Long-Range Chromatin Interactions Drive Mutant TERT Promoter Activation

Semih Can Akıncılar, Ekta Khattar, Priscilla Li Shan Boon, Bilal Una, Melissa Jane Fullwood, and Vinay Tergaonkar

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ABSTRACT

Cancer-specific TERT promoter mutations (-146C>T and -124C>T) have been linked to reactivation of the epigenetically silenced telomerase reverse transcriptase gene (TERT). Understanding how these single-nucleotide alterations drive TERT reactivation is a fundamental unanswered question and is key for making successful therapeutics. We show that unlike wild-type promoters, recruitment of the transcription factor GABPA specifically to mutant TERT promoters mediates long-range chromatin interaction and enrichment of active histone marks, and hence drives TERT transcription. CRISPR-mediated reversal of mutant TERT promoters, or deletion of its long-range interacting chromatin, abrogates GABPA binding and long-range interactions, leading to depletion of active histone marks, loss of POL2 recruitment, and suppression of TERT transcription. In contrast, de novo introduction of a TERT promoter mutation enables GABPA binding and upregulation of TERT via long-range interactions, acquisition of active histone marks, and subsequent POL2 recruitment. This study provides a unifying mechanistic insight into activation of mutant TERT promoters across various human cancers.

SIGNIFICANCE: This study identifies a key mechanism by which cancer-specific mutant TERT promoters cause reactivation of TERT. Because the mechanism uncovered here is not utilized by promoters that drive TERT in normal cells, this mechanism could be exploited to make inhibitors which have the potential to block telomerase function and hence the progression of up to 90% of human cancers. Cancer Discov; 6(11); 1276–91. ©2016 AACR.

See related commentary by Min and Shay, p. 1212.

INTRODUCTION

Telomerase is a reverse transcriptase that elongates telomeres and thus maintains genomic integrity (1–3). It is minimally composed of the catalytic protein component TERT and a template forming RNA component, TERC. Although TERC is ubiquitously expressed (4), TERT is epigenetically silenced in most adult somatic cells, limiting their replicative lifespan. Up to 90% of human cancers reactivate TERT expression transcriptionally to reconstitute telomerase enzyme activity, which subsequently enables replicative immortality (5).

Recently, two cancer-specific somatic mutations in the TERT promoter were identified (6, 7). These mutations are particularly common in a subset of cancers including melanoma (74%), glioblastoma (83%), hepatocellular carcinoma (44%), and urothelial bladder carcinomas (53.5%; refs. 6–10). These mutations cause -146 promoter (-146C>T) have been linked to reactivation of the epigenetically silenced telomerase reverse transcriptase gene (TERT). Understanding how these single-nucleotide alterations drive TERT reactivation is a fundamental unanswered question and is key for making successful therapeutics. We show that unlike wild-type promoters, recruitment of the transcription factor GABPA specifically to mutant TERT promoters mediates long-range chromatin interaction and enrichment of active histone marks, and hence drives TERT transcription. CRISPR-mediated reversal of mutant TERT promoters, or deletion of its long-range interacting chromatin, abrogates GABPA binding and long-range interactions, leading to depletion of active histone marks, loss of POL2 recruitment, and suppression of TERT transcription. In contrast, de novo introduction of a TERT promoter mutation enables GABPA binding and upregulation of TERT via long-range interactions, acquisition of active histone marks, and subsequent POL2 recruitment. This study provides a unifying mechanistic insight into activation of mutant TERT promoters across various human cancers.

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RESULTS

Mutant TERT Promoters Display Active Histone Marks and Long-Range Chromatin Interactions

Cancer-specific mutations in the TERT promoter (-146C>T and -124C>T) create consensus-binding sites for
ETS transcription factors (14). ETS transcription factors are autoinhibited, and binding of these factors alone is not sufficient to drive transcriptional activation, and it is known that they need to heterodimerize with other factors for activating transcription (15–17). GABPA and ETS1 have been reported to specifically interact with the proximal mutant TERT promoters (11, 18), but the mechanism(s) by which they activate the mutant promoters is not completely understood. It is known that levels of these factors do not dramatically differ between normal and cancer cells. Furthermore, there are several ETS/GABPA binding sites on the WT promoter which remain dormant in most cell types. Therefore, there are several ETS/GABPA binding sites on the WT promoter which remain dormant in most cell types.

To address these issues, we first asked if cancer-specific genetic changes on the mutant TERT promoters are not fully explain the mechanism of TERT reactivation by these single-nucleotide changes. Are there uncharacterized events specifically initiated by the mutant sites and their chromatin context which drive TERT transcription from mutant TERT promoters? To address these issues, we first asked if cancer-specific point mutations alone are associated with activating epigenetic changes on the mutant TERT promoters. Figure 1A shows the cell lines used in the study along with TERT promoter mutation status. Figure 1B shows the schematic outline of the TERT promoter. In this study, we divide the promoter into three distinct regions: proximal promoter (up to -1 kb), distal promoter (up to -5 kb), and long-distance elements (beyond -5 kb), upstream of the TSS. The most common TERT promoter mutations are shown at the -146C>T and -124C>T positions with respect to the ATG translation start site. We examined the active histone marks H3K4Me3 and H3K9Ac in BLM, A375, T98G (bearing -146C>T mutation in TERT promoter), and U251 (bearing -124C>T mutation in TERT promoter) cell lines up to -5 kb upstream of the TERT gene. Interestingly, we observed enrichment of both H3K4Me3 and H3K9Ac in the proximal TERT promoter region of these mutant cell lines (Fig. 1C; Supplementary Fig. S1A). We next performed similar experiments in primary melanocytes with the WT TERT promoter (Fig. 1D), and additionally we investigated multiple cancer cells, including PC3 (prostate cancer), Fadu (head and neck cancer), and the HCT116 colon cancer cell line (Fig. 1D; Supplementary Fig. S1B), wherein TERT is driven by a WT promoter. We observed a trend of lower enrichment of the active histone marks in the active WT TERT promoter from primary melanocytes and other cancer cells (Fig. 1D). It must be noted that average TERT expression levels from a panel of cells with mutant and active WT TERT promoter are not correlated with the mutational status of the promoter as reported previously (Supplementary Fig. S1C; refs. 13, 19). These results suggest that comparing TERT levels and epigenetic marks across cancer cell types is not ideal, and the mechanistic insights regarding the functioning of the mutant and WT TERT promoters could only be inferred using a set of isogenic lines wherein the cause and effect of making these alterations can be evaluated.

