Supplementary Materials

Materials and Methods

Patient tissues and human cell lines

Patient tumor tissue samples were collected from various participating centers with approval from the Research Ethics Board of the Hospital for Sick Children (Toronto, Canada). Detailed description of the patient tumors including source tissue block (Frozen or FFPE), histology, and tumor grade information (if available) is shown in Supplemental File 1. DNA from patient tumor tissues were extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. Human cell lines were acquired through ATCC and cultured under specified conditions (ATCC).

Genomic DNA preparation and bisulfite PCR amplification

Genomic DNA was isolated from each sample (blood, tissue, or cell pellets) using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. Genomic DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research) in accordance to the manufacturer's protocol. HotStarTaq Plus Master Mix Kit (Qiagen) was used to PCR amplify target amplicons from bisulfite-converted DNA using primer sets completely devoid of CpG sites (Supplemental Table 1). For NGS using the MiSeq platform, the resulting PCR products were prepared in accordance to the 16S Metagenomic Sequencing Library Preparation guide (15044223 B, Illumina) and were sequenced following the manufacturer's instructions for the MiSeq Reagent Kit v3 (Illumina) to obtain single-end 125 nucleotide read lengths. For NGS using the HiSeq platform, the PCR products were first purified using the QIAquick Gel Purification Kit (Qiagen),

followed by sequencing library preparation using Nextera XT DNA Library Preparation Kit (Illumina) according to standard single-end sequencing protocol provided and sequenced following the manufacturer's instructions for the HiSeq 2500 Rapid Run (Illumina) mode to obtain 126 nucleotide read lengths.

Sequencing read alignment and methylation analysis

Sequence reads were identified using standard Illumina base-calling software. Adapter sequences were trimmed and sequencing reads containing at least one base with a Phred quality score below 20 were discarded prior to analysis. FastQ files were aligned against the reference genome (GRCh37/hg19) using BS-Seeker 2 (default parameters) with a mapping efficiency of above 96.6%. The methylation level of each sampled cytosine was calculated as the number of reads reporting a C, divided by the total number of reads reporting either a C or T.

Reporter gene expression analysis

All reporter gene expression analyses include data from experimental triplicates. The significance of reporter gene expression was assessed with unpaired t-test.

Functional analysis of THOR on *TERT* promoter activity

The effect of THOR on *TERT* promoter activity was assessed using Luciferase-based assay. 5' truncation analysis of *TERT* promoter was performed by PCR amplification of several fragments in ~100-bp increment (from the distal end of THOR, exact differences between each amplicon is outlined in Figure 3A). These fragments were ligated into a promoter-less *firefly* luciferase vector pGL4B (Promega). Finalized constructs were transformed in DH5α and amplified using QIAprep Spin MiniPrep Kit (Qiagen). Constructs were then transiently transfected into the cell lines using

PolyFect Transfection Reagent (Qiagen). Control plasmid pRL *Renilla* luciferase vector (Promega) was co-transfected to normalize the readings. After 24 hours of incubation, luciferase measurements were made using Dual-Glo® Luciferase Assay System (Promega) and a single-tube luminometer (Berthold Technologies).

Effect of *in vitro* methylation of THOR on *TERT* promoter activity

rTHOR was PCR amplified and cloned into multiple cloning site of a CpG-free reporter backbone vector (pCpGfree-promoter-Lucia (Invivogen)) using FastDigest BcuI (SpeI) and NsiI (Thermo Fisher Scientific). Constructs were transformed into *E. coli* containing PIR1 (One Shot™ PIR1 Chemically Competent *E. coli,* Invitrogen) and amplified using Qiaprep Spin MiniPrep Kit (Qiagen). The vector DNA was then exposed to *in vitro* methylation using *M. SssI* CpG Methyltransferase (NEB) for methylation of rTHOR region. Both un-methylated (control) and methylated forms of the construct were transiently transfected into HeLa and HT1080 cancer cell lines. After 48 hours, light signal produced by synthetic luciferase *Lucia* (CpG-free synthetic luciferase) was measured using QUANTI-Luc™ (Invivogen) and a single-tube luminometer (Berthold Technologies).

