

Multiple ETS Factors Participate in the Transcriptional Control of *TERT* Mutant Promoter in Thyroid Cancers

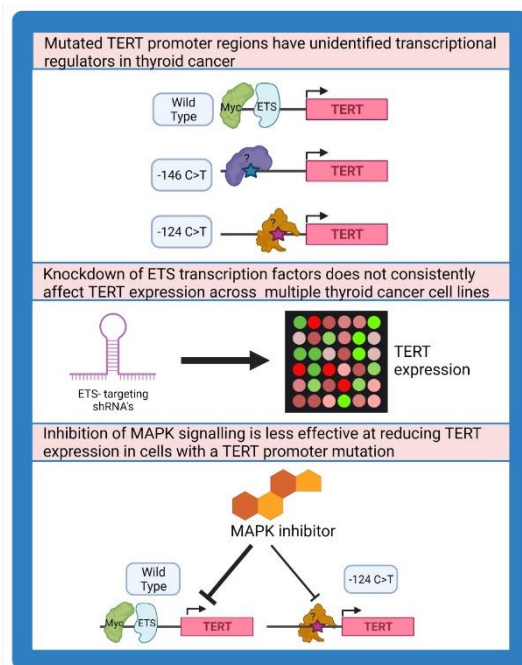
Caitlin E.M. Thornton^{1,2,#}, Jingzhu Hao^{1,2,#}, Prasanna P. Tamarapu³ and Iñigo Landa^{1,2,*}

1. Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts;
 2. Harvard Medical School, Boston, Massachusetts.
 3. Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, New York;
- # These authors contributed equally to this work
- * Correspondence: ilanda@bwh.harvard.edu;

Abstract

Hotspot mutations in the *TERT* (telomerase reverse transcriptase) gene are key determinants of thyroid cancer progression. *TERT* promoter mutations (TPM) create *de novo* consensus binding sites for the ETS ("E26 transforming sequence") family of transcription factors. In this study, we systematically knocked down each of the 20 ETS factors expressed in thyroid tumors and screened their effects on *TERT* expression in seven thyroid cancer cell lines with defined TPM status. We observed that, unlike in other TPM-carrying cancers such as glioblastomas, ETS factor GABPA does not unambiguously regulate transcription from the *TERT* mutant promoter in thyroid specimens. In fact, multiple members of the ETS family impact *TERT* expression, and they typically do so in a mutation-independent manner. In addition, we observe that partial inhibition of MAPK, a central pathway in thyroid cancer transformation, is more effective at suppressing *TERT* transcription in the absence of TPMs. Taken together, our results show a more complex scenario of *TERT* regulation in thyroid cancers compared to other lineages, