RESEARCH COMMUNICATION

Mutation of the *TERT* promoter, switch to active chromatin, and monoallelic *TERT* expression in multiple cancers

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Somatic mutations in the promoter of the gene for telomerase reverse transcriptase (TERT) are the most common noncoding mutations in cancer. They are thought to activate telomerase, contributing to proliferative immortality, but the molecular events driving *TERT* activation are largely unknown. We observed in multiple cancer cell lines that mutant *TERT* promoters exhibit the H3K4me2/3 mark of active chromatin and recruit the GABPA/B1 transcription factor, while the wild-type allele retains the H3K27me3 mark of epigenetic silencing; only the mutant promoters are transcriptionally active. These results suggest how a single-base-pair mutation can cause a dramatic epigenetic switch and monoallelic expression.

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The telomerase ribonucleoprotein (RNP) complex maintains telomeric DNA in normal stem cells as well as in most cancer cells. This telomere maintenance is necessary to perpetuate indefinite cellular proliferation. Most human cells express the telomerase RNA subunit hTR, while normal somatic cells other than stem cells do not express telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase. In such somatic cells, *TERT* gene expression is epigenetically silenced at the transcriptional level (Atkinson et al. 2005; Liu et al. 2007; Zhu et al. 2010).

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The failure to discover recurrent mutations or gene rearrangements that activate *TERT* expression was incongruous with its fundamental role in cancer. This situation changed when two cancer-specific somatic mutations in the *TERT* promoter were identified (Horn et al. 2013; Huang et al. 2013). These *TERT* promoter mutations occur more commonly than any other observed mutation in a number of cancers, including melanomas, glioblastomas (GBMs), hepatocellular carcinomas (HCCs), and urothelial carcinomas (UCs) (Killela et al. 2013; Kinde et al. 2013; Vinagre et al. 2013). The mutations are uniformly C>T transitions, predominantly located either −124 base pairs (bp) or −146 bp upstream of the *TERT* translational start site (ATG). Data based on reporter constructs suggest that *TERT* promoters with these mutations are about twofold more effective at driving expression than wild-type promoters (Horn et al. 2013; Huang et al. 2013; Rachakonda et al. 2013). The mutations are associated with functional increases in TERT protein, telomerase activity, and telomere length (Borah et al. 2015) and decreased survival for cancer patients (Rachakonda et al. 2013; Griewank et al. 2014; Borah et al. 2015). Genome editing of the *TERT* promoter at −124 suggests that the mutations are causative for increased *TERT* expression in normal bladder stem cells and bladder cancer cells (Li et al. 2015; Xi et al. 2015) and are capable of preventing *TERT* silencing upon differentiation of stem cells (Chiba et al. 2015).

Both the −124 and −146 promoter mutations create consensus binding sites for E-twenty-six (ETS) family transcription factors, of which there are 27 members, many of which are predicted to bind to the same sequence (Hollenhorst et al. 2011). A recent study implicated GABPA as a relevant ETS factor (Bell et al. 2015). However, the pathway by which an epigenetically silenced *TERT* gene is activated by the promoter mutation remains largely unknown. In the current study, we addressed this question and observed monoallelic expression of *TERT* from the promoter bearing the −124 mutation. Thus, *TERT* provides a model system for investigating central questions about how subtle genetic mutations can drive major epigenetic alterations.

Results and Discussion

The chromatin state of wild-type and mutant TERT promoters is different

As *TERT* promoter mutations are almost always heterozygous (Fig. 1A), we explored the possibility that the allele bearing a promoter mutation is selectively active. Initially, we chose to test this hypothesis in HCC-derived cell lines and, in preparation, used PCR and Sanger sequencing as well as next-generation sequencing to identify lines that are heterozygous for *TERT* promoter mutations at −124 bp from the ATG (66 bp from the transcriptional start site [TSS]) (Fig. 1A; Supplemental Table S1). The cell lines spanned a very large range of *TERT* expression

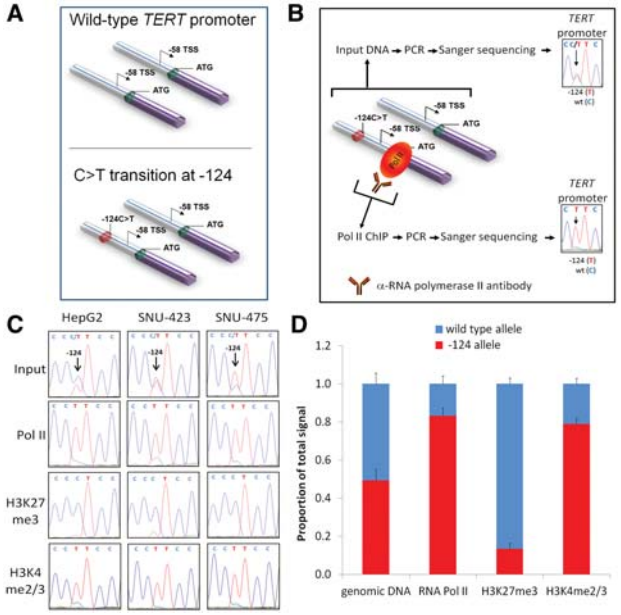

Figure 1. *TERT* promoter mutations reside in open, transcriptionally active chromatin. (*A*) Schematic of the *TERT* promoter indicating the position of the mutation relative to the TSS and the ATG. Purple represents the gene body. (*B*) Schematic illustrating the experimental approach using chromatin immunoprecipitation (ChIP) to test for allele-specific binding of proteins (such as RNA polymerase II [Pol II]) at the *TERT* promoter. (*C*) Sequence data obtained from ChIP for the indicated proteins followed by PCR for the *TERT* promoter in three HCC lines heterozygous for the mutation at −124. (*D*) Summary of quantification of fluorescence signal intensities of DNA sequencing chromatogram peaks at −124 from SNU-423, SNU-475, and HepG2. Quantification was performed for three independent replicate ChIP experiments for each cell line; peak heights in ChIP sequences were significantly different from input sequence peak heights. Paired *t*-test assuming heteroscedasticity: Pol II, $P = 0.005$; H3K27me3, $P = 0.004$; and H3K4me2/3, $P = 0.005$.

and telomerase activity, with no clear difference between lines with and without promoter mutations (Supplemental Tables S1, S2; Supplemental Fig. S1A–C). The lack of a genotype–phenotype correlation differs from results obtained in UC cell lines (Borah et al. 2015), but the number of cell lines studied here may be insufficient to reveal an association, if there is one, in HCC.

We selected HepG2, SNU-423, and SNU-475 as lines with promoter mutations and detectable telomerase and performed chromatin immunoprecipitation (ChIP) with antibodies to RNA polymerase II (Pol II) (e.g., Fig. 1B). Sequencing of PCR products obtained from these ChIP experiments indicated that Pol II had a strong preference for occupying the mutant *TERT* promoter in all three lines (Fig. 1C,D). We confirmed that these results were not due to a bias introduced by PCR (Supplemental Fig. S2).

Pol II recruitment to DNA frequently correlates with an open chromatin state that can be identified by post-translational modifications on nucleosomal histone proteins. Dimethylation or trimethylation of the first lysine of histone 3 (H3K4me2/3) is established by the Trithorax group of proteins and is associated with gene activation (Schuettengruber et al. 2011). Consistent with the results from the Pol II ChIP, sequencing revealed a strong preference for the H3K4me2/3 mark on the mutant compared with the wild-type allele (Fig. 1C,D).

The Polycomb-repressive complex PRC2 is thought to be solely responsible for deposition of H3K27me3, a hallmark of facultative heterochromatin containing repressed genes (Steffen and Ringrose 2014). We found high levels of H3K27me3 at the *TERT* promoter in telomerase-negative human primary cells compared with the HCC line SNU-475 (Supplemental Fig. S3A,B), consistent with a previous report (Atkinson et al. 2005) that *TERT* is epigenetically silenced in telomerase-negative somatic cells. We then examined the allele specificity of the H3K27me3 mark in HCC lines with *TERT* promoter mutations by PCR and sequence analysis of DNA immunopurified with an antibody against H3K27me3. We found a substantial depletion of the H3K27me3 mark on the promoter mutant allele relative to the wild-type allele (Fig. 1C,D). We obtained similar results for two UC-derived lines and one GBM-derived line (Supplemental Fig. S4). Thus, the *TERT* allele with a promoter mutation has an active chromatin mark, while the wild-type allele in the same cell bears an epigenetic hallmark of inactive chromatin.