***TERT* expression analysis by quantitative real-time RT-PCR**

Total RNA was extracted from each tissue or cell line using Trizol reagent (Invitrogen) and 1 μg of total RNA was reverse transcribed to cDNA with Superscript III and IV (Invitrogen). Each cDNA sample (60ng) was analyzed in triplicate with LightCycler 480 system (Roche Applied Science) using SYBR Green Master Mix (Roche Applied Science) according to Manufacturer's instructions. The endogenous housekeeping gene *HPRT* was used as a normalization control. The

TERT expression levels of different tissues and cell lines were normalized to *TERT* levels observed in WI38 human fibroblast cells.

Detection of ALT phenotype

ALT was detected by screening for c-circles using two different detection methods. The first was detection of c-circles by dot blot as previously described (1) and the second was detection using a telomere-specific qPCR assay (2).

Quantitative and Statistical Analyses

Defining the THOR boundaries

Methylation levels of CpG sites within THOR were assessed using multiple sequencing platforms (Bisulfite pyrosequencing/NGS - HiSeq and MiSeq). The boundaries of THOR (located at chr5:1,295,321–1,295,753, GRCh37/hg19) were based on cut-off value of 22%. The methylation level average for normal samples started to exceed 22% at the distal end of THOR, while methylation level average for cancer cell lines started to fall below 22% at the proximal end of THOR.

Defining the THOR hypermethylation cut-off

We have calculated the cut-off for the THOR hypermethylation signature to be 16.1% THOR methylation by adding +2 SD of the average THOR (UTSS) methylation of the normal samples (7.9%, $n = 80$).

Bioinformatics analysis of THOR methylation

Using a modified version of interactive tool Methylation plotter (3), unsupervised clustering analyses of normal primary cell lines/tissues (n=43) and cancer cell lines (n=18) or tumor samples

(*n*=87) were performed. The significances of the mean differential methylation at each CpG site within THOR between normal primary cell lines/tissues and cancer cell lines or tumor samples were assessed using Methylation plotter (Kruskal-Wallis test).

