SUPPLEMENTARY MATERIAL

Methods

Representative formalin-fixed paraffin-embedded (FFPE) tissues from 23 individual melanocytic tumors were analyzed. Tumor tissues were isolated by manual microdissection guided by a corresponding H&E slide to obtain at least 50% lesional tissue for DNA extraction. The samples were obtained from 21 pediatric and 2 adult patients for whom follow-up data were available (Table S1).

TERT Promoter Amplicon Library Construction and Methylation Sequencing

Genomic DNA was isolated from FFPE tissue sections by using the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega) and converted by using the MethylEdgeTM Bisulfite Conversion System (Promega) as per manufacturer's protocol. DNA methylation analysis was performed by bisulfite PCR, using the GoTaq® Long PCR Master Mix (Promega) with primers forward GGGAAGTGTTGTAGGGAGGTATTT and reverse AAAACCATAATATAAAAACCCTAAA for 45 cycles at 95°C for 15 s, 53°C for 20 s, and 68°C for 30 s.

DNA libraries were prepared from individual amplicons (50 ng) by using the NextFlex DNA Sequencing kit (Bioo Scientific) and 0.6 μM high-throughput DNA barcodes (Bioo Scientific). Final libraries were checked individually for quality and quantification by quantitative PCR and an Agilent 2200 bioanalyzer. Equimolar concentrations of amplicon libraries were pooled, denatured, and sequenced on an Illumina MiSeq by using 300 cycle reagents (PE150; version 2) and a Nano sequencing kit.

Analysis of *TERT* Promoter, *BRAF,* and *NRAS* Mutations

Genomic DNA was extracted from FFPE tissue sections and screened for hotspot mutations in the *TERT* promoter, *BRAF* (exon 15), and *NRAS* (exons 2 and 3), as previously described (Lu *et al.*, 2015)

TERT mRNA In Situ Hybridization

mRNA ISH, a novel method to detect mRNA in FFPE tissues (Wang *et al.*, 2012), was performed for *TERT* mRNA on a Discovery Ultra automation system (Ventana Medical Systems, Inc.) by using RNAscope® VS Reagent Kit – RED (Advanced Cell Diagnostics). VS Probe – Hs-*TERT* (Cat#605516) specific to the sequence spanning nucleotide 2164 to 3231, encoding the *TERT* transcript, was applied according to the manufacturer's instructions. Briefly, 5-µm FFPE tissue sections were pretreated in citrate buffer with heat, followed by protease digestion before hybridization with the target oligo probes. The slides were hybridized sequentially with target probes incubated at 43°C for 2 h and 32 min, preamplifier at 53°C for 32 min, amplifier at 53°C for 32 min, and label probes at room temperature for 12 min. Between the hybridization steps, slides were washed with Ribowash buffer (0.1× saline sodium citrate). Hybridization signals were detected by chromogenic development with Fast Red, followed by counterstaining with hematoxylin. Each sample was quality controlled for RNA integrity with an RNAscope probe for PPIB RNA and for background with a probe for bacterial dapB RNA. The specific RNA staining signal was identified as intracellular red punctate dots. mRNA ISH was performed on FFPE sections from 2 melanomas in GCN and 2 proliferative nodules in GCN.

Bisulfite-Treated Read Alignment and Methylation Calls

The ends of reads with a quality score less than 20 were trimmed before adaptor removal. The adaptor matching part of the read was removed if it aligned with the Illumina adapter sequence by at least 1 bp and had a mismatch error rate of 0.1 or less. Reads were aligned to chromosome 5 by using the BSAMP2.74 with a maximum gap size of 3. Only uniquely aligning reads were reported (Xi and Li, 2009). To calculate the methylation ratio for a base position C at a CpG site, the base position needed to have at least a 5× coverage. The number of bisulfite-converted Cs (T, representing unmethylated Cs) and nonconverted Cs (C, representing methylated Cs) were recorded for each C position in a CpG context. The methylation ratio (Beta-value) at each position was calculated as the total number of reads with C divided by the total number of reads with C and T. The methylation status was defined as follows: >0.7, hyperemethylated (Figure 1, red); 0.5–0.7, partially methylated (orange); 0.3 to <0.5, partially unmethylated (cyan); and <0.3, unmethylated (blue).