Transcriptional up-regulation of GABPB1 and allele-specific binding of GABPA in HCC

The *TERT* promoter mutation creates a consensus binding site for an ETS family transcription factor, leading to the prediction that the *TERT* promoter mutation drives telomerase in response to the recruitment of such a factor. RNA sequencing (RNA-seq) analysis was performed on a panel of nine HCC lines, five of which were heterozygous for the *TERT* promoter mutation (Supplemental Tables S1, S2). Many of the 27 ETS factors were expressed among the cell lines (Supplemental Table S3), but only GABPB1 was expressed at significantly higher levels in lines with promoter mutations compared with those without ($P = 0.005$) (Fig. 2A,B; Supplemental Table S3).

GABPB1 contains a transcriptional activation domain and forms a heterodimer with the ETS factor GABPA (LaMarco et al. 1991; Gugneja et al. 1995). The latter protein performs the sequence-specific DNA-binding function and was also expressed in all HCC lines tested (Supplemental Table S3). Using ChIP, we found that GABPA bound to the *TERT* promoter in both HCC and UC lines (Fig. 2C). This binding was specific for the mutant promoter in the two heterozygous HCC lines that we tested (Fig. 2D). In support of allele-specific binding, ENCODE ChIP-seq (ChIP combined with deep sequencing) data for two mutant cell lines show GABPA bound at the *TERT* promoter, while two wild-type cell lines did not (Supplemental Fig. S5). Using siRNA knockdown, we found that GABPA contributed to TERT transcription (Supplemental Fig. S6). While our work was in preparation, Bell et al. (2015) reported that GABPA exhibited an allele bias in binding to the *TERT* promoter in several different cancers. Our results extend this conclusion to new HCC cell lines and indicate an associated up-regulation of GABPB1, but not GABPA or GABPB2, in the mutant promoter HCC cells. The selective up-regulation of the transactivating subunit GABPB1 provides some insight into how these cells specifically enhance expression of GABPA-bound genes such as *TERT* despite the apparent redundancy for similar consensus binding sequences among the 27 ETS transcription factors.

Many of the ETS transcription factors are predicted to bind to the same sequence (Hollenhorst et al. 2011). The