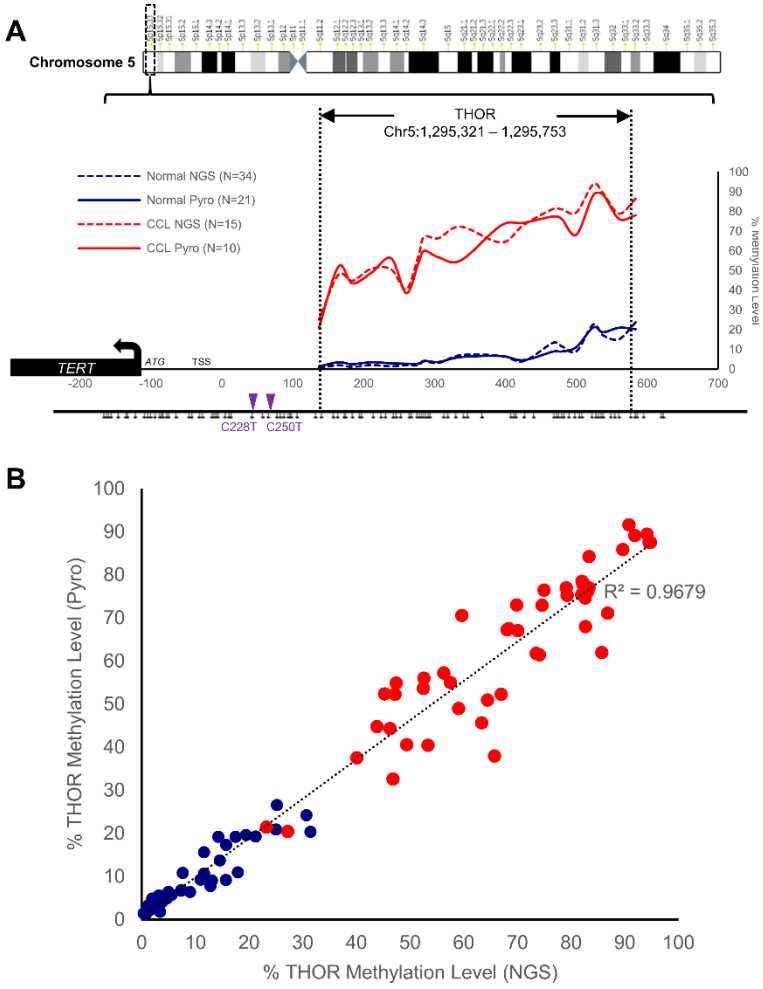
Statistical analysis

General statistical analyses were done with Fisher's exact test and chi-square test for categorical variables and two-tailed t-test (Graphpad Prism version 6.0) for continuous variables. Detailed statistical tests used in each experiment is described in each figure legends. P-values < 0.05 was considered statistically significant (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