RNA Sequence Read Alignment and Gene Expression Calculations

Paired-end reads from stranded RNA sequence were aligned to the following 4 database files by using BWA (0.5.10) aligner (Li and Durbin, 2010): (1) the human GRCh37-lite reference sequence, (2) RefSeq, (3) a sequence file representing all possible combinations of non-sequential pairs in RefSeq exons, and (4) AceView database flat file downloaded from the UCSC Genome Browser Database, which represented transcripts constructed from human ESTs. The mapping results from (2) to (4) were aligned to human reference genome coordinates. In addition, they were aligned by using STAR 2.3.0 to the human GRCh37-lite reference sequence without annotations. The final BAM file was constructed by selecting the best alignment among

the 5 mappings. HTSeq (Anders *et al.*, 2015) was used to count the number of fragments that mapped to each gene (Gencode v 15), where each gene is considered as the union of all its exons. The count was then normalized to fragments per kilobase of exons per million fragments mapped as the expression value of the gene. Further, the expression value was log2 transformed.

Statistical Analyses

Statistical analyses were performed using the R statistical package (http://www.r-project.org/ version 2.15.1). Logistic regression was used to compare the fraction of methylated Cs in the sequenced melanomas in GCN and the remaining samples, with age and gender as covariates. Linear regression was applied to evaluate the association between gene expression and *TERT* promoter methylation. A multivariate logistic regression analysis that considered age, gender, and disease subtype as covariates showed that the methylation ratio for the melanomas arising in GCN (n = 3) was significantly higher than that for the other subtypes (n = 20), with an estimated Beta-value of 2.6815 ($P = 2 \times 10^{-16}$).

Transcription Factors with Potential Binding Sites within the *TERT* Promoter

To identify which transcription factors have potential binding sites in the amplicon region of the *TERT* promoter, we used the data in TRANSFAC® (http://www.biobase-international.com) and the Transcription Factor ChIP-seq from ENCODE. Using the UCSC genome browser, we integrated the data to visualize the binding motifs for the transcription factors with potential binding sites in the region. Among several transcription factors whose binding sites overlaid in the bisulfite amplicon region, 3 were

previously known to repress the transcriptional activity of *TERT*, namely CTCF (Renaud *et al.*, 2007), SIN3A (Xu *et al.*, 2008), and MAZ (Xu *et al.*, 2013) (Figure S1).

References

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Xi Y, Li W (2009) BSMAP: whole genome bisulfite sequence MAPping program. *BMC Bioinformatics* 10:232.

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Xu M, Luo W, Elzi DJ*, et al.* (2008) NFX1 interacts with mSin3A/histone deacetylase to repress hTERT transcription in keratinocytes. *Mol Cell Biol* 28:4819-28.

