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Alternative arrangements of telomeric recognition sites regulate the binding mode of the DNA-binding domain of yeast Rap1

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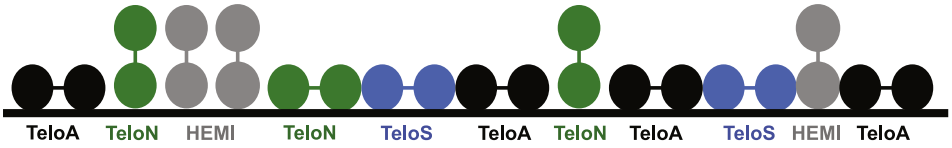
HIGHLIGHTS

- Yeast telomeres contain a heterogeneous distribution of possible Rap1 binding sites.
- Rap1 binds to all these different arrangements of sites using different binding modes.
- Rap1 binds to an isolated half-site using only one of its two Myb-like domains.
- At telomeres Rap1 forms a heterogeneous population of bound-states.

GRAPHICAL ABSTRACT



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ABSTRACT

The function of yeast Rap1 as an activator in transcription, a repressor at silencer elements, and as a major component of the shelterin-like complex at telomeres requires the known high-affinity and specific interaction of the DNA-binding domain (DBD) with its recognition sequences. In addition to a high-affinity one-to-one complex with its DNA recognition site, $Rap1^{DBD}$ also forms lower affinity complexes with higher stoichiometries on DNA. We proposed that this originates from the ability of $Rap1^{DBD}$ to access at least two DNA-binding modes. In this work, we show that $Rap1^{DBD}$ binds in multiple binding modes to recognition sequences that contain different spacer lengths between the hemi-sites. We also provide evidence that in the singly-ligated complex $Rap1^{DBD}$ binds quite differently to these sequences. $Rap1^{DBD}$ also binds to a single half-site but does so using the alternative DNA-binding mode where only a single Myb-like domain interacts with DNA. We found that all arrangements of Rap1 sites tested are represented within the telomeric sequence and our data suggest that at telomeres Rap1 might form a nucleoprotein complex with a heterogeneous distribution of bound states.

1. Introduction

The Rap1 protein from budding yeast *Saccharomyces cerevisiae* is an important regulator of transcription and genomic integrity. Rap1 is responsible for activating transcription of ribosomal protein genes and silencing transcription at HM silent mating-type loci [1,2]. A highly abundant protein [3], Rap1 is also found in large numbers at telomeres where it is involved in establishing architectural nucleoprotein complexes [4], silencing transcription at telomeres [5,6], and acts as a negative regulator of telomere length [2,7,8]. As a consequence of its wide genomic activities, the *RAP1* gene is essential [9]. Loss of function mutations in the Rap1 protein leads to improper telomere elongation [2], telomere fusion [10], and failure to silence target genes and telomeres [5,11].

Rap1 is a DNA-binding protein that recruits other protein factors to carry out its diverse genomic functions. The DNA-binding domain (DBD) of Rap1 contains two Myb-type motifs and is centrally positioned within the 827 amino acid sequence [12]. Crystal structures of $Rap1^{DBD}$ bound to DNA show that the two Myb-type motifs of the DBD are bound

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with the two half-sites of a Rap1 recognition sequence in a one-to-one complex [12–14]. Most of the interactions with other protein factors occur with the Rap1 C-terminal domain (RCT) [15,16]. Proteins that interact directly with the RCT include members of the silent information regulators (SIR) Sir3 and Sir4, forming a complex with Rap1 on DNA during gene silencing [6]. The RCT domain also binds the Rap1 interacting factors Rif1 and Rif2, forming the core scaffold of the shelterin-like complex at telomeres [15]. The Rap1–Rif complexes are recruited to the repetitive T(G_{1-3}) arrays of telomeric DNA forming a "cap" at the chromosome ends together with other protein complexes like Ku70–Ku80 and Cdc13–Stn1–Ten1 [17–19]. This nucleoprotein complex shelters the chromosome ends from being recognized as double-strand breaks, suppressing the DNA damage response pathway [20].

Rap1 plays an important role in regulating telomere length homeostasis. It has been proposed that Rap1 is a central component of a counting mechanism where the cell monitors and responds to the number of Rap1 molecules (or Rap1–Rif2) present at the telomere as a way of regulating access to telomerase [21,22]. The recent crystal structure of a $Rap1^{RCT}$–Rif2 complex suggests that Rif2 may in fact bind two Rap1 molecules from two different binding surfaces [23].

Crystal structures of $Rap1^{DBD}$ bound to DNA have added to the current available model where the two Myb-type motifs of the DBD bind simultaneously and with high affinity with the two half-sites of a Rap1 recognition sequence [12–14]. However, previous work from Del Vescovo et al. showed that Rap1 at a minimum is capable of binding a single hemi-site [24]. Recently we provided evidence that while the DBD indeed binds its recognition sequence in a high-affinity singly-ligated complex, it can also access higher stoichiometry complexes on both telomeric and non-telomeric dsDNA substrates [25]. We proposed that the ability of $Rap1^{DBD}$ to achieve stoichiometries on DNA higher than the expected one-to-one is due to its ability to switch between at least two DNA-binding modes. In one mode, observed in the crystal structures, both Myb-like domains bind with high affinity to both hemi-sites in the recognition sequence; in the alternative mode only one Myb-like domain interacts with lower affinity with DNA. In this work, we extended our study to other recognition sequences that contain different spacer lengths between the hemi-sites or a single half-site. All of the sequences tested are potential Rap1 recognition sites found in the heterogenous yeast telomeres [26–28]. The ability of $Rap1^{DBD}$ to bind to a single half-site within a short dsDNA substrate provides direct evidence that a single myb-like domain is sufficient for interaction and that the alternative binding mode can be populated. We propose that the ability of Rap1 to bind different arrangements of sites at telomeres and its ability to access different binding modes could lead to the formation of a heterogeneous nucleoprotein complex.