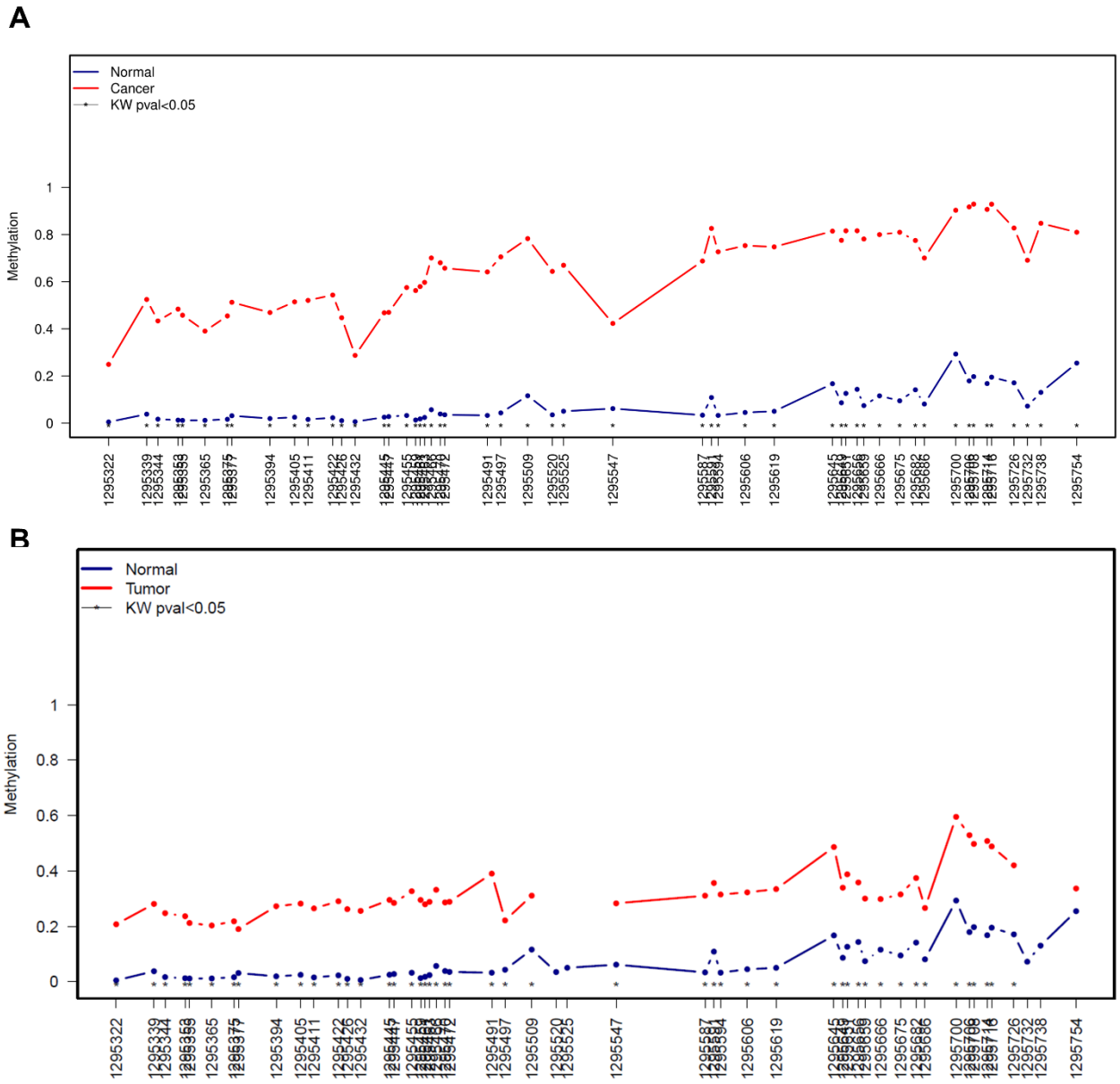
Supplemental References

1. Henson JD, et al. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. *Nat Biotechnol.* 2009;27(12):1181-5.
2. Lau LM, et al. Detection of alternative lengthening of telomeres by telomere quantitative PCR. *Nucleic Acids Res.* 2013;41(2):e34.
3. Mallona I, Diez-Villanueva A, and Peinado MA. Methylation plotter: a web tool for dynamic visualization of DNA methylation data. *Source Code Biol Med.* 2014;9:11.