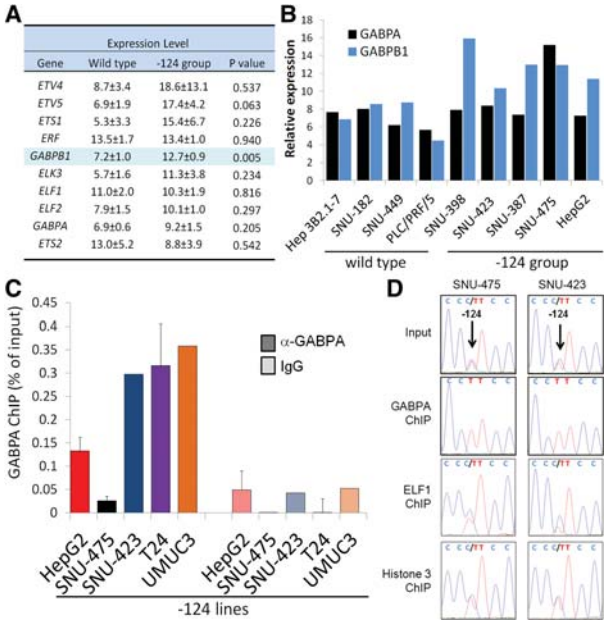

Figure 2. ETS transcription factor expression and binding of GABPA to the *TERT* promoter in HCC and UC cell lines. (*A*) The 10 most highly expressed ETS transcription factors in the tested HCC lines using RNA-seq; *P*-values were derived from a two-tailed *t*-test assuming heteroscedasticity. Expression levels are ±SEM. (*B*) GABPA and GABPB1 expression levels from RNA-seq analysis of HCC lines with and without *TERT* promoter mutations. (*C*) Quantitative ChIP for GABPA occupancy at the *TERT* promoter in HCC (HepG2, SNU-475, and SNU-423) and UC (T24 and UMUC3) cell lines with promoter mutations. Error bars indicate ±SEM. $n = 1–5$; $P = 0.03$ from a one-tailed paired *t*-test between each ChIP and its IgG control. (*D*) ChIP followed by PCR and Sanger sequencing of the *TERT* promoter in two HCC lines (SNU-423 and SNU-475).

ETS factor ELF1 was recently implicated in melanoma progression; evaluation of promoter mutations in the cancer-associated gene succinate dehydrogenase (*SDHD*) suggested that they disrupt ELF1 binding (Weinhold et al. 2014). When we compared the proposed ELF1-binding site in *SDHD* with the *TERT* promoter sequence, we found that these promoters share a similar bipartite consensus ETS site (Supplemental Fig. S7). This sequence similarity led us to test whether ELF1 occupied the *TERT* promoter. ChIP showed ELF1 bound at the *TERT* promoter in HCC and UC cell lines, but, in contrast to GABPA, ELF1 did not demonstrate a preference for the mutated allele in the lines that we examined (Fig. 2D; Supplemental Figs. S8, S9).

We reasoned that if this bipartite sequence (Supplemental Fig. S7) responds functionally to GABPB1 up-regulation, one prediction is that other genes showing elevated expression in our RNA-seq experiment may also harbor these signatures. Thus, we examined the proximal promoters and 5′ untranslated regions (UTRs) of the most differentially expressed genes in the HCC lines based on our RNA-seq data. Several candidate promoters were identified that also contained these promoter signatures, although the spacing between the ETS motifs varied (Supplemental Fig. S7; Supplemental Tables S4, S5). We found that GABPA bound to these genes, including *SDHD* (Supplemental Fig. S10), and this is corroborated by ENCODE ChIP-seq data in multiple cell lines (Supplemental Fig. S11). These data are consistent with the notion that GABPA/GABPB1 binds to bipartite sequences (e.g., Bell et al. 2015), likely as a dimer of heterodimers (Sawada et al. 1994), and that subtle differences in consensus sequences may guide the binding of different ETS factors.

TERT is expressed monoallelically from mutant promoters

Reporter genes driven by *TERT* promoters with a mutation at −124 show modestly higher expression than wild-type promoters (Horn et al. 2013; Huang et al. 2013; Rachakonda et al. 2013). However, the allele-specific phenotypes that we observed at the endogenous *TERT* promoter (Figs. 1C, 2D) suggest that such comparisons of exogenous plasmids may underestimate the contribution of the promoter mutation. If the mutation acts as a switch and is a key component of activating *TERT* expression, then the mutated allele should be largely or solely responsible for TERT mRNA expression in these cancer cells.

To test this, we used two tools to identify from which allele transcripts are derived. First, using UC cells that are heterozygous for promoter mutations, we searched their exome sequence data for naturally occurring heterozygous single-nucleotide polymorphisms (SNPs) in the *TERT* gene body (Supplemental Table S6). UMUC3 and T24 cells both bear a SNP in one copy of *TERT* in exon 2 (rs2736098). PCR and sequencing of genomic DNA demonstrated that these cells are indeed heterozygous for rs2736098, while sequencing of the RT–PCR products indicated that, in each case, the cDNA exhibited a single variant of the SNP (Fig. 3A), although the expressed variant differed for the two lines. As controls, we detected no amplification in samples where reverse transcriptase was omitted, indicating that the products were indeed derived from cDNA (Supplemental Fig. S12). In addition, we were able to detect biallelic expression of heterozygous SNPs in the transcripts of STAG1 and RNase H (in UMUC3 cells) and p53 and NBN (in SNU-475 cells), indicating faithful amplification of these variant cDNA templates (Supplemental Fig. S13).