2. Materials and methods

2.1. Reagents and buffers

All chemicals used were reagent grade. All solutions were prepared with distilled and deionized Milli-Q water (18 MΩ at 25 °C). All oligonucleotides were purchased from Integrated DNA Technology (IDT, Coralville, IA). The oligonucleotides used for binding experiments were all HPLC purified, suspended in TE buffer (10 mM Tris–HCl, pH 8.3 and 0.1 mM EDTA) and the concentration was determined spectrophotometrically using the extinction coefficients provided. The sequence composition of the "top" strand of the oligonucleotides used is shown in Table 1 and the position of the FAM or Cy3 fluorescent labels is indicated in the text. All annealed duplex dsDNAs were prepared by mixing equimolar concentrations of each oligonucleotide strand in 20 mM HEPES (pH 7.4), 50 mM NaCl, 10% *v*/*v* glycerol, 2 mM $MgCl_2$ and incubated in a pre-heated 95 °C water bath, followed by slow cooling to room temperature.

Table 1
Sequence of the "top" strands of the dsDNA substrate used. The half-site of the Rap1 recognition sequence at telomeres is in bold.

	L_{bp}	S_{bp}	h_{bp}	5′-3′ "top" strands
TeloA	21	3	4	CCGC**ACACC**CAC**ACACC**AGTG
$TeloA_{h3}$	19	3	3	CGC**ACACC**CAC**ACACC**ATG
$TeloS_{h5}$	21	1	5	CGCGC**ACACCC**A**CACC**AGTGG
TeloS	19	1	4	CCGC**ACACCC**A**CACC**AGTG
$TeloS_{h2}$	15	1	2	CC**ACACCC**A**CACC**AG
$TeloS_{h1}$	13	1	1	C**ACACCC**A**CACC**G
TeloN	18	0	4	CCGC**ACACCACACC**AGTG
$TeloN_{h5,6}$	21	0	5,6	CGCGC**ACACCACACC**AGTAGG
HEMI	13	–	4	CCGC**ACACC**AGTG
HEMI-L	13	–	1,7	C**ACACC**AGTCGCG
HEMI-R	13	–	7,1	CAGTCGC**ACACC**G

2.2. Purification of Rap1 constructs

The DNA-binding domain of Rap1 comprising residues 358–601 [14] was cloned and overexpressed in *Escherichia coli* Rosetta2(DE3)pLysS (EMD Chemicals, Novagen, Gibbstown, NJ). The $Rap1^{DBD}$ was purified with a two-column purification protocol as described [25]. Briefly, following cell lysis the clarified supernatant was incubated with 0.3% *v*/*v* polyethyleneimine, recovered in the supernatant and incubated overnight with Glutathione Sepharose 4 Fast Flow GST-affinity resin. After cleavage of the GST-tag the $Rap1^{DBD}$ was directly loaded on a Poros 50 HE Heparin and eluted at 600 mM NaCl. Purified $Rap1^{DBD}$ was dialyzed against Storage Buffer (20 mM HEPES (pH 7.4), 400 mM NaCl, 40% *v*/*v* glycerol, 1 mM DTT, and 0.5 mM EDTA) and then stored at −80 °C. Before the experiments, $Rap1^{DBD}$ was dialyzed against Buffer HN_{50} (20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 10% *v*/*v* glycerol) and the concentration was determined using an extinction coefficient of 24,870 M^{-1} cm^{-1} [29,30].

2.3. Analysis of telomeric sequences

We analyzed a series of telomeric sequences that range in size from 34 nt to 363 nt in total length. These include: Tel270 and Telo80 [26]; wt1–7 [28]; M34310–M34313 (GenBank, [27]); TEL01L-TR, TEL01R, TEL03L, TEL03R, TEL04L, TEL06R, TEL08L-TR, TEL08R, TEL09L, TEL10L, TEL10R, TEL11L, TEL11R, TEL12L, TEL13L, TEL13R and TEL14R (SGD, strain S288C). In order to identify all possible unique arrangements of Rap1 binding sites, we applied a few simple rules. First, we assumed that Rap1 sites are occupied from the telomeric end and moved inward. Second, the order of binding follows a simple affinity rule based on the spacing between half-sites, where 3 bp > 2 bp > 1 bp > 0 bp > half-site. Finally, no overlap between identical sites was allowed when assuming that only the site proximal to the telomeric end is occupied.

2.4. Analytical ultracentrifugation

All sedimentation experiments were collected on an Optima XL-A analytical ultracentrifuge using a An60Ti rotor (Beckman Coulter, Brea, CA). Sedimentation velocity experiments with 2 μM Cy3-labeled DNA were performed using Epon charcoal-filled double-sector centerpieces at 55,000 rpm with 0.03 cm spacing and recording scans every 8 min at 545 nm. Sedimentation equilibrium experiments were performed using Epon charcoal-filled six-sector centerpieces at the appropriate rpm with 0.001 cm spacing, scanned every 4 h, averaged from 10 replicates and recorded at 545 nm. Sedimentation velocity and equilibrium data were processed and analyzed with SedFit/SedPhat (Peter Schuck). The apparent molecular weights of the complexes were determined as described [25].

2.5. Equilibrium fluorescence titrations and Electrophoretic Mobility Shift Assays

All fluorescence titrations were performed with an L-format PC1 spectrofluorimeter (ISS, Champaign, IL) equipped with Glenn-Thompson polarizers. Measurements of the anisotropy and total fluorescence intensity of FAM-labeled DNA were recorded using excitation and emission wavelengths of 480 nm and 530 nm respectively, as described [25]. Electrophoretic Mobility Shift Assays (EMSA) of Rap1^{DBD} with labeled or unlabeled dsDNAs were performed on 8% acrylamide/bisacrylamide 1X TBE mini gels with running buffer pre-chilled at 4 °C. Gels were scanned using a Typhoon 9400 Variable Mode Imager (Amersham BioSciences, GE Healthcare Bio Sciences, Piscataway, NJ) or for unlabeled DNAs stained with GelRed (Phenix Research, Candler, NC) and scanned on an Alpha Imager HP imager (Protein Simple, Santa Clara, CA).

3. Results

3.1. Rap1^{DBD} binds to a wide array of telomeric sequences that differ in the spacing between the half-sites of the recognition sequence

The canonical DNA recognition sequence for Rap1 binding is comprised of 13 bp with two hemisites spaced by 3 bp [31,32] and at telomeres the half-site is characterized by the sequence ACACCC. We examined the distribution of all possible Rap1 sites with different bp spacing or just a single half-site for 30 telomeric sequences that contain more than one possible Rap1 site (see Materials and methods). We made the simple assumptions that the sites are being filled from the telomeric end inward (3′ to 5′ of the GT-rich strand) and with no overlap (i.e. unique sites). Moreover, we assumed that the available sites are bound in order, following the affinity rule of 3 bp > 2 bp > 1 bp > 0 bp >> half-site. Fig. 1a shows the observed distribution of all possible unique sites for the examined sequences. As expected, of all the sites that contain a Rap1 recognition sequence, the 3 bp spacing between the two half-sites dominates over the 1 bp. Interestingly, we also found a significant distribution of potential unique sites that contain no spacing (0 bp) between the half-sites. Most strikingly we found that following the above rules, a large fraction of the telomeric sequences also contain non-overlapping single half-site sequences. Recently we studied the binding of the DNA-binding domain of Rap1 (Rap1^{DBD}) to a DNA substrates that contains the canonical 3 bp spacing between the half-sites [25]. Given the distribution of possible sites observed in Fig. 1a, in this work we studied how Rap1^{DBD} binds to model dsDNAs containing variable lengths in the spacer between the two direct repeats (ACACC) of Rap1 telomeric sites, or merely a single hemi-site (see below). The sequences of the DNA substrates are listed in Table 1. We used substrates where either the handle sequence was maintained at a length of 4 bp and the spacing (S) was varied, therefore decreasing the total DNA length, or where the length of the handle region was varied together with the inter-site spacing (S) to maintain a constant length of the DNA (Fig. 1b).

Fig. 1c shows an electrophoretic-mobility shift assay (EMSA) at 30 nM TeloS (labeled at the 5′-end of the top strand with 6-carboxyfluorescein (FAM)) with increasing concentrations of Rap1^{DBD}. Consistent with the reported ability of Rap1 to bind to a substrate with a 1 bp spacing [13], Rap1^{DBD} binds to this 19 bp TeloS substrate (Table 1). As observed for the 21 bp TeloA [25], at higher concentrations of TeloS (300 nM) binding of Rap1^{DBD} is also accompanied by detectable super-shifts (Fig. 1d), showing that Rap1^{DBD} can access higher stoichiometries also on a substrate with a shorter inter-site spacer. The same behavior is also observed for the 18 bp TeloN (Fig. 1c,d), where there is no spacer between hemi-sites (Table 1). Sedimentation velocity (data not shown) and equilibrium experiments with TeloS and TeloN (labeled with Cy3 at the 5′-end of the top strand) in the presence of saturating concentrations of Rap1^{DBD} show that ~three molecules of protein can bind in solution (Table 2). Independent of the spacer length, Rap1^{DBD} is capable of forming a 3:1 complex on telomeric substrates that contain

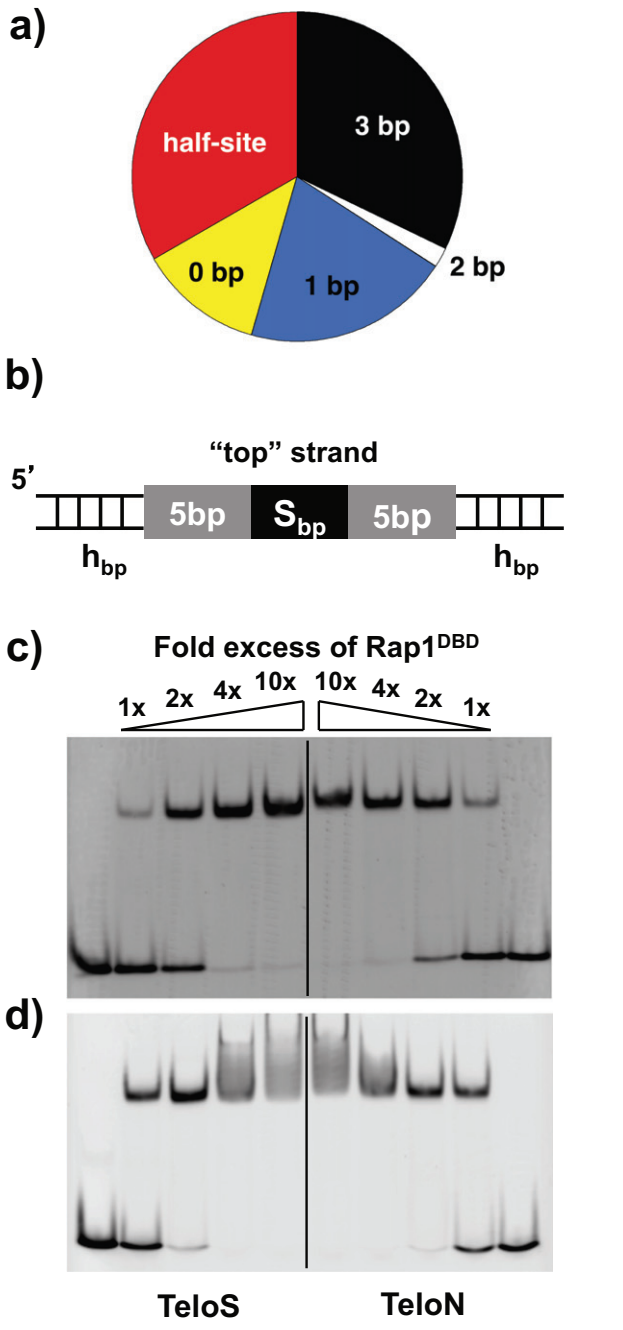

Fig. 1. Rap1^{DBD} binds to a wide array of telomeric sequence arrangements. (a) Distribution of telomeric sequences that contain a given number of base-pairs separating two tandem repeats. (b) Schematic of the model dsDNA substrate used in this work. (c) EMSA as a function of fold-excess Rap1^{DBD} for 30 nM TeloS (left) and TeloN (right) labeled at the 5′-end of the top strand with FAM (Table 1). (d) Same experiments as in (c) but at 300 nM DNA.

two hemi-sites, even on a dsDNA as small as 18 bp (i.e. TeloN). Finally, similar to what we observed with TeloA [25], the weaker binding of the third Rap1^{DBD} molecule to TeloS and TeloN is highly sensitivity to NaCl concentration. At 150 mM NaCl only the 2:1 complex is populated, indicating that at this higher NaCl concentration binding of the third molecule of Rap1^{DBD} is effectively abolished in solution (Table 2).

3.2. Rap1^{DBD} binds differently to recognition sequences containing a varying number of base-pairs in the spacer between the two half-sites

We showed that binding of Rap1^{DBD} to FAM-labeled DNA is accompanied by a large change in fluorescence anisotropy or relative total

Table 2
Molecular weight of Rap1–DNA complexes determined by equilibrium analytical sedimentation in Buffer HN_{50}.

	Mw_{obs} (kDa)[a]	P/D[b]
5′-Cy3-TeloS	88.8 ± 0.2 (59.5 ± 0.2)[c]	2.56 ± 0.02 (1.58 ± 0.01)[c]
5′-Cy3-TeloN	86.7 ± 0.2 (65.9 ± 0.3)[c]	2.51 ± 0.02 (1.82 ± 0.02)[c]
5′-Cy3-$TeloS_{h1}$	56.5 ± 0.3	1.61 ± 0.02
5′-Cy3-HEMI	62.3 ± 0.3	1.80 ± 0.03

[a] $\nu_{P3D} = 0.7$ mL/g, w-avg using $\nu_P = 0.726$ mL/g and $\nu_D = 0.527$ mL/g.
[b] Based on the calculated Mw_{dsDNA} and $Mw_{DBD} = 29.8 \pm 0.15$ kDa.
[c] Observed Mw and stoichiometry for experiments in Buffer HN, 150 mM NaCl.

fluorescence intensity depending on the position of the fluorophore on the substrate [25]. We used the same signal changes to study the binding of $Rap1^{DBD}$ to substrates of the same length (19 bp) but containing either 1 bp (TeloS) or 3 bp ($TeloA_{h3}$) spacing between the two direct repeats in the Rap1 recognition sequence. Fig. 2a shows the change in fluorescence anisotropy (upper panel) and relative total fluorescence intensity (lower panel) of TeloS and $TeloA_{h3}$ (with FAM at the 5′-end of the top strand) as a function of the total $Rap1^{DBD}$ to DNA concentrations in Buffer HN_{50}. It is clear that the change in the monitored signals is dramatically different when the spacing between the half-sites is changed.

The change in anisotropy clearly shows that three molecules of $Rap1^{DBD}$ can bind to TeloS at saturation. This is consistent with the stoichiometry determined from the sedimentation equilibrium experiments (Table 2). Interestingly, for TeloS the anisotropy increases linearly, while for $TeloA_{h3}$ formation of the singly-ligated species shows little change in anisotropy. This is similar to what we reported for the 21 bp TeloA [25]. The large difference in the anisotropy of the singly-ligated species formed on TeloS and $TeloA_{h3}$ suggests that when the spacing between the half-sites is changed different complexes are formed. We also note that for $TeloA_{h3}$ binding of the first molecule leads to an initial small fluorescence increase followed by ~20% quenching upon binding of the next $Rap1^{DBD}$ molecules (Fig. 2a, lower panel). This behavior on $TeloA_{h3}$ is different from TeloS, suggesting that also the multiply-ligated species might be in a different conformation. Moreover, the fluorescence change for $TeloA_{h3}$ is also different from the one we observed on the 21 bp TeloA, where binding of the first $Rap1^{DBD}$ molecule does not lead to a change in fluorescence intensity and binding of the second and third ones lead to a fluorescence increase [25]. For these two substrates the handle regions differ by one base-pair (Table 1) and for these lengths of the handles the crystal structures of $Rap1^{DBD}$ with DNA do not show interaction with this region [12–14]. The different changes in fluorescence intensity suggest that the signal is sensitive not only to the spacing between the half-site but also to the total length of the substrate. A similar phenomenon is also observed for TeloS when the handle region is changed (see Supporting Information).

Finally, different signal responses of the complexes of $Rap1^{DBD}$ with TeloS and $TeloA_{h3}$ are also observed when the label is placed at the 3′-end of the top strand of the substrates (Fig. 2b). While binding of the first $Rap1^{DBD}$ molecule to $TeloA_{h3}$ leads to little change of fluorescence, binding of the first molecule to TeloS leads to a large ~50% fluorescence increase. We note that in this case the signal is dominated by the binding of the first molecule; binding of the next two molecules leads to a complex behavior in the fluorescence intensity.

3.3. $Rap1^{DBD}$ binds differently to a substrate containing no spacing between the half-sites

Electrophoretic-mobility shift assays in Fig. 1c show that $Rap1^{DBD}$ can bind to a dsDNA substrate that has no spacing between the two half-sites of the recognition sequence. Interestingly, with TeloN binding is observed at 30 nM DNA suggesting that removal of the spacing does not dramatically affect the apparent affinity for the singly-ligated complex. Fig. 3a shows the change in fluorescence anisotropy as a function

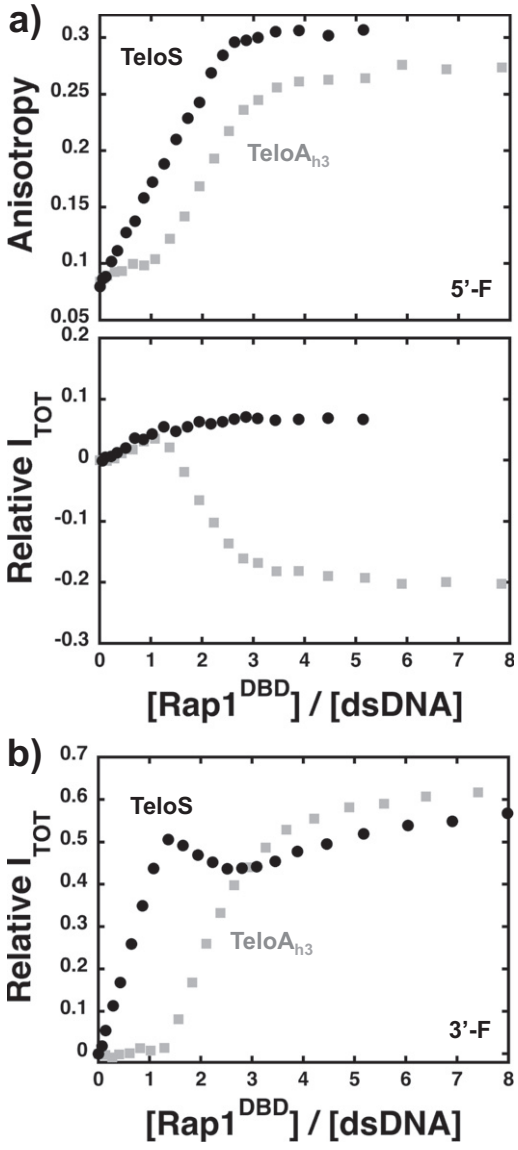

Fig. 2. $Rap1^{DBD}$ binds to a substrate containing 1 bp spacing between the two half-sites. (a) Change in fluorescence anisotropy as a function of $Rap1^{DBD}$/DNA ratio for 255 nM TeloS (circles) or $TeloA_{h3}$ (squares) labeled at the 5′-end of the top strand. (b) Same experiments as in (a) but monitoring the change in relative total intensity for the same dsDNAs FAM-labeled at the 3′-end of the top strand.

of $Rap1^{DBD}$/DNA for TeloN substrates with FAM either at the 5′- or 3′-end of the top strand. When the label is at the 5′-end of the top strand the change in anisotropy plateaus at ~1.2 $Rap1^{DBD}$/DNA. This would suggest that when there is no spacing between the half-sites, $Rap1^{DBD}$ forms a simple one-to-one complex with the substrate. However, when the label is at the 3′-end position on TeloN the change in anisotropy is similar to what we observed for $TeloA_{h3}$ (Fig. 2a). Moreover, when the label is at the 3′-end of the same strand, the relative total fluorescence intensity shows no change up to a ratio of two $Rap1^{DBD}$/DNA, but is then followed by an increase in fluorescence as the protein concentration is increased (data not shown). These signals are not consistent with formation of a simple 1:1 complex. Indeed, the EMSAs in Fig. 1 show that on TeloN more than one molecule of $Rap1^{DBD}$ can bind at saturation. This is further confirmed by the 3:1 stoichiometry determined from the sedimentation equilibrium experiments performed at much higher DNA concentration (Table 2). The behavior of the anisotropy for TeloN labeled at the 5′-end can be intuitively understood if the signal is dominated by the binding of the first molecule and

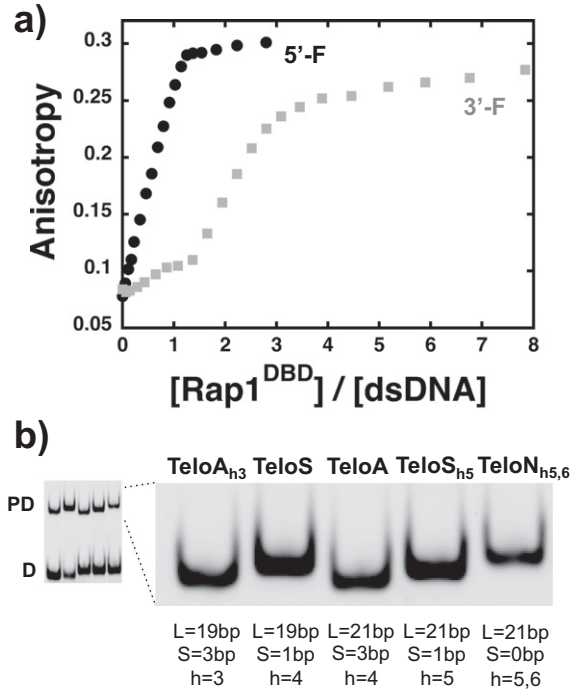

Fig. 3. Rap1^{DBD} binds to substrates that do not contain any base-pair spacing between the half-sites. (a) Change in fluorescence anisotropy as a function of Rap1^{DBD}/DNA ratio for 255 nM TeloN labeled with FAM at the 5′-end (black circles) or the 3′-end (black squares) of the top strand. (b) EMSA of Rap1^{DBD}:DNA complexes formed at a 1:1 ratio with 300 nM DNA substrates containing a different spacing between the half-sites for two total lengths of the DNA.

binding of the second and third ones occur with lower affinity, as suggested by the EMSAs in Fig. 2. In this case, at the DNA concentration of the equilibrium titrations the fraction of the doubly- and triply-ligated species would be very small and thus contribute little to the observed signal.

In addition to the spacing between the half-sites, the fluorescence signals are also sensitive to the total length of the dsDNA substrate. A similar effect is also true for TeloN (Supporting Information, Fig. S3). It is evident that binding of Rap1^{DBD} to the substrates examined is accompanied by distinct signal signatures, strongly suggesting that different singly-ligated complexes must be formed. This is further supported by differences in electrophoretic-mobility of the singly-ligated Rap1^{DBD}–DNA complexes. Fig. 3b shows EMSAs targeting the one-to-one complexes (formed at 300 nM DNA with 300 nM Rap1^{DBD}) on substrates labeled at the 5′-end of the top strand with FAM. We found that despite the DNA being shorter, the singly-ligated species formed on TeloS (19 bp) migrates slower than the one formed on TeloA (21 bp). However, because of the difference in length, the charge on the DNA is also different for these complexes. Complexes formed on DNAs of the same length but with either 3 bp or 1 bp between the half-sites (e.g. TeloA vs TeloS$_{h5}$ or TeloA$_{h3}$ vs TeloS) still show distinguishable differences in electrophoretic-mobility. Moreover, the complex formed on a substrate with no base-spacing between the half-sites migrates different than any other dsDNA of the same length (TeloA vs. TeloS$_{h5}$ vs. TeloN$_{h5,6}$). These observations corroborate our suggestion that the singly-ligated species of the DNA-bound Rap1^{DBD} is different depending on the spacing between the half-sites.

3.4. Rap1^{DBD} can bind to a substrate containing just a single half-site but it does so in a different binding mode

Analysis of the distribution of the possible Rap1 binding sequences at telomeres (Fig. 1a) shows that there is a large fraction of the telomeric sequence that contains potential single half-sites. The ability of Rap1 to bind to a substrate containing a single half-site was first reported by Del Vescovo et al. and interpreted as a possible means to bind non-canonical recognition sequences [24]. In the context of our proposal that Rap1^{DBD} can bind to DNA in at least two binding modes, we wondered whether binding to a single half-site might be due to the alternative, lower affinity binding mode we proposed [25]. At 300 nM DNA Rap1^{DBD} binds to the 13 bp HEMI (labeled at the 5′-end of the top strand with FAM, Table 1) and the presence of super-shifted bands is not as evident as for the other substrates studied (Fig. 4a). This suggests that either a 1:1 complex is formed on HEMI, or that if more than one molecule of Rap1^{DBD} can bind, it does so with much lower affinity than on any of the other substrates with the same size handles. This second possibility appears to be the case. EMSAs performed with 1 μM HEMI clearly show the presence of super-shifts (Fig. 4b). Moreover, sedimentation equilibrium experiments performed with 2 μM Cy3-labeled HEMI indeed show that at saturation two Rap1^{DBD} molecules can bind (Table 2). This indicates that for a short 13 bp dsDNA substrate, only ~5–6 bp are sufficient for interaction, similar to what observed with the 13 bp TeloS$_{h1}$ containing two half-sites (Table 2). Next we asked whether the apparent lower affinity for a second Rap1^{DBD} molecule on HEMI may be due to the central position of the half-site. If this were the case, it would be expected that by moving the half-site to one side of the DNA, the transition to higher stoichiometry would be favored, due to an increased number of base-pairs allowing for the non-specific binding of a second molecule of Rap1^{DBD}. To this end we used HEMI-L (FAM-labeled at the 5′-end of the top strand) where the ACACC sequence is moved towards the 5′-end of the top strand. For this substrate, formation of a super-shift becomes evident at 300 nM DNA (Fig. 4a) and even more so at 1 μM DNA (Fig. 4b), consistent with the expectation that when the half-site is centrally positioned the higher affinity for this site hampers the binding of a second molecule of Rap1^{DBD}. Interestingly, the affinity of the singly-ligated complex for HEMI-L is diminished compared to HEMI, strongly suggesting that the base-pairs flanking the half-site provide extra stabilization of the complex [24]. We note that similar effects on the apparent affinity of the singly-ligated complex are also present for the TeloS substrates, when the size of the handle region is decreased (see Supporting Information).

Based on the crystal structures of the Rap1^{DBD} bound to its recognition sequences [12–14] it is difficult to rationalize how the DBD would be able to bind on the 13 bp HEMI with both Myb-like domains interacting with the DNA. We next tested whether differences in binding modes of Rap1^{DBD} are present on these short 13 bp substrates when the DNA contains either two (TeloS$_{h1}$) or one half-site (HEMI). Binding of Rap1^{DBD} to HEMI (labeled at the 5′-end of the top strand with FAM) leads to a large change in anisotropy, with the signal being dominated by the binding of the first Rap1^{DBD} molecule (plateau at ~1.2 P/D) (Fig. 4c). This is the same behavior observed for TeloN (see above) although once again, it is clear that on these substrates more than one Rap1^{DBD} molecule can bind at saturation (Table 2). Binding of the first molecule of Rap1^{DBD} to TeloS$_{h1}$, containing two half-sites separated by 1 bp, is instead accompanied by a much smaller change in anisotropy as compared to HEMI, followed by an additional contribution to the signal from the low-affinity binding of a second Rap1^{DBD} molecule. For HEMI-L, where the half-site is directly adjacent to the fluorophore, the anisotropy plateaus at ~1.7 P/D suggesting that when the half-site is positioned to this end of the DNA, the signal becomes more sensitive to the binding of the second Rap1^{DBD} molecule.

Differences in the formed complexes on these substrates are amplified when the label is placed at the 3′-end of the DNA and the total fluorescence intensity is monitored (Fig. 4d). Binding of the first molecule of Rap1^{DBD} to HEMI is not accompanied by significant changes in fluorescence intensity, and at this concentration of DNA, the signal is only sensitive to the low-affinity binding of the second molecule. Instead, for TeloS$_{h1}$ binding of the first Rap1^{DBD} molecule leads to a large fluorescence increase (~80%) and dominates the

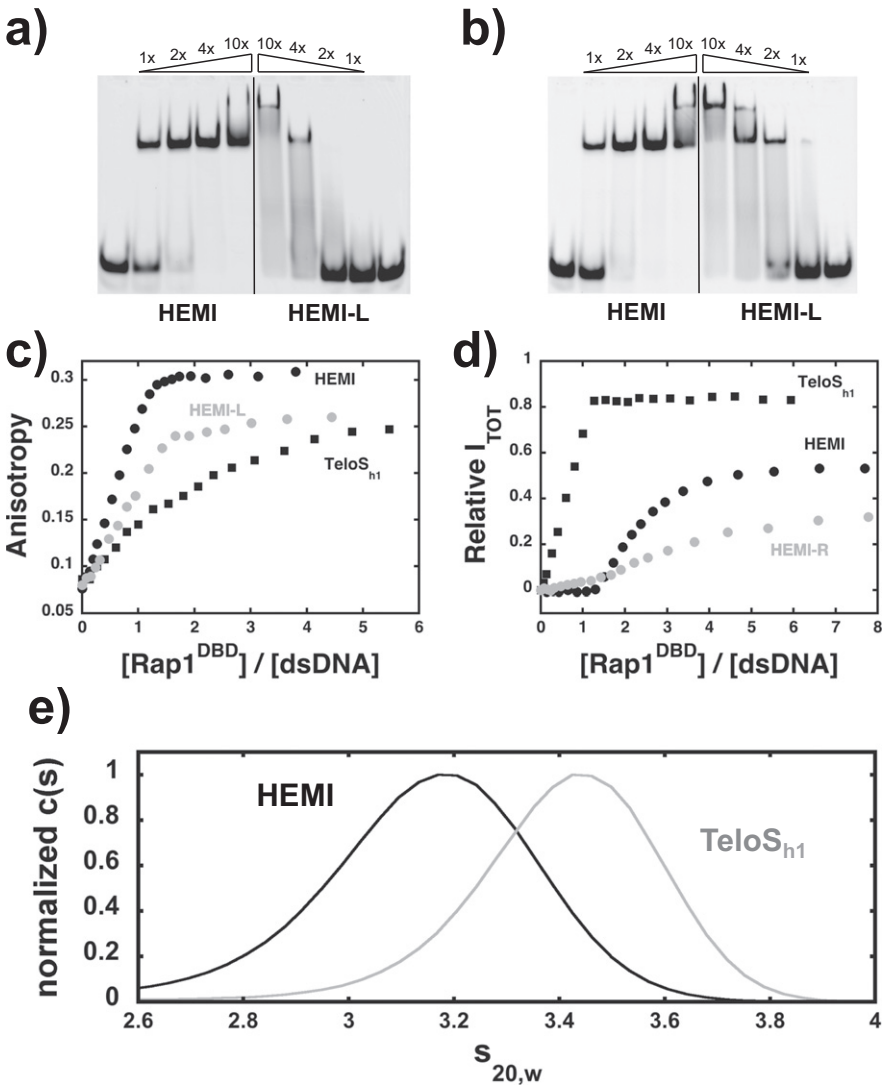

Fig. 4. Rap1^{DBD} binds to substrates that contain a single half-site. (a) EMSA as a function of fold-excess Rap1^{DBD} for 300 nM HEMI (left) and HEMI-L (right) labeled at the 5′-end of the top strand with FAM (Table 1). (b) Same experiments as in (a) but performed with 1 μM DNAs. (c) Change in fluorescence anisotropy as a function of Rap1^{DBD}/DNA ratio for 255 nM HEMI (black circles), HEMI-L (gray circles) and TeloS$_{h1}$ (black squares) FAM-labeled at the 5′-end of the top strand. (d) Change in relative total intensity as a function of Rap1^{DBD}/DNA ratio for 255 nM HEMI (black circles), HEMI-R (gray circles) and TeloS$_{h1}$ (black squares) FAM-labeled at the 3′-end of the top strand. (e) Sedimentation coefficient distributions from velocity experiments of 2 μM Cy3-labeled HEMI (black) and TeloS$_{h1}$ (gray) in Buffer HN$_{50}$ at equimolar Rap1^{DBD}/DNA loading ratio. For clarity the region corresponding to free DNA has been excluded.

total signal. This is similar to what we observed for all of the substrates containing two half-sites spaced by 1 bp (see above). We note that for TeloS$_{h1}$ the half-site proximal to the label is followed by a single base-pair compared to HEMI. To test whether this might contribute to the observed differences between HEMI and TeloS$_{h1}$, Fig. 4d also shows the relative fluorescence change for HEMI-R, where the single half-site has been moved proximal to the label at the 3′-end of the top strand (Table 1). For this substrate, binding of the first molecule of Rap1^{DBD} is not accompanied by a significant increase in fluorescence. This strongly suggests that the change in fluorescence intensity for TeloS$_{h1}$ must be due to the formation of a distinct singly-ligated complex as compared to HEMI.

The presence of distinct Rap1^{DBD} complexes formed with 13 bp dsDNAs containing either a single or two half-sites is further supported by analytical sedimentation velocity experiments. Fig. 4e shows the distribution of sedimentation coefficients of 2 μM HEMI or TeloS$_{h1}$ (labeled with Cy3 at the 5′-end of the top strand) in the presence of a 1:1 loading ratio of Rap1^{DBD}. The observed difference in sedimentation coefficient (3.18 S vs 3.44 S) indicates that binding to substrates containing either one or two half-sites is accompanied by a significant conformational change of the bound protein.

4. Discussion

In *S. cerevisiae* telomeric DNA ends are heterogeneous in sequence, leading to a distribution of potential Rap1 binding sites that can have variable spacing between half-sites [26–28]. The number of Rap1 binding sites at telomeres has been estimated largely by considering canonical Rap1 recognition sequences with half-sites spaced by 3 bp [26,27]. More recently, work from Williams et al. [33] showed that indeed the predicted number of Rap1 molecules can be observed both with synthetic telomeric arrays and natural sequences. However, these assays were performed under conditions where the number of protein molecules never exceeded the number of potential canonical Rap1 sites [33]. Analysis of telomeric sequences in Fig. 1a to include all possible Rap1 recognition sites shows that although the 3 bp and 1 bp spacing between the ACACC direct repeats are dominant, a substantial fraction of potential sites contains zero bp spacing, and an even larger fraction

contains of a single hemi-site. This suggests the possibility that if Rap1 can bind all of these sequences, then the potential number of Rap1 molecules at telomeres could be greater than previously anticipated. In this work we showed that Rap1 can indeed bind to all of these potential arrangements of the recognition sequence within short model dsDNA substrates. The crystal structure of the DBD bound to a telomeric recognition sequence with 1 bp spacing shows that Rap1 adopts a conformation similar to the one with 3 bp spacing, although the contacts are different [13]. The data in this work show that in solution, the spacing between half-sites affects the formation of the singly-ligated species and possibly the transition to the second, alternative binding mode. Indeed EMSAs at 300 nM DNA show that the super-shifts on TeloS become evident at even lower $Rap1^{DBD}$ concentrations than on TeloA [25]. We recently showed that when $Rap1^{DBD}$ binds to lower affinity recognition sequences it appears to be more prone to access the higher stoichiometry complexes [25]. The observation in Fig. 1d that super-shifts appear at lower $Rap1^{DBD}$ concentrations is consistent with a lower affinity for TeloS that in turn, would also favor transition to the multiply-ligated states. Similar behavior is also observed for TeloN suggesting the possibility that reducing the spacing between telomeric hemi-sites (from 3 bp to 1 bp to 0 bp) makes the transition to a higher-order complex (i.e. switch between binding modes) more favorable, and at the same time likely weakens the apparent affinity of the singly-ligated complex.

We proposed that formation of the high stoichiometry complex of $Rap1^{DBD}$ on model DNA substrates occurs through a switch in binding mode: from two Myb-like domains bound to the recognition sequence with high affinity, to a single Myb bound with lower specificity [25]. Part of this argument was based on the proposal that $Rap1^{DBD}$ does not bind to either the handle region or the ends of the dsDNA substrate. The data where the handle region in the substrate was varied strongly support this proposal (see Supporting Information). Moreover, the distinct signal signatures for substrates of the same length but with different spacing between the half-site also indicate that binding of the additional two $Rap1^{DBD}$ molecules does not occur at the ends of the duplex DNA substrate. The different spectroscopic signals also suggest that binding of Rap1 to recognition sequences with varying length of the inter-site spacer leads to different $Rap1^{DBD}$–DNA complexes, both in the singly- and multiply-ligated states. Interestingly, we found that the distinct behaviors of the anisotropy and total fluorescence when the label is placed at different positions on the substrate are sensitive not only to the spacing between the half-sites but also to the total length of the substrate. Yet, for the same length of DNA the data suggest that the singly-ligated complex formed on these substrates is still sensitive to the presence of 3 bp or 1 bp spacing and that it is in a different conformation. This is further supported by the observation that these complexes show different electrophoretic-mobility. However, $Rap1^{DBD}$ complexes with substrates of the same length but with either 3 bp or 1 bp spacing do not show major differences in the sedimentation coefficient of the singly-ligated species (data not shown). This suggests that the observed changes in electrophoretic-mobility and the distinct signatures in the spectroscopic signals are not the result of large conformational changes. Indeed, the crystal structures of the DBD bound to either a TeloA or a TeloS substrate are very similar [13]. It is possible that the changes we observe represent either different conformations of the Rap1–DNA complex that are accessible in solution or differences in the design of the substrates where the DBD is less prone to accommodate the contacts observed in the crystal structure.

To our surprise, analysis of telomeric sequences shows that a large fraction of half-sites might be available for Rap1 to bind. Work from Del Vescovo et al. first pointed out the ability of Rap1 to bind to a single hemi-site and suggested it as a possible means to bind non-canonical recognition sequences [24]. We propose an alternative interpretation. The data in this work show that Rap1 can bind a single ACACC site centrally located within a 13 bp dsDNA. Based on the available crystal structures, with such a short DNA duplex it is difficult to rationalize how $Rap1^{DBD}$ would be able to bind with both Myb-like domains interacting with the DNA. Rather, our data suggest that on this substrate $Rap1^{DBD}$ must bind in a very different mode as compared to substrates containing two half-sites. This is corroborated by the observation that binding of $Rap1^{DBD}$ to 13 bp dsDNAs containing either one or two half-sites has very different spectroscopic signatures, suggesting that distinct complexes must be formed. Indeed the observed difference in sedimentation coefficient (Fig. 5c) suggests that binding to HEMI and $TeloS_{h1}$ is accompanied by a large conformational change of the complex. In the context of our model that $Rap1^{DBD}$ can bind to DNA in at least two binding modes [25], we propose that Rap1 binds to the single half-site using the alternative, lower-affinity binding mode where only one Myb-like domain interacts with the hemi-site.

Finally, analysis of telomeric sequences in Fig. 1a also shows that a fraction of possible Rap1 sites contains no spacing between the two direct repeats. The EMSA at 30 nM DNA shows that $Rap1^{DBD}$ can indeed bind to the TeloN substrate, suggesting that the affinity has not been reduced as much as for HEMI, containing a single hemi-site. For this TeloN substrate, it is plausible that either both Myb-like domains bind to the two adjacent direct repeats or if a single Myb-like domain binds it does so similar to an isolated repeat but with optimal contacts, as suggested by the higher affinity. Indeed, the anisotropy and fluorescence changes for TeloN with the label at different positions are quite similar to the ones observed with HEMI (single hemi-site) rather than to the other substrates containing two half-sites.

In summary, telomeric sequences contain an array of possible Rap1 binding sites ranging from two direct repeats (half-sites) spaced by 3, 1, or zero base-pairs to a large fraction of isolated potential half-sites. We showed that $Rap1^{DBD}$ can bind to all these arrangements of sites within short dsDNA substrates. For sites containing 3 bp or 1 bp spacing between the direct repeats, $Rap1^{DBD}$ binds in the high-affinity mode where both Myb-like domains interact with the DNA, although the formed complexes exist in different conformations depending on the length of the spacer sequence. For isolated half-sites, and also possibly for sites containing two repeats without spacing, we propose that $Rap1^{DBD}$ binds with the alternative binding mode where a single Myb-like domain interacts with DNA. The ability of the DBD to access different binding modes on DNA appears to be a general property of the protein (RG, unpublished), maintained also in larger constructs containing the N-terminus region, with the single BRCT domain, and the C-terminus region (RCT), the domain involved in interaction with other proteins. However, little is currently known of possible linkage between DNA binding and conformational transitions of the full-length protein. It is possible that the presence in Rap1 of these additional regions alters the specific complexes formed on different recognition sequences. On

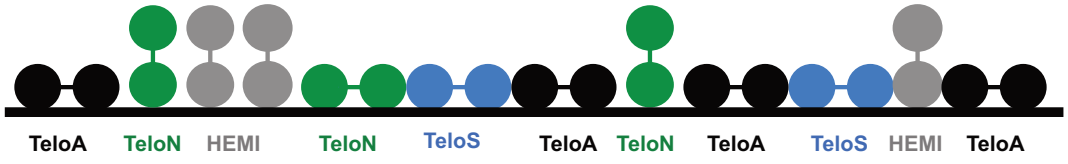

Fig. 5. At telomeres Rap1 can form a nucleoprotein complex with heterogeneous distribution of bound states. Cartoon model of a possible distribution of Rap1 bound states at different binding sites with variable spacing between the half-site, 3 bp (black), 1 bp (blue), none (green) or isolated half-site (gray).

substrates containing two hemisites the presence of these additional domains might contribute to the proper orientation of the DNA-bound complex. However, on DNA substrates containing isolated sites, where a single Myb-like domain binds, we speculate that the additional domains of Rap1 contribute little to the formed complex. We propose that at telomeres, binding of Rap1 in different modes would lead to a heterogeneous distribution of bound states rather than to a simple array of Rap1 molecules all in the same conformation (Fig. 5). It has been suggested that at telomeres Rif proteins can bind the Rap1 C-terminal domains of two adjacent Rap1 molecules, providing a bridge that would stabilize the nucleoprotein complex [23]. However, little is known of the allosteric coupling between the binding of Rif proteins (or any other Rap1 interacting protein) with the RCT domain and the DBD binding to DNA. We speculate that if Rap1 at telomeres indeed forms a heterogeneous distribution of bound states, this might affect the ability of the interacting partners to bind the RCT. This could also have implications for the ability of the telomere to fold-back [34], and/or establish the molecular determinants that lead to the distinction between long and short states of the telomere [35].

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