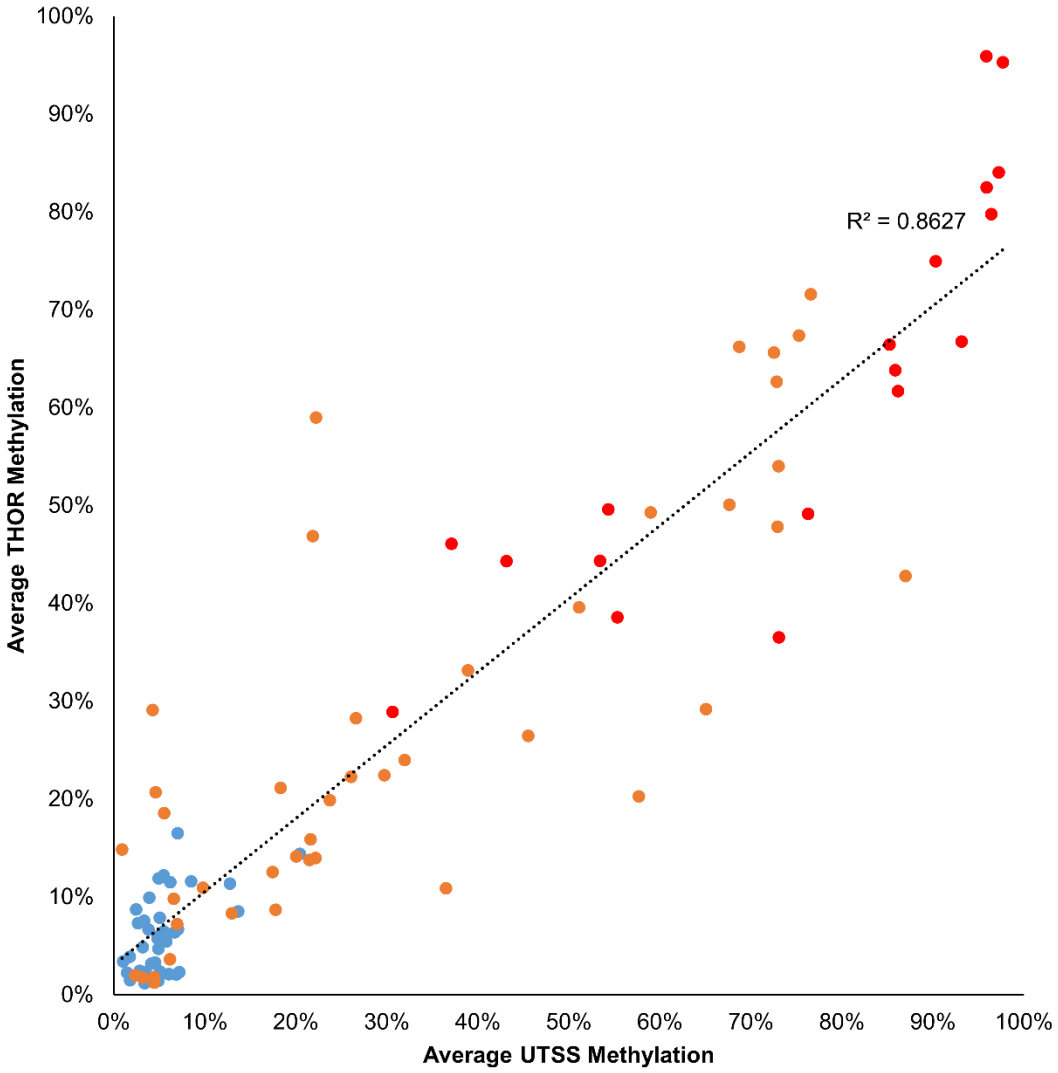
Supplemental Figures



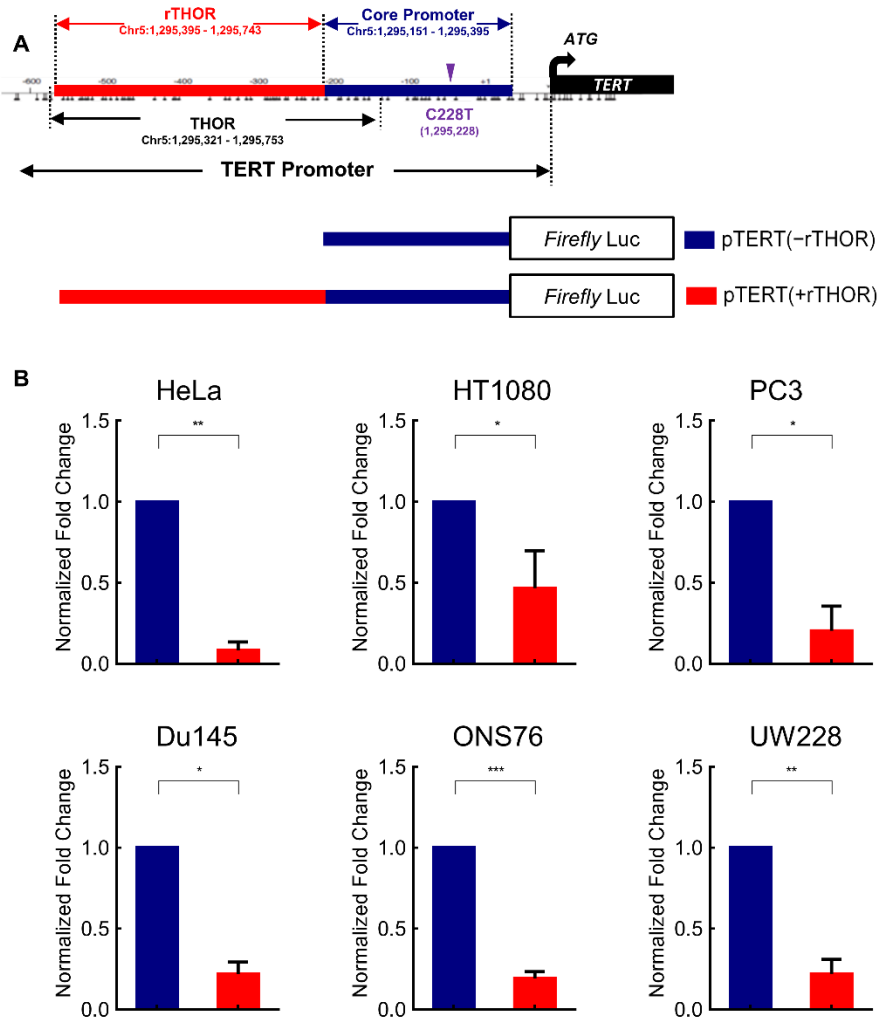
Supplemental Figure 1. Correlation between pyrosequencing and next-generation sequencing techniques for THOR methylation assessment. (**A**) Average DNA CpG methylation of THOR in normal cell lines/tissues (blue) and *TERT* expressing cancer cell lines (red) comparing data obtained through pyrosequencing (solid line) with data obtained through next-generation sequencing (dotted line). (**B**) Linear regression analysis displaying average methylation level at each of the 52 CpG sites (represented as a single data point) within THOR for normal samples (*n*=43) (blue) and cancer cell lines (*n*=18) (red) from data obtained with both pyrosequencing (Y-axis) and NGS (X-axis) techniques.



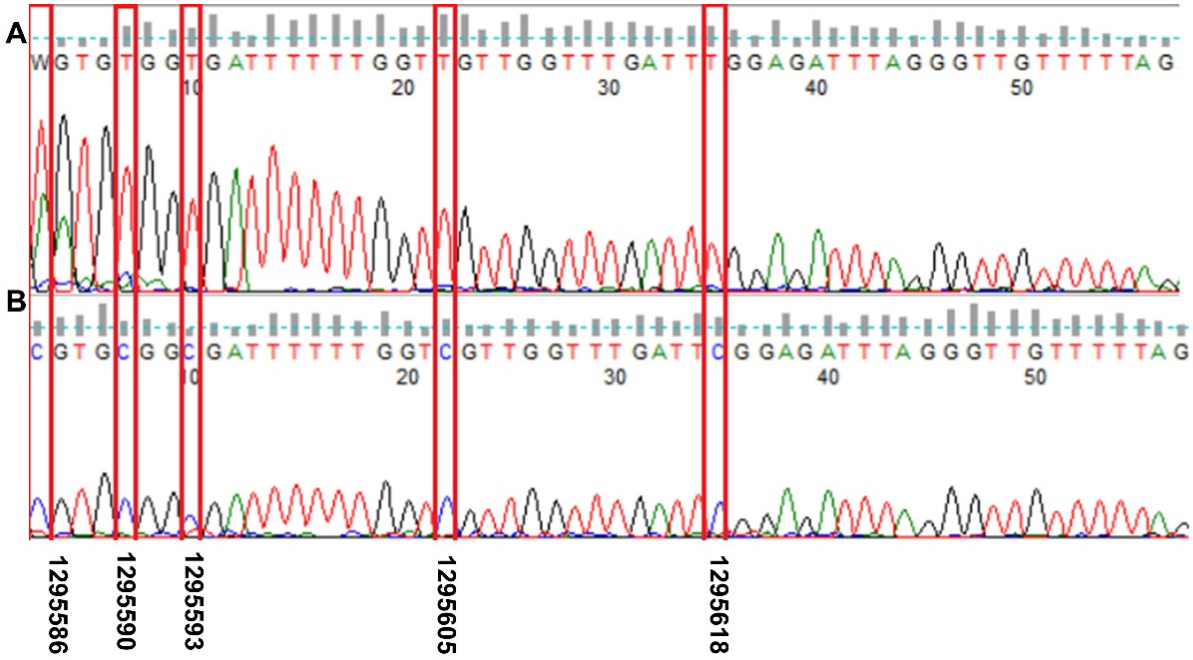
Supplemental Figure 2. Significance of differential methylation at individual CpG sites within THOR. Average methylation level at every CpG site within THOR between normal cell lines/tissues (*n*=43) (blue) and (**A**) cancer cell lines (*n*=18) (red) and (**B**) tumor samples (*n*=87) (red). Kruskal-Wallis test shows significant differences in CpG methylation throughout THOR (**P*<0.05). Numbers in x-axis indicate coordinates of CpG sites on chromosome 5 (GRCh37/hg19).