A recent study by Barutcu and colleagues suggested that GABP was specifically enriched in Topologically Associated Domains (TAD) in breast cancer cells, suggesting that it may also mediate interactions between domains at long distances (20). To investigate if GABPA bound to mutant TERT promoter could engage in long-range chromatin interactions, we performed the Circular Chromosome Conformation Capture (4C) assay (21). Figure 1E depicts the intrachromosomal interactions of the TERT promoter (chromosome 5) in BLM and A375 cell lines as brought forth by the 4C assay. The y axis indicates the read counts and the x axis indicates distance upstream and downstream of the TERT promoter which are aligned with respect to the dashed red line. The top panel (Fig. 1E) indicates the RefSeq genes on chromosome 5. Significant interactions are represented in different shades of red and blue dots for A375 and BLM cells, respectively. Darker dots indicate more significant interactions, indicated as q values in the color-coded legends. We observed multiple significant interactions occurring along chromosome 5 upstream and downstream of -146C>T TERT promoter. Taken together, these findings suggest that mutant TERT promoter displays active histone marks and long-range chromatin interaction.

Reversing the -146C>T Mutant TERT Promoter Site to WT Specifically Reverses the Proximal Histone Marks and Alters Long-Range Chromatin Interactions

To address if the histone marks and chromatin interactions observed on the mutant promoters are specifically driven by the point mutation in proximal mutant promoters, we reversed the mutated nucleotide to the WT residue by genome editing of promoters using the CRISPR/Cas9 system (Fig. 2A). The mutant TERT promoter (-146C>T labeled as red color) was targeted by single-guide RNA adjacent to the mutant region. The WT nucleotide was introduced using a repair template containing homology regions as shown in Fig. 2A. We generated isogenic lines with the mutant TERT promoter (-146C>T), hereafter referred to as BLM6, and with the WT TERT promoter (-146C), hereafter referred to as...
Regulation of Mutant TERT Promoter

**A**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>Promoter status</th>
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<tbody>
<tr>
<td>BLM</td>
<td>Melanoma</td>
<td>-146C&gt;T</td>
</tr>
<tr>
<td>A375</td>
<td>Melanoma</td>
<td>-146C&gt;T</td>
</tr>
<tr>
<td>T98G</td>
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<td>Head and neck</td>
<td>WT</td>
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</tr>
<tr>
<td>U2OS</td>
<td>Osteosarcoma</td>
<td>WT-ALT</td>
</tr>
</tbody>
</table>

**B**

**Long-distance elements**

<table>
<thead>
<tr>
<th>Distance (bp) from the viewpoint</th>
<th>ZDHHC11</th>
<th>BRD9</th>
<th>TRIP13</th>
</tr>
</thead>
</table>

**C**

-146C>T TERT promoter

% Input

-5 kb -2.5 kb -1 kb -0.8 kb -TSS

**D**

-146C TERT promoter

% Input

-5 kb -2.5 kb -1 kb -0.8 kb -TSS

**E**

-5 kb -2.5 kb -1 kb -0.8 kb -TSS

**q-value**

-5.0 0.0001 0.0001 0.0001 0.001 0.01 >q-value <= 0.05 0.1 >q-value <= 0.2 0.2 >q-value <= 0.5 q-value >0.5

**Distance (bp) from the viewpoint**

-4e+05 -2e+05 0 2e+05 4e+05
A

Long-distance elements
-5,000 -2,500 -1,000 -800
-46C>T BLM6 (-146C>T TERT promoter)
-1 kb -0.8 kb -TSS
Repair template
-5 kb -1 kb -0.8 kb
CRISPR/Cas9 editing

Long-distance elements
-5,000 -2,500 -1,000 -800

B

BLM6 (-146C>T TERT promoter) BLM14 (-146C TERT promoter)

P < 0.01

P < 0.001

P < 0.05

P < 0.01

P < 0.01

P < 0.01

C

BLM6 rep 1

Count (vst)

14

16

12

10

8

6

4

2

0

BLM6 rep 2


D

BLM14 rep 1

Count (vst)

14

16

12

10

8

6

4

2

0

BLM14 rep 2


E

Log2 fold change

1.6

1.4

1.2

1.0

0.8

0.6

0.4

0.2

0.0

BLM6 rep 1

BLM6 rep 2

BLM14 rep 1

BLM14 rep 2

F

G

Interaction fold change

P < 0.05
as BLM14. Genetic status was confirmed by Sanger sequencing as shown in Fig. 2B (top left). As compared with the BLM6, TERT expression was dramatically reduced in the BLM14 cells (Supplementary Fig. S2A). Reversal of mutant to WT TERT promoter also leads to reduced telomerase activity (Supplementary Fig. S2B). Early-passage BLM cells showed not show significant differences in telomere length; however upon long-term culture, significant telomere attrition was observed in engineered cells (Supplementary Fig. S2C and S2D), suggesting the functionality of this mutation reversal. We also analyzed BLM14 cells for the Alternative Lengthening Telomers (ALT) phenotype. There was no colocalization between TRF2 and PML in BLM6 or BLM14 cells (Supplementary Fig. S2E). U2OS was used as the positive control where we observed very strong colocalization as reported previously for ALT cell lines (22). We performed RNA sequencing from BLM6 and BLM14 cells in order to eliminate possible off-target effects of the CRISPR/Cas9 method. No significant differences were detected in genes that were predicted to have off-target effects for guide RNA used in CRISPR/Cas9 editing by RNA sequencing (Supplementary Table S1). Next, we investigated the epigenetic status of the proximal TERT promoter in these cell lines. Interestingly, we observed that the proximal active histone marks were significantly reduced upon single-nucleotide reversal (Fig. 2B).

Next, we mapped the long-range intrachromosomal interactions in these cell lines using the 4C assay. Fig. 2C–E show differential intrachromosomal interactions of the TERT promoter between BLM6 and BLM14 cells. Red dots indicate significant and black dots indicate nonsignificant interactions between mutant and WT TERT promoters. The intrachromosomal interactions were dramatically reduced in BLM14 (Fig. 2D) as compared with BLM6 cells (Fig. 2C). Fold interaction differences are indicated as a log2 scale that is shown in a lower panel together with RefSeq genes that are located on chromosome 5 (Fig. 2E). As 4C is a sem-quantitative method, to accurately quantify the differences of these chromatin interactions, we selected 12 of the interactions that were obtained from 4C analysis and performed chromosome conformation capture (3C)-qPCR assays. Figure 2F shows the strongest five regions interacting with the mutant TERT promoter. The x axis indicates the distance of the regions from -146C>T site of the TERT promoter, which is represented as “0” along with its coordinates below, and the y axis indicates the binding strength in log2 scale. The strongest interaction was observed between the TERT promoter and a region 300 kb upstream of promoter (chr5:1,556,087–1,558,758), hereafter referred to as T-INT1 (Fig. 2F). The 3C-qPCR assay verified that the mutant -146C>T mutant TERT promoter in BLM6 cells showed significantly higher interaction with the T-INT1 intergenic region when compared with the -146C WT TERT promoter in BLM14 cells (Fig. 2G). Supplementary Table S2 shows the list of regions with genomic coordinates that were found to differentially interact with the mutant TERT promoter in BLM6 cells. Because a GABPA binding site is the only difference between WT and mutant TERT promoters, we speculated that histone marks and long-range interactions uniquely apparent on the mutant promoters might be initiated/mediated by GABPA binding to this site in the proximal promoter.

Silencing GABPA Expression Dampens the Active Chromatin Marks As Well as Long-Range Interactions in Mutated TERT Promoter

As the mutant TERT promoter creates a binding site for the ETS family member GABPA transcription factor, we analyzed the GABPA recruitment in BLM6 and BLM14 cells. As shown in Fig. 3A, GABPA was specifically enriched at the proximal mutant TERT promoter in BLM6 cells. A similar enrichment pattern was obtained for RNA POL2 (Fig. 3B). We next explored the possibility that GABPA recruitment specifically drives the epigenetic marks in the proximal TERT promoter region. We depleted GABPA by siRNA in BLM6 cells (Fig. 3C). Chromatin immunoprecipitation (ChIP) was performed in GABPA-depleted cells, which showed reduced recruitment of GABPA to the mutant TERT promoter along with reduced POL2 recruitment (Fig. 3D). This was associated with reduced TERT expression in GABPA-depleted cells (Fig. 3E). Most importantly, GABPA depletion was associated with reduced enrichment of active histone marks in the proximal TERT promoter (Fig. 3F).

Mutant TERT promoter showed long-range interaction as observed using 4C assays (Fig. 2C–E), and GABP has been shown to have the potential for mediating long-range interactions (20). To investigate if GABPA is a key mediator, we performed the 3C-qPCR assay with GABPA-depleted BLM6 cells. We observed significant reduction of intrachromosomal interaction between the TERT promoter and the T-INT1 region upon GABPA depletion (Fig. 3G). To understand the molecular basis of how GABPA could mediate this long-range interaction, we analyzed the T-INT1 region for the presence of potential GABPA motifs using the transcription factor motif

**Figure 2.** Reversing the TERT promoter mutation to WT reverses the active chromatin marks and alters long-range chromatin interactions. A, CRISPR/Cas9-mediated reversal strategy of mutated -146C>T residue (shown as red color) to WT -146C residue (shown as orange color) by repair template harboring -146C residue. We obtained BLM cells that have undergone the CRISPR process and are mutant for -146C residue and are labeled as BLM6. BLM cells that have undergone the CRISPR process and mutated back to WT for -146T residue to -146C residue are labeled as BLM14. B, DNA chromatograms spanning the TERT promoter region in BLM6 and BLM14 are shown. ChIP was performed in BLM6 and BLM14 cells against histone marks (H3K4Me3 and H3K9Ac). Graph shows qPCR analysis with % input obtained across various regions of TERT promoter with distances indicated in boxes below the x axis. Error bars indicate the mean ± SD of the two independent experiments. P values were calculated by two-tailed Student t test method. C–E, plot generated by FourCseq of the differential intrachromosomal interactions of the TERT promoter between replicates of BLM6 and BLM14. The green line shows the distance-dependent fit and the blue dashed line indicates the fit of z-score > 2. Interactions detected by z-score > 2 (P-adjusted value < 0.05) for at least one replicate are shown as red dots. Fragments not called as interactions and that do not show significant change between conditions are shown as black dots. vst, variance stabilizing transformation. E, the calculated fold change is shown above the hg 19 RefSeq genes in the regions 1 megabase from the TERT promoter. F, quantifications of 5 interactions, obtained from C–E, were measured by 3C-qPCR in BLM6 and BLM14 cells. Error bars indicate the mean ± SD of the three independent experiments. P values were calculated by two-tailed Student t test method.
**A** Anti-GABPA

**B** Anti-POL2

**C** GABPA expression

**D** TERT promoter

**E** TERT expression

**F** TERT promoter

**G** 3C assay

**H** Anti-BRD4

**I** Anti-BRD4

**J** BRD4 expression

**K** GABPA expression

**L** GABPA promoter

**M** TERT expression

**N** TERT promoter

**O** 3C assay
Regulation of Mutant TERT Promoter

We found multiple GABPA motifs in this region (Supplementary Fig. S3A). It is well known that GABPA factors work as tetramers (15–17). As this region is about 2.5 kb long and GABPA sites are spread along the whole sequence, we designed ChIP qPCR primers specific to the 5′ region, central region, and 3′ region. GABPA was specifically enriched at the 5′ region (data not shown). We examined the GABPA binding to this region in BLM6 and BLM14 cells and found that it was significantly increased in BLM6 cells (Supplementary Fig. S3B). Overall, these results suggest that GABPA dimers/tetramers bound at long distance, the T-INT1 region (particularly GABPA sites located in the 130 to 290bp region according to chr5:1,556,087 and -146C-T mutant site in the TERT promoter provide the stable binding required for GABPA-mediated transcription, and this might be the molecular basis of the specific interaction of the mutant TERT promoter with the 300 kb region upstream of its TSS. Indeed, the presence of several GABPA sites in 5′ of the T-INT1 region may explain why this region might specifically interact with the mutant TERT promoter. Because the WT TERT promoter does not possess the GABPA motif at the -146 position, such long-range interaction does not take place and the chromatin interaction around the TERT promoter does not get stabilized for further modification by epigenetic factors. To our knowledge, this is the first report showing GABPA can indeed function as a mediator of long-distance interactions.

Active histone marks are known to recruit chromatin remodelers such as BRD4 (24), so we analyzed whether BRD4 is associated with active TERT promoter in BLM6 cells. We observed strong enrichment of BRD4 at the TERT promoter in BLM6 cells as compared with BLM14 cells (Fig. 3H). Similar patterns of BRD4 recruitment were observed upon reducing GABPA expression, suggesting the essential role for GABPA in initiating the establishment of the chromatin marks on this promoter (Fig. 3I).

The BET domain family inhibitor JQ1 has been shown to regulate GABPA expression (25). Among BET family members, BRD4 has been shown to be the most potent target of JQ1 inhibition. Thus, to investigate whether BRD4 affects GABPA expression, we knocked down BRD4 in BLM6 and BLM14 cells. Figure 3J shows the knockdown efficiency in BLM6 and BLM14 cells. We observed significant reduction in GABPA expression, suggesting that BRD4 regulates GABPA expression independent of TERT promoter status (Fig. 3K).

In addition, we assessed direct BRD4 recruitment to the GABPA promoter and observed that it was highly enriched, suggesting that BRD4 indeed directly regulates GABPA expression (Fig. 3L). However, reducing BRD4 reduced TERT expression in BLM6 cells (Fig. 3M). This was corroborated with reduction in telomerase activity upon knocking down GABPA or BRD4 in BLM6 cells (Supplementary Fig. S4A). Because there is no GABPA recruitment on TERT proximal promoter of BLM14, GABPA reduction due to BRD4 knockdown had no effect on BLM14 cells. Moreover, the epigenetic status of the TERT promoter affects the proliferation rate in BLM6 but not BLM14 cells with WT TERT promoter (Supplementary Fig. S4B and S4C). Furthermore, knocking down BRD4 led to significant reduction in active histone marks in BLM6 cells (Fig. 3N). We also investigated the long-range interaction changes in the TERT promoter upon BRD4 knockdown. We observed significant reduction in association of the proximal TERT promoter with the T-INT1 region as measured by the 3C-qPCR assay, when BRD4 levels were reduced (Fig. 3O). As a control, we also analyzed the effect of BRD4 knockdown on TERT expression in Fadu cancer cells bearing active WT TERT promoter. TERT expression remained unaffected upon BRD4 knockdown in Fadu cells (Supplementary Fig. S4D and S4E). There was no effect of knocking down BRD4 or GABPA on alternate splicing of TERT mRNA (Supplementary Fig. S4F). This suggests that depletion of GABPA or BRD4 affects TERT transcription from the mutant TERT promoter. These results could be used to guide our toward therapeutic modalities.

**Reversing -146C-T TERT Promoter Mutation to WT in A375 Melanoma and T98G Glioblastoma Cells Reduces the Active Chromatin Marks and Affects Long-Range Chromatin Interactions**

To further validate the novel molecular mechanism mediated by long-range interactions observed in our assays, and to find the general significance of these findings, we performed CRISPR-mediated reversal of -146C-T mutation in A375 melanoma cells to generate mutant WT TERT promoter-containing cells (A375 -146C). Reversal of TERT promoter mutation in A375 cells reduced the TERT expression and telomerase activity and significantly diminished the active

**Figure 3.** Silencing GABPA expression dampens the active chromatin marks as well as long-range interactions in mutated TERT promoter. A and B, Chip was performed in BLM6 and BLM14 cells against GABPA and POL2 followed by qPCR with primers specific for TERT promoter region proximal to TSS. Graph shows qPCR results with fold recruitment over IgG or Si input method as indicated in y axis. C, GABPA expression analysis in BLM6 cells transfected siControl and siGABPA. D, Chip was performed in BLM6 cells transfected with siCont and siGABPA against IgG, GABPA, and POL2 followed by qPCR with primers specific for TERT promoter region proximal to TSS. E, TERT expression analysis in BLM6 cells transfected with siCont and siGABPA. F, Chip was performed in BLM6 cells transfected siCont and siGABPA against histone marks (H3K4Me3 and H3K9Ac) followed by qPCR with primers specific for TERT promoter region indicated in boxes below x axis. G, quantification of TERT promoter interaction with 300 kb upstream (chr5:1,556,087–1,558,758) DNA region measured by 3C-qPCR in BLM6 cells upon GABPA knockdown. H, Chip was performed in BLM6 and BLM14 cells against IgG and BRD4 by qPCR with primers specific for TERT promoter region proximal to TSS. I, BRD4 expression analysis in BLM6 cells transfected with siCont and siBRD4. J, GABPA expression analysis in BLM6 and BLM14 cells transfected with siCont and siBRD4. K, Chip was performed against histone marks (H3K4Me3 and H3K9Ac) followed by qPCR with primers specific for proximal TERT promoter region in BLM6 cells transfected with siCont and siBRD4. L, Chip was performed against histone marks (H3K4Me3 and H3K9Ac) followed by qPCR with primers specific for proximal TERT promoter region in BLM14 cells transfected with siCont and siBRD4. M, TERT expression analysis in BLM6 and BLM14 cells transfected with siCont and siBRD4. N, Chip was performed against histone marks (H3K4Me3 and H3K9Ac) followed by qPCR with primers specific for proximal TERT promoter region in BLM6 cells transfected with siCont and siBRD4. O, quantification of TERT promoter interaction with 300 kb upstream T-INT1 region measured by 3C-qPCR in BLM6 and BLM14 cells with BRD4 knockdown. Error bars indicate the mean ± SD of the two independent experiments. P values were calculated by two-tailed Student t test method.
Reversing the TERT promoter mutation to WT in A375 melanoma and T98G glioblastoma cell lines reduces the active chromatin marks and affects long-range chromatin interactions. We obtained A375 cells which have undergone the CRISPR process and are mutant for -146C residue and are labeled as A375 -146C.T. A375 cells which have undergone the CRISPR process and mutated back to WT for -146T residue to -146C residue are labeled as A375 -146C.T and T98G -146C cells. Quantification of TERT promoter interaction with chr5:1,556,087–1,558,758 region (T-INT1) is shown. H-I, ChiP was performed in T98G -146C-T and T98G -146C cells against histone marks (H3K4Me3 and H3K9Ac), POL2, GABPA, and BRD4. Graph shows ChiP analysis with primers specific to TERT promoter region proximal to TSS. Results were calculated with % input or fold recruitment over IgG as indicated in y axis. G, 3C-qPCR assay was performed in A375 -146C-T and A375 -146C cells. Quantification of TERT promoter interaction with T-INT1 region is shown. H-I, ChiP was performed in T98G -146C-T and T98G -146C cells against histone marks (H3K4Me3 and H3K9Ac), POL2, GABPA, and BRD4. Graph shows ChiP analysis with primers specific to TERT promoter region proximal to TSS. K, 3C-qPCR assay was performed in T98G -146C-T and T98G -146C cells. Quantification of TERT promoter interaction with chr5:1,556,087–1,558,758 region (T-INT1) is shown. n=3 for all the experiments, error bars indicate mean ± SD of three independent experiments. P values were calculated by Student t test method.

Histone marks (Fig. 4A–C). ChiP-qPCR analysis showed that POL2 binding was significantly reduced in A375 -146C cells (Fig. 4D). Furthermore, GABPA and BRD4 were significantly enriched in A375 -146C-T cells as compared with A375 -146C cells (Fig. 4E and F). We performed the 3C-qPCR assay in these cells, and the A375 -146C cells showed significant reduction in interaction with the T-INT1 region (Fig. 4G). We also generated WT TERT promoter–containing glioblastoma cell line T98G (T98G -146C) using the CRISPR/Cas9 method (18). T98G cells naturally contain a -146C mutation in the TERT promoter. We found that T98G -146C cells showed reduced active histone marks, POL2, GABPA, and BRD4 in the proximal TERT promoter region (Fig. 4H–J). The 3C-qPCR assay revealed that the WT TERT promoter in T98G -146C cells showed significant reduction in interaction with the T-INT1 region as compared with mutant TERT promoter (Fig. 4K). We conclude that the mechanism by which -146C>T mutation operates is most likely conserved across cancer types.
Introduction of -146C>T Mutant Site in the WT TERT Promoter Generates Long-Range Chromatin Interactions and Increases Proximal Histone Marks

To investigate if the cancer-associated single-point mutations are sufficient for causing the changes described above, we next introduced the -146C>T mutation in HCT116 colon cancer cells with the WT TERT promoter to create isogenic lines (HCT116 -146C and HCT116 -146C>T; Fig. 5A). Cell lines were verified by sequencing, and the HCT116 -146C>T cell line was homozygous (data not shown). Introduction of the TERT promoter mutation in HCT116 cells increased the TERT expression and Telomeric Repeat Amplification Protocol (TRAP) activity, and significantly enriched the active histone marks (Fig. 5B–D). ChIP analysis showed that POL2 binding was significantly increased at the mutant TERT promoter (Fig. 5E). Furthermore, GABPA and BRD4 were significantly enriched in the mutant TERT promoter as compared with the WT TERT promoter (Fig. 5F and G). GABPA was also strongly associated with the T-INT1 region in HCT116 -146C>T cells (Supplementary Fig. S5). We further investigated whether HCT116 -146C and HCT116 -146C>T cells show differential long-range interaction. The 3C-qPCR assay revealed that the mutant TERT promoter in HCT116 -146C>T cells showed significant interaction with the T-INT1 region (Fig. 5H), which was also the strongest interaction observed in 3C analysis of BLM cells (Fig. 2G).

As summarized in Fig. 5I, reversing -146C>T mutation to -146C WT in BLM, A375, and T98G cells causes reduction in GABPA recruitment and loss of long-range interaction. This is concomitant with reduction in active histone marks, POL2 recruitment, TERT expression, and telomerase activity. In contrast, introduction of -146C>T mutation in HCT116 cells causes an increase in GABPA recruitment and formation of long-range interaction. This is concomitant with an increase in active histone marks, POL2 recruitment, TERT expression, and telomerase activity as compared with the WT isogenic counterpart. We conclude that the -146C>T TERT promoter mutation could be sufficient to initiate long-range interaction of this promoter, and it could also be sufficient for activating this promoter via epigenetic modifications initiated and promoted by stabilizing long-range interaction with the T-INT1 region of the TERT promoter (Fig. 1B).

Deletion of the T-INT1 Region Abrogates Transcription from Both -124C>T and -146C>T Mutant TERT Promoters

To verify whether mutant TERT promoters are indeed activated via long-range interaction with the T-INT1 region, we created cells wherein we deleted the T-INT1 region while keeping the proximal mutant TERT promoter intact. We also investigated the generality of our observations by also including a cell line with -124C>T mutation in these assays. Figure 6A shows the location of the mutation site (-146C>T or -124C>T) on the TERT promoter and the location of the T-INT1 region. Putative GABPA motifs in the T-INT1 region are shown as red dots. CRISPR-mediated removal of the interacting region from three cell lines (BLM and A375 cell lines which have the -146C>T mutation in the TERT promoter, and U251 cell line which has the -124C>T mutation in the TERT promoter) led to the generation of respective isogenic lines that do have the T-INT1 region (T-INT1 WT) or cells where the T-INT1 region is deleted (T-INT1 KO; Fig. 6A). Genetic status was confirmed by genotyping PCR as shown in Supplementary Fig. S6A. In BLM cells, upon removal of the T-INT1 region, TERT expression and telomerase activity reduced dramatically (Fig. 6B and C) without any change in GABPA expression (Supplementary Fig. S6B). We observed a significant reduction in the active histone mark H3K4me3 in the proximal TERT promoter (Fig. 6D). Also POL2, GABPA, and BRD4 recruitment to TERT promoter was also reduced significantly (Fig. 6E and F; Supplementary Fig. S6C). Removal of the T-INT1 interaction region in A375 was also associated with reduced TERT expression and telomerase activity (Fig. 6G and H). The active histone mark H3K4me3 was reduced significantly in the proximal TERT promoter of A375 (Fig. 6I). POL2 and GABPA recruitment to the TERT promoter was also reduced significantly (Fig. 6J and K).

Interestingly, we observed a dramatic reduction in TERT expression and telomerase activity in the U251 T-INT1 KO cell line which harbors the -124C>T mutation (Fig. 6L and M). Similar to melanoma cells, deletion of the T-INT1 region decreased the enrichment of H3K4me3, POL2, and GABPA in the proximal TERT promoter of U251 cells (Fig. 6N–P).

These results demonstrate that GABPA recruitment to the mutant TERT promoter enables long-range interaction of the proximal TERT promoter with the T-INT1 region and facilitates generation of associated epigenetic changes that drive its transcription. These results also suggest that this is a general mechanism used by both the -124C>T and the -146C>T mutant promoters. Our results are presented in the form of a model in Figure 7.

DISCUSSION

Cancer-specific TERT promoter mutations offer an invaluable starting point to understand the mechanistic basis of activation of the dormant TERT promoter in cancers. Our study reveals a novel mechanism utilized by GABPA to activate TERT transcription. Using the 3C assay, we have shown that GABPA mediates long-range chromatin interaction to form a stable complex that drives TERT transcription. Reversal of the mutation in the TERT promoter or removal of the mutant TERT promoter–interacting region (T-INT1) using genome editing results in loss of GABPA binding, depletion of epigenetic marks, and reduction of TERT transcription. Interestingly, the introduction of the -146C>T TERT promoter mutation in HCT116 cells could specifically enrich histone marks and enable long-range interaction. Our findings suggest that GABPA binding to the mutant TERT promoter is an initiating and sufficient event to drive the epigenetic status of the TERT promoter.

The introduction of the -146C>T and -124C>T TERT promoter mutations in human embryonic stem cells and their differentiation of them into fibroblasts and neural progenitor cells revealed that these mutations were capable of overcoming epigenetic silencing (26). Moreover, the TERT expression in these cells was comparable to that found in cancer cells and could significantly delay telomere shortening–induced senescence. Furthermore, the high frequency of mutations in the TERT promoter mostly at two nucleotide positions (-146C>T, -124C>T).
Figure 5. Introducing -146C>T TERT promoter mutation in HCT116 cells increases the active chromatin marks and enables long-range chromatin interaction. A, CRISPR/Cas9-mediated conversion of -146C residue (orange color) to mutant -146C>T residue (red color) by repair template harboring -146T nucleotide is shown. We obtained HCT116 -146C cells which have undergone the CRISPR process and are WT for -146C residue and are labeled as HCT116 -146C. HCT116 cells which have undergone the CRISPR process and are mutated for -146C residue to -146T residue and are labeled as HCT116 -146C>T. B, graph shows qPCR analysis of TERT expression normalized to actin levels. C, graph shows telomerase activity (TRAP) in HCT116 -146C and HCT116 -146C>T cells. D–G, ChIP was performed in HCT116 -146C and HCT116 -146C>T cells against histone mark H3K4Me3, H3K9Ac, POL2, GABPA, and BRD4. Graph shows qPCR analysis with % input obtained with primers specific to TERT promoter region proximal to TSS. H, 3C-qPCR assay was performed in HCT116 -146C and HCT116 -146C>T cells. Quantification of TERT promoter interaction with T-INT1 region is shown. n = 3 for all the experiments, error bars indicate mean ± SD of three independent experiments. P values were calculated by Student t test method. I, figure summarizes the results of GABPA enrichment, formation of long-range interaction, enrichment of active histone marks and POL2, TERT expression and telomerase activity that were obtained from isogenic cell lines generated by CRISPR/Cas9 editing. ↑ indicates increase, and ↓ indicates decrease.
Removal of T-INT1 region in melanoma and glioblastoma cell lines reverses the active chromatin marks and decreases TERT expression and telomerase activity. A, removal of T-INT1 region strategy in -124C>T mutant cell line is shown by CRISPR/Cas9 editing (right). Red dots indicate putative GABPA motifs in T-INT1 region. We obtained A, Figure 6. B, T-INT1 KO cells which have undergone the CRISPR process and interaction region Chr5:1,556,087–1,558,758 was removed are labeled as T-INT1 KO. T-INT1 WT cells which have undergone the CRISPR process and are WT for Chr5:1,556,087–1,558,758 region (T-INT1) and are labeled as T-INT1 WT.

ChIP was performed in U251 T-INT1 WT and U251 T-INT1 KO cells against H3K4Me3 and POL2 followed by qPCR with primers specific to TERT promoter region proximal to TSS. Results were analyzed with % input method.

Figure 6. Removal of T-INT1 region in melanoma and glioblastoma cell lines reverses the active chromatin marks and decreases TERT expression and telomerase activity. A, removal of T-INT1 region strategy in -124C>T mutant cell line is shown by CRISPR/Cas9 editing (right). Red dots indicate putative GABPA motifs in T-INT1 region. We obtained T-INT1 WT cells which have undergone the CRISPR process and are WT for Chr5:1,556,087–1,558,758 region (T-INT1) and are labeled as T-INT1 WT. T-INT1 KO cells which have undergone the CRISPR process and interaction region Chr5:1,556,087–1,558,758 was removed are labeled as T-INT1 KO. B, graph shows qPCR analysis of TERT expression normalized to actin levels in A375 T-INT1 WT and A375 T-INT1 KO cells. C, graph shows telomerase activity (TRAP) in BLM T-INT1 WT and BLM T-INT1 KO cells. D–F, ChIP was performed in BLM T-INT1 WT and BLM T-INT1 KO cells against H3K4Me3, POL2, and GABPA followed by qPCR with primers specific to TERT promoter region proximal to TSS. Graph shows qPCR results with % input method. G, graph shows qPCR analysis of TERT expression normalized to actin levels in A375 T-INT1 WT and A375 T-INT1 KO cells. H, graph shows telomerase activity (TRAP) in A375 T-INT1 WT and A375 T-INT1 KO cells. I and J, ChIP was performed in A375 T-INT1 WT and A375 T-INT1 KO cells against H3K4Me3 and POL2 followed by qPCR with primers specific to the TERT promoter region proximal to TSS. Graph shows qPCR results with % input method. K, ChIP was performed in A375 T-INT1 WT and A375 T-INT1 KO cells against GABPA followed by qPCR with primers specific to TERT promoter region proximal to TSS. Results were analyzed with fold recruitment over IgG with primers specific to TERT promoter region proximal to TSS. L, graph shows qPCR analysis of TERT expression normalized to actin levels in U251 T-INT1 WT and U251 T-INT1 KO cells. M, graph shows telomerase activity (TRAP) in U251 T-INT1 WT and U251 T-INT1 KO cells. N and O, ChIP was performed in U251 T-INT1 WT and U251 T-INT1 KO cells against H3K4Me3 and POL2 followed by qPCR with primers specific to TERT promoter region proximal to TSS. Results were analyzed with % input method. P, ChIP was performed in U251 T-INT1 WT and U251 T-INT1 KO cells against GABPA followed by qPCR with primers specific to TERT promoter region proximal to TSS, and results were analyzed with fold recruitment over IgG. N = 3 for all the experiments, error bars indicate mean ± SD of three independent experiments. P values were calculated by Student t test method.
-124C>T) strongly implicates them as driver events, appearing upon tumor initiation or possibly later in tumor development (27, 28).

