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A comprehensive map of hotspots of de novo telomere addition in *Saccharomyces cerevisiae*

Katrina Ngo[1], Tristen H. Gittens[1], David I. Gonzalez[1], E. Anne Hatmaker[1,2], Simcha Plotkin[1], Mason Engle[1],

Geofrey A. Friedman[1], Melissa Goldin [1], Remington E. Hoerr[1], Brandt F. Eichman[1,3], Antonis Rokas[1,2],

Mary Lauren Benton[4] and Katherine L. Friedman[1*]

1. Department of Biological Sciences, Vanderbilt University

2. Evolutionary Studies Initiative, Vanderbilt University

3. Department of Biochemistry, Vanderbilt University

4. Department of Computer Science, Baylor University

Addresses:

1,2 and 3. 465 21st Avenue S, 1210 MRBIII, Nashville, TN 37232

4. One Bear Place #97141, Waco, TX, 76798

*Corresponding author: Katherine.friedman@vanderbilt.edu

Running head: Telomere addition hotspots in *S. cerevisiae*

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Abstract

Telomere healing occurs when telomerase, normally restricted to chromosome ends, acts upon a

double-strand break to create a new, functional telomere. De novo telomere addition on the

centromere-proximal side of a break truncates the chromosome but, by blocking resection, may allow

the cell to survive an otherwise lethal event. We previously identified several sequences in the baker's

yeast, *Saccharomyces cerevisiae,* that act as hotspots of de novo telomere addition (termed Sites of

Repair-associated Telomere Addition or SiRTAs), but the distribution and functional relevance of SiRTAs

is unclear. Here, we describe a high-throughput sequencing method to measure the frequency and

location of telomere addition within sequences of interest. Combining this methodology with a

computational algorithm that identifies SiRTA sequence motifs, we generate the first comprehensive

map of telomere-addition hotspots in yeast. Putative SiRTAs are strongly enriched in subtelomeric

regions where they may facilitate formation of a new telomere following catastrophic telomere loss. In

contrast, outside of subtelomeres, the distribution and orientation of SiRTAs appears random. Since

truncating the chromosome at most SiRTAs would be lethal, this observation argues against selection for

these sequences as sites of telomere addition per se. We find, however, that sequences predicted to

function as SiRTAs are significantly more prevalent across the genome than expected by chance.

Sequences identified by the algorithm bind the telomeric protein Cdc13, raising the possibility that

association of Cdc13 with single-stranded regions generated during the response to DNA damage may

facilitate DNA repair more generally.

Introduction

The maintenance of DNA integrity is essential for cell function. To maintain genomic integrity and prevent sequence loss, most eukaryotic chromosomes terminate with nucleoprotein structures termed telomeres that protect chromosomes from end-to-end fusion and block excessive nucleolytic resection. Telomeres contain a characteristic, repetitive sequence that is rich in thymine and guanine (TG-rich) on one strand (Blackburn 1991). While the majority of the telomere is double-stranded, the TG-rich strand extends past the complementary cytosine and adenine (CA)-rich strand to create a 3' overhang. Regeneration of this 3' overhang after each round of DNA replication results in progressive sequence loss, but in cells that maintain telomere length over successive generations, this end-replication problem is counterbalanced through extension of the 3' strand by telomerase (reviewed in Lingner et al. 1995; Osterhage & Friedman, 2009; Bonnell et al. 2021). Telomerase uses an intrinsic RNA molecule as template for synthesis of the TG-rich strand (Greider & Blackburn, 1989; Singer & Gottschling, 1994) while the lagging strand polymerase machinery subsequently fills in the complementary, CA-rich strand (reviewed in Gilson & Géli, 2007; Pfeiffer & Lingner, 2013).

Because telomeres are, by definition, the end of a DNA molecule, they resemble a DNA double-strand break (DSB). Indeed, similar to telomeres, enzymatic resection at a DSB generates 3' overhangs that can serve as substrates for homologous recombination. The specific sequence of the 3' overhang at telomeres distinguishes it from 3' overhangs generated by resection at a double strand break, thereby enforcing different outcomes at these otherwise similar structures (telomere elongation versus DNA repair, respectively; reviewed in Casari et al. 2022; Doksani & de Lange, 2014). However, rarely, the 3' overhang generated at a DSB is recognized by telomerase, resulting in addition of a new or de novo telomere (reviewed in Hoerr et al. 2021; Pennaneach et al. 2006). De novo telomere addition (dnTA), also termed telomere healing, causes loss of sequences distal to the site at which the telomere is added but prevents additional resection that would ultimately be lethal.

Several human diseases (e.g. Phelan/Mcdermid syndrome and α-thalassemia) are associated

with terminal truncations generated by dnTA (Bonaglia et al. 2011; Guilherme et al. 2015; Lamb et al.

1993; Nevado et al. 2022). The observation of recurrent telomere addition events within a small

chromosome region suggests that sequences associated with these diseases are unusually prone to

telomerase action. This phenomenon is not limited to human cells, and has been observed in other

eukaryotic organisms including *S. cerevisiae* (Mangahas et al., 2001; Ouenzar et al., 2017; Stellwagen et

al., 2003). Although dnTA events are generally very rare, the *S. cerevisiae* genome contains hotspots

where dnTA occurs at frequencies estimated to be at least 200-fold above background (Obodo et al.

2016; Epum et al. 2020). These sequences present a unique opportunity to use yeast as a model to study

the consequences of such sequences for genome stability and evolution.

Telomeres in *S. cerevisiae* have a 3' terminating strand that consists of irregular repeats

containing a pattern of a single T followed by one, two, or three Gs. Despite this heterogeneity, the

telomere contains recognition sites for several sequence-specific DNA binding proteins that associate

with the double-stranded portion of the telomere and the single-stranded TG-rich overhang (Rap1 and

Cdc13, respectively; reviewed in Wellinger & Zakian, 2012). Rap1 participates in telomere length

homeostasis, telomere capping, and formation of telomeric chromatin (Hardy et al. 1992; Kyrion et al.

1993; Marcand et al. 1997; Negrini et al. 2007; Pardo & Marcand, 2005; Teixeira et al. 2004;

Vodenicharov et al. 2010), while Cdc13 interacts with the Est1 component of telomerase to recruit

telomerase to telomeres (Evans & Lundblad, 1999; Pennock et al. 2001, Chen et al. 2018). Cdc13

additionally interacts with Stn1 and Ten1 to limit nucleolytic resection and promote fill-in synthesis by

the lagging strand polymerase machinery (Pennock et al 2001; Lin et al. 2021).

Sequences serving as hotspots of dnTA in yeast were first observed as sites of telomere healing

in response to an induced DSB on chromosome VII (Mangahas et al. 2001). Subsequently, spontaneous

truncations of chromosome V occurring as a result of dnTA were shown to cluster in a small

chromosomal region (Myung et al. 2001; Stellwagen et al. 2003; Pennaneach et al. 2006). Following

structure/function analysis of the sequence on chromosome V and an additional hotspot on

chromosome IX, we named these sequences Sites of Repair-associated Telomere Addition or SiRTAs.

SiRTAs contain two TG-rich sequence tracts. One tract (the Core) serves as the direct substrate for

telomere addition by telomerase while the second tract (the Stim, located 5' to the Core on the TG-rich

strand) is required for high levels of dnTA at the Core sequence (Obodo et al. 2016). The Stim can be

functionally replaced with canonical Cdc13 binding sites or with a sequence designed to artificially

recruit Cdc13 (Obodo et al. 2016). Together, these observations support a model in which resection of

the 5'-terminating strand following a DSB exposes TG-rich sequences on the 3' overhanging strand that

are bound by Cdc13, with subsequent recruitment of telomerase. Telomere addition is favored at SiRTAs

even when the initiating break is artificially induced 2-3 kilobases distal to the eventual site of telomere

addition, suggesting that SiRTAs stimulate repair rather than serving as fragile sites per se (Obodo et al.

2016).

For the SiRTAs described above, the TG-rich sequence is on the same strand that terminates as a

TG-rich 3' overhang at the nearest telomere, a property we refer to as the "TG-orientation." On the left

arm of a chromosome, SiRTAs in this orientation are TG-rich on the bottom (3' to 5' or minus) strand

while on the right arm, the TG-rich sequence is on the top (5' to 3' or plus) strand. Telomere addition at

a SiRTA in the TG-orientation requires a DSB distal to the SiRTA and stabilizes the centromere-containing

side of the break. If the SiRTA is distal to all essential genes on that arm (as is true for the SiRTAs on

chromosomes V and IX), the resulting terminal deletion is compatible with viability, even in a haploid

strain. However, not all characterized SiRTAs are TG-oriented. We recently described a SiRTA in the

opposite or "CA-orientation" that promotes cell survival under sulfate-limiting conditions by facilitating

formation of an acentric fragment containing *SUL1*, encoding the primary sulfate transporter (Hoerr et

al. 2023). Despite identification of several SiRTAs in addition to those described above (Ngo et al. 2020),

understanding of the genome-wide frequency and distribution of these sequences is lacking.

Here, we validate the use of a computational algorithm, the Computational Algorithm for

Telomere Hotspot Identification (CATHI), to predict SiRTA function based on similarity with the TG$_{1-3}$

pattern of the yeast telomeric repeat. In parallel, we develop and validate a high-throughput sequencing

method that dramatically increases the number of putative sequences that can be characterized while

simultaneously yielding information about the site of telomerase action. Together, we use these

approaches to determine the overall locations and orientations of SiRTAs on a genome-wide scale. All

but one of the subtelomeric repetitive regions (defined as X and Y' elements; Louis et al. 1994; Louis &

Haber, 1992) contain at least one SiRTA in the TG-orientation. However, outside of the subtelomeric

regions, there is no apparent bias in the location or orientation of predicted SiRTAs, although these

sequences occur more frequently than expected by chance. SiRTA function correlates with the ability of

a sequence to bind Cdc13, but overall binding affinity is insufficient to explain all variation in the

frequency of dnTA. This work provides a foundation for developing a fuller understanding of how sites

with a propensity to stimulate dnTA impact genomic stability and evolution.

Methods

Strain construction

Strains were constructed in the S288C background as described (Ngo et al. 2020; Hoerr et al.

