rif2 (% WT)
D727A	WT	–	+	51	10	76
Patch 4	WT	–	+++	47	0	42
H709A	WT	WT	+	65	65	0
R747A	WT	WT	++	83	63	5
Patch 2b	WT	WT	++	101	43	−1
K796A	WT	WT	+	111	54	126
M817A	–	WT	++	29	2	159
R804A M817A	–	WT	+++	21	2	178
M763A	–	WT	–	0	67	6
M763A M817A	–	WT	WT	−1	71	120

Minus signs (−) indicate a decrease in silencing or telomere length. Plus signs (+) indicate an increase in telomere length. Binding is expressed as a percentage of the range between background and wild-type values. WT, wild type.

missense mutants had been tested for both HM and telomeric silencing defects. Two, L736M and H810Y, showed defects in telomeric silencing, while the third, G726E D727N, showed a defect in HM silencing with a slight increase in telomeric silencing.[15] A later study showed that increased telomere length decreases HM silencing, and the authors suggested that the HM loci and telomeres compete for limited pools of Sir proteins, specifically that Sir4 is limiting in mating-type silencing and that Sir3 is limiting in telomeric silencing.[12] Our data provide further evidence for this limiting-protein hypothesis, although we cannot rule out other context-dependent differences as the cause of the differences between the two silenced regions.

The different roles Rap1 plays at the HM loci and telomeres may result in part from the different sets of proteins that provide redundant means of Sir3 and Sir4 recruitment at the two loci. Each of the four silencers at the HM loci has a binding site for the origin recognition complex (ORC), which recruits Sir4 indirectly through interactions with Sir1.[21] The HMR-E silencer examined in our assays also has a binding site for Abf1, which has been recently shown to recruit Sir3 (P. Moretti, P. Yuan, R. Sternglanz, and D. Shore, personal communication). Wild-type strains therefore have a redundant means of recruiting both Sir3 and Sir4. Because HM silencing is so robust, functional mutants are difficult to isolate, and we therefore used the previously reported hmrΔA strain,[3] which has a functional Abf1 binding site and a mutated ORC binding site at the HMR-E silencer. This leaves a redundant means for Sir3 recruitment but no redundant means for Sir4 recruitment. It is possible that the observed HM silencing defects in our assays are due to impairment in the ability of Rap1 to recruit Sir4. The telomeres, in contrast, have a redundant means for Sir4 recruitment via interaction with the Ku complex,[22] but no redundant means for Sir3 recruitment. As mentioned above, all of the

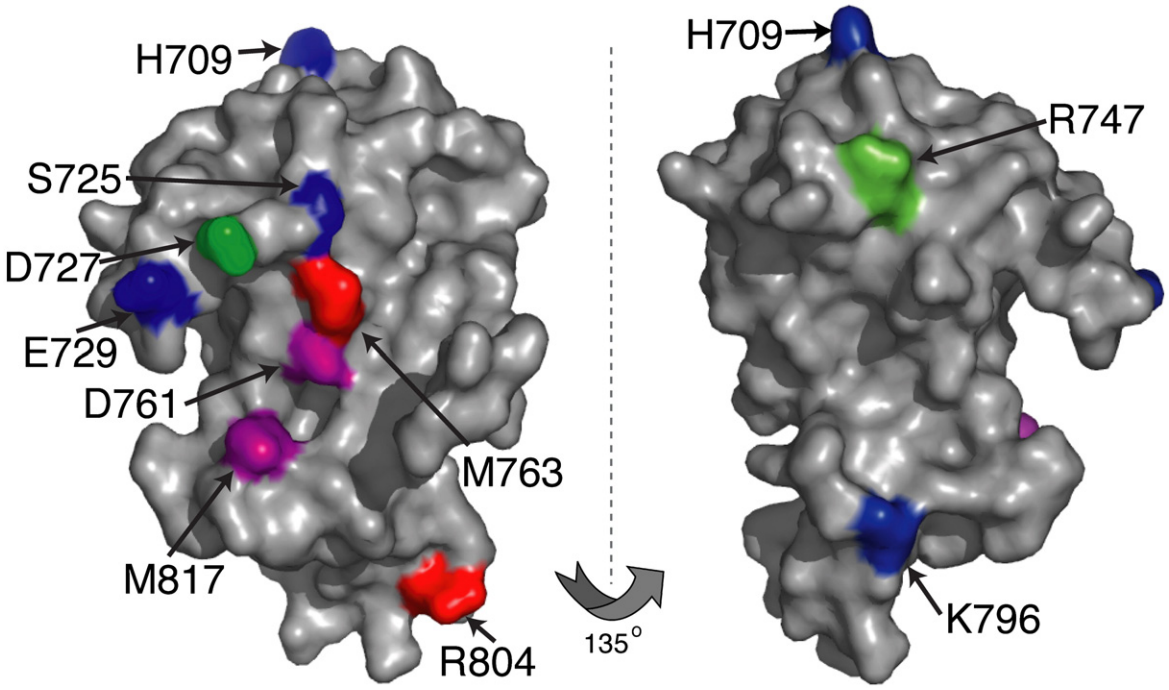

Fig. 5. Residues at which mutation causes a loss of Rap1 function. Side chains are colored in the same color scheme as in Fig. 4. Left, the M817 cluster plus R804; right, the R747 ridge.

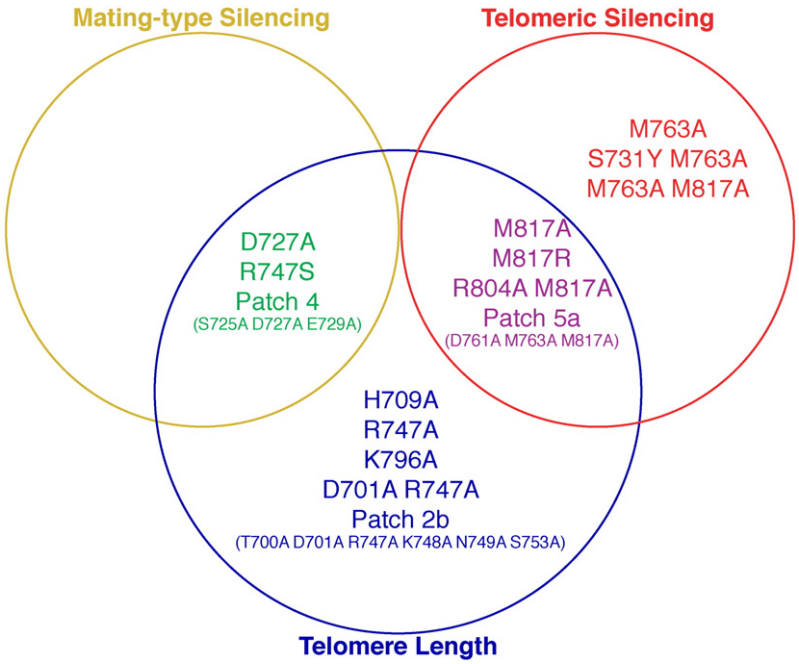

Fig. 6. Overlapping phenotypes of Rap1 loss-of-function mutants. Side chains are colored in the same color scheme as in Fig. 4. With the exception of Patch 2b, patch mutants with defects of comparable magnitude to those of mutants with fewer substitutions are not shown.

telomeric silencing mutants isolated in this study had marked defects in Sir3 recruitment, while the HM silencing mutants did not. Thus, in our assays, the essential function of Rap1 at the telomeres may be Sir3 recruitment, while the essential role of Rap1 at the HM loci may be Sir4 recruitment.

Differences in the number of Rap1 binding sites at the HM loci and at telomeres could also give rise to distinct roles for Rap1 in transcriptional silencing at each of these regions. The HM loci have only one Rap1 binding site per silencer,[23] while the telomeres each have as many as 20 tandem Rap1 binding sites.[24] Tandem binding of Rap1 at the telomeres could bring neighboring Rap1 molecules into close proximity, which could affect the conformation of Rap1 or mask surfaces of Rap1 important for interactions with other proteins. Rap1 molecules bound in tandem could also bind cooperatively to silencing proteins. One Rap1 molecule could interact with the Sir3 component of the Sir complex, while an adjacent Rap1 molecule interacts with the Sir4 component of the same complex. Interactions with adjacent Rap1 proteins could also explain why residues important for interactions with Rif2 map to opposite sides of the Rap1 C-terminal domain (Fig. 5). A single Rif2 molecule may simultaneously bind the H709-R747 surface of one Rap1 molecule and the M763 surface of a neighboring Rap1 molecule. Further studies of the effect of different DNA templates on Rap1 interactions will be needed to sort out what role, if any, cooperative interactions may play in recruiting silencing proteins. We note that the yeast two-hybrid system used in the present study more closely mimics Rap1 binding to telomeres, since these experiments used a reporter strain with eight tandem binding sites for the LexA/Rap1 fusion. If tandem Rap1 binding affects interactions with other proteins, the yeast two-hybrid results would better reflect Rap1 interactions at telomere than those at the HM loci.

Separation of telomeric silencing and length regulation functions

In this study, we found new separation-of-function mutants that have defects in only telomeric silencing or only telomere length regulation, as well as mutants with both telomeric silencing and telomere length defects. Mutants with long telomeres and functional telomeric silencing as well as those with long telomeres and defective telomeric silencing have been reported,[15] but the molecular basis for these phenotypes was not known. We found that defects in only telomere length regulation correlate with impaired Rif2 interactions, while defects in both telomeric silencing and telomere length regulation correlate with impaired Sir3 and Rif1 interactions (Table 1). We also found a new class of mutants, including a M763A single mutant and a M763A M817A double mutant, with telomeric silencing defects and short or wild-type telomeres. Like the M817A and R804A M817A mutants with both telomeric silencing and length defects, these mutants had impaired Sir3 interactions. Interestingly, the Sir3 interaction was perturbed to a greater degree with the M763A mutants than with those that had both silencing and length defects, yet the M763A mutant has a much milder loss of silencing than either the M817A or R804A M817A mutant, suggesting that additional factors contribute to the loss of telomeric silencing. Our separation-of-function mutants partially reveal the molecular basis for the various telomere phenotypes and will provide a tool for future studies.

Conservation of the Rap1 protein-interaction domain

Rap1 homologues in yeast species closely related to *S. cerevisiae* have high sequence similarity to *S. cerevisiae* Rap1,[25–27] so we expect the protein-interaction

domain of these homologues to have the same structure as *S. cerevisiae* Rap1. Despite this overall structural conservation, many of the surface residues that we found to be important for Rap1 function are not conserved among yeast species. A few residues, such as R747 and M817, are similar in all yeast homologues, while other residues, including H709, are not conserved at all. This lack of conservation is not surprising because the proteins with which Rap1 interacts are also poorly conserved among yeast species. We predict that changes on the Rap1 surface compensate for changes in the Rap1-interacting proteins.

In contrast to yeast Rap1 homologues, Rap1 homologues in higher eukaryotes share only poor sequence similarity with *S. cerevisiae* Rap1. This poor sequence conservation is consistent with the different Rap1 protein interactions in yeast and higher eukaryotes. Higher eukaryotes have no known Sir3, Sir4, or Rif2 homologues, and human Rap1 is not thought to interact with human Rif1. A sequence alignment of the human and *S. cerevisiae* protein-interaction domains shows only 25% identity.[28] Published analysis has shown that pairwise alignments with sequence identity lower than 30% are often inaccurate.[29] To test the accuracy of this alignment, we used both ClustalX[30] and M-Coffee[31] for alignments and found that the two programs generated different alignments (data not shown); this suggests that the sequence similarity is too low to draw conclusions about structural homology. Thus, a structure of the Rap1 C-terminus of higher eukaryotes must be determined independently.

