Rap1 Binds Single-stranded DNA at Telomeric Double- and Single-stranded Junctions and Competes with Cdc13 Protein

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The ends of eukaryotic chromosomes are protected by specialized telomere chromatin structures. Rap1 and Cdc13 are essential for the formation of functional telomere chromatin in budding yeast by binding to the double-stranded part and the single-stranded 3’ overhang, respectively. We analyzed the binding properties of Saccharomyces castellii Rap1 and Cdc13 to partially single-stranded oligonucleotides, mimicking the junction of the double- and single-stranded DNA (ds-ss junction) at telomeres. We determined the optimal and the minimal DNA setup for a simultaneous binding of Rap1 and Cdc13 at the ds-ss junction. Remarkably, Rap1 is able to bind to a partially single-stranded binding site spanning the ds-ss junction. The binding over the ds-ss junction is anchored in a single double-stranded hemi-site and is stabilized by a sequence-independent interaction of Rap1 with the single-stranded 3’ overhang. Thus, Rap1 is able to switch between a sequence-specific and a non-specific binding mode of one hemi-site. At a ds-ss junction configuration where the two binding sites partially overlap, Rap1 and Cdc13 are competing for the binding. These results shed light on the end protection mechanisms and suggest that Rap1 and Cdc13 act together to ensure the protection of both the 3’ and the 5’ DNA ends at telomeres.

Telomeres are specialized chromatin structures that protect the ends of chromosomes from being detected as double-stranded breaks and from end-to-end chromosome fusions. In most eukaryotes, the telomeres are composed of repetitive duplex DNA ending in a single-stranded 3’ overhang. The assembly of the telomere chromatin is nucleated by the sequence-specific binding of the telomere proteins, which interact with telomere-associated proteins to form a higher order structure, the telosome. In mammalians, the single-stranded 3’-end is further protected by its invasion of the duplex telomeric repeats, resulting in a t-loop structure (1). Although such loop structures have not been identified in budding yeast, formation of a fold back structure is believed to form a telomere protective cap of the yeast chromosome ends. The primary protein binding to the double-stranded telomere DNA in Saccharomyces cerevisiae is Rap1. Rap1 executes a negative regulation of telomere length via its interacting partners Rif1 and Rif2 in a “protein counting” mechanism, where a higher amount of bound Rap1-Rif complexes will inhibit the telomere extension (2, 3). Rap1 binds as a monomer along the length of the telomeric sequence with an average spacing of ~18 base pairs (4, 5). The crystal structure of the Rap1-DBD in complex with a telomeric DNA sequence revealed that the DNA-binding domain (DBD)2 of Rap1 is formed by two similar subdomains, which are structurally related to the Myb DNA-binding motifs and the homeodomains (6). The Myb-like domains are positioned in tandem on the DNA, and the N and C termini are located close together so that the DNA is completely enclosed within the protein. This is corroborated by transmission electron microscopy where Rap1 is imaged as a ring- or C-shaped structure (4). In this way, the Rap1 protein efficiently wraps and protects the length of the telomeric DNA and also supplies the platform for the formation of the higher order structure by its C-terminal interaction with the Sir3 and Sir4 proteins (7).

# Results

Rap1 binds partially single-stranded DNA and influences binding of Cdc13 to 3’ overhangs.

**Conclusion:** Rap1 and Cdc13 provide protection of the respective 3’- and 5’-ends and compete for binding at telomere ds-ss junctions.

**Significance:** Knowledge of the telomeric DNA-protein interactions is crucial for understanding the molecular mechanisms of the telomerase extension.

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2 The abbreviations used are: DBD, DNA-binding domain; nt, nucleotide; MBS, minimal binding site; ds, double-stranded; ss, single-stranded.
with the same permutation, and the CA-rich strands in human telomeres have a preferred end permutation, indicating that these are well regulated processes (13, 14). However, the molecular mechanisms of the end trimming processes still remain unresolved. The 3’ overhangs are bound by DNA-binding proteins that form a cap to protect the chromosome ends from being recognized as damaged DNA, and loss of a functional cap structure leads to activation of the DNA damage response pathways and eventually cell cycle arrest, senescence, or apoptosis (15, 16). In S. cerevisiae, the 3’ overhangs are bound by Cdc13, which recognizes DNA via an oligonucleotide/oligosaccharide-binding fold domain (OB-fold) (17). Cdc13 serves a dual function in telomere length regulation, on the one hand as a positive regulator because it recruits telomerase to the 3’ overhangs by interaction with the Est1 subunit and on the other hand as a negative regulator because it interacts with Stn1 and Ten1 to form the Cdc13-Stn1-Ten1 (CST) complex and thereby blocks telomerase recruitment (18, 19). It is depicted that the DNA polymerase α-primase complex interacts with the CST complex to promote the synthesis of the CA-rich complementary strand (16, 20–22).

The budding yeast Saccharomyces castellii is a beneficial system for the analysis of telomere-binding proteins because it has regular 8-mer telomeric repeats (5’-TCTGGGTG-3’) that provide the possibility of predicting the positioning of the telomere-binding proteins along the telomere repeated array (23). The full-length S. castellii Rap1 protein (scasRap1) binds a 12-nucleotide (nt) minimal binding site (MBS) within the double-stranded telomeric DNA (5’-GGGTGTCTGGGT-3’), thus corresponding to 1.5 telomeric repeats, and its demarcated DNA-binding domain was shown to retain the binding properties of the full-length protein (24, 25). Within this MBS, the sequence specificity of scasRap1 is dependent on the interaction with two 5-nt motifs separated by 1 nt, thus specifying the hemi-sites bound by the respective homeodomains (underlined, 5’-GGGTGTCTGGGT-3’) (24). ScasRap1 binds sequentially to sites having a 16-bp center-to-center spacing, thus effectively covering the length of the telomere sequence array (25). The Cdc13 homolog in S. castellii (scasCdc13) binds an 8-mer MBS on the 3’ overhangs by making highly sequence-specific contacts with four of the nucleotides (underlined, 5’-GTGTCTGGGT-3’) (26). Intriguingly, three of these four nucleotides coincide with the nucleotide positions most important for the sequence-specific binding of scasRap1, leading to a concerted action of these two proteins in the conservation of the telomeric DNA sequence during evolution (24).