2023). The parental strain contains a *URA3* marker distal to an HO recognition site on chromosome VII

(YKF1975 *MATa::ΔHOcs::hisG hmlαΔ::hisG HMRa::NAT ura3Δ851 trp1Δ63 leu2Δ::KAN^R ade3::GAL10::HO*

Chr VII, 15828-16027 (*adh4*)::HOcs::*HYG^R pau11*::*URA3*). 300 bp sequences to be tested for SiRTA

function were inserted using the CRISPR/Cas9 system as described in Anand et al. (2017) using a guide

sequence of 5'-TGCGGCAAGTTCATCTTCCA located ~2kb centromere-proximal to the HO recognition site.

PCR products for recombinational insertion were generated as follows. Forward primers were designed

by including 40 bases upstream of the gRNA recognition site

(5'TTTCTTTGGAAAACGTTGAAAATGAGGTTCTATGATCTAC) followed by the first ~20 bases on the 5' end

of the sequence of interest. Reverse primers were constructed by taking the reverse complement of the

40 bases downstream of the gRNA site (5'-AGAACATAGAATAAATTTGGTACTGGAACGTTGATTAACT)

followed by the last ~20 bases of the sequence of interest. Sequences tested are listed in Supplementary

File 1. The DNA fragment needed to insert SiRTA 6R210(+) onto chromosome VII using the CRISPR

system was synthesized and inserted into the pMX plasmid using Invitrogen GeneArt Gene Synthesis

services (Thermo Fisher Scientific). The 6R210(+) DNA fragment was amplified from the plasmid using

PCR designed as described above. One step gene replacement using template DNA from pFA6a-TRP1

(Longtine et al. 1998) was used to replace *RAD52*.

For testing on chromosome IX, the *URA3* marker and HO cleavage site were integrated on

chromosome IX to create strain YKF1752 (*MAT**a**::ΔHOcs::hisG hmlαΔ::hisG HMRa::NAT ura3Δ851*

trp1Δ63 leu2Δ::KAN^R ade3::GAL10::HO Chr9;35050-41450::HOcs::HPH^R *soa1::URA3*) as described in

Obodo et al. (2016). Yeast strains containing the BS Mut1 and BS Mut2 mutations are described in

Obodo et al. (2016) as YFK1610 and YFK1652, respectively.

HO cleavage assay

The HO cleavage assay was performed as described (Ngo et al. 2020; Hoerr et al. 2023). Briefly,

cells were grown in synthetic dropout media lacking uracil (SD-Ura) + 2% raffinose to an optical density

at 600nm (OD600) of 0.6-1.0. Cells were serially diluted and plated on yeast extract peptone medium

with either 2% dextrose (YEPD) or 2% galactose (YEPgal). After incubation at 30°C for three days, colony

number was determined on at least two plates of each condition. The frequency of survival on YEPgal

was calculated as: (average number of colonies per plate on YEPgal x dilution factor)/(average number

of colonies per plate on YEPD x dilution factor). At least 100 colonies surviving on YEPgal were patched

to medium containing 1 mg/mL 5-fluoroorotic acid (5-FOA) to select for cells in which the *URA3* marker

was lost [gross chromosomal rearrangement (GCR) events]. The frequency of GCR events was

determined as: (frequency of survival on YEPgal*frequency of clones demonstrating 5-FOA resistance).

Thirty clones that displayed growth on medium containing 5-FOA were selected and inoculated in liquid

YEPD for genomic DNA extraction using the MasterPure™ Yeast DNA Purification Kit (Lucigen). Multiplex

PCR was used as described in Ngo et al. (2020) to map the approximate site of dnTA in relationship to

the sequence of interest. Primers for chromosome VII and IX are listed in Supplementary File 1. Colonies

where the DNA loss event mapped within the sequence of interest were tested for telomere addition

using one primer centromere proximal to the putative telomere addition site and a second primer

complementary to the telomeric repeat (Supplementary File 1).

Pooled Tel-seq

Thirty 5-FOA resistant clones isolated as described above were separately inoculated in 200 μL

of YEPD in a 96-well culture plate and incubated overnight at 30°C to reach saturation. Equal volumes (at

least 30 μL) of each culture were pooled and DNA was extracted using the YeaStar™ genomic DNA kit

(ZYMO research).

Libraries were prepared using 50 ng of genomic DNA and a modified protocol using the Twist

Library Preparation kit (Twist Bioscience 106543). Denatured DNA templates in a 96-well plate were

randomly primed with 5' barcoded adapters. Samples were pooled, captured on streptavidin coated

magnetic beads, and washed to remove excess reactants. A second 5' adapter tailed primer with a

strand-displacing polymerase was utilized to convert the captured templates into dual adapter libraries.

Beads were washed to remove excess reactants. Four cycles of PCR were utilized to amplify the library

and incorporate the plate barcode in the index read position. Libraries were sequenced using the

NovaSeq 6000 with 150 bp paired end reads targeting 13 to 15 million reads per sample. Real Time

Analysis software (version 2.4.11; Illumina) was used for base calling and data quality control was

completed using MultiQC v1.7.

Sequencing data are available from the NIH Sequence Read Archive (SRA) under BioProject ID

PRJNA939836. Reads mapping to a 300 bp control sequence located in the essential gene *BRR6* (Chr VII:

36933 to 37233) or to the 300 bp sequence of interest [inserted on chromosome VII or at the

endogenous location of SiRTA 9L44(-)] were identified using Bowtie2 (Galaxy Version 2.5.0+galaxy0) with

the sensitive local setting (Langmead et al. 2009; Langmead and Salzberg 2012). Any remaining library

primer sequences were removed using the Trimmomatic tool (Galaxy Version 0.38.0; Bolger et al. 2014)

and reads mapping to the putative SiRTA that also contain telomere sequence (match to 5'-GGGTGTGG

or 5'-CCACACCC) were identified and tabulated. The number of individual reads with evidence of

telomere addition was normalized to the number of control reads at *BRR6* by expressing the number of

telomere reads as a percentage of control reads. Where applicable, sites of telomere addition were

mapped to the original SiRTA sequence to determine the location of the event.

Purification of Cdc13-DBD

Cdc13-DBD was expressed in *E. coli* using pET21a-Cdc13-DBD-His6, a gift from the Wuttke lab.

Purification was done as described (Anderson et al. 2002; Obodo et al. 2016).

Fluorescence Polarization binding assays

Binding assays were conducted using a fixed concentration of a 5'-6-carboxyfluorescein (FAM)

labeled tel-11 oligonucleotide (25 nM). Cdc13-DBD was added at final concentrations of 0, 6.25, 12.5,

25, 37.5, 50, 62.5 and 75 nM. Competition binding assays were conducted at fixed concentrations of

Cdc13-DBD (30 nM) and 5'-6-FAM labeled tel-11 oligonucleotide (25 nM). Unlabeled oligonucleotides of

75 bases each were used at final concentrations of 0, 6.25, 12.5, 25, 50, 150 and 200 nM.

Oligonucleotide sequences are listed in Supplementary File 1. Labeled and unlabeled oligonucleotides

and protein were mixed in binding buffer (50 µM Tris pH 8, 1 µM EDTA pH 8, 15% glycerol, 75 µM NaCl,

75 µM KCl) in a final volume of 80 µL and incubated at 4˚C for 30 minutes. Each reaction was measured

in triplicate (25 µl per measurement) in a Corning 384 well assay plate using the BioTek Synergy H1

hybrid reader. This procedure was repeated at least three times for each competitor. The relative

polarization (ΔP) was determined using the following equation: $\Delta P = P_0 - P_x$, where P_0 is the polarization

value at a competitor concentration of 0 and P_x represents the polarization value at x competitor

concentration. For each experiment, technical replicates were averaged and the averaged data were fit

to the following equation: $\Delta P = (P_{max}[\text{competitor}])/(K_{i,app}[\text{competitor}])$, where P_{max} is the maximal

polarization value and $K_{i,app}$ is the apparent inhibition constant (Anderson et al. 2008; Vaasa et al. 2009).

An unlabeled 75mer containing the Tel11 sequence at the center of the oligonucleotide (Tel11-75) was

included in each experiment and normalized $K_{i,app}$ values are reported as the fold change relative to this

control ($K_{i,app}$ of Tel11-75/$K_{i,app}$ of experimental oligonucleotide)

Implementation of the CATHI algorithm

Initially, the program generates a series of sliding windows to be utilized in the score calculation.

The window and step size of the sliding windows can be customized using the --window and --step

options. For each window, the program searches for strings of at least 4 characters that begin with a G

and consist of only Gs and Ts. These become the set of candidate scoring regions. From these

candidates, regions that consist only of Gs are removed. The program then scans candidate regions for

any consecutive Ts, or four or more consecutive Gs. If either are encountered, that candidate region is

truncated after the first T or the third G, respectively. Once the set of candidates has been filtered, the

number of nucleotides remaining in the candidate set is counted and any applicable scoring penalties

are subtracted. There are no penalties applied by default, but users can choose to apply them. The --

penalty option imposes score deductions for any GGTGG sequences, and the --ttpenalty imposes score

deductions for Ts that flank the candidate regions.

CATHI is implemented in Python (version 3) using the BioPython (Cock et al. 2009), NumPy

(Harris et al. 2020), Pandas (McKinney 2010), and pybedtools (Dale et al. 2011) libraries. CATHI can

perform in two modes: (1) score mode; and (2) signal mode. The default score mode will return the

maximum CATHI score for each input sequence. For each sequence in the provided FASTA file, CATHI

will generate sliding windows and calculate the CATHI score for each window, returning only the

maximum score per sequence. In signal mode, CATHI will generate sliding windows and return the

genomic coordinates and CATHI score for each window. CATHI output is printed to the screen for easy

redirection and can be optionally printed in BED format. Code can be obtained from

https://github.com/bentonml/cathi. In this work, both strands of each chromosome in *S. cerevisiae*

were separately scanned in signal mode using a step size of 1 and window size of 75. Perfect telomeric

repeats representing *bona fide* terminal telomeres were trimmed prior to analysis (if present).

Coordinates used for each chromosome are in Supplementary File 2.

Overlapping and adjacent windows meeting or exceeding the threshold value can be merged

into a single region using the --cluster option, where the beginning is the start coordinate of the most

upstream window and the ending is end coordinate of the most downstream window. The score of the

merged region is the maximum CATHI score across all merged windows.