Supplementary Figures

Figure S1

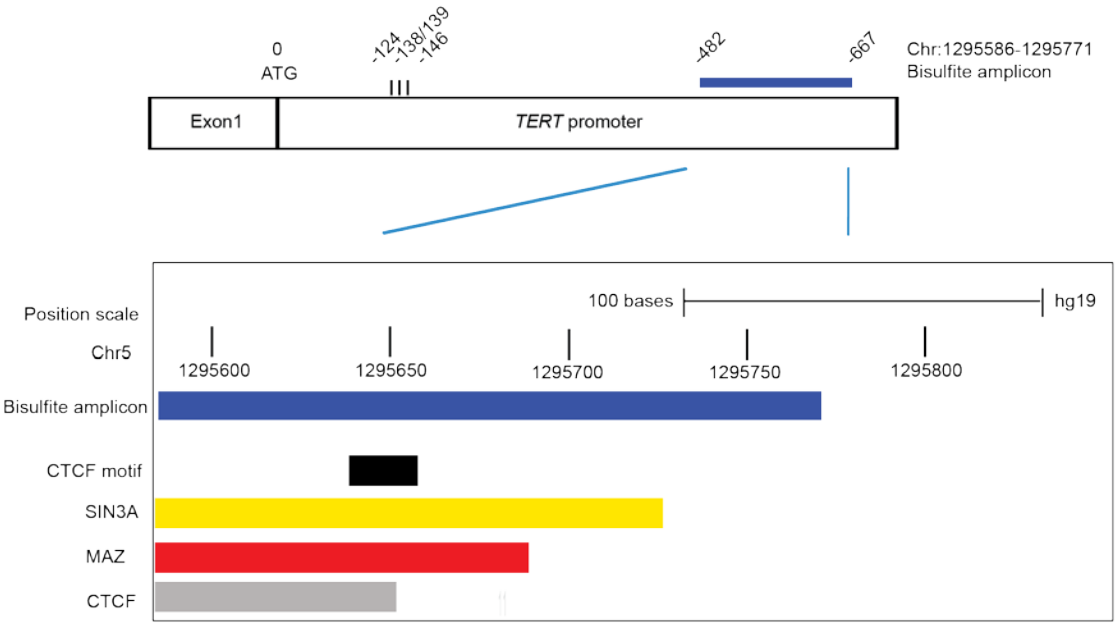

Figure S1−Schema of the *TERT* promoter and the putative transcriptional repressor binding sites. The *TERT* promoter (top panel) and the potential binding sites for transcription repressors (bottom panel). The top panel from left to right shows the position of hotspot mutations C228T at –124, CC242/243TT at –138/–139, and C250T at –146 from the ATG start site, and the position of the bisulfite amplicon sequence (blue bar). The amplicon sequence encompasses 26 CpG ([chr5:1295586−1295771 (GRCh37/hg19)] from nucleotide positions –482 to –667, according to the ATG start site. Potential transcription binding sites in the bisulfite amplicon were analyzed by using TRANSFAC® and the Transcription Factor Chip-seq data from ENCODE. The binding sites for 3 transcriptional repressors SIN3A, MAZ and CTCF overlay within the bisulfite amplicon region. By using the UCSC genome browser, the position of the CTCF motif (black bar) was visualized.