Model of functional protein interactions at the HM loci and telomeres

Our findings suggest a model in which multiple Sir protein recruitment events and competition between Sir and Rif proteins determine the degree of silencing at the HM loci and telomeres and of telomere elongation or shortening (Fig. 7). In this model, the formation of stable silencing complexes at the HM loci and telomeres requires that the Sir complex be recruited by more than one chromatin-associated molecule to become stably associated with and silence that region of chromatin. At the silent mating-type loci, each of the four silencers has at least two distinct DNA-binding proteins, each of which can recruit Sir3 or Sir4. At telomeres, tandemly bound molecules of Rap1 or a combination of Rap1 and Ku70 could recruit Sir3 and Sir4. We favor the former hypothesis since previous studies have shown that Sir4 does not associate with telomeres in the absence of Rap1.[32] Previous studies have shown that Sir4 can associate with the HM loci and telomeres independent of other Sir proteins[32–34] and that Sir3 and Sir4 can interact directly.[9,35] Our finding that Rap1 mutations that disrupt Sir3 interactions cause a loss of telomeric silencing suggests that indirect recruitment of Sir3 via interactions with Sir4 is not sufficient for stable association of Sir3 with chromatin. We propose that multiple recruitment events, requiring interactions between multiple chromatin-associated molecules and Sir proteins, stabilize the Sir complex and facilitate chromatin silencing. Competition between Sir and Rif proteins for interaction with Rap1 could also affect both silencing and telomere length. Impaired Sir binding could free telomere-bound Rap1 molecules for Rif binding, while impaired Rif binding could free telomere-bound Rap1 molecules for Sir binding. However, additional factors, including cooperative binding by and interactions between Sir3 and Sir4 or Rif1 and Rif2, as well as changes in telomere length, also contribute to the net effect of impaired Rap1 interactions. Together with our data, this model opens new avenues to explore how the Sir complex binds in the context of chromatin and what effect competition from Rif1 and Rif2 has on Sir complex binding.

Materials and Methods

Expression and purification of Rap1 protein

A DNA fragment encoding residues 672–827 of Rap1 was amplified using the PCR from *S. cerevisiae* genomic DNA with primers containing added sequences encoding an N-terminal tobacco etch virus (TEV) protease cleavage site and a C-terminal hexahistidine tag, each flanked by attB recombination sites. Sequential recombination reac-

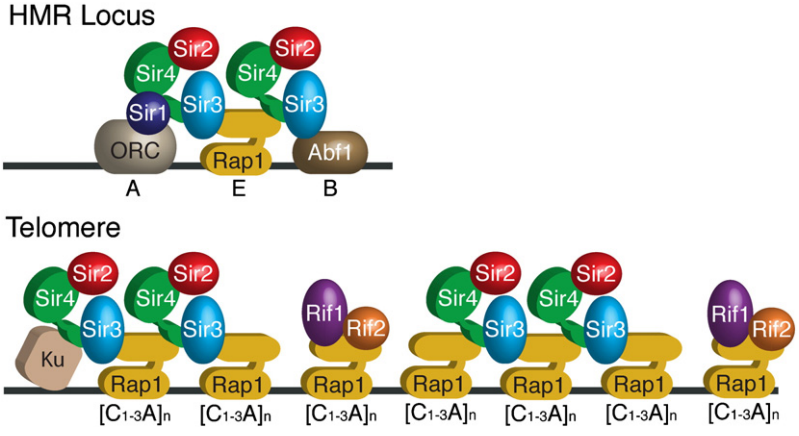

Fig. 7. Multiple recruitment model for mating-type and telomeric silencing. In this model, the Sir complex is recruited via multiple interactions with either ORC, Rap1, and Abf1 at the HM loci or Rap1 and Ku70 at the telomeres. Sir3 and Sir4 compete with Rif1 and Rif2 for binding to Rap1 at the telomeres.

tions were used to insert the PCR product into a pMALc2g vector (NEB) adapted for use in the Gateway system according to the Gateway Technology manual (Invitrogen). The resulting plasmid, pMAL/Rap672H, encoding residues 672–827 of Rap1 with a TEV-cleavable N-terminal maltose binding protein tag and a C-terminal hexahistidine tag, was transformed into *Escherichia coli* BL21 (DE3) pLysS cells for expression.