Second, to assess whether the TERT transcripts from T24 and UMUC3 were derived from the allele bearing the *TERT* promoter mutation, we performed PCR and sequencing of a region that encompasses both the *TERT* promoter mutation and the SNP in exon 2. We used template DNA isolated by either RNA Pol II ChIP of UMUC3 cells or H3K27me3 ChIP of T24. Because the RNA Pol II ChIP precipitated the transcribed allele from UMUC3, while the H3K27me3 ChIP isolated the nontranscribed allele from T24, we conclude that the allele harboring the *TERT* promoter mutation also contains the variant observed by RT–PCR (Fig. 3B,D).

We expected that cancer cells with no *TERT* promoter mutation would show expression of both alleles of *TERT*. Indeed, TERT cDNA from the colon cancer cell line HCT-116, which does not bear any known mutation in the *TERT* promoter, exhibited an allelic ratio similar to genomic DNA based on RT–PCR analysis of a naturally occurring SNP in exon 2 (Fig. 3C).

With few exceptions, such as imprinted genes and X-chromosome inactivation, monoallelic expression as dramatic as that observed here for *TERT* is highly unusual. Pedigree analysis of inherited disease-associated *TERT*

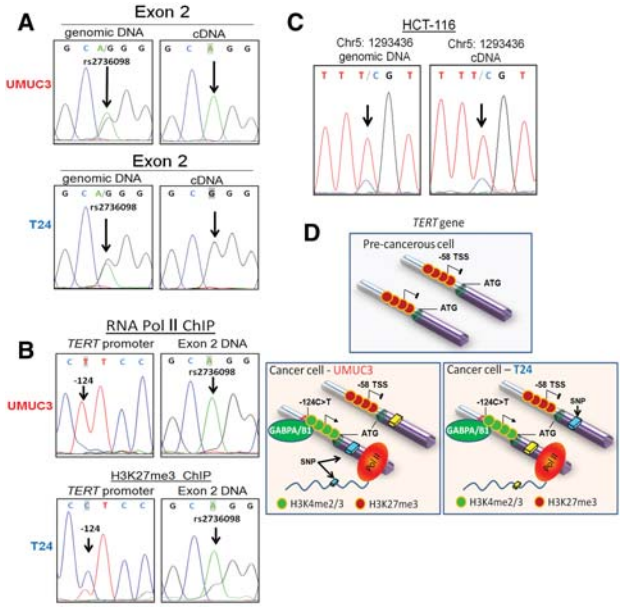

Figure 3. Monoallelic expression of *TERT* in tumor-derived cell lines with mutations in the *TERT* promoter. (*A*) PCR and Sanger sequencing show a heterozygous coding SNP (rs2736098) in exon 2 of *TERT* in two UC lines (genomic DNA), but only one sequence is expressed (cDNA) (see the model in *D*). (*B*) Linking the *TERT* promoter mutation with the exon 2 SNP. PCR and sequencing were performed for a region spanning the promoter mutation and rs2736098; PCR template DNA was derived from allele-specific ChIP for RNA Pol II or H3K27me3 (e.g., Fig. 1B). (*C*) No allelic bias in expression of *TERT* in HCT-116 colon carcinoma cell line with no known *TERT* regulatory mutations. The ratio of the two sequences is the same in genomic DNA and cDNA; the cause of the different peak heights is unknown. Chromosomal coordinates are for hg38. (*D*) Model of the *TERT* promoter state in precancerous cells versus cancer cells with *TERT* promoter mutations. The SNP rs2736098 is found on different alleles in T24 and UMUC3, as shown by the allele-specific ChIP in *B*.

mutations (Armanios et al. 2005) does not support maternal or paternal imprinting. For nonimprinted genes, the allelic bias in gene expression is typically on the order of twofold (Yan et al. 2002; Li and Clevers 2010).