Figure S2

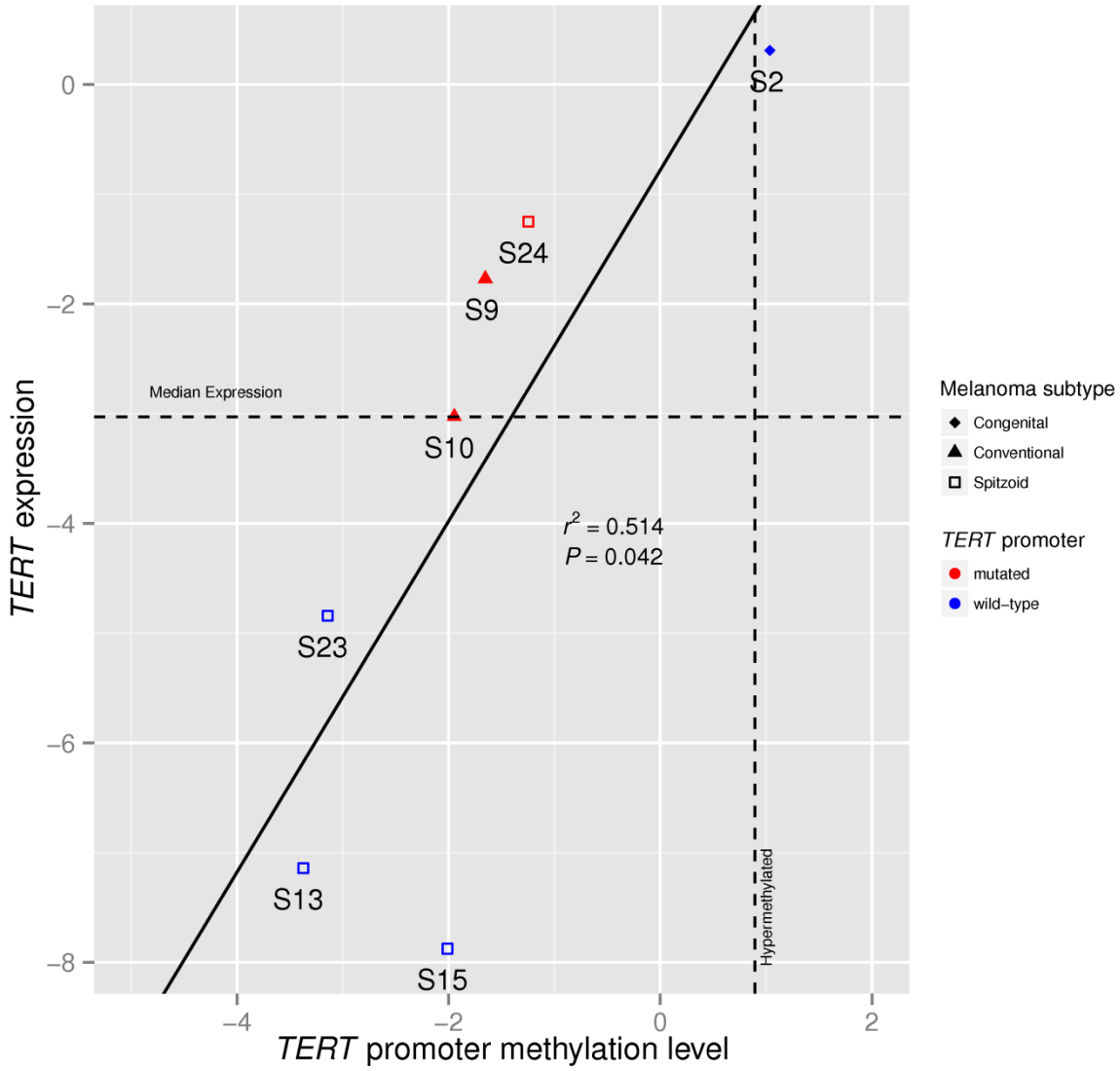

Figure S2– Association between *TERT* expression on the RNA sequence read and the *TERT* promoter methylation level. A strong positive correlation was observed between *TERT* expression and *TERT* promoter methylation level (adjusted r^2 = 0.5145, P = 0.042). S, sample identification number.

Supplementary Tables

Table S1– Demographic data for the 23 patients with melanocytic neoplasms.

Sample no.	Age	Sex	Primary site	Sample site	Outcome	F/U time (months)
S1	5 y	M	trunk	Primary	DOD	14
S2	3 y	M	scalp	Primary	DOD	8
S4	19 y	F	face	metastatic	DOD	36
S5	16 y	M	face	metastatic	DOD	45
S6	15 y	F	trunk	metastatic (LN)	AWD	61
S7	17 y	F	trunk	metastatic (LN)	AWD	31
S8	14 mo	F	trunk	Primary	NED	62
S9	16 y	M	scalp	metastatic	DOD	70
S10	14 y	F	unknown	metastatic	DOD	21
S11	1 mo	F	trunk	Primary	NED	372
S13	2 y	M	lower extremity	Primary	NED	8
S14	9 y	M	ear	Primary	NED	11
S15	4 y	F	trunk	Primary	NED	40
S16	2 y	M	lower extremity	Primary	NED	72
S18	48 y	F	scalp	metastatic (LN)	DOD	25
S19	11 y	F	lower extremity	metastatic (LN)	DOD	24
S20	4 y	F	lower extremity	Primary	NED	13
S21	7 y	M	trunk	Primary	DOD	25
S22	38 y	F	upper extremity	metastatic (LN)	DOD	36
S23	13 y	F	lower extremity	Primary	NED	17
S24	14 y	M	trunk	metastatic	DOD	18
S26	5 y	F	trunk	Primary	NED	6
S27	15 y	F	trunk	Primary	NED	36