Selenomethionine-substituted protein was expressed using the methionine-suppression method.[36] *E. coli* cells were grown at 37 °C to an OD_{600} of 0.5, and Rap1 expression was induced with 1 mM IPTG at 20 °C. Cells were harvested by centrifugation 20 h after induction, and cell pellets were stored at −80 °C until lysis. Cells were resuspended in 50 mM sodium phosphate (pH 7.4) with 0.5 M NaCl, 1 mM DTT, 20% glycerol, 20 mM $MgCl_2$, 200 μl DNase I (Roche), and Complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche) and lysed with a microfluidizer. The lysate was cleared by centrifugation, and the supernatant was filtered.

The double-tagged Rap1 protein was separated from other soluble proteins by affinity chromatography with HisTrap (Amersham) and amylose (NEB) columns. The N-terminal maltose binding protein tag was cleaved with TEV protease, and the resulting single-tagged Rap1 was purified by anion-exchange chromatography with a MonoQ column (Amersham) and size-exclusion chromatography with a Superdex 75 column (Amersham). Purified protein was concentrated to 7.5 mg/ml in a buffer containing 20 mM Hepes (pH 7.4) with 1 mM Tris(2-carboxyethyl)phosphine hydrochloride and 1 mM EDTA.

Crystallization

Crystals were grown at 4 °C by the hanging-drop vapor-diffusion method using a 1:1 ratio of protein to well buffer containing 0.1 M Mes (pH 6.4) and 42% 2-methyl-2,4-pentanediol (MPD). The initial, irregular crystals were crushed using a SeedBead (Hampton) for microseeding into preequilibrated drops with well buffer containing 0.1 M Mes (pH 6.2–6.6) and 36–42% MPD. This seeding process was repeated using the resulting crystals as seeds until single crystals were obtained that were then harvested and frozen in liquid nitrogen.

Structure determination

Diffraction data to 1.85 Å resolution were recorded from frozen crystals at GM/CA-CAT, Advanced Photon Source, Argonne National Laboratory, on a MARmosaic300 detector and processed using HKL2000.[37] The crystals formed in space group $P2_1$ with unit cell dimensions $a = 48.01$ Å, $b = 47.98$ Å, $c = 72.56$ Å, $\alpha = \gamma = 90°$, $\beta = 94.23°$ and two molecules of protein per asymmetric unit. Phases were determined by single-wavelength anomalous dispersion using SOLVE,[38] and initial maps were calculated using RESOLVE.[39] Chain tracing with ARPw/ARP[40] yielded an initial model with 90% of the protein backbone fit to the density. The model was subjected to rigid-body and simulated annealing refinement with CNS,[41] and multiple rounds of rebuilding and refinement were done with O,[42] CNS, Coot,[43] and RefMac.[40] The final model includes residues 674–822 of Rap1 molecule A, residues 674–824 of Rap1 molecule B with Arg674 and Lys824 modeled as alanines, two molecules of Mes, three molecules of MPD, and 303 water molecules. The model has an overall *B*-factor of 21.9 $Å^2$. The Ramachandran plot shows 94.5% of residues in most favored regions and the remaining 5.5% in generously allowed regions. Statistics for the final model are presented in Table 2.

Mutagenesis and yeast strain construction

The wild-type pRS313/Rap1 plasmid[4] has been described previously. Mutations in the pRS313/Rap1 plasmid were introduced with the QuikChange II or QuikChange Multi Site-Directed Mutagenesis Kits (Stratagene) using complementary or sense oligonucleotide primers. Wild-type or mutant pRS313/Rap1 plasmids were introduced into yeast strains with deletions of the chromosomal RAP1 gene by the plasmid shuffle method.[44] For HMR silencing and telomere length assays, Rap1 plasmids were transformed into YLS34, a W303-1B-based strain with an hmrΔA mutation and TRP1 marker in the HMRE silencer,[3] yielding the YEF strains. For telomeric silencing assays, Rap1 plasmids were transformed into the GK28-3b strain, in which the URA3 gene has been inserted at the VII_L telomere,[15] yielding the CLY strains.