Generation of a shuffled yeast genome

A set of five randomized versions of the *S. cerevisiae* (sacCer3) genome was generated to

evaluate the number of SiRTAs expected when applying the CATHI algorithm to a null model. DNA

sequence was downloaded from the sacCer3 reference genome using the BedTools (version 2.30.0)

'getfasta' command (Quinlan and Hall 2010) after adjusting the start and end coordinates of each

chromosome to exclude subtelomeric regions (Supplementary File 2). Nucleotides were randomly

shuffled within each adjusted chromosome using Python's built-in randomization library. This procedure

maintains the nucleotide composition and length of each chromosome while randomizing the actual

DNA sequence.

Enrichment for genomic annotations in putative SiRTAs

Overlap between putative SiRTAs and other genomic annotations was determined using a

permutation-based enrichment test. Enrichment for SiRTAs with several different genomic annotations

was determined: (1) essential and non-essential genomic regions (Giaever et al. 2002); (2) Est2 binding

sites (Pandey et al. 2021); (3) Pif1 binding sites (Paeschke et al. 2011); (4) γH2AX binding sites (Capra et

al. 2010); (5) G-quadruplex regions (Capra et al. 2010) and (6) Rap1 binding sites (Rhee and Pugh 2011).

When the original dataset included strand information (as in the case of G4-sites) that information was

considered in the analysis.

Enrichment between the SiRTAs and the annotations was calculated as the fold change between

the observed and expected overlap. To ensure meaningful overlaps between the SiRTAs and the

genomic annotations, at least 50% of the binding site was required to overlap with the SiRTA or at least

50% of the SiRTA was required to overlap with the essential/non-essential region. To create the

distribution of expected overlap, 1000 permutations were performed by randomly shuffling regions

throughout the genome and calculating the amount of SiRTA overlap. Shuffled regions are non-

overlapping, length- and strand-matched (G4 sequences only) with the annotations. When specified,

telomeric and/or subtelomeric regions were excluded. Subtelomeric regions are defined in

Supplementary File 2. For G4 sites, overlap was only recorded if the G4-forming sequence and SiRTA are

on the same strand. An empirical p-value is calculated for the overlap using the expected distribution;

where relevant, p-values are corrected for multiple comparisons using the Bonferroni method.

Determining overlap with genes

The location of predicted SiRTAs was compared to the location of genes within the *S. cerevisiae*

genome to determine the number of predicted SiRTAs in both inter- and intragenic regions. Coordinates

for genes and subtelomeres (defined as X and/or Y' elements) were obtained from the *S. cerevisiae*

S288C annotation available from NCBI (accession numbers NC_001133.9, NC_001134.8, NC_001135.5,

NC_001136.10, NC_001137.3, NC_001138.5, NC_001139.9, NC_001140.6, NC_001141.2, NC_001142.9,

NC_001143.9, NC_001144.5, NC_001145.3, NC_001146.8, NC_001147.6, NC_001148.4); FASTA and

GFF3 files for the reference assembly of strain S288C (GCF_000146045.2) were downloaded from NCBI's

RefSeq database. The RefSeq genome annotation is identical to that in the *Saccharomyces* Genome

Database (SGD).

Coordinates for predicted SiRTAs were obtained from the CATHI program and manually

converted into GFF3 files, one for each chromosome. Overlap between predicted SiRTAs and genes was

calculated using the "intersect" function within bedtools v2.30.0 (Quinlan 2014) for each chromosome.

Predicted SiRTAs within annotated genes were manually assigned to the template or coding strand using

chromosome visualization in Geneious Prime v2020.1.2.

Modeling SiRTA distribution

Python programs to model the expected distribution of telomere addition events within a region

and to model the random distribution of SiRTAs between the forward and reverse strand are available at

https://github.com/geofreyfriedman/sirta. For the latter, random strand distributions were generated

for each chromosome based on the observed number of SiRTAs on each strand. The expected

distribution of SiRTAs between the forward and reverse strands was quantified by 1) determining the

number of consecutive SiRTAs on the same strand (run length) or 2) summing the number of times that

consecutive SiRTAs are found on different strands (number of strand switches). To avoid "edge effects"

generated at the ends of each chromosome, 10,000 iterations were generated for each chromosome

and run lengths or strand switches were summed across the 16 chromosomes (sum of iteration 1, sum

of iteration 2, etc). In each case, the observed value was compared to the random distribution

generated from 10,000 iterations.

Results

High throughput sequencing of pooled samples accurately measures de novo telomere addition

Measurement of the propensity for de novo telomere addition (dnTA) across the genome is

complicated by varied chromosome context (which can affect the frequency of competing repair

pathways) and our inability to capture dnTA addition events at sequences that are proximal to essential

genes and/or in the CA-orientation. To circumvent these limitations, we previously developed a "test

site" on the left arm of chromosome VII. CRISPR/Cas9 is used to insert sequences (typically 300 bp)

oriented such that the TG-rich sequence of interest is on the bottom (3' to 5') strand. A recognition site

for the homothallic switching (HO) endonuclease is located ~2kb distal to the CRISPR/Cas9 integration

site. A *URA3* marker located further distal to the HO cleavage site facilitates selection for cells carrying a

truncated chromosome VII-L (Figure 1a and b). Importantly, *RAD52* is deleted to prevent homology-

directed repair between the sequence inserted on chromosome VII and that same sequence at its

endogenous location.

Cells are plated on solid medium containing galactose to induce expression of the HO

endonuclease. To escape persistent cleavage and generate a colony, a cell must incur a mutation at the

HO site that blocks nuclease recognition or lose the HO site completely through formation of a gross

chromosomal rearrangement (GCR). To identify the latter, which include dnTA events, 100 clones arising

on the galactose plate are screened for loss of the *URA3* marker via growth on medium containing 5-

fluoroorotic acid (5-FOA). Thirty 5-FOA-resistant clones are then analyzed to determine the nature of the

resulting GCR event. In past work, we utilized a clone-by-clone mapping strategy that employed multiple

PCR reactions to identify the approximate location of each GCR event (Figure 1a). For colonies in which

the event maps to the sequence of interest, Southern blotting or PCR utilizing a telomeric primer is

utilized to determine if the event involved telomere addition (Figure 1b).

The efficiency of SiRTA function is expressed as the percent of 5-FOA-resistant clones (from a

total of 30) that contain a telomere-addition event within the sequence of interest at the insertion site

on chromosome VII. Typically, the experiment is done 2-3 times and the average values of the biological

replicates are reported. The 300 bp sequence analyzed represents ~1.4% of the 21,922 bp region within

which a GCR event can be recovered [between the HO cleavage site and the first essential gene on VII-L

(*BRR6*)]. To determine a threshold for SiRTA activity, we modeled the expectation for random repair

within this region (*Materials and Methods* and Supplementary Figure 1). Assuming random distribution

of 30 GCR events, two or more would be expected to occur within the 300 bp test sequence in 6.2% of

trials, while three or more would be expected in only 0.78% of trials. Therefore, we chose to define a

SiRTA as a sequence in which the average efficiency of dnTA is >6.6% (an average of greater than 2 out

of 30 clones containing dnTA within the 300 bp test sequence). Sequences tested are named using the

following scheme: chromosome number, chromosome arm (L for left and R for right), distance from the

left telomere rounded to nearest kilobase, and the strand on which the SiRTA is located [(+) for the

forward strand and (–) for the reverse strand].

To increase the throughput of this analysis pipeline and to map dnTA events with nucleotide

precision, we developed the Pool-Tel-seq (PT-seq) method (*Materials and Methods* and Figure 1c). As in

our original approach, a single inoculum is plated on a medium containing galactose to induce HO

cleavage and thirty 5-FOA resistant colonies are identified that have lost the chromosome VII terminus.

The 30 colonies are grown separately to saturation in liquid medium and equal volumes of each culture

are pooled to generate a single genomic DNA sample for library construction and high through-put

sequencing. The resulting sequence reads (>12 million) are analyzed for those that align at least partially

to the 300 bp putative SiRTA and show evidence of telomere addition (TG_{1-3} or $C_{1-3}A$ sequence). To

account for differences in read depth between experiments, the number of reads meeting these criteria

is normalized to the number of reads mapping to a 300 bp sequence within *BRR6,* an essential gene on

chromosome VII that lies centromere-proximal to the site at which the putative SiRTA is integrated

(Figure 1c). At least two biological replicates are generated for each strain. To benchmark SiRTA

efficiency based on our previous method, we applied PCR-based mapping and PT-seq to multiple 30-

colony samples derived from SiRTAs of a range of efficiencies. Using linear regression, we find a strong

correlation between the two methods (r^2=0.97), allowing us to use this standard curve to estimate the

number of colonies within a 30-colony sample that underwent dnTA at the putative SiRTA (Figure 1d,

closed triangles; Supplementary File 3). This method also yields information about the relative frequency

of telomere addition at each nucleotide position.

370   To verify that this method is applicable at other locations in the genome, we utilized PT-seq to

test SiRTA 9L44(-) at its endogenous location on chromosome IX. *Cis-* and *trans*-acting mutations with

effects on the efficiency of dnTA at SiRTA 9L44(-) were used to compare the PCR and PT-seq

methodologies over a range of SiRTA efficiencies. Again, results of the two methods are strongly

correlated (r^2=0.95; Figure 1d, open circles; Supplementary File 3). The slopes of the standard curves

generated at both chromosome locations are statistically indistinguishable (p = 0.76 by analysis of

covariance), suggesting that the percent of GCR events incurring dnTA (SiRTA efficiency) can be

accurately estimated from PT-seq results regardless of chromosome location.

Putative SiRTAs are accurately identified using a computational method

Visual inspection of sequences found to function as SiRTAs revealed similarity to yeast telomeric

sequences, consistent with prior work demonstrating that association of Cdc13 with the Stim sequence

is required for dnTA (Obodo et al. 2016). The Core sequence is also TG-rich, likely reflecting required

complementarity to the *TLC1* template sequence and (perhaps) the ability to associate with Cdc13. We

postulated that SiRTA function could be predicted by considering not only the TG-richness of a sequence

but also its similarity to the pattern of the yeast telomeric repeat (TG$_{1-3}$). SiRTA function does not require

a perfect match to the telomeric sequence, so we developed a strategy to score similarity to a telomeric

repeat while allowing divergence from that pattern. The Computational Algorithm for Telomere Hotspot

Identification (CATHI) identifies strings of consecutive Gs and Ts, awards one point for each base in that

string, and subtracts 1.5 points for each instance of GGTGG, a sequence that is not found in yeast

telomeres (Figure 2a). Calculations are done in a sliding window that can be varied in size (*Materials and*

Methods).