Abbreviations: DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease; LN, lymph node; y, years old; mo, months old

Table S2– Methylation ratios (Beta-value) in the range of 0 to 1 at each CpG site for the 23 melanocytic tumors.

chr	C position in CpG context (CpG site)	S1	S2	S4	S5	S6	S7	S8	S9	S10	S11
5	1295586	1	0.76	0	0	0	0.061	0	0.043	0.556	0
5	1295590	1	0.68	0	NA	0.242	0	0	NA	0	NA
5	1295593	0.042	0.48	0	0	0	0.061	0	0.12	0	0
5	1295605	1	0.692	0.286	0	0	0.086	0	0.074	0	0
5	1295618	NA	0.714	NA	NA	NA	0.216	NA	0.071	NA	NA
5	1295644	1	0.515	0.273	0.271	0.258	0.278	0	0.163	0.154	0
5	1295648	1	0.694	0	0.266	0.231	0.158	0	0.167	0.233	0.034
5	1295650	1	0.692	0	0.284	0.284	0.288	0.023	0.319	0.424	0
5	1295655	1	0.722	0	0	0.108	0.353	0	0.107	0.353	0
5	1295658	1	0.722	0	0	0.054	0.088	0	NA	0.059	0
5	1295665	1	0.722	0	NA	0.316	0.182	0	0.107	0.059	0
5	1295674	1	0.737	0	0.353	0.243	0.323	0	0.25	0.235	0
5	1295681	1	0.737	0	0.406	0.378	0.387	0	0.214	0.111	0
5	1295685	1	0.722	0	0	0.25	0.233	0.1	0.074	NA	0
5	1295699	1	0.647	0	0.312	0.472	0.467	0.15	0.222	0.353	0
5	1295705	1	0.706	0	0.533	0.343	0.379	0.053	0.269	0.353	0
5	1295707	1	0.765	0.167	0.333	0.6	0.429	0.105	0.346	0.412	0
5	1295713	1	0.647	NA	0.533	0.306	0.148	0.053	0.333	0.059	0
5	1295715	1	0.706	0	0.333	0.057	0.556	0.053	0.538	0.412	0.091
5	1295725	1	0.647	0	0	0.371	0.296	0	0.37	0.118	0.273
5	1295731	1	0.562	0	0	0.171	0.192	0	0.222	0	0
5	1295737	1	0.625	0	0.357	0.343	0.385	0	0.385	0.267	0
5	1295753	1	0.688	0	0.28	0.355	0.48	0.053	0.4	0.333	0
5	1295759	1	0.625	0	0.32	0.172	0.083	0.053	0.292	0	0
5	1295761	1	0.625	0	0.261	0.552	0.417	0.053	0.5	0.154	1
5	1295771	1	0.714	0.188	0.263	0.56	0.5	0.118	0.316	0.083	0