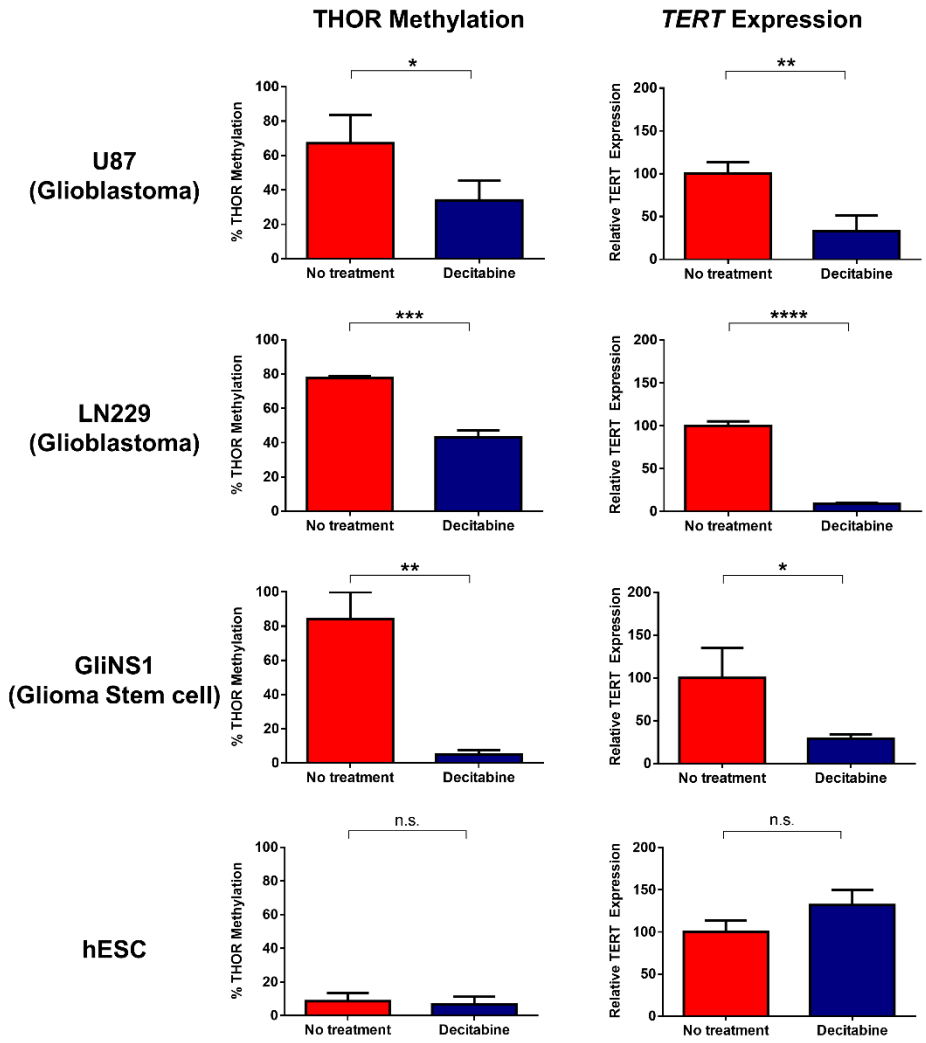
Supplemental Figure 3. Correlation between average THOR and UTSS methylation levels. Linear regression analysis displays the correlation between average UTSS methylation (5 CpG sites) (X-axis) and average THOR methylation (52 CpG sites) (Y-axis) for normal samples (blue), tumor samples (orange) and cancer cell lines (red).