The algorithm was developed through an iterative process in which sequences were identified

and tested for SiRTA function. This dataset included several previously published SiRTAs, sequences

identified during the work described here, and negative control sequences that were not expected to

function as SiRTAs. To standardize measurements of SiRTA efficiency, all the tested sequences were

assayed on chromosome VII by inserting a 300 bp region encompassing the putative SiRTA. If boundaries

of the SiRTA sequence were previously established, the SiRTA was centered within the 300 bp region. All

sequences were tested at least in duplicate and the average percent of clones undergoing telomere

addition within the test sequence was determined in comparison to the chromosome VII standard curve

(Figure 1d). During initial testing, data were obtained for 37 sequences, seven of which had an average

SiRTA efficiency above the 6.6% cutoff. To optimize the algorithm, we calculated a score for each of the

37 sequences using varying window sizes (25 to 150 bp) and penalties (0 to 3) to identify a combination

generating the best fit by linear regression (Supplementary File 4). A window size of 75 and a penalty of

1.5 for GGTGG sequences yielded the highest correlation between CATHI score and SiRTA efficiency (r^2 =

0.63 for all sequences and 0.69 for the seven sequences exceeding the 6.6% cutoff for SiRTA function).

Testing of additional sequences after the algorithm parameters were established resulted in a

final dataset of 47 sequences (13 active as SiRTAs) that are graphed relative to CATHI score in Figure 2b

(Supplementary File 3). Using the threshold of 6.6% to define an active SiRTA, a CATHI score of 20

effectively separates active and inactive sequence with a false positive rate of ~2% (1/47) and a false

negative rate of ~4% (2/47). We conclude that the algorithm can be used to accurately identify

sequences with a propensity to stimulate dnTA. For those sequences with a CATHI score of 20 or

greater, the score is moderately predictive of SiRTA efficiency (r^2=0.43; p=0.015). The sequences with

the four highest CATHI scores tested are also the most efficient. However, scores between 20 and 30 are

less predictive of efficiency, suggesting that some aspects of SiRTA function are not captured by the

algorithm (see *Discussion*).

Distribution of SiRTAs across the yeast genome

Using the algorithm parameters established above, the 16 chromosomes (excluding any

terminal TG_{1-3} telomeric sequences; see Supplementary File 2 for coordinates) were scanned as a series

of 75 bp sliding windows with a step size of 1. Overlapping windows with scores of 20 or greater were

merged such that the starting and ending coordinates of a predicted SiRTA represent the maximum

distance between the first and last window meeting the threshold value. The final score assigned to a

set of overlapping windows is equivalent to the highest CATHI score in that set. The algorithm was

separately applied to the top and bottom strands and strand information was retained. Overall, we

identified 728 sequences in the *S. cerevisiae* genome with a CATHI score of 20 or greater

(Supplementary File 4).

We examined the overall distribution of these 728 sequences within the 16 yeast chromosomes

(Figure 3). SiRTAs on the top strand (5' to 3'; 342) are shown in blue and those on the bottom (3' to 5';

386) strand are shown in red (Figure 3b and c). The centromere of each chromosome is depicted as a

black circle. The overall distribution between the two strands is not different from random (p=0.25 by

chi-square test). However, there is a minor but significant tendency for the SiRTAs to cluster on the

same strand. This effect is quantified by measuring the number of times a SiRTA is found on the

opposite strand from its neighbor (number of "strand switches"). We observe 322 strand switches

across the genome, significantly fewer than the number expected by chance (351.8 ± 13.0; p=0.013;

Supplementary Figure 2a). This difference is driven almost entirely by a reduction in the number of

"singlet" SiRTAs compared to what would be expected by chance (148 observed versus 187.7 ± 15.0

expected; p=0.0043; Supplementary Figure 2b). In contrast, runs of longer length do not deviate

significantly from expectation. We conclude that there is a minor tendency for SiRTAs to cluster on the

same strand, but only with the nearest neighbor.

In a haploid cell, chromosome truncation proximal to the last essential gene on a chromosome

arm will be lethal. Therefore, we were interested in determining whether the distribution of putative

SiRTAs is different in essential versus nonessential regions. For our analysis, we defined the nonessential

region on each chromosome arm as comprising sequences distal to the last essential gene (Figure 3a;

Supplementary File 2). The last essential gene, in turn, is the most telomere-proximal gene for which

single gene deletion was reported to cause lethality during the systematic knockout of each open

reading frame in *S. cerevisiae* (Giaever et al. 2002). This definition does not take synthetic lethality into

account; some nonessential regions may be smaller than defined here if the combined loss of one or

more genes in that region results in cell death. In Figure 3b, nonessential regions are highlighted in gray;

those same regions are shown in expanded form in Figure 3c. The nonessential regions are divided into

sequences that are unique (in most cases) among the different chromosome arms and the highly

repetitive subtelomeric X and Y' elements found immediately adjacent to the telomeric repeats (Figure

3a). All chromosome arms contain at least a portion of the X element while some also contain one or

more Y' elements (~6 kb each; Louis and Haber 1990; Zhu and Gustafsson 2009). The transition to

subtelomeric sequence is shown on each chromosome arm as a black line (Figure 3c). Diagrams in Figure

3 are based on the published sequence of reference strain S288C. Recent long-read sequencing analyses

confirm that some subtelomeric regions contain additional terminal sequences (primarily Y' elements)

that were not included in the published reference genome (Yue et al. 2017), so our analysis likely

underestimates the number of subtelomeric SiRTAs.

To test the hypothesis that SiRTAs are preferentially located in nonessential terminal regions,

we utilized a permutation-based enrichment test to compare the distribution of SiRTAs between

essential and non-essential regions to that of randomly shuffled sequences matched in number and

length to the sequences identified by the CATHI algorithm. This analysis shows significant enrichment for

SiRTAs in the nonessential regions of the genome (p<0.01) but this enrichment disappears when the

subtelomeric regions (X and Y' elements) are excluded from the analysis (Figure 4a). We conclude that

SiRTAs are disproportionately enriched in nonessential regions, but that this effect is limited to the most

distal, highly repetitive subtelomeric sequences. Seventy-five of the 728 putative SiRTAs lie within the

subtelomeric sequences. Nine of those 75 sequences consist of perfect telomeric (TG_{1-3}) repeats located

between X and Y' elements and seven of these perfect repeats constitute the top scoring sites in the

genome (Figure 4b and Supplementary File 5). The remaining predicted SiRTAs in subtelomeric regions

are not comprised of perfect telomeric repeats and lie within the X or Y' elements. We were concerned

that the tracts of perfect telomeric repeats might affect our analysis. However, when the nine perfect

telomeric repeats are excluded, there remains significant enrichment for SiRTAs within the nonessential

regions (p=0.001; Supplementary Figure 3). Notably, all chromosome ends [with one exception: 6R]

contain at least one region predicted to function as a SiRTA (Figure 3c). We conclude that SiRTAs are

disproportionately found within the subtelomeric regions but are otherwise not significantly enriched

within nonessential sequences.

As described in the *Introduction*, the strand on which a SiRTA is located has important

implications for the consequence of dnTA. SiRTAs in the TG-orientation (those oriented to stabilize the

centromere-containing fragment when a break occurs distal to the site) are on the bottom strand for the

left arm of a chromosome and on the top strand for the right arm. SiRTAs in the genome as a whole do

not show a bias for the TG- versus CA-orientation (372 versus 356; p=0.675 by chi-square test). To

examine the distribution more carefully, we examined SiRTA orientation within the nonessential regions,

where an excess of SiRTAs was already noted (Figure 4a). Interestingly, enrichment within nonessential

regions is observed only for SiRTAs in the TG-orientation (p<0.01), while SiRTAs in the CA-orientation are

not significantly enriched (Figure 4c). The same result is observed when the nine perfect TG_{1-3} repeats

are removed from the analysis (p=0.001; Supplementary Figure 3). As expected, enrichment is no longer

observed when subtelomeric sequences are excluded (Figure 4c). This differential distribution is

visualized in a plot showing the CATHI score of predicted SiRTAs within the nonessential chromosome

regions. In non-subtelomeric regions, distributions are indistinguishable for the two orientations (Figure

4b). In contrast, SiRTAs in the X and Y' elements are much more likely to be in the TG-orientation than in

the CA-orientation (p<0.0001 by chi-square test; Figure 4b). The enrichment of TG-oriented SiRTAs

within subtelomeres is also visually apparent in the clustering of red symbols at or near the subtelomere

junction on all left arms and of blue symbols on nearly all right arms (except 6R; Figure 3c).

We tested the ability of three sequences identified in subtelomeric regions to stimulate dnTA

using the HO cleavage assay. Two of these sequences overlap with an X element [14L07(-) and

15R1084(+)] and one overlaps with a Y' element [7R1089(-)]. All three sequences function as SiRTAs,

stimulating de novo telomere addition at frequencies of 10.0%, 13.3% and 36.6%, respectively

(Supplementary Figure 4). Although 7R1089(-) functions well as a SiRTA in our assay, it is worth noting

that it is in the CA orientation. Because it is part of a conserved Y' sequence, a very similar sequence

occurs at multiple chromosome ends (also in the CA orientation). The X element sequences are TG-

oriented and represent sequences found in two distinct regions of the X element sequence. Taken

together, these results support the interesting possibility that sequences capable of functioning as

SiRTAs have been retained near chromosome termini to facilitate chromosome rescue in the event of

telomere loss.

TG-rich sequences identified by the algorithm are overrepresented in the yeast genome

We were curious whether sequences predicted to stimulate dnTA are found in yeast at the

expected frequency given the nucleotide composition of the yeast genome. To address this question, we

generated five scrambled genomes identical in sequence composition to the yeast genome. To avoid

potential biases introduced by the subtelomeric regions, this analysis was done on sequences from

which the subtelomeric X and Y' elements were excluded (see *Materials and Methods*). The scrambled

genomes contain, on average, 283.2 ± 18.3 sequences that score 20 or higher compared to 653

sequences observed in the yeast genome, an excess of 2.3-fold. The differential becomes increasingly

apparent at higher scores with an excess of 1.8-fold at a score of 20 and an excess of 5.2-fold at a score

of 25. Among the scrambled genomes, an average of fewer than one sequence has a score of 30 or

higher (range 0 to 2), while 21 sequences scoring 30 or higher are observed in the *S. cerevisiae* genome

(Figure 5a and Supplementary File 6). In Figure 5b, putative SiRTAs with scores of 27 or higher are shown

to emphasize the strikingly different distributions in the simulated versus actual genomes. To address

whether the excess of higher scores might be functionally related to SiRTA function, we examined the

predicted and actual occurrence of CATHI scores less than 20, which are unlikely to stimulate increased

levels of dnTA (see Figure 2b). For sequences with CATHI scores of 15-19, we still observe an excess in

the actual genome, although the excess is less pronounced (1.3-fold, with 2703 ± 16.3 predicted

compared to 3485 observed; Figure 5c).