Table S2– Cont'd

CpG site	S13	S14	S15	S16	S18	S19	S20	S21	S22	S23	S24	S26	S27
1295586	0	0.029	0.02	0.095	0.433	0	NA	0.824	0.722	0	0.13	0	0.053
1295590	NA	0.108	NA	0	0.469	0	0.105	0.765	0.722	0.045	0.087	0	0.05
1295593	0.024	0.051	0.038	0.048	0.471	0	0.143	0.824	0.611	0	0.261	0	0.05
1295605	0	0.062	0.018	0	0.417	0	0.087	0.824	0.722	0	0.16	0	0.05
1295618	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.148	NA	0.048
1295644	0.38	0.1	0.118	0.2	0.491	0	0.167	0.773	0.68	NA	0.258	0	0.125
1295648	0.04	0.036	0.09	0.086	0.475	0	0.139	0.741	0.581	0	0.235	0	0.172
1295650	0.027	0.093	0.3	0.081	0.474	0	0.079	0.759	0.677	0	0.306	0	0.323
1295655	0	NA	0.169	0.333	0.419	0	0	0.769	0.312	0.125	0.235	0	0.267
1295658	0.029	0.095	0.133	0	0.323	0	0	0.769	0.438	0.062	0.235	0.125	0.267
1295665	0.088	0.214	0.246	0.062	0.438	0	0.067	0.615	0.625	0.133	0.235	0.067	0
1295674	0.111	0.238	0.115	0	0.412	0	0	0.769	0.588	0.214	0.353	0	0.067
1295681	0.056	0.3	0.222	0	0.441	0	0.059	0.786	0.529	0.143	0.353	0	0.333
1295685	0.028	0.125	0.169	NA	NA	0	0	0.786	0.529	0	0.235	0	0
1295699	0.083	0.317	0.345	0.312	0.548	NA	0.056	0.786	0.733	0.143	0.471	0	0
1295705	0.029	0.3	0.283	0.438	0.5	0	0.056	0.643	0.6	0.357	0.529	0.125	0.2
1295707	NA	0.375	0.396	0.125	0.531	0	NA	0.786	0.8	0.5	0.471	0.125	0.2
1295713	0.086	0.19	0.321	0.294	0.471	0	0.056	0.643	0.6	0	0.444	0.125	0.062
1295715	0.088	NA	NA	0.438	NA	0	0.412	0.786	0.467	0.429	NA	0.062	NA
1295725	0.083	0.167	0.154	0.294	0.353	0	0.056	0.786	0.75	0	0.312	NA	0.062
1295731	0.143	0.025	0.113	0	0.324	0	0	0.786	NA	0.077	0.125	0	0.067
1295737	0.029	0.368	0.28	0.167	0.485	0	0.118	0.786	0.438	0.083	0.375	0	0.2
1295753	0.406	0.188	0.4	0.118	0.562	0	0	0.714	0.733	0.083	0.6	0.312	0.308
1295759	0.031	0.103	0.163	0	0.406	1	0.067	0.786	0.467	0.25	0.267	0.067	0.071
1295761	0.062	0.241	0.317	0.529	0.667	0	0.133	0.786	0.733	0.417	0.467	0.067	0.143
1295771	NA	0.364	0.371	0.214	0.68	0	0.2	0.846	0.667	0	0.615	0	0

Abbreviations: Chr, chromosome; NA, not available.

Table S3– The total number of methylated Cs and unmethylated Cs in the sequenced region for each sample.

Sample no	M	UM	Gender	Age	Disease
S1	528	23	M	2to10	Melanoma in GCN
S2	365	178	M	2to10	Melanoma in GCN
S21	301	92	M	2to10	Melanoma in GCN
S4	23	484	F	10to20	Other
S5	175	617	M	10to20	Other
S6	248	701	F	10to20	Other
S7	220	629	F	10to20	Other
S8	16	567	F	<2	Other
S9	165	519	M	10to20	Other
S10	89	343	F	10to20	Other
S11	16	330	F	<2	Other
S13	79	819	M	2to10	Other
S14	163	847	M	2to10	Other
S15	259	1044	F	2to10	Other
S16	69	395	M	2to10	Other
S18	381	435	F	>20	Other
S19	11	329	F	10to20	Other
S20	41	405	F	2to10	Other
S22	262	163	F	>20	Other
S23	42	371	F	10to20	Other
S24	148	351	M	10to20	Other
S26	17	414	F	2to10	Other
S27	56	381	F	10to20	Other

Abbreviations: M, number of methylated Cs in the region (26 CpGs); UM, number of unmethylated Cs in the region (26 CpGs); GCN, giant congenital nevus