Human TERT promoter harbors a G-rich region of 12 sequential G-tracks, encompassing three SP1 binding sites, and has the ability to form multiple G-quadruplexes. Masking of the SP1 binding sites by G-quadruplex structure is predicted to produce significant inhibition of TERT promoter activity (29). The G-quadruplex structure may also have an important role in the regulation of the mutant TERT promoter, and it is suggested that TERT promoter mutations may have an impact on this predicted secondary structure and that the complex relationship between secondary structure and GABPA recruitment could also play a role in regulating TERT expression (11). The complex regulatory cross-talk between long-range interaction and G-quadruplex structure in regulating mutant versus WT TERT promoters needs further investigation.

Furthermore, it would also be interesting to understand what cofactor(s) cooperate with GABPA or other ETS family members on the -124C>T and -124C>T mutant sites. Clearly, having the know-how and the ability to target distinct mechanisms which operate in cancer cells which are driven by the mutant TERT promoter would be a step forward in designing therapeutics against telomerase in cancer. Targeting mutant TERT promoter–specific pathways that do not impinge on the WT TERT promoter will help in designing drugs that are less toxic and can be taken over a longer term at a higher dose. With the realization that telomerase inhibition could yield a magic bullet to treat most human cancers of any origin and carrying any genetic alteration, many pharmaceutical companies have developed and tested various versions of telomerase inhibitors over the last decade. Many refinements were made, and the most successful inhibitor, by Genron, was believed to reach the clinic. But this inhibitor, which inhibits enzymatic function of telomerase, failed clinical trials early last year. Based on the understanding of how mutant TERT promoters operate, a new set of inhibitors that limit TERT transcription only in cancer cells with -124C>T and -124C>T mutations could be designed rapidly. Because these new transcriptional inhibitors will work to limit levels of TERT and hence telomerase (indirectly limiting the activity) only in cells with mutation, namely the cancer cells, they may not cause toxicity to stem cells or other normal somatic cells where continued telomerase activity is necessary for physiology.

**METHODS**

**Cell Lines and Reagents**

The A375 and HCT116 cell lines were originally purchased from the ATCC and were a gift from Dr. Shang Li (DUKE-NUS Medical School, Singapore). Fadu cells were obtained from the ATCC. BLM cells and primary melanocytes were a gift from Dr. Birgitte Lane (Institute of Medical Biology, Singapore), and T98G-WT (CRISPR-engineered WT TERT promoter) was produced previously (18). PC3 was a gift from Dr. Ernesto Guccione (Institute of Molecular and Cell Biology, Singapore). All cells were maintained in DMEM except PC3, which was maintained in RPMI and supplemented with 10% FBS (Hyclone), penicillin and streptomycin (Gibco) using standard tissue culture techniques. Cell lines were not authenticated by us.

**Genomic DNA Isolation and Sanger Sequencing**

Genomic DNAs of cell lines were isolated with tail lysis buffer supplemented with Proteinase K. DNA was precipitated with isopropanol, and the TERT promoter was sequenced as described previously (18).

**Reversal of the TERT Promoter Mutation by CRISPR/Cas9 Editing**

The -124C>T TERT promoter mutation in the BLM and A375 cell lines was converted back to the WT promoter sequence (-146C), and
Regulation of Mutant TERT Promoter

-146C was mutated to -146C>T in HCT116 cells as described previously (38). Briefly, guide RNA specific to the TERT promoter was cloned into pX458 (GFP) plasmid and was cotransfected with repair template (130bp) containing WT (-146C) or mutant (-146C>T) TERT promoter sequence. GFP-positive cells were seeded into 96-well plates (1 cell/well) by FACS after 48 hours, and each clone was screened by PCR and Sanger sequencing.

TERT Interaction Region Removal by CRISPR/Cas9 Editing

pX458-GFP plasmid was modified by removing Cas9 and GFP with EcoRI-AgeI restriction enzymes and inserting Ds-Red monomer in order to prevent off-target effect of excess Cas9 enzyme in the cells (36) and also to be able to observe transfection efficiency of both plasmids. Guide-RNA-1 CACCGCGCAACAGGATGCCTGACAT and Guide-RNA-2 CACCCGGCGTCGGAGCAATTCCAC targeting TERT interaction region (Chr5:1,555,087–1,558,758) were cloned into pX458 (GFP) and pX458 (Ds-Red) plasmids, respectively. Cells were transfected in 6-well plate by X-tremeGENE 9 transfection reagent (Roche). GFP and Ds-Red-positive cells were sorted into 96-well plates (1 cell/well) after 48 hours by FACS, and each clone was genotyped by PCR and Sanger sequencing.

RNA Sequencing

The RNA-sequencing library was prepared from two replicates of the BLM6 and BLM14 cell lines by the Illumina TruSeq Total RNA Sequencing Kit according to the manufacturer's instructions.

RNA-sequencing Analysis

Raw reads were aligned to the human genome hg19 by TopHat2. Differential gene expression was performed by cufflinks and cuffdiff with default parameters.