TG-dinucleotide repeats stimulate dnTA and are among the strongest SiRTAs in the genome

In analyzing predicted sites of dnTA, our attention was particularly drawn to SiRTA 6R210(+).

With a score of 61, this sequence represents the strongest predicted site outside the subtelomeric

regions (see outlier in Figure 5b). SiRTA 6R210(+) contains a nearly perfect 62 nucleotide TG-

dinucleotide repeat and is the longest TG-dinucleotide repeat in the *S. cerevisiae* genome (the next

longest is 41 nt; Supplementary File 5). Interestingly, the sequence is in the TG-orientation but lies

centromere-proximal to the last essential gene on the left arm of chromosome VI, implying that repair

by dnTA at this site in a haploid cell would be lethal.

To determine the efficiency of dnTA at SiRTA 6R210(+), we inserted the TG-dinucleotide repeat

(centered within a 300 bp region) at the test site on chromosome VII. Most strains that we monitor for

SiRTA function on chromosome VII generate GCR events at a frequency of ~0.001%, equivalent to a

negative control strain lacking a SiRTA. In contrast, a strain containing the 62 bp TG-dinucleotide repeat

generates 5-FOA resistant colonies at a 10-fold higher frequency of ~0.01% (Figure 6a). By PT-seq, 86%

of GCR events involve dnTA addition within the inserted sequence (Figure 6b). Therefore, although the

percent of events at the SiRTA that involve dnTA is similar in strains carrying the TG-dinucleotide repeat

versus the efficient SiRTA 14L35(-) (76%), the overall frequency with which dnTA occurs at the 62-nt

dinucleotide repeat is at least 10 times higher. We conclude that SiRTA efficiency alone (defined as the

percentage of GCR events in which telomere addition occurred within the sequence of interest)

underestimates the propensity of a sequence to stimulate telomere addition when SiRTA activity is very

high. For this reason, we did not include SiRTA 6R210(+) in the comparison of CATHI score and SiRTA

efficiency in Figure 2b.

Using the PT-seq results, we mapped the sites at which dnTA occurred at SiRTA 6R210(+) (Figure

6c). Each arrow corresponds to the last nucleotide that aligns between the chromosome and at least

one PT-seq read, representing the 3'-most nucleotide at which synthesis by telomerase may have

initiated. Given that ~86% of the 60 individual strains represented in the analysis contain evidence of

dnTA, these results correspond to the mapping of 50-52 independent telomere addition events. Sites

identified in a larger fraction of reads were likely targeted by telomerase in multiple independent

clones. Consistent with our prior observation that a 5' Stim sequence is required to stimulate telomere

addition in a 3' Core sequence (polarity relative to the TG-rich strand), the vast majority of telomere

addition events occur in the 3' half of the dinucleotide repeat or in sequences immediately downstream.

Two events occurred at least 50 bases downstream of the TG-repeat, consistent with prior reports that

TG-rich sequences can stimulate dnTA within neighboring sequences (Kramer and Haber 1993;

Mangahas et al. 2001).

Excluding the subtelomeric regions, TG-dinucleotide repeats comprise 11 of the 21 CATHI scores

of 30 or greater (Figure 6d). SiRTA 7L69(-) (CATHI score = 34) contains a 34-nt perfect TG repeat and was

identified by the Zakian laboratory as a site capable of stimulating dnTA in response to a DSB induced

more than 50kb distal to the eventual site of telomere addition (Mangahas et al. 2001). When

integrated at the test site on chromosome VII, this 34-nt repeat stimulates dnTA with an efficiency of

47.9% by PT-seq (Supplementary File 3). Together, these observations focus attention on TG-

dinucleotide repeats as potential mediators of genome instability.

Sequences that function to stimulate de novo telomere addition bind Cdc13 in vitro

Previous studies demonstrated that Cdc13 binding at the Stim sequence is required to promote

dnTA. We hypothesized that sequences with CATHI scores of 20 or greater will bind Cdc13 with greater

affinity than sequences with lower scores. Additionally, we predicted that the two sequences identified

as false negatives in our initial analysis (Figure 2b) should bind Cdc13 with higher affinity than the single

sequence identified as a false positive. To test these predictions, we utilized fluorescence polarization to

measure the ability of unlabeled, 75-base oligonucleotides to reduce association of the Cdc13 DNA

binding domain (Cdc13-DBD) with a 6-carboxyfluorescein (FAM) labeled 11-mer containing the canonical

Cdc13 binding site (5'-GTGTGGGTGTG; referred to here as Tel11). Cdc13-DBD binds with similar

sequence specificity and affinity to the Tel11 sequence as full-length Cdc13 (Lewis et al. 2014). The goal

of these analyses was not to identify individual Cdc13 binding sites, but rather to measure the relative,

cumulative ability of each sequence to bind Cdc13.

We first established that the FAM-labeled Tel11 oligonucleotide binds Cdc13-DBD

(Supplementary Figure 5a) and selected concentrations of 30 nM Cdc13-DBD and 25 nM labeled Tel11

for the competition analyses. The apparent inhibition constant ($K_{i,app}$) is defined as the concentration of

each competitor required to reduce binding to the FAM-labeled Tel11 by half. An unlabeled 75-mer

containing the Tel11 sequence at the center of the oligonucleotide (Tel11-75) was included in each

experiment and normalized $K_{i,app}$ values are reported as fold change relative to this control ($K_{i,app}$ of

Tel11-75/$K_{i,app}$ of experimental oligonucleotide). Sequences flanking the Cdc13 consensus binding site in

Tel11-75 lack any TG or GG motifs to minimize additional association of Cdc13-DBD. Oligonucleotides

used in these assays are found in Supplementary File 1 and representative competition curves are

shown in Supplementary Figure 5b. To validate the method, we determined the normalized $K_{i,app}$ of a

double-stranded version of Tel11-75 (0.5 +/- 0.3) and the inverse complement of the Tel11-75 sequence

(0.4 +/-0.2), both of which show the expected reduction in binding relative to Tel11-75 (Figure 7a). A 75-

mer sequence from chromosome VI previously shown to lack SiRTA activity (CATHI score=5) also

competes very weakly for Cdc13-DBD association (normalized $K_{i,app}$ = 0.5 +/- 0.2; Figure 7a). Finally, as

expected, a 2xTel11-75 sequence that contains two adjacent Tel11 sequences competes twice as well as

the Tel11-75 control oligonucleotide (normalized $K_{i,app}$ = 2.2 +/- 0.2; Supplementary Figure 6a).

We chose to test several sequences with CATHI scores over 20 that had been previously shown

to stimulate dnTA. Oligonucleotides were designed to correspond to the 75 bases with the highest

CATHI score within the 300bp sequence tested for SiRTA function. Both 14L35(-) and 14R131(+) compete

in a manner indistinguishable from the Tel11-75 control sequence (normalized $K_{i,app}$ of 1.0 +/- 0.3 and

0.9 +/-0.2, respectively) and bind more robustly than the negative control sequences (Figure 7a). The

two sequences identified as false negatives in Figure 2b [2R780(-) and 14R306(+)] both compete more

effectively than the Tel11-75 control sequence (normalized $K_{i,app}$ of 1.6 +/- 0.9 and 1.4 +/- 0.6,

respectively; Figure 7a). This observation is consistent with the ability of these sequences to stimulate

dnTA and suggests that the algorithm fails to predict Cdc13 binding in some cases. We also tested the

false positive sequence [12R330(+)] with a CATHI score of 22 and an average dnTA frequency of 2.3%

(below our cut-off for SiRTA function). This 75-base sequence has a normalized $K_{i,app}$ of 0.7 +/- 0.2,

intermediate to that of the Tel11-75 control sequence and the negative controls (Figure 7a).

Given the extremely high SiRTA activity of the 62-nt TG-dinucleotide repeat described above, we

tested the ability of a 75-mer containing this repeat to compete for Cdc13-DBD binding. The normalized

$K_{i,app}$ of 4.9 +/- 2.9 measured for this sequence is considerably higher than any other sequence tested

(Figure 7b). The first, second, and fourth base of the canonical Cdc13 binding site (5'-**G**T**GT**GGGTGTG)

contribute most strongly to Cdc13 affinity, comprising a GxGT motif that recurs in the TG-dinucleotide

motif. The 62-nt dinucleotide repeat is predicted to accommodate approximately five 11-mer binding

sites, remarkably close to the observed 4.9-fold increase in competition compared to the Tel11-75

control oligonucleotide with a single binding site.

Our prior analysis of SiRTA 2R780(-) presented an additional opportunity to test the correlation

between Cdc13 binding and SiRTA efficiency (Hoerr et al. 2023). SiRTA 2R780(-) contains a Stim

sequence of ~50 bases, deletion of which abrogates dnTA. We previously showed that mutation of

either one of two GxGT motifs located in this 50 nt Stim sequence greatly diminishes SiRTA function, an

effect that we attributed to reduced Cdc13 association (Hoerr et al. 2023). Consistent with this

hypothesis, we find that mutation of one or both motifs significantly reduces the ability of the

oligonucleotide to compete for Cdc13-DBD binding (Figure 7c).

The experiments described above provide evidence that sequences capable of stimulating dnTA

associate more robustly with Cdc13 than sequences that do not function as SiRTAs, although our ability

to distinguish borderline cases is limited. While there appears to be a threshold of binding required for

SiRTA function, the cumulative "affinity" of a sequence measured in this assay is not fully predictive of

SiRTA efficiency [e.g. 14L35(-) and 14R131(+) compete equivalently, but differ by a factor of two in SiRTA

efficiency; 80.5% versus 30.7%]. This discrepancy may result in part from our choice of 75-mer sequence

to test in each case, but also likely reflects the specific number, affinity, and distribution of Cdc13

binding sites within the sequence.