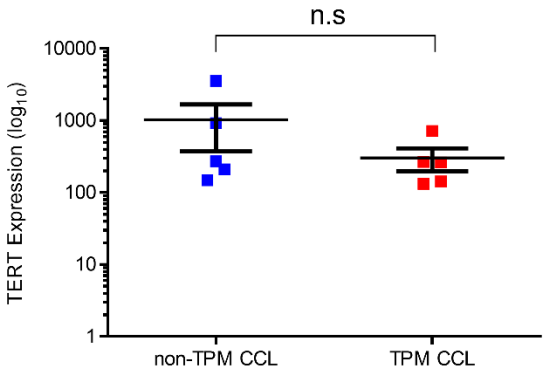
Supplemental Figure 4. Effect of unmethylated rTHOR addition on the reporter gene expression. For the data shown, each experiment was performed in triplicates. (**A**) Schematic representation of the *TERT* promoter is shown. Repressive THOR (rTHOR, red) is a transcriptional regulatory element located within THOR, upstream of the *TERT* core promoter (blue). (**B**) Normalized fold changes in *TERT* promoter activity are shown before (blue) and after (red) the addition of rTHOR to the *TERT* core promoter. (Unpaired *t*-test, **P*<0.05)



Supplemental Figure 5. Efficiency of *in vitro* methylation of rTHOR. (**A**) Bisulfite-sanger sequencing of the rTHOR from CpG-free vector when (A) untreated (pCpG(+rTHOR)) or (B) treated (pCpG(+rTHOR^{Meth})) with in vitro methyltransferase enzyme (M.sssl). Representative UTSS region (5 CpG sites) is shown in the chromatogram. Y-axis numbers indicate chromosomal coordinates of CpG sites (GRCh37/hg19). Methylated Cytosine at CpG sites are not converted into Thymine from bisulfite treatment.



Supplemental Figure 6. Correlation between THOR demethylation and reduced *TERT* expression in cancer cells. For the data shown, each experiment was performed in triplicates. Human glioma cell lines (U87 and LN229), glioma stem cells (GliNS1), and embryonic stem cell line (hESC) were treated with demethylating agent Decitabine (5-azacytidine). Bar graphs on the left represent THOR methylation levels before and after treatment, assessed using bisulfite pyrosequencing. The bar graphs on the right show consequent changes in *TERT* expression, normalized to the expression level of untreated control (Unpaired *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).



Supplemental Figure 7. Difference in *TERT* expression between TPM and non-TPM cancer cell lines that commonly exhibit THOR hypermethylation. Dot plot shows difference in *TERT* expression between cell lines with TPM (*n*=5) and without TPM (*n*=5) present, normalized to *TERT* expression of human primary fibroblast line WI38 (Unpaired *t*-test, mean±SEM).

Supplemental Table 1. List of primers used in PCR and sequencing

Name	Use	Primer Sequence (5' → 3')
TERT-Full	HiSeq	F: GGGAAGTGTTGTAGGGAGGTATT R: CCAACCCTAAAACCCCAAA
TERT+Full	HiSeq	F: TTAGTTTTGGGGTTTTAG R: AAAAAATATTACAAAAAAACACT
TERT+A1	MiSeq	F: GGAGGGGTTGGGAGGGTT R: CCTACCCCTTCACCTT
TERT+A2	MiSeq	F: AGTTGGAAGGTGAAGGGGTAGG R: AACTCCCAATAAATTC
TERT+A3	MiSeq	F: GAATTTATTGGGAGTT R: TCCCTACACCCTAAAAA
TERT+A4	MiSeq	F: GTTTAGGTTGTGGGGTAATT R: CTAAAAACAACCCTAAATC
pTERT-R	THOR functional analysis	CAGCGCTGCCTGAAACTC
pTERT-562-F	THOR functional analysis	CAATGCGTCCTCGGGTTC
pTERT-456-F	THOR functional analysis	GAGGCAGCCCTGGGTCTC
pTERT-347-F	THOR functional analysis	GGCCGATTCGACCTCTCT
pTERT-214-F	THOR functional analysis	CCGGGCTCCCAGTGGATT
pTERT-132-F	THOR functional analysis	GTCCTGCCCCTTCACCTT
TERT-Exp	*TERT* expression	F: GCCTTCAAGAGCCACGTC R: CCACGAACTGTCGCATGT