RNA Interference and Gene Expression

For siRNA treatment, cells were transfected when they reached approximately 60% confluence with siControl (Dharmacon; D001810-10), siBRD4 (Dharmacon; L011662), or siBRD4 (Dharmacon; L-004937) using X-tremeGENE siRNA transfection reagent (Roche). GFP and Ds-Red-positive cells were sorted into 96-well plates (1 cell/well) after 48 hours by FACS, and each clone was genotyped by PCR with primers surrounding the T-INT1 region: Forward: GTCTG CATTAAGTCGTC and reverse: GGGGACATTTATGTCTTGC. PCR product was approximately 4530bp, and T-INT1 KO PCR product was 550bp.

ChIP Assays

ChIP was performed as described previously (32). Anti-H3K4Me3 (Millipore; 04-745), anti-H3K9Ac (Millipore; 07-352), anti-H3K14Ac (Millipore; 07-353), anti-POL2 (Santa Cruz Biotechnology; sc-899), anti-GABPA (Santa Cruz Biotechnology; sc-22180), anti-BRD4 (Bethyl; A301-985A), and IgG (Santa Cruz Biotechnology) antibodies were used for the immunoprecipitation. All antibodies were used at a concentration of 1 µg per 1 million cells. After elution, chip eluate was used for ChIP-qPCR with primers (33) targeting TSS, 0.8 kb, 1 kb, 2.5 kb, and 5 kb upstream regions of the TERT promoter and/or TERT interaction region. Primers are indicated in Supplementary Table S3.

4C Assay

4C was performed as described previously (34). Briefly, 10^5 cells were fixed with 1% formaldehyde for 10 minutes, and nuclei pellets were isolated after cell lysis with cold lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40, 1% Triton X-100) supplemented with protease inhibitors. First step digestion was performed overnight at 37°C with 200 unit HindIII enzyme. Digestion efficiency was measured by RT-qPCR with HindIII site-specific primers. After phenol-chloroform extraction, DNA was ligated overnight at 16°C by T4 DNA ligase (Fermentas). Following de-crosslinking, DNA was processed for second digestion with 30 units of DpnII enzyme overnight at 37°C. After final ligation, 4C template DNAs were quantified by the Qubit dsDNA High Sensitivity Kit (Thermo Fisher) and preceded for library preparation for sequencing using TERT promoter-specific primers (Supplementary Table S3) with Illumina Nextera adapters.

4C Sequencing Analysis

The quality of the FASTQ files was evaluated using FastQC v0.11.3 (35). Illumina Nextera adaptor sequences were removed using scythe v0.991 (36). Tagdust2.3 was used to extract reads that were sandwiched between the bait region (TERT promoter + HindIII) and a DpnII cut site (37). The extracted reads were aligned to the hg19 genome using Bowtie 2 (38) with very sensitive parameters in unpaired mode. The aligned reads were filtered for mapping qualities of >30. r3CSeq (39) was used in batch mode to call interactions for A375 and BLM cells. Plots were generated by FourCSeq of the differential intrachromosomal interactions of the TERT promoter between replicates of BLM6 CRISPR mutant and BLM14 CRISPR WT (40). The green line shows the distance-dependent fit, and the blue dashed line indicates the fit of z-score > 2. Interactions detected by z-score > 2 (P-adjusted value < 0.05) for at least one replicate are shown as black dots. Fragments not called as interactions and that do not show significant change between conditions are shown as black dots. The calculated fold, fold change is shown above the Hg19 ReSeq genes in the regions 1 megabase from the TERT promoter.

3C Assay

3C was performed as described previously (41). Cells were processed in the same way as described in 4C. Following HindIII digestion and ligation, 3C template DNAs were quantified by the Qubit dsDNA High Sensitivity Kit (Thermo Fisher). In order to optimize real-time PCRs and to estimate the minimal amount of 3C template for quantification in a linear range, we prepared control template including equal amounts of target regions that we obtained in 4C analysis. These regions were amplified by region-specific primers and purified from gel and mixed in equimolar amounts. After digestion of these fragments with HindIII, fragments were ligated by T4 DNA ligase. 3C DNA templates were used for normalization of 3C-qPCRs. 3C-qPCR reactions were performed by the Sybr-Greener Kit (Invitrogen), and samples were normalized by the levels of TERT promoter region. 3C-qPCR primers are indicated in Supplementary Table S3.

Real-Time TRAP

Real-time TRAP assay was performed as described previously (42).

Colony Formation Assay

Colony formation assay was performed by crystal violet BLM6, and BLM14 cells were transfected with siControl, siGABPA, and siBRD4. After 24 hours, 500 cells were seeded into a 6-well plate. Culture media were refreshed every 2 days, and after 7 days, cells were fixed with 75% ethanol for 30 minutes and stained with 0.2% crystal violet dye.

Immunofluorescence

ALT-associated PML bodies were visualized via TRF2 and PML immunofluorescence staining. Cells were grown on chamber slides (Millipore EZ slides) and fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes.
at room temperature and were blocked with PBS supplemented with 0.2% Triton X-100 and 1% BSA (blocking buffer) for 30 minutes at room temperature. Following incubation, cells were incubated with the primary antibodies TRF2 (Millipore; 05-521) and PML (Santa Cruz Biotechnology; sc-5621), 1/200 and 1/150 dilutions, respectively, in blocking buffer overnight at 4°C. Cells were washed 4 times with blocking buffer for 8 minutes and incubated with secondary antibodies AlexaFlour 488 and AlexaFlour 555 (1/2,000 dilution; Invitrogen; A10001 and A21428) for 1 hour at room temperature. After cells were washed 4 times, image acquisition was performed with Zeiss LSM800, Plan-Apochromat 63x/1.40 apertures of the objective lenses.

Statistical Analysis
The Student’s t test (two-tailed) was performed to determine the significance of difference for ChIP-qPCR, qPCR, and RT-TRAP experiments. Results of each ChIP, gene expression, 4C, and 3C assays were obtained from at least two or three independent experiments, as indicated in the figure legends.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
# Long-Range Chromatin Interactions Drive Mutant *TERT* Promoter Activation

Semih Can Akincilar, Ekta Khattar, Priscilla Li Shan Boon, et al.


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