As another approach to benchmark the effect of high affinity Cdc13 binding on SiRTA function,

we tested the ability of either a single canonical Cdc13 binding site or two tandem sites to stimulate

dnTA at the test site on chromosome VII. One copy of the Tel11 site stimulated telomere addition in only

four or five of 60 GCR events analyzed by PT-seq (7.5%; Supplementary Figure 6b). Remarkably, adding a

second Tel11 (2xTel11) site increases the frequency GCR events undergoing dnTA to 83.3%

(Supplementary Figure 6b). Together these results demonstrate the importance of Cdc13 binding sites

for stimulating dnTA at SiRTAs.

SiRTA distribution is not strongly associated with known protein binding sites or chromosome landmarks

To gain insight into factors that may contribute to dnTA, we examined whether putative SiRTAs

preferentially overlap with the binding sites of proteins related to telomere addition such as Est2 (a

component of telomerase recently shown to associate with internal chromosome sites; Lendvay et al.

1996; Pandey et al. 2021), Rap1 (a transcription regulator that also binds telomeric repeats and affects

telomere length homeostasis; Conrad et al. 1990; Rhee & Pugh, 2011), and Pif1 (a helicase that

negatively regulates telomerase at telomeres and DNA double-strand breaks; Schulz and Zakian 1994;

Paeschke et al. 2011). We also examined the correlation between predicted SiRTAs and fragile sites,

identified as regions that associate with γH2AX even in the absence of exogenous damage (Downs et al.

2000; Capra et al. 2010), and between SiRTAs and sequences predicted to form G-quadruplex structures

(Capra et al. 2010). Using a permutation-based enrichment test under conditions that require overlap

with at least half of the predicted SiRTA sequence, we find statistically significant enrichment among

SiRTAs for Est2, Rap1, γH2AX binding sites, and G4-forming sequences (Supplementary Figure 7a and b).

However, overlap never exceeds 20% of the putative SiRTAs, arguing against a strong functional

relationship. Although the actual number of putative SiRTAs overlapping with a Pif1 binding site is the

highest of all characteristics tested (18.4%), this extent of overlap is not significant, likely because the

regions reported to bind Pif1 by chromatin immunoprecipitation are relatively broad. To determine

whether the predicted strength of the SiRTA affects these results, we divided putative SiRTAs into

tertiles based on CATHI score but found no strong relationship between CATHI score and the

significance of overlap (Supplementary Figure 7c). There is also no obvious enrichment among

sequences that are known to function as SiRTAs. Of the 14 SiRTAs known to be active, only one overlaps

with an Est2 binding site and one overlaps with a Rap1 binding site. Overall, these results fail to identify

any overlapping binding sites with evidence of strong functional significance and suggest that fragile

sites and G-quadruplex forming sequences are not strongly correlated with predicted hotspots of dnTA.

SiRTAs are predominantly found within coding regions

We were interested in determining whether SiRTAs are preferentially excluded from genic

regions due to higher levels of evolutionary constraint. Each of the 728 SiRTAs was categorized as genic

(any part of the SiRTA overlapped with a gene as defined by the start and stop codon of each annotated

gene) or intergenic (Supplementary File 9). Seventy-eight percent of all SiRTAs overlap with coding

regions and only 22% are exclusively found in intergenic regions (Supplementary Figure 8). Given that

approximately 30% of the yeast genome is intergenic (Hurowitz and Brown 2003; Lynch et al. 2008), we

conclude that SiRTAs are not excluded from expressed regions. There are two exceptions. When a SiRTA

contains long (>20 nt) TG-dinucleotide repeats, those repeats are virtually never found within a coding

region. This result is not surprising since expansion or contraction of a dinucleotide repeat is expected to

disrupt the open reading frame. Second, intergenic SiRTAs are disproportionately found in the

subtelomeric X and Y' elements. While only 8% of all SiRTAs are in the subtelomeric regions, 37% of

intergenic SiRTAs are subtelomeric, consistent with the presence of few transcribed regions in the

subtelomeres (Supplementary File 9). Interestingly, for those SiRTAs that overlap with open reading

frames, 58% are located on the template strand, which is different from the expectation of random

distribution (Supplementary Figure 8; $p < 0.05$ by Chi square test) and suggests that the presence of these

sequences within genes may, in some cases, have consequences for cellular fitness.

Discussion

Prediction of SiRTA function

In this work, we predict the distribution of SiRTAs in the yeast genome, an important step in

understanding the role of these sequences in genome stability and function. The Zakian laboratory

initially proposed that hotspots of dnTA addition contain tracts of 15 or more nucleotides consisting

exclusively of T and G in a "telomere-like" pattern (Mangahas et al. 2001). Our subsequent analysis of

SiRTAs on chromosome V and IX revealed that these requirements are too strict. For example, SiRTA

5L35(-) (formerly called 5L-35) stimulates dnTA in response to both spontaneous and induced DSBs

(Stellwagen et al. 2003; Obodo et al. 2016; Ngo et al. 2020), but the longest uninterrupted string of TG

sequence is 14 nucleotides, including several instances of a TT motif that never occurs within telomeric

repeats.

Given this information, we set out to develop a method that could reliably predict whether a

particular sequence has the capacity to stimulate unusual levels of dnTA (Figure 2). The algorithm

described here prioritizes "telomere-like" sequences, but provides flexibility for some deviation from

that pattern. With a single exception (discussed below), sites previously identified to stimulate dnTA are

predicted by the algorithm to function as SiRTAs. For example, the Zakian lab identified three sites on

chromosome VII that stimulate dnTA following an induced DSB (Mangahas et al. 2001). Two of these

sites, now renamed SiRTA 7L67(-) and 7L69(-) (CATHI scores of 28 and 34, respectively), were originally

found to stimulate dnTA at a distance of more than 50 kb from the induced DSB. In the standardized

conditions of our chromosome VII test site where the break is induced ~2 kb from the sequence of

interest, these SiRTAs stimulate dnTA with efficiencies of 49.1% and 47.9% (Figure 2b and

Supplementary File 3). The third site identified by the Zakian lab lies within the *URA3* gene, integrated at

an ectopic location internal to the induced break. Although we did not test this sequence in our assay, it

has a CATHI score of 22 and is annotated as SiRTA 5R117(+) to reflect the native location of *URA3* on

chromosome V (Supplementary File 4).

Overall, the algorithm presented here correctly predicts SiRTA function (yes or no) with an

accuracy close to 95% (44/47). Because false positives and false negatives occur at similar frequency, our

estimate of ~650 SiRTAs (excluding subtelomeric X and Y' elements) is likely quite accurate, based on the

definition of a SiRTA proposed here.

Sequences that stimulate dnTA associate with Cdc13

Because prior work suggests that dnTA is stimulated by association of Cdc13 with single-

stranded DNA generated after a DSB, the CATHI algorithm likely identifies sequences with affinity for

Cdc13. To test this hypothesis, we developed a fluorescence polarization competition assay in which

sequences are tested for their relative ability to compete with a labeled oligonucleotide for binding to

the purified Cdc13 DNA binding domain. A 75-mer oligonucleotide containing two tandem copies of the

canonical Cdc13 binding site competes twice as well as an oligonucleotide containing a single site,

suggesting that the assay is sensitive to Cdc13 binding (Supplementary Figure 6a). Our goal is to

measure overall association of Cdc13, which arises as a combination of the number and affinity of

binding sites. We find that 75-mer oligonucleotides containing sequences that stimulate dnTA compete

as well or better than a 75-mer containing a single match to the telomeric consensus Cdc13 binding

sequence. In contrast, sequences that fail to support dnTA compete less well (Figure 7a). Importantly,

mutations previously demonstrated to reduce SiRTA function also reduce Cdc13 binding (Figure 7c).

Together with our previous demonstration that dnTA is stimulated through the artificial recruitment of

Cdc13 to the Stim sequence of a SiRTA (Epum et al. 2020; Hoerr et al. 2023; Obodo et al. 2016), these

results are consistent with the requirement for a threshold level of Cdc13 in stimulating dnTA.

Despite the observation at a single SiRTA that Cdc13 association correlates well with SiRTA

efficiency, the apparent overall affinity for Cdc13 measured by fluorescence polarization poorly predicts

SiRTA efficiency. For example, SiRTA 14L35(-) competes equivalently with the control sequence but

stimulates dnTA more strongly than most other SiRTAs, including those that compete more effectively

for Cdc13 binding. This apparent discrepancy may reflect an effect of the distribution or spacing of

Cdc13 binding sites on SiRTA function. In prior work, we observed that deletion of the ~30 nt spacer

region between the Stim and Core sequences of a SiRTA dramatically increases SiRTA efficiency (Obodo

et al. 2016). The highly efficient SiRTA 14L35(-) contains an unusually long region of TG-rich sequence

that likely acts as both a Stim and Core region with little or no spacer, a property that may account for

its ability to stimulate dnTA strongly despite an overall lower affinity for Cdc13.

Limitations to the predictive capacity of the CATHI algorithm

The well-characterized and functional SiRTA 2R780(-) has a CATHI score of only 13, despite

stimulating dnTA with an efficiency of 31.11% (Hoerr et al. 2023). By fluorescence polarization, this

sequence competes for Cdc13 binding more effectively than many sequences with higher CATHI scores,

suggesting that the failure of the algorithm to predict SiRTA function (at least in this case) is primarily a

failure to predict Cdc13 binding. One possible explanation is that the CATHI algorithm does not prioritize

matches to the GxGT motif identified as particularly impactful for Cdc13 binding (Anderson et al. 2002).

Indeed, we find that mutation of even one GxGT motif in SiRTA 2R780(-) strongly reduces Cdc13 binding

and nearly eliminates SiRTA function (Figure 7c) (Hoerr et al. 2023). The 226 bp minimal sequence of

SiRTA 2R780(-) contains seven GxGT sequences that may account for the ability of this sequence to

stimulate dnTA despite lacking sufficiently long/abundant telomere-like tracts to be identified by the

algorithm.