Model for an epigenetic switch

In normal somatic cells other than stem cells, *TERT* expression is repressed, and telomerase activity is not detectable (Kim et al. 1994; Wright et al. 1996; Meyerson et al. 1997; Atkinson et al. 2005). Whether cancers are derived from normal stem cells (with active telomerase) or transiently proliferating cells (with inactive telomerase) is currently unknown (for review, see Li and Clevers 2010). Our data are consistent with a model in which cancer cells bearing one of the recurrent *TERT* promoter mutations are derived from cells (perhaps transiently proliferating ones) in which *TERT* is normally silenced. Such cells gain a de novo binding site for the common and abundant transcription factor GABPA/B1 heterodimer (Fig. 3D) by virtue of the *TERT* promoter mutation. The higher expression of GABPB1 in the promoter mutant cell lines is consistent with the idea that these cells were predisposed to activation by the mutation. Our data indicate that the mutation results in binding of GABPA in HCC cells, leading to recruitment of Pol II either concomitant with or subsequent to an epigenetic shift from a repressed to an active chromatin state. At the same time, the remaining wild-type allele in the same cell remains silent, residing in inactive chromatin.

Introduction of *TERT* promoter mutations by genome editing in human embryonic stem cells indicates that the mutations have the capacity to prevent the programmed silencing that *TERT* normally undergoes upon terminal differentiation (Chiba et al. 2015) and suggests another mechanism by which such mutations may contribute to oncogenesis. Our data are equally consistent with such a model, where tumors arise from stem cells with active telomerase, and the *TERT* promoter mutation causes that allele to remain selectively active while the other allele becomes repressed. In short, the tumor cell lines show the result of an epigenetic switch, but future experiments will be required to determine whether the mutant allele was switched on or the wild-type allele was switched off.

Other important questions remain to be answered. The prevalence of these mutations, together with the critical function of TERT in telomere maintenance, suggests that they may function as gatekeepers to cancer development. If these mutations convert *TERT* from a repressed state to an expressed state, what is the temporal order of events? In the scenario that the mutation is the initiating event, as suggested by the genome-editing studies in UCs (Li et al. 2015), recruitment of sequence-specific pioneering factors to the mutated site could constitute the secondary event. What are these pioneering factors? These recruitment events may lead to the methylation of H3K4 on the mutant allele, which in turn drives H3 acetylation (Crump et al. 2011). Histone modifications such as these promote the transition of Pol II from an initiating form to an elongating form (Stasevich et al. 2014), resulting in gene expression. That many mutations concomitantly form allele-specific transcription factor-binding sites and associate with epigenetic changes (Kilpinen et al. 2013; McVicker et al. 2013) suggests that genetic changes can indeed drive epigenetic changes.

Because many ETS factors are reported to bind to similar consensus sequences, do ETS factors other than GABPA/B1 also bind and activate mutant *TERT* promoters? Conversely, given the ubiquitous nature of the ETS factors, do other family members discriminate among their target genes using a different bipartite sequence? For example, the bipartite sequence identified by Bell et al. (2015) differs significantly from the proposed binding site in the *SDHD* promoter (Supplemental Fig. S7). Identifying the mechanisms controlling GABPA/B1 activity and expression likely will be important to understand *TERT* expression in these cells. Finally, *TERT* promoter mutations distinguish cancer cells from normal telomerase-expressing cells. Thus, from a translational point of view, full understanding of the mechanistic differences in the transcription of *TERT* among these cell types may provide a therapeutic approach or a biomarker for stratifying tumors.

Materials and methods

Cell lines

HCC lines and UMUC3 and T24 were obtained from American Type Culture Collection. UMUC3 and T24 were grown as described (Guin et al.

2014) except as noted below and were authenticated by the University of Colorado Cancer Center Protein Production Shared Resource using an Applied Biosystems Profiler Plus kit, which analyzed nine STR loci (Life Technologies, 4303326). After authentication, cells were frozen within 1–2 wk. Vials of cells were resuscitated <2 mo prior to being used in experiments in this study. All cells were cultured in DMEM (VWR Scientific) with GlutaminePlus (Atlanta Biologicals), 10% FBS (Seradigm), penicillin/streptomycin (GIBCO), glutamax (GIBCO), and sodium pyruvate (GIBCO), except HepG2 cells were cultured in EMEM (American Type Culture Collection) without sodium pyruvate.