Transcriptional silencing assays

Silencing at the HMR mating-type locus was assayed in YEF strains, in which HMR silencing and TRP1 expression are dependent on functional Rap1.[3] As an initial screen, YEF strains were streaked for single colonies and replica-plated on SC-Trp. Strains that grew in this initial screen as well as other strains of interest were diluted to an OD_{600} of 0.5 and plated in serial fivefold dilutions on SC-Trp as a test for loss of HMR silencing and on synthetic complete medium lacking histidine (SC-His) as a growth control.

Silencing at the telomeres was assayed in CLY strains, in which telomeric silencing and URA3 repression are dependent on functional Rap1.[15] CLY strains were diluted to an OD_{600} of 0.5 and plated in serial 10-fold dilutions on synthetic complete medium with 5-FOA (SC+5-FOA) as a

Table 2. Crystallographic statistics

Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	48.01, 47.98, 72.56
α, β, γ (°)	90.00, 94.23, 90.00
Wavelength (Å)	0.9797
Resolution (Å)	50–1.85 (1.92–1.85)
R_{sym} (%)	11.0 (26.1)
$I/\sigma(I)$	24.5 (7.5)
Completeness (%)	99.5 (95.9)
Redundancy	7.3 (5.9)
Refinement	
Resolution (Å)	1.85
No. reflections	28,210
R_{work}/R_{free} (%/%)	17.5/19.9
No. atoms	
Protein	2466
Solvent	48
Water	303
B-factors	
Protein	20.9
Solvent	25.5
Water	29.2
r.m.s.d.	
Bond lengths (Å)	0.007
Bond angles (°)	1.71

Numbers in parentheses indicate statistics for the outermost shell.

test for functional telomeric silencing and on SC-His as a growth control.

Telomere length assays

Genomic DNA from YEF strains was prepared by the glass bead method[45] and digested with XhoI. Telomere length was determined by Southern blotting with a ^{32}P-labeled poly(dC-dA) probe.[7]

Western blots

Whole-cell lysates from YLS and YEF strains were separated by SDS-PAGE and electroblotted to nitrocellulose membranes. Membranes were probed with a rabbit polyclonal anti-Rap1 antibody (Santa Cruz) followed by a horseradish-peroxidase-coupled donkey anti-rabbit antibody (Amersham). Signal was visualized using enhanced chemiluminescence (GE Healthcare). Membranes were also probed with a mouse anti-actin antibody (Abcam) followed by a horseradish-peroxidase-coupled sheep anti-mouse antibody (Amersham) as a protein loading control.

Yeast two-hybrid studies

The L40 reporter strain (Invitrogen) was used for all yeast two-hybrid studies. LexA, LexA/Rap1(635–827), and LexA/Rap1(679–827) were expressed from pBTM116 and a pBTM116-derived plasmids.[9] Mutations in the LexA/Rap1(679–827) fusions were generated with the QuikChange Multi Site-Directed Mutagenesis Kits (Stratagene) using sense oligonucleotide primers. LexA/Rap1(679–827) was used in all interaction studies with Rap1 mutants; LexA/Rap1(635–827) was used only for evaluating the interaction between Rap1 and Sir4. GAD and GAD fusion proteins were expressed from pACT2 and pACT2-derived plasmids. GAD/Rif1(1614–1916),[9] GAD/Rif2(14–395),[10] and GAD/Sir3(356–978)[9] have been described previously. β-Galactosidase activity was quantitated using the Yeast β-Galactosidase Assay Kit (Pierce). In each assay, a minimum of three independent colonies of each mutant were tested in duplicate. Values presented represent the average of all samples for a given mutant.

Protein Data Bank accession codes

Coordinates have been deposited at the Protein Data Bank with accession code 3CZ6.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.04.078

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