Although the presence of GxGT motifs is an attractive explanation for the activity of SiRTA

2R780(-), attempts to incorporate the motif into the algorithm did not improve the accuracy with which

SiRTA function could be predicted and instead increased the number of false positive results. For

example, the false negative SiRTA 14R306(+) (CATHI score = 15.5) contains five GxGT motifs (two of

which overlap), but the false positive SiRTA 12-330(+) (CATHI score = 22) also contains five distinct GxGT

motifs. There are at least three (non-exclusive) explanations for remaining discrepancies between the

predictive algorithm and measured rates of dnTA. First, it remains unclear how Cdc13 affinity is affected

by deviation from the consensus telomere binding site. Although extensive mutagenesis has been

conducted *in vitro*, these studies either altered single nucleotide sites (showing that positions 2 and 5-11

are tolerant of single changes) or simultaneously mutated the seven 3'-most nucleotides (showing that

the GxGT motif is insufficient; Lewis et al. 2014; Glustrom et al. 2018). Neither approach fully

recapitulates the sequences that Cdc13 will encounter at internal sites exposed by resection. Second, as

described above, the distance between Cdc13 binding sites likely contributes strongly to SiRTA function.

We have attempted to account for this property by using a window size of 75. However, some effects on

SiRTA efficiency likely arise from varied distributions of Cdc13 binding sites that we cannot fully capture

with the algorithm. Third, we suspect that dnTA can be stimulated either by a small number of high-

affinity Cdc13 binding sites or by a larger number of low-affinity sites. SiRTAs at either extreme of this

continuum may be difficult to identify using the current strategy.

SiRTAs do not colocalize strongly with binding sites for other telomere/telomerase-associated proteins

769   Our co-localization analysis failed to identify additional proteins that strongly impact SiRTA

function. Overall, we consider it unlikely that the observed enrichment represents a functional

relationship. For example, Rap1 binding sites are overrepresented among SiRTAs, but this result is not

surprising given that the consensus binding site for Rap1 is also TG-rich. In previous work, we showed

that Rap1 association *per se* is not required for SiRTA activity (Obodo et al*.* 2016). Our results suggest

that Est2 binding in undamaged conditions is not required for a sequence to function as a SiRTA. Only

8.1% of predicted SiRTAs overlap with experimentally determined sites of Est2 enrichment and only one

of the fourteen active SiRTAs is also an internal Est2 binding site.

777   Consistent with our observation that SiRTAs stimulate dnTA even when located several kilobases

from an induced DSB, we observe only a modest correlation between sequences that function as SiRTAs

and sites enriched for phosphorylated H2A (γH2AX), a marker of DNA damage. Since enrichment was

determined in undamaged cells, these sites represent regions of the genome that are prone to

spontaneous damage, likely due to difficulties encountered during DNA replication. It will be interesting

to determine whether SiRTAs that overlap with fragile sites are more likely to stimulate the spontaneous

formation of gross chromosomal rearrangements. For example, we have proposed that the generation

of acentric fragments through dnTA on chromosome II is facilitated by an unusually high density of

inverted repeats in this region combined with errors in the resolution of stalled replication forks (Hoerr

et al. 2023). In this light, it is interesting to note that sequences required to stimulate dnTA on

chromosome II [SiRTA 2R780(-), coordinates 779784 to 780009] overlap with a region of enhanced

γH2AX association (779987-780040; Capra et al. 2010).

Interestingly, we find a statistically significant tendency for predicted SiRTAs, if found within an

open reading frame, to be oriented with the TG-rich sequence on the template strand. We propose that

the presence of the TG-rich sequence within the exposed strand of the transcription bubble may be

deleterious. Interestingly, this bias is opposite to that observed for G-quadruplex forming sequences in

mammalian cells, which are more likely to be found on the coding strand (Agarwal et al. 2014; Rhodes

and Lipps 2015; Kim 2019). In yeast, Replication protein A (RPA)-bound single-stranded DNA at stalled

transcription complexes has been implicated as a major signal of DNA damage (Wang and Haber 2004;

Tapias et al. 2004; Fanning 2006). Conceivably, competition for binding to single-stranded DNA by Cdc13

could interfere with this process, leading to selection against Cdc13 target sequences on the exposed

coding strand. Despite the frequent presence of SiRTAs within genes, transcription does not appear to

be required for SiRTA function since the test site that we developed on chromosome VII is contained

within an intragenic region, more than 1.5 kilobases from the 3' end of the *ADH4* gene.

Implications of SiRTA distribution in the yeast genome

The compact and well-annotated yeast genome presents an opportunity to assess evidence of

selective pressures that might act upon sequences with a propensity to stimulate dnTA. Based on the

hypothesis that dnTA within the nonessential terminal region of a chromosome arm might provide a

selective advantage by allowing a cell to survive a persistent DSB, we examined the distribution of

predicted SiRTAs in essential and nonessential chromosome regions. As predicted, we found a significant

enrichment of putative SiRTAs in nonessential terminal regions. Furthermore, as expected for a role in

chromosome stabilization, only SiRTAs in the TG-orientation are overrepresented. However, both effects

disappear when the subtelomeric X and Y' elements are removed from the analysis (Figure 4a and c).

Nine of the TG-oriented SiRTAs in the subtelomeric regions correspond to stretches of TG_{1-3}

(perfectly "telomeric") sequence that are located predominantly between tandem Y' elements.

However, the vast majority, while TG-rich, deviate substantially from the TG_{1-3} pattern. Whether these

sequences are vestiges of ancient telomeric repeats is unclear. Because the Y' and X elements are similar

between chromosome ends, many of the subtelomeric SiRTAs have similar or identical CATHI scores and

represent a small number of unique sequences. Given the near ubiquity of TG-oriented SiRTAs within

subtelomeric regions (identified at 31 of 32 chromosome ends), we speculate that these sequences are

conserved, at least in part, due to an ability to stimulate dnTA in the event of catastrophic telomere loss,

most likely due to replication errors within the telomeric repeats. The subtelomeric region on the right

arm of chromosome VI contains a truncated X element followed immediately by TG_{1-3} telomeric repeats

and therefore lacks sequences predicted to function as a SiRTA (Figure 3c). This subtelomeric structure

may have resulted from a prior dnTA event within the X element. In the future, it would be interesting to

determine whether the right arm of chromosome VI is more sensitive to catastrophic loss of telomeric

repeats than other chromosome termini that contain intact X element repeats.

While the spatial distribution and orientation of putative SiRTAs outside the subtelomeres are

not strongly skewed, the number of sequences with potential to act as SiRTAs is significantly higher than

predicted by chance (Figure 5). This excess is observed at both low and high scores, but is increasingly

pronounced at higher CATHI scores. Scores of 30 or greater are approximately 20-fold overrepresented

in the yeast genome compared to the random expectation (Figure 5b). Our data do not provide evidence

that SiRTA function *per se* is driving this excess, particularly because we also observe an excess of scores

below 20, representing sequences that are not likely to stimulate dnTA at unusually high levels (Figure

5c). Since many SiRTAs are located within coding regions, we considered the possibility that codon bias

might explain this pattern. However, codons consisting of only G and T (or only C and A; TTT and AAA

excluded) collectively are not overrepresented among all codons (Supplementary File 10; Nakamura et

al. 2000). It is possible that particular amino acid repeats could result in this effect. For example, poly-

proline tracts consisting of CCA and CCC codons can generate a SiRTA signature. However, only a small

fraction of poly-proline tracts are also identified as potential sites of dnTA.

An intriguing possibility is that association of Cdc13 with single-stranded DNA revealed by

resection may be important to stimulate fill-in synthesis of the resected strand. At telomeres, Cdc13, in

association with its binding partners Stn1 and Ten1, recruits polymerase α-primase to facilitate

resynthesis of the 5' recessed strand (Grandin 2001; Rice and Skordalakes 2016; Ge et al. 2020). In

mammalian cells, the complex of Ctc1, Stn1, and Ten1 fulfills the same role at both telomeres and

double-strand breaks (Chastain et al. 2016; Giraud-Panis et al. 2010; Wang et al. 2007). In this model,

TG-rich sequences may be retained in the genome at a higher-than-expected frequency to facilitate DNA

repair, with elevated rates of dnTA resulting as a rare byproduct.

Persistence of sequences such as the long TG-dinucleotide repeat on chromosome VI that

support extremely high levels of dnTA is surprising since it seems likely that such sequences would

interfere with normal repair. Future work will address whether dnTA is inhibited at SiRTAs in some

contexts (for example, the SiRTA on chromosome VI may be less capable of stimulating dnTA in its

endogenous location than when that same sequence is integrated at the test site on chromosome VII).

Alternatively, the deleterious consequences incurred by dnTA at such a sequence may be insufficient to

result in purifying selection or the TG-dinucleotide repeat may contribute to cell fitness through some

other mechanism.

This work provides, for the first time, a genome-wide map of sites predicted to stimulate dnTA.

With the exception of sites clustered in subtelomeric regions, the largely random distribution and

orientation of SiRTAs throughout the genome stands in interesting contrast to the observation that

sequences with this capability are found much more often than expected by chance. The tools

presented here will facilitate studies to address this apparent contradiction and to determine the impact

of these sequences on genome stability and evolution.

Data availability

Strains and plasmids are available upon request. Code to run the CATHI algorithm can be found

at https://github.com/bentonml/cathi. Code to model random distribution can be found at

https://github.com/geofreyfriedman/sirta. Data summarized in Figures 1 and 2 are found in

Supplementary File 3. Data summarized in Figure 3 are found in Supplementary Files 2 and 4. Data

summarized in Figure 4 are found in Supplementary Files 2 and 5. Data summarized in Figure 5 are

found in Supplementary File 6. Data summarized in Figure 6 are found in Supplementary Files 3 and 5 .

Data summarized in Figure 7 are found in Supplementary File 7. Locations of data presented in

supplementary figures are referenced within the corresponding figure legends. Sequencing data are

available from the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA939836.

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Conflicts of Interest

A.R. is a scientific consultant for LifeMine Therapeutics, Inc.

Figure legends

Figure 1. Validation of Pooled Telomere sequencing (PT-seq) as a method to quantify de novo telomere

addition. a) Diagram depicting the structure of chromosome VII and the strategy for mapping GCR

events resulting from a DSB generated by the HO endonuclease. Locations of the essential gene *BRR6*

and the sequence to be tested (SiRTA) are shown. A *URA3* marker inserted distal to the HO cleavage

sites allows selection for cells that lose the chromosome terminus following HO cleavage (see text for

additional explanation). The approximate locations of PCR products utilized to map GCR events are

shown. Product 1 amplifies sequences within *BRR6* and is utilized as a positive control. Products 2 and 3

amplify regions internal to the putative SiRTA or across the SiRTA, respectively, and are used to identify

clones in which a GCR event occurred within the SiRTA. Product 4 is used to verify loss of the

chromosome terminus. b) Diagram of a GCR event in which telomere addition occurred in the putative

SiRTA. Addition of telomeric DNA at the SiRTA is verified by the presence of PCR product 5, generated

with a forward primer proximal to the SiRTA and a reverse primer complementary to the telomeric

repeat. c) Flow chart representing the PT-seq methodology. D) Correlation of the PCR-based and PT-seq

methodologies (Supplementary File 3). Results on chromosome VII (black triangles; r^2=0.97) and

chromosome IX (open circles; r^2=0.95) were analyzed by linear regression (lines are nearly

superimposed). Line equations (VII: y=3.961x-2.039 and IX: y=3.8022x-0.5505) are statistically

indistinguishable (p=0.94) by analysis of covariance.