RNA extraction and cDNA preparation

Following RNA extraction with Trizol (Life Technologies), reverse transcription was performed by treating of 10 µg of RNA with 5 U of RQ1 DNase (Promega) according to the manufacturer's protocol, followed by phenol extraction (pH 7), chloroform extraction, and then ethanol precipitation. The cDNA was generated from 2 µg of RNA synthesized using random hexamers, oligo$(dT)_{20}$, and SuperScript III (Life Technologies). Following treatment with RNase H (New England Biolabs), quantitative PCR was performed with iQ SYBR Green PCR mix (Bio-Rad) using a Roche LightCycler 480 with the program 10 min at 98°C, 30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C, and 5 min at 72°C, followed by quantification using the Roche LightCycler 480 software. Melt curve analyses were examined to ensure the uniformity of relevant PCR amplicons.

Primers for rs2736098 in UMUC3 and T24 were forward (5′-CGTGGT TTCTGTGTGGTGTC-3′) and reverse (5′-CCTTGTCGCCTGAGGAG TAG-3′). Primers for assessing HCT-116 *TERT* SNP (COSM3136609) were forward for both cDNA and genomic DNA (5′-GCAGGTGTA CGGCTTCGT-3′), reverse for genomic DNA (5′-CTCCTCACCTGG GCTCCT-3′), and reverse for cDNA (5′-CAGGATCTCCTCACGCAGA-3′). The heterozygous SNP in HCT-116 was first identified using the COSMIC cell line browser (Forbes et al. 2014).

ChIP

ChIP was performed as previously described (Schwartz et al. 2012; Davidovich et al. 2013) with the exceptions noted in the Supplemental Material. For immunoprecipitation, 5–25 µg of solubilized chromatin was used with 2 µg of α-RNA Pol II antibody (EMD Millipore, catalog no. 05-623), α-H3 (Abcam, ab-1791), α-H3K4me2/3 (Abcam, ab-6000), α-H3K27me3 (EMD Millipore, 07-449), or 4 µg of α-GABPα (Santa Cruz Biotechnology, H-180 sc-22810) and nutated overnight at 4°C.

All replicates reported in this study represent independent biological samples.

PCR and sequence analysis of the TERT promoter, TERT expression, and telomerase activity

Quantitative PCR for the *TERT* promoter was performed on a Roche LightCycler 480 using iQ SYBR Green PCR mix (Bio-Rad) with primers for the *TERT* promoter forward (5′-GTCCTGCCCCTTCACCTT-3′) and reverse (5′-AGCGCTGCCTGAAACTCG-3′), and the reaction was supplemented with 5% 7-deaza-2′-deoxyguanosine (Roche) using the program 10 min at 95°C, followed by 17 step-down cycles of 1 min at 95°C, 1 min at 70°C–54°C, and 2 min at 72°C; 40 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C; and 10 min at 72°C. Temperature ramp rates for primer annealing were 1°C/min and extension at 2°C/min. TERT mRNA expression (Borah et al. 2015) and telomerase activity (Stern et al. 2012) were analyzed as previously described. See the Supplemental Material for further details.

RNA-seq expression analysis

Total RNA was isolated from cell lines using Qiagen reagents and following the recommended protocol. RNA-seq libraries were constructed using TrueSeq RNA kit from Illumina according to the manufacturer's protocol. The major steps in the protocol were (1) depletion of rRNA with the use of probes complementary to rRNA sequences, (2) generation of cDNA, and (3) generation of next-generation sequencing libraries. The libraries were sequenced on a HiSeq 2500 (Illumina). The sequencing data were matched to the human reference genome version hg19 using the CASAVA pipeline (Illumina) with the ELAND algorithm set for RNA analysis. The expression profiles were compiled on a Genome Studio RNA expression module (Illumina) using reads that passed the chastity quality filter by Illumina. The data are reads per kilobase per million mapped reads. Data for each gene are normalized for the length of the transcripts.

Competing interest statement

T.R.C. is on the board of directors of Merck.

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