The boundary of the double-stranded and single-stranded telomeric DNA (the ds-ss junction) constitutes a functionally important region of the telomere and thus a particularly interesting position for the investigation of telomere chromatin assembly. Just like the single-stranded 3’ overhangs, the 5’-end requires protection from nuclease activity, which in principle could be carried out by the general main telomere-binding proteins binding close to the ds-ss junction. It has been shown that the human telomere-binding protein Trf2 localizes preferentially to the ds-ss junction and stimulates the t-loop formation (1, 27, 28). In S. cerevisiae it has been demonstrated that loss of Cdc13 leads to extensive degradation of the CA-rich strand, indicating that Cdc13 is involved in the protection of the 5’-end (16). In this study, we have analyzed the recruitment of S. castellii Rap1 and Cdc13 to partially single-stranded oligonucleotides that mimic the ds-ss junction of the telomere. Our investigation of the protein binding to defined DNA compositions revealed the minimal 3’ overhang required for Cdc13 binding and the characteristics of the terminal binding of Rap1. Based on this, we have established the minimal DNA structure for obtaining simultaneous binding of Rap1 and Cdc13, as well as the optimal setup for the high affinity binding of both proteins. Surprisingly, we found that Rap1 is capable of binding over the ds-ss junction by interacting with the single-stranded 3’ overhang and thereby influences the ability of Cdc13 to bind to the 3’ overhang. Our results imply a dynamic interplay of Rap1 and Cdc13 at the ds-ss junction and suggest that they act together to provide an efficient capping of the telomere ds-ss junction.

EXPERIMENTAL PROCEDURES

Cloning and Protein Purification—Cloning of the S. castellii genes RAP1, RAP1-DBD, and CDC13 in the pGEX-6p-1 vector, and protein purifications have previously been described (24–26). The recombinant proteins were produced as GST fusion proteins, and the GST tag was then cleaved off with PreScission protease (GE Healthcare). A negative control extract was obtained by using an empty vector and was prepared in parallel with the recombinant protein extracts. The empty vector negative extract did not give any detectable shift in the EMSA together with the respective D19 (double-stranded, 5’-GTG TCTGGGTGTCCTGG) or S16 (single-stranded, 5’-CTG GGTGTCTGGGTG) telomeric oligonucleotides (see Fig. 2A).

Electrophoretic Mobility Shift Assay—The binding abilities of Rap1, Rap1-DBD, and Cdc13 were analyzed by EMSA. Various partially single-stranded probes were used in direct binding studies together with one or two of the proteins (see Fig. 1). All of the oligonucleotides include a 14-nt non telomeric part (box) to direct the annealing and are named according to the number of double-stranded (D) and single-stranded (S) nucleotide positions in the telomeric sequence part. The reverse strand of the respective probe was radioactively labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs). The probes were annealed with the complementary strand (in 1 mM Tris-HCl, pH 8, 0.1 mM MgCl₂) by boiling for 2 min and slow cooling down. In the EMSA reactions 5 or 10 fmol of probe, 1.5 μg of nonspecific competitor (equal amounts of Escherichia coli DNA, yeast tRNA, and salmon sperm DNA), binding buffer (final concentration, 10 mM Tris-HCl, pH 7.5, 7 mM MgCl₂, 8% glycerol), and different amounts of protein were incubated for 15 min at 25 °C. Crude extracts were used for Rap1 and Rap1-DBD, because a higher binding activity was obtained with crude extracts as compared with purified protein, and they produced the same shifted signal (supplemental Fig. S1). The typical range of protein amount used in the reaction was ~0.3–3 μg for Rap1 and ~0.15–1.5 μg for Rap1-DBD. For Cdc13, the affinity chromatography purified protein was used in a range from ~2 to 5 μg. The binding capacity of the extracts were analyzed by EMSA, and the fraction of protein binding was titrated for each extract to use similar amounts of active protein in mixed reac-
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tions and parallel comparative reactions. In experiments where simultaneous binding of two proteins were investigated, the first protein was prebound to the probe for 15 min at 25 °C, and then the second protein was added and followed by an incubation for 15 min at 25 °C. The free probes and the controls were similarly incubated for two times 15 min at 25 °C. The samples were loaded on a 6% nondenaturing polyacrylamide gel and run in 1× TBE (89 mm Tris-borate, 2 mm EDTA, pH 8.0), 150 V at 4 °C. After drying of the gel, the signal was analyzed using a BioRad Molecular Imager FX PhosphorImager.

Half-time of DNA-Protein Complexes—The half-times (t1/2) of the complexes were analyzed by allowing the proteins to bind telomeric oligonucleotides D13S13, D19 (double-stranded, 5’-GTGTCTGAGGTCTGTTG-3’) for Rap1 and D13S13, S15 (5’-GGGTGTCTGAGGTCTGTTG-3’) for Cdc13 for 15 min at 25 °C and followed by the addition of excess amounts (×1000) of nonlabeled specific competitor. The samples were incubated (1–40 min), directly placed on ice, loaded within 2 min, and run on a 6% nondenaturing polyacrylamide gel as above. The signals were quantified using Quantity One software (Bio-Rad) and normalized against a control that was generated by adding the nonlabeled competitor prior to protein addition. The fraction of bound probe was calculated and plotted against the incubation time.

RESULTS

Rap1 and Cdc13 Bind to Oligonucleotides Having a 3’ Overhang—All of the telomeres end with a single-stranded 3’ overhang in vivo, but how the DNA-binding proteins assemble to form the telomere chromatin at the junction of the double- and single-stranded DNA is still not well understood. To illuminate this, we wanted to analyze the respective binding of Rap1 and Cdc13 to partially single-stranded oligonucleotides (ds-ss oligonucleotides) and to characterize the criteria for their simultaneous binding. We have previously determined the MBS for the S. castellii Cdc13, as well as the MBS for the full-length Rap1 protein and for its demarcated DNA-binding domain (Rap1-DBD) (Fig. 1A) (24, 26). Using this information, we designed a series of telomeric ds-ss oligonucleotides containing binding sites for Rap1 and Cdc13 at various positions in relation to the ds-ss junction (Fig. 1B). First, we tested our recombinant extracts of full-length Rap1 and Rap1-DBD by EMSA with the fully double-stranded D19 oligonucleotide. Although the extracts of the recombinant proteins gave distinct band shifts (Fig. 2A, lanes 2 and 3), the control extracts made by using empty vector did not show any shift (Fig. 2A, lane 4). Second, the recombinant Cdc13 protein gave a distinct shift with the fully single-stranded S16 oligonucleotide (Fig. 2A, lane 6), whereas no shift was seen with the control extracts made from empty vector (Fig. 2A, lane 7). Next, we performed EMSA on the D17S17 oligonucleotide, which contains a double-stranded part including a full Rap1 MBS (bold), followed by two double-stranded nt positions and a 17-nt-long single-stranded 3’ overhang (Fig. 1B, D17S17). Our EMSA analyses showed that Rap1 and Rap1-DBD, as well as Cdc13, bind very well to this ds-ss oligonucleotide in vitro and thus demonstrated the feasibility of performing the DNA binding analyses on these types of substrates (Fig. 2B).

Rap1 Can Bind Over Short Stretch of Single-stranded DNA—Rap1 has tandem binding sites on the duplex telomeric DNA and should in theory, depending on the permutation of the complementary strand, be able to bind close to the ds-ss junction. We wanted to determine exactly how close to the junction Rap1 was able to bind, and for this purpose we used a series of ds-ss oligonucleotides where the MBS was progressively moved toward the junction. As expected, Rap1 was able to bind directly adjacent to the junction, with the last 3’ nucleotide of the MBS positioned as the last double-stranded nucleotide position of the ds-ss oligonucleotide (supplemental Fig. S2). Surprisingly, however, Rap1 was also able to bind a ds-ss oligonucleotide where two of the nucleotide positions in the Rap1 MBS are single-stranded (Fig. 1B, D13S13). Rap1 generated a pronounced shifted band in the binding reaction with the D13S13 oligonucleotide, thus demonstrating that Rap1 can bind over the ds-ss junction (Fig. 3A, lanes 2–4). To determine whether this is a feature preserved in the DNA-binding domain of Rap1, we performed the experiment using the demarcated Rap1-DBD. Because Rap1-DBD also generated a pronounced shifted band with this oligonucleotide (Fig. 3A, lanes 6–8), we conclude that the DBD of Rap1 harbors the property to bind over short stretches of single-stranded DNA at the telomeric ds-ss junction.

The Single-stranded 3’ Overhang Is Used to Stabilize Binding of Rap1 over ds-ss Junctions—The unexpected finding of Rap1 binding over the ds-ss junction prompted us to investigate whether Rap1 would still be able to bind telomeric DNA if the single-stranded part of its MBS would be even more extended. We constructed an oligonucleotide with the same 5’-3’ strand sequence as the D13S13, but in contrast leaving the second Rap1 MBS hemi-site fully single-stranded (Fig. 1B, D10S16). Thus, the double-stranded part of the D10S16 oligonucleotide only covers one single Rap1 MBS hemi-site followed by one linker nucleotide (position 7). Remarkably, the addition of Rap1 in 2-fold increasing amounts to the binding reaction with D10S16 resulted in a shift, demonstrating that Rap1 is able to bind directly to this oligonucleotide (Fig. 3B, lanes 4–6). However, a quite weaker shift was obtained, which indicates that the affinity to this oligonucleotide is lower than to D13S13 (with only 2 nt of the MBS single-stranded). Rap1 binds DNA in bi-partite manner via two Myb-like homeodomains (6, 24). Because our results show that Rap1 is able to bind a telomeric oligonucleotide containing only one double-stranded hemi-site, we wanted to elucidate whether a single Rap1 hemi-site would indeed be sufficient to support the binding. We therefore tested binding of Rap1 to the blunt end oligonucleotide D10, containing the same double-stranded sequence as D10S16 but totally lacking the single-stranded 3’ overhang. Thus, only one hemi-site of the Rap1 MBS is present in this oligonucleotide (Fig. 1B, D10). We found that Rap1 was not able to bind D10, because no shift was observed in our EMSA analysis, even though very high protein amounts were added (Fig. 3B, lane 8). Together, our results show that Rap1 needs more than one hemi-site to facilitate binding and that Rap1 is dependent on an interaction with the single-stranded overhang when binding over the ds-ss junction.
We considered the possibility that binding over the ds-ss junction would be independent of which strand that would be used to create the single-stranded overhang. We therefore tested binding of Rap1 to an oligonucleotide having a CA-rich single-stranded 5' overhang instead of the previously used TG-rich 3' overhang (Fig. 1B, D10S11-5'). However, Rap1 did not produce any detectable shift with the D10S11-5' oligonucleotide in the EMSA. This shows that binding to the single hemi-site only can be stabilized by an interaction with the 3' overhang (Fig. 3B, lanes 10–12).

Furthermore, we wanted to investigate whether the interaction of Rap1 with the single-stranded 3' overhang at the ds-ss junction is sequence-dependent. We designed an oligonucleotide with the same structure as the D10S16, i.e., with a single double-stranded hemi-site of the Rap1 MBS but instead with a random nontelomeric sequence in the 3' overhang.

![Table showing binding results](image)
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In summary, our results demonstrate that Rap1 can bind to a single-stranded 3’ overhang in a sequence-independent manner. A, EMSA analysis of D13S13, having two single-stranded nucleotide positions in the DNA sequence of the 3’ overhang; D10, having only a single blunt-ended hemi-site of the Rap1 MBS; D10S11-5’, having a single double-stranded hemi-site and a single-stranded CA-rich 5’ overhang. Parallel reactions with similar amounts of Rap1 bound to D10S16 with a telomeric 3’ overhang, versus D10S16(NT) with a nontelomeric 3’ overhang. Protein was added in 2-fold increasing amounts.

hang, D10S16(NT) (Fig. 1B). The two oligonucleotides were assayed in parallel on the same EMSA gel, using equal amounts of Rap1 in the reaction series (Fig. 3C). The oligonucleotides shifted equally as well, showing that Rap1 is able to bind to D10S16(NT) with a similar affinity as to the D10S16 oligonucleotide (Fig. 3C). Thus, the Rap1 binding is not dependent on the DNA sequence of the 3’ overhang.

In summary, our results demonstrate that Rap1 can bind to a single double-stranded hemi-site of its MBS if the binding is stabilized by a single-stranded 3’ overhang. The interaction of Rap1 with the single-stranded DNA is not sequence-specific, but a 5’-3’ orientation of the overhang is required.

Rap1 and Cdc13 Bind Simultaneously to an Oligonucleotide with a 3’ Overhang—Cdc13 is essential for protection of the single-stranded 3’ overhangs in vivo, and it is believed that Rap1 and Cdc13 will bind simultaneously to the telomere at certain times during the cell division cycle (29, 30). To investigate the requirements for a simultaneous binding at the ds-ss junction, we used the ds-ss oligonucleotide D17S17 in a mixed reaction with both Rap1 and Cdc13 (Fig. 4, A and B). The D17S17 oligonucleotide contains the respective full MBS of both Rap1 and Cdc13, and as a control the respective proteins were tested in separate reactions and were found to bind this oligonucleotide with high affinity (Fig. 4A, lanes 2 and 3). In the first experiment, Rap1 was preincubated with the oligonucleotide, and Cdc13 was subsequently added to the reaction in increasing amounts (Fig. 4A, lanes 4–7). A ternary DNA-Rap1-Cdc13 complex was formed when Cdc13 was added to the prebound DNA-Rap1 complex, which was observed as a second shifted band with a lower mobility (Fig. 4A, lanes 4–7, top band). The signal of the ternary complex (top band) increases when Cdc13 is added in increasing amounts to the prebound D17S17-Rap1 complex. To investigate whether the formation of the ternary complex is dependent on the order of protein addition, we performed the reverse experiment. Also in this experiment, a ternary complex was formed, when Rap1 was added in increasing amounts to the prebound DNA-Rap1 complex, which was observed as a second shifted band with a lower mobility (Fig. 4B, lanes 4–7, top band). Similarly, the signal of the ternary complex (top band) increases as more Rap1 is added into the reaction.

To elucidate whether the ability of the full-length Rap1 protein to bind simultaneously as Cdc13 is inherent in its DBD, the above experiments were repeated using the Rap1-DBD extract. Correspondingly, a ternary complex was formed, which shows that Rap1-DBD and Cdc13 can bind simultaneously to D17S17 (Fig. 4C, lanes 4–6). Moreover, the formation of the ternary complex was again independent of the order of protein addition (Fig. 4C, lanes 9–11). We conclude that both full-length Rap1
and Rap1-DBD can bind simultaneously with Cdc13 to a ds-ss oligonucleotide containing the Rap1 and Cdc13 MBS on the respective double- and single-stranded part of the oligonucleotide. Moreover, the formation of a ternary DNA-Rap1-Cdc13 complex is independent of the order of protein addition.

**Rap1 and Cdc13 Can Bind Simultaneously over ds-ss Junctions**—We demonstrated above that Rap1 can bind to the D10S16 oligonucleotide, which has one double-stranded hemi-site and one single-stranded hemi-site of its MBS (Fig. 3A, lanes 4–6). This oligonucleotide also contains an MBS for Cdc13, which overlaps with the binding site of Rap1 by two nucleotides (Fig. 1B, D10S16). We were interested in finding out whether Rap1 and Cdc13 would be able to bind simultaneously to D10S16 despite this overlap (Fig. 4, A and B). Indeed, a ternary DNA-protein complex was formed when Cdc13 was added in increasing amounts to the prebound D10S16-Rap1 complex (Fig. 5, lanes 4–6). The ternary complex is observed independently of the order of the protein addition, as it also appears in the reverse experiment where Cdc13 is prebound to D10S16 and Rap1 is added into the binding reaction (Fig. 5, lanes 9–11). Remarkably, these results show that Rap1 and Cdc13 are capable of simultaneously binding an oligonucleotide containing partially overlapping binding sites. This, together with our results showing the lack of sequence specificity of the Rap1 interaction with the single-stranded 3’ overhang, indicates that Rap1 probably uses a more narrow binding site on the overhang than it does on the double-stranded DNA.

**Cdc13 Requires a 9-nt 3’ Overhang to Bind at ds-ss Junctions**—As shown above, Cdc13 is able to bind the 3’ overhang of oligonucleotide D17S17 where the 8-mer Cdc13 MBS is located with a four nucleotides spacer to the ds-ss junction (Figs. 1 and 4A, lane 2). However, depending on the permutation of the telomeric sequence at the ds-ss junction, a presumptive Cdc13 binding site could in theory be situated even closer to the junction. Thus, in theory, the shortest 3’ overhang that could be protected would correspond to a single 8-mer MBS for the Cdc13 protein. We therefore wanted to investigate whether Cdc13 can bind a ds-ss oligonucleotide containing exclusively the 8-mer Cdc13 MBS on the 3’ overhang.

First, to investigate whether Cdc13 is able to bind directly at the ds-ss junction, we used D13S13, which contains the Cdc13 MBS immediately adjacent to the double-stranded part and is followed by five additional nucleotides (Fig. 1B, D13S13). A single shifted band was observed when Cdc13 was added into a binding reaction with D13S13, thus demonstrating that Cdc13 was indeed able to bind directly at the ds-ss junction (Fig. 6, lane 2). Next we wanted to test whether Cdc13, like Rap1, would be able to bind over junctions. To this end, Cdc13 was challenged to bind an oligonucleotide where the two 5’-most nucleotide positions in the 8-mer Cdc13 MBS are double-stranded (Fig. 1B, D15S11). As Cdc13 failed to bind D15S11 in an EMSA reaction, we conclude that Cdc13 cannot bind over the telomeric ds-ss junctions (Fig. 6, lane 4).

Having confirmed that Cdc13 exclusively binds the single-stranded 3’ overhang and is able to bind directly at the ds-ss junction, we next analyzed whether Cdc13 could bind a ds-ss oligonucleotide containing exclusively the 8-mer Cdc13 MBS on the 3’ overhang (Fig. 1B, D13S8). However, no band shift appeared when EMSA was performed on Cdc13 and the D13S8 oligonucleotide, indicating that a longer single-stranded 3’ overhang is needed for Cdc13 binding (Fig. 6, lane 6). We therefore tested binding of Cdc13 to progressively longer 3’ overhangs having 1–2 additional nucleotides after the MBS (Fig. 1B, D13S9 and D13S10). A clear shifted signal was indeed observed with the 9-nt overhang, demonstrating that 9 nt is the minimal length required to obtain Cdc13 binding to the 3’ overhang (Fig. 6, lanes 8–10). However, a much stronger shift was obtained for the 10-nt overhang when equal amounts of Cdc13 protein were used, indicating a more stable binding interaction (Fig. 6, lanes 11–14). The signal obtained for the binding to the 10-nt overhang is comparable with that obtained for the 13-nt overhang, indicating that a 10-nt 3’ overhang is sufficient to support a high affinity binding of Cdc13 (Fig. 6, lanes 2 and 13).

In summary, binding of Cdc13 is accomplished when the Cdc13 MBS is situated immediately at the ds-ss junction but is abolished if the MBS is partly double-stranded, showing that Cdc13 can bind directly adjacent to, but not over a junction. A minimal length of 9 nt is required to facilitate Cdc13 binding on the 3’ overhang, but the binding is further stabilized when the 3’ overhang reaches a length of 10 nt.

**Rap1 and Cdc13 Compete for Binding at ds-ss Junctions**—Our results above show that Rap1 and Cdc13 are able to simultaneously bind the oligonucleotide D10S16, which has overlapping binding sites for the two proteins. However, the low affinity of Rap1 to this oligonucleotide prompted us to test for...
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simultaneous binding to the oligonucleotide D13S13, which Rap1 binds with high affinity (Fig. 3A, lane 2). In D13S13, as in D10S16, two single-stranded nucleotides of the Rap1 MBS overlap with the Cdc13 MBS. However, in D13S13, with the exception of these two overlapping positions, the rest of the Rap1 MBS is double-stranded (Fig. 1B). First, Rap1 was prebound to D13S13, and Cdc13 was added into the binding reaction in increasing amounts. Surprisingly, no ternary complex was formed in this reaction, demonstrating that no simultaneous binding is possible to this oligonucleotide (Fig. 7A, lanes 4–8). Instead a band corresponding to the D13S13-Cdc13 complex appears, and as the Cdc13 amount increases in the reactions, the signal of the upper band corresponding to the D13S13-Rap1 complex decreases. This shows that Cdc13 is competing with Rap1 for its binding site when added in excess amounts.

To test whether the order of protein addition could be of importance, we performed the reverse experiment with Cdc13 prebound to D13S13 and Rap1 addition in increasing amounts (Fig. 7A, lanes 11–13). Neither in this experiment, any ternary complex was formed when Rap1 was added into the binding reaction with the preformed Cdc13-D13S13 complex. Thus, no simultaneous binding to the D13S13 oligonucleotide is possible. Interestingly, however, when Rap1 is added in excess amounts, the upper band corresponding to the D13S13-Rap1 complex enhances. Concurrently, the signal from the lower band corresponding to the D13S13-Cdc13 complex is decreasing. This demonstrates that Rap1 is able to compete for the binding site when added in excess amounts. Thus, we have demonstrated that both Rap1 and Cdc13 compete for their partially overlapping binding sites at the telomeric ds-ss junction when added in excess amounts.

To investigate whether this competitive feature is inherent in the DNA-binding domain of Rap1, we repeated the experiment with Rap1-DBD and Cdc13. Rap1-DBD was allowed to bind the oligonucleotide D13S13 before Cdc13 was added into the reaction in increasing amounts. Neither in this case, any ternary complex was formed, showing that the proteins cannot bind simultaneously to the oligonucleotide (Fig. 7B). However, Cdc13 is able to compete for the binding site, because the lower band corresponding to D13S13-Rap1-DBD is fading as more Cdc13 is added into the reaction (Fig. 7B, lanes 4–6). Subsequently, the reverse experiment was carried out, with Cdc13 prebound to D13S13 and Rap1-DBD added into the reaction. In the same way as for the full-length protein, Rap1-DBD is able to compete for the binding site when added in excess amounts into a reaction with the D13S13-Cdc13 complex (Fig. 7B, lanes 9–11). Thus, the full-length Rap1 and the Rap1-DBD give the same results, which demonstrates that the ability to compete for binding with Cdc13 is retained within the DNA-binding domain of Rap1.

Because Rap1 and Cdc13 both compete for the binding at the ds-ss junction, the stability of the D13S13-Rap1 and D13S13-Cdc13 complexes, respectively, would be predicted to have an effect on the outcome of the competition. For this purpose, we compared the stability of the respective DNA-protein complexes by time course measurements. In separate reactions, Rap1 versus Cdc13 were prebound to a telomeric oligonucleo-
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FIGURE 8. The minimal and optimal requirements for the simultaneous binding of Rap1 and Cdc13 at the ds-ss junction. A, upper panel, Cdc13 was prebound to D10S12 followed by the addition of Rap1 in 2-fold increasing amounts. Lane 2, only Rap1 added; lane 3, only Cdc13 added. Lower schematic, model of the simultaneous Rap1 and Cdc13 binding to the minimal ds-ss junction. The Rap1 binding is directed by a single double-stranded hemi-site and stabilized by the interaction with the single-stranded 3’ overhang. Despite the interaction with Rap1, Cdc13 is allowed access its binding site on the 3’ overhang. B, upper panel, Cdc13 was prebound to D15S16 followed by the addition of Rap1 in 2-fold increasing amounts. Inversely, Cdc13 was prebound to D21S10 followed by the addition of Rap1 in 2-fold increasing amounts. Lanes 2 and 6, only Rap1 added; lanes 3 and 9, only Cdc13 added. Lower schematic, model of the simultaneous Rap1 and Cdc13 binding to the optimal DNA setup at the ds-ss junction. This DNA configuration allows for high affinity binding sites for both proteins, where Rap1 binds to a fully double-stranded MBS. The 6-nt spacer is dictated by the 8-mer telomeric repeat and may be either double- or single-stranded. Bold letters denote the Rap1 MBS, and blue letters denote the Cdc13 MBS.

denote the Rap1 MBS, and denote the Cdc13 MBS.

tide. An excess amount of cold competitor was then added, which was followed by an incubation of the reactions for different amounts of time (1–40 min). The reactions were run on EMSA, and the signals were quantified and then normalized to a sample where the cold competitor was added prior to protein addition. The fraction of bound probe was plotted against the reaction time, and the t1/2 (half-time of the complexes) was determined as the time point when 50% of the initial bound fraction was still bound. For the D13S13-Cdc13 complex, t1/2 was estimated as 2.5 min, and for the D13S13-Rap1 complex, t1/2 was estimated as ≤1 min (Fig. 7C). We considered the possibility that a more stable DNA-Rap1 complex could be formed if Rap1 was allowed to bind a telomeric oligonucleotide with a full double-stranded MBS. We therefore tested the stability of Rap1 binding to the D19 oligonucleotide, which contains the full MBS of Rap1 (“Experimental Procedures”). However, the D19-Rap1 complex was estimated to the same t1/2 of ≤1 min (Fig. 7C). Furthermore, we also tested the stability of Cdc13 binding to the single-stranded telomeric oligonucleotide S15, containing the full MBS of Cdc13 (“Experimental Procedures”), which gave a t1/2 of 4.5 min for the S15-Cdc13 complex. These results show that both the Rap1 and Cdc13 proteins form DNA-protein complexes with very short t1/2. Because the dissociation of one of the proteins will give the opportunity for the other one to bind, the concentration of the respective proteins will have an influence on the outcome of the competition. Thus, we suggest the following model for the observed competition (Fig. 7D). The very short t1/2 values of the respective DNA-Rap1 and DNA-Cdc13 complexes mean that the bound proteins will dissociate frequently. When the site is vacant, the protein present at the highest concentration will have a higher probability for binding the site (Fig. 7D).

In conclusion, because of the ability demonstrated here of Rap1 to bind over the ds-ss junction, it is possible to generate a site for competitive binding between Rap1 and Cdc13. Because the half-time of the DNA-protein complex is low for both Rap1 and Cdc13 to an oligonucleotide containing a 2-nt overlap of their respective MBS, both proteins are able to compete for their binding site when added in excess amounts.

Minimal and Optimal Requirements for Rap1 and Cdc13 Binding at the ds-ss Junction—Here we showed that, remarkably, Rap1 and Cdc13 bind simultaneously at the ds-ss junction, even though their respective MBS partially overlap. Together with our determination of the 9-nt minimal single-stranded overhang required for Cdc13 binding, we can now make a prediction of the theoretical minimal DNA substrate required for simultaneous binding of Rap1 and Cdc13 at the telomeric ds-ss junction. In this prediction, a sequence stretch including a single duplex hemi-site of the Rap1 MBS, followed by an 8-nt MBS for Cdc13 plus one additional 3’ nucleotide, would be sufficient to support the assembly of both proteins (Fig. 8A). To test this theory, we designed the D10S12 oligonucleotide, which is similar to D10S16, except that it has a shorter single-stranded overhang (Fig. 1B). In this experiment, Cdc13 was prebound to D10S12, and Rap1 was then added in increasing amounts. A ternary complex was indeed formed, which shows that the D10S12 oligonucleotide meets the minimal requirements for simultaneous binding of the Rap1 and Cdc13 proteins at the ds-ss junction (Fig. 8A, lanes 4–6).
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However, our binding analyses show that Rap1 binds with higher affinity to a binding site with a fully double-stranded MBS compared with a binding site where the second hemi-site is single-stranded as in the above case (supplemental Fig. S3). Moreover, above we demonstrated that Cdc13 binds with a higher affinity to a 3’ overhang consisting of the MBS +2 additional nucleotides. Thus, even though both Rap1 and Cdc13 bind very well to the D10S12 oligonucleotide (Fig. 8A, lanes 2 and 3), these sites appear to be less favorable because both proteins bind with a somewhat reduced affinity to these types of sites. Thus, we would predict that the optimal scenario for a highly stable simultaneous binding of both proteins would be a telomeric sequence including the full double-stranded MBS of Rap1 and the MBS of Cdc13 followed by two additional nucleotides (Fig. 8B). To meet these criteria, the respective binding sites will inevitably be located 6-nt apart, because of the 8-nt repeated sequence of the S. castellii telomeric DNA. However, because Rap1 and Cdc13 can both bind directly adjacent to the ds-ss junction, this 6-nt spacer could in theory be either single-stranded or double-stranded. To test these predictions, we therefore designed two oligonucleotides, one where the Rap1 MBS and the Cdc13 MBS were separated by a 6-nt single-stranded stretch (D15S16) and one where the two respective MBS were separated by six double-stranded positions (D21S10). Cdc13 was prebound to either D15S16 or D21S10, and Rap1 was added in increasing amounts. The formation of a ternary complex in both cases shows that Cdc13 and Rap1 are able to bind simultaneously to both types of substrates (Fig. 8B, lanes 4–6 and 10–12). Also in the reverse experiment, with Rap1 prebound and Cdc13 added in increasing amounts, a ternary complex was formed (supplemental Fig. S4).

In conclusion, we have shown that the optimal setting for a simultaneous high affinity binding of Rap1 and Cdc13 at the telomeric ds-ss junction of S. castellii is obtained when their respective binding sites are separated by 6 nt of either single-stranded or double-stranded DNA (Fig. 8B). Furthermore, we have determined that Cdc13 needs a minimal 3’ overhang of 9 nt for binding directly at the ds-ss junction. Surprisingly, however, we found that Rap1 is able to bind a site spanning over the telomeric ds-ss junction by using the single-stranded 3’ overhang for the stabilization of this interaction. When the single-stranded part of the Rap1 MBS is limited to 2 nt, Rap1 and Cdc13 compete for the DNA interaction at the ds-ss junction, and the protein with the highest concentration will form the most abundant DNA-protein complex. However, in a configuration where the single-stranded part of the Rap1 MBS is longer, a simultaneous binding of Rap1 and Cdc13 is facilitated, thus defining the minimal DNA requirements for the assembly of telomeric chromatin (Fig. 8A).

DISCUSSION

To maintain functional telomeres to protect the chromosomes, the length of the telomeric DNA needs to be regulated. During the S phase of the cell cycle, short telomeres are preferably elongated by telomerase before long telomeres, and there are several models proposed on how telomere length could be regulated (31). A model by Shore and Bianchi (22) proposes that short telomeres are more extensively resected by a 5’ exonuclease than long ones. This will result in an increased single-stranded 3’ overhang length that can be bound by a larger number of Cdc13 proteins and hence recruit telomerase to the end. Still, however, the molecular procedure for the 5’-end resection of the CA-rich strand is largely unknown. The 5’-end resection takes place at the junction of the double- and single-stranded telomeric DNA (ds-ss junction), and it could be inferred that the proteins binding close to the ds-ss junction will play an important role in the complementary strand maintenance. The sequence permutation of the CA-rich complementary strand terminus will determine whether and where the DNA binding proteins will be allowed to interact with the ds-ss junction, which in turn will interfere with the access of the resection machinery to the 5’-end. Hence, to be able to pinpoint the details of these molecular mechanisms, it is of importance to elucidate the binding properties of the telomere proteins at this specific location.

In this study, we used partially single-stranded oligonucleotides that mimic the telomeric end, to determine the interplay of Rap1 and Cdc13 when establishing DNA interactions at the ds-ss junction in vitro. As previously demonstrated, S. castellii Rap1 binds with high sequence specificity to a 12-mer MBS of the double-stranded telomeric DNA (5’-GGGTGCTCTGGGT-3’) (24). This is a bipartite binding site where each of the two Myb-like homeodomains binds to a 5-nt hemi-site (Fig. 1A). To our surprise, however, we here discovered that Rap1 is able to establish a DNA interaction with a partially single-stranded site spanning over the ds-ss junction. This DNA-protein complex is stabilized by an interaction of Rap1 with the single-stranded 3’ overhang. Although the single-stranded DNA needs to be in the 5’ to 3’ orientation toward the end for this interaction to take place, the interaction is not sequence-specific. Because we found that only a single hemi-site of the MBS needs to be double-stranded to establish binding over the ds-ss junction, this implies that the second Myb-like homeodomain of Rap1 is able to switch between a sequence-specific binding of double-stranded DNA and a nonspecific binding of single-stranded DNA. This is very intriguing, and we speculate that the protein domain may be able to adopt two distinct modes of interaction when binding to a double-stranded site or a single-stranded site, respectively. Although this is an unpredicted feature, S. cerevisiae Rap1 is known to be very versatile in its binding because it functions as a transcriptional activator and/or repressor at a huge amount of different promoters throughout the genome (32, 33). Thus, S. cerevisiae Rap1 has been reported to have a loosely defined consensus sequence and has also been shown to have a high flexibility in its binding both to promoters and to telomere DNA (34–36). However, the new mode of binding over the ds-ss junction presented here is unprecedented and adds a new level of complexity to the functionality of Rap1.

The ability of S. castellii Rap1 to switch between qualitatively different modes of binding is further supported by the fact that the two oligonucleotides D13S13 and D10S16 give different outcomes in our test for simultaneous binding of Rap1 and Cdc13. In both of these oligonucleotides, the respective Rap1 and Cdc13 MBS overlap with two positions, whereas they differ in the extent of double-stranded positions in the Rap1 MBS.
D13S13, all except the two overlapping positions are double-stranded, whereas only the 5′-most hemi-site is double-stranded in D10S16. Our results show that Rap1 and Cdc13 cannot bind simultaneously to the D13S13 oligonucleotide but instead compete for the binding site at the ds-ss junction. In contrast, Rap1 and Cdc13 bind simultaneously to D10S16, which indicates that Rap1 is not contacting all of the nucleotides included in the MBS. This decrease in sequence length used for the binding, together with our demonstration that the interaction is sequence-independent, supports our notion that the Rap1 protein is able to conform into a separate mode of interaction when binding single-stranded DNA. Intriguingly, this resembles the property of the human telomere-binding protein Trf2. In addition to the sequence-specific Myb-like domain, Trf2 contains a basic domain that directs a sequence-independent binding to DNA junctions (37). Furthermore, this Trf2 binding property is reminiscent of the sequence-independent binding properties of the p53 protein (38). In addition to its highly sequence-specific binding mode, p53 has the ability to bind in a sequence-nonspecific manner to the junctions of stalled replication forks. In this way, p53 stabilizes chicken foot intermediates and is suggested to act by protection of stalled forks and by preventing further fork movement until the cause of the replication arrest is repaired, thus avoiding deleterious rearrangements. We hypothesize that the ability of Rap1 to bind sequence-independently to single-stranded DNA may similarly convey the potential for Rap1 to protect complex DNA structures at the telomere. It would therefore be highly interesting to further analyze the binding of Rap1 to various complex DNA structures.

The budding yeast gene CDC13 is essential, and loss of function leads to cell cycle arrest and subsequent cell death (15, 16). Cdc13 serves a critical role in the telomere elongation mechanism by binding to the single-stranded 3′ overhangs and thereby recruiting telomerase via its interaction with the telomerase Est1 subunit (19, 39). However, Cdc13 has also been implicated in the regulation of the synthesis of the CA-rich complementary strand, because a loss of Cdc13 function leads to extensive resection of the CA strand and thereby grossly extended 3′ overhangs (16). Here we have demonstrated that Cdc13 can bind to the ds-ss junction even when its MBS is located directly next to the 5′-end. This implies that Cdc13 may fulfill a protective function at the 5′-end by sterically hinder the access of nucleases to the 5′-end.

An additional function of Cdc13 is to form a protective cap on the 3′ overhang, shielding the 3′-end from degradation and preventing chromosome end-to-end fusions. Here we determined that a minimal length of 10 nt is needed to achieve high affinity Cdc13 binding to the 3′ overhang (Fig. 6). This DNA setup corresponds to the MBS located directly at the ds-ss junction followed by two additional nucleotides in the 3′-end (Fig. 8C). In S. cerevisiae, the single-stranded 3′ overhangs are extended to >30 nt in length in late S phase, but during the rest of the cell cycle they were determined to be only 14–16 nt (12). This means that the binding site for the Cdc13 protein would be rather limited during most parts of the cell cycle. However, our results show that a similar short length would conveniently provide a binding site for a single Cdc13 protein in S. castellii. A 16-nt overhang would in all occasions provide a binding site, whatever the permutation would be of the 5′-end of the CA-rich strand (Fig. 8C). However, a 14-nt overhang length would be somewhat dependent on the permutation of the 5′-end. In human telomeres, there is a prevalence for the CA-rich strand ending with the same permutation (14). It would thus be very interesting to determine whether the permutation of the 5′-end is similarly regulated in S. castellii and to correlate this with the location of the Cdc13 binding site. Presumably, the permutation of the 3′-end, which is released from the telomerase extension, may also be relevant for the discussion. The S. castellii telomerase enzyme produces 8-mer repeats (40). It is noteworthy that the extension product released from telomerase corresponds to the sequence permutation CTGGGTGT-3′, which in itself does not support Cdc13 binding (40). Therefore, to create a binding site for Cdc13, telomerase would need to add this 8-mer repeat onto 3′ overhangs of at least four nucleotides, in this way creating a 12-nt minimal 3′ overhang that can sustain Cdc13 binding (GTGTCCTGGGTGT-3′).

Normally, we do not consider Rap1 and Cdc13 to be rivals for the same binding site, because they bind to the double-stranded DNA and single-stranded DNA, respectively. However, in this work we demonstrate that Rap1 has the capability of binding over the ds-ss junction with a high affinity even when the two 5′-most positions of its MBS are single-stranded (Fig. 7A). Because those two positions are also used by Cdc13 for its binding to the 3′ overhang, this opens up for the possibility of a binding site competition between Rap1 and Cdc13 (Fig. 7C). Our results show that Rap1 and Cdc13 do indeed compete for binding at this ds-ss binding site, because no ternary complex formation is observed when adding both proteins into the binding reaction (Fig. 7A). We considered that the outcome of the competition would be influenced by the stability of the DNA-protein complexes as well as the concentration of the proteins. Our time course experiments show that the half-time for Rap1 in complex with this ds-ss junction (D13S13) is the same as for that formed with a fully double-stranded DNA (D19). Both complexes have very short half-times (≈1 min), which are in agreement with that shown for S. cerevisiae Rap1 in complex with a fully double-stranded telomeric oligonucleotide (4). Likewise, Cdc13 in complex with either the single-stranded DNA (S15) or the ds-ss junction (D13S13) has similarly short half-times (<4.5 min). Because both Rap1 and Cdc13 form short-lived complexes with telomeric DNA, we would therefore predict that the concentration of the respective protein would play a major role in the outcome of the binding competition. In support for this, our competition experiments show that as excess amounts of one of the proteins are added, a DNA-protein complex will form with this protein at the expense of the other protein. This notion is further supported by the fact that our competitions were performed with preformed complexes. Thus, both Rap1 and Cdc13 are able to compete for the binding site at the ds-ss junction, and which one of the proteins will form the majority of the DNA-protein complexes will depend on the concentration of the respective proteins.

Besides being a negative regulator of telomere length, Rap1 functions as a transcriptional activator and/or repressor (32). Rap1 is present in abundant amounts in S. cerevisiae (sce), and...
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Based on expression levels, there is an approximately three times higher nuclear concentration of scerRap1 than scerCdc13 in the cell (41). Even though this may not correlate with the concentration levels at the telomeres, one could still speculate that scerRap1 might exist in higher amounts because there are predicted to be more scerRap1 binding sites than scerCdc13 binding sites on an average telomere. In *S. cerevisiae*, the amounts predicted are: 14–20 scerRap1 on 300-bp telomeres (scerRap1 binds every ~18 bp) versus 1 scerCdc13 per telomere on 14–16-nt 3’ overhangs (MBS is 11 nt) (5, 12, 42). In addition, scerRap1 is also more consistently present at the telomeres throughout the cell cycle, in comparison with scerCdc13, which has a more varied presence and only accumulates during late S phase when the 3’ overhangs are elongated (29, 30). In the light of this, we speculate that Rap1 has the potential for having a regulatory role on the Cdc13 binding of telomeres in the case of short 3’ overhangs and overlapping binding sites. Only in the late S phase would the Cdc13 protein concentration be able to reach levels that would give Cdc13 the possibility for competing at such sites.

Because Cdc13 has been shown to interact with the catalytic Pol1 subunit of DNA polymerase α-primase, and loss of Cdc13 function leads to massive unregulated 5’-end resection, the current model depicts that Cdc13 (within the CTS complex) plays an important role in promoting the replication of the CA-rich strand. Following telomerase-mediated extension of the TG-rich strand, the CTS complex would recruit the DNA polymerase α-primase complex and thus terminate telomerase action (21, 22). Here we have shown that Cdc13 is able to bind a site located precisely at the ds-ss junction. Such a positioning of Cdc13 would make it highly probable to be physically intervening with the resection machinery and thereby would argue for a role of Cdc13 in the protection of the 5’-end. However, the effectiveness of the protection would be predicted to depend on the permutation of the 5’-end, because this would define where the Cdc13 binding site is situated in respect to the ds-ss junction. On the other hand, in the light of our results, a similar protective role could also be argued for Rap1. When binding over the ds-ss junction, Rap1 would most probably sterically hinder the access of other proteins to the 5’-end, and hence this property would argue for the role of Rap1 as a general protector of the 5’-end, and moreover, as a possible candidate for the regulation of the access of the 5’-end processing machinery. Thus, according to our results, both Rap1 and Cdc13 hold the property to bind at the ds-ss junction and may thus confer redundant functions in the protection of the 5’-end.

Because we demonstrate that Rap1 has the possibility to bind to the absolute terminal telomere repeat even though merely a single double-stranded hemi-site of its MBS is present, this new and remarkable feature also puts Rap1 in a new light as a protector of the very extreme terminus of the 3’-end. When forming a complex with Rap1, the single-stranded 3’ overhang will be protected from attacks by nuclease. This trait bears resemblance to the properties of the human telomere-binding protein Trf2, which has been suggested to be able to protect single-stranded overhangs by binding over the ds-ss junctions (27, 28). This means that in situations where very short 3’ overhangs are present at the telomeres, Rap1 will have the potential to protect the overhang from degradation. In fact, this would allow for a safety mechanism where, on 3’ overhangs too short for Cdc13 to bind, Rap1 may take over the function of the Cdc13 protein as a protector of the 3’ overhangs and thereby may prevent degradation and end-to-end fusions.

In conclusion, our demonstration of the binding characteristics of Rap1 and Cdc13 defines the criteria for chromatin assembly at the critical ds-ss junction of the telomeric DNA. This knowledge will facilitate in the elucidation of the molecular mechanisms of the telomere replication in yeast. Our results indicate a dynamic interplay between Rap1 and Cdc13 to protect the end structures, and we are picturing a model where the two proteins have partially redundant functions. In our model, the protein that will take major action at a particular time point would depend on the DNA structure displayed at the ds-ss junction, as well as the length of the 3’ overhang and the protein concentration. If these parameters are highly regulated in the cell, an explicit scenario will be crystallized as those parameters are unraveled and defined. On the other hand, if these parameters are less tightly regulated, the redundancy of Rap1 and Cdc13 binding at the ds-ss junction will ensure that a cooperative protection is obtained at all events.

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