Figure 2. Computational Algorithm for Telomere Hotspot Identification (CATHI) predicts SiRTA function.

a) Summary of methodology used to generate CATHI score. An example corresponding to SiRTA

14R131(+) is shown (300 bp sequence beginning at chromosome XIV nucleotide 131308). Although

multiple 75 bp windows within this sequence surpass the threshold score of 20, the calculation is shown

only for the highest-scoring window, starting at nucleotide 131471 (bold, bracketed text). Underlined

sequences correspond to strings of 4 or more guanine or thymine nucleotides conforming to the

patterns described in the flowchart and in more detail in *Materials and Methods*. Each underlined

nucleotide is awarded one point. Subsequently, each occurrence of a GGTGG pentanucleotide incurs a

1.5-point penalty to generate the final score. b) Correlation of CATHI score and the percentage of GCR

events that result from de novo telomere addition within the sequence of interest. Each value is the

average of at least two experiments, each with 30 GCR events. The standard curve for chromosome VII

(Figure 1d) is used to convert PT-seq values to the percentage of GCR events undergoing dnTA in the

SiRTA. Horizontal dashed line indicates a minimum telomere-addition efficiency of 6.6% used to define

an active SiRTA (see text for detail). Thirty-two of the sequences fall below this threshold and 15 are

above this threshold. Vertical dashed line illustrates a CATHI score of 20 that effectively separates active

and inactive sequences. Thirty-three sequences fall below this threshold and 14 are above this threshold

(Supplementary File 3). The open circles are false negatives or false positives. Linear regression analysis

on SiRTAs with a CATHI score of 20 or more yields a p-value of 0.01 (r^2 =0.43).

Figure 3. Summary of predicted SiRTAs across the *S. cerevisiae* genome. a) Diagram of chromosome

landmarks. Predicted SiRTAs are listed in Supplementary File 4. Non-essential regions (grey boxes) are

sequences located between the last essential gene (most distal gene on the chromosome arm that

causes lethality in a haploid strain when deleted) and the telomere on each chromosome arm.

Subtelomeres are located immediately adjacent to the telomeric repeats within the nonessential regions

and are composed of a single complete or partial X element (all telomeres) and one or more Y' elements

(a fraction of telomeres). Genomic coordinates are listed in Supplementary File 2. Location of the CA- or

TG-oriented sequences are indicated for the left and right chromosome arms (see text). b) Distribution

of SiRTAs on each of the 16 yeast chromosomes; distance in base pairs from the left telomere is

indicated at the bottom of the figure and corresponds to coordinates in the S288C reference genome.

Black circles mark the centromere position and nonessential regions are highlighted in grey. Blue lines in

the top half of each bar represent SiRTAs on the top (plus) strand and red lines in the bottom half of

each bar refer to SiRTAs on the bottom (minus) strand. c) Distribution of SiRTAs in the nonessential

regions of the left and right arms of each chromosome as defined in Supplementary File 2. The transition

between the subtelomeric X element and the remainder of the nonessential region is shown as a

horizontal black line. Red and blue lines are as described in (b). Diagrams in (a) and (b) were generated

using shinyChromosome (Yu et al. 2019).

Figure 4. SiRTAs are enriched in subtelomeric regions. a) Using a permutation strategy as described in

Materials and Methods, the enrichment of SiRTAs (Log2 fold change) was determined for the

nonessential and essential chromosome regions. Analysis utilized all genomic sequences (except the

terminal telomeric repeats) or genomic sequences from which the subtelomeric regions were excluded,

as indicated (coordinates in Supplementary File 2). *p-value <0.01 by chi-squared test with Bonferroni's

correction. b) Distributions of CATHI scores (20 and greater) for putative SiRTAs in the TG- or CA-

orientations. Analysis is presented separately for SiRTAs in nonessential regions (subtel excluded) versus

those in the subtelomeric repeats (subtel only). Nine SiRTAs containing perfect telomeric (TG_{1-3}) repeats

are indicated with open circles. ****p <0.0001 by chi-squared test. Coordinates of telomeric repeats are

found in Supplementary File 5. c) Enrichment analysis was conducted separately for SiRTAs in the TG or

CA orientation (see text and Figure 3a for definitions) as described in part (a). Results for the

nonessential regions are shown. *p-value<0.01 by chi-squared test with Bonferroni's correction.

Figure 5. CATHI scores are significantly elevated in the *S. cerevisiae* genome relative to expectation. a) As

described in *Materials and Methods*, the algorithm was applied to the *S. cerevisiae* genome (excluding

subtelomeric regions) and the number of sequences at each score (15 or higher; rounded down to the

nearest integer) was graphed (solid bars). Genomic sequences (excluding subtelomeric regions) were

scrambled five times and the identical procedure was applied (Supplementary File 6). Data are

presented as the average and standard deviation of the five trials (open bars). b) Distribution of CATHI

scores of 25 and above in the *S. cerevisiae* genome (closed circles) and shuffled genomes (open circles).

Subtelomeric sequences were excluded. c) As in (b), but data are shown for CATHI scores ranging from

15-19.

Figure 6. A 62 bp TG-dinucleotide repeat [SiRTA 6R210(+)] supports high levels of dnTA. a) Strains in

which a 300 bp sequence encompassing SiRTA 6R210(+) or 14L35(-) was integrated on chromosome VII

were subjected to the HO-cleavage assay as described in *Materials and Methods*. The percent of cells

that survived on galactose-containing medium and acquired 5-FOA resistance [cells containing a gross

chromosomal rearrangement (GCR)] is shown. A strain lacking any insertion (No SiRTA) was utilized as a

control. Error bars are standard deviation. b) The percent of GCR events that involve de novo telomere

addition in the sequence of interest was determined by PT-seq for each strain described in (a). Each data

point was generated by analysis of 30 GCR events. Average and standard deviation are shown. c) The

300 bp sequence encompassing 6R210(+) is shown. Sequence reads generated by PT-seq from a total of

60 GCR events [corresponding to the experiments shown in (b)] were filtered for those containing

evidence of de novo telomere addition within the 300 bp sequence. Sites at which de novo telomere

addition was observed are indicated (arrows). Arrow width indicates the percent of telomere-containing

reads that map to that particular site. d) CATHI scores are shown for all non-subtelomeric SiRTAs with

scores of 30 or higher, separated by CA- or TG-orientation. Open circles correspond to SiRTAs containing

TG-dinucleotide repeats (also listed in Supplementary File 5).

Figure 7. Sequences that function as SiRTAs bind Cdc13 *in vitro.* a) A competition fluorescence

polarization assay was utilized to measure the relative association of the Cdc13 DNA binding domain

with the indicated sequences (Supplementary File 1). Relative $K_{i,app}$ was determined as described in

Materials and Methods. Each point represents an independent measurement; error bars are standard

deviation (Supplementary File 7). The dotted line indicates normalization of values to the $K_{i,app}$ of a tel11-

75 oligonucleotide included in each experiment. b) Same as in (a). $K_{i,app}$ of the TG-dinucleotide repeat

analyzed in Figure 6 is shown [6R210(+)]. Data for 14L35(-) are repeated from (a) for comparison. c)

Same as in (a). 2R780(-) and its mutated variants are described in Hoerr et al. (2023).

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Figure 1

Figure 2

(a)

Within 75bp windows, identify strings of 4 or more bases containing consecutive Gs (1-3) and a T (underline); the highest scoring window (bold) from overlapping windows is reported.

14R131(+)
TTTAATTTCTTGTGTGGACGAGGATACTTTTCAAATAATGAGGGCGATTGAGGAAGTAATGGTGATGTTACAGCTGATTTGAT
TTGCGATGCCGGCATCATAGGTGGAGCTGGCGGAATGACGCCATCAGTAGATGAAGATAATACAGATGGTAGCGGCGGCG
[GTGGTGGTGTTTCTCCCTTGGGTTTGCCGAACAATGCCATGGGTGGAGGAGGAGGTGGTGGTGGTGGAGGGGGAG]G
AGCAGGAGAATCCCCAGTAGTTTCTTGCTTGACAGTGTCGTCTTCCGACTTTTTCTCTTTCT

↓

Starting at the first G award 1 point for each G and T

<u>GTGGTGGTGT</u>TTCTCCCTT<u>GGGT</u>TTGCCGAACAATGCCAT<u>GGGTGG</u>AGGAGGA<u>GGTGGTGGTGGTGG</u>AGGGGGAG
　　　10　　　　　　　　　　　4　　　　　　　　　　　　　　6　　　　　　　　14

↓

Subtract 1.5 points for any instances of GGTGG

<u>GTGGTGGTGT</u>TTCTCCCTT<u>GGGT</u>TTGCCGAACAATGCCAT<u>GGGTGG</u>AGGAGGA<u>GGTGGTGGTGGTGG</u>AGGGGGAG
　　-1.5　　　　　　　　　　　　　　　　　　　-1.5　　　　-1.5
　　　　　　　　　　　　　　　　　　　　　　　　　　　-1.5
　　　　　　　　　　　　　　　　　　　　　　　　　　　　-1.5
　　　　　　　　　　　　　　　　　　　　　　　　　　　　　-1.5

CATHI=25

(b)

Figure 3

(a)

Top: CA orientation
Bottom: TG orientation

Top: TG orientation
Bottom: CA orientation

Telomere   Last essential gene   Centromere   Last essential gene   Telomere

5'

3' Non-essential   Essential   Non-essential

X   Y'

Subtelomere

(b)

(c)

Distance from left end (bp)

Distance from left end (bp)

Distance from right end (bp)

Figure 4

(a)

(c)

(b)

Figure 5

Figure 6

Figure 7

(a)

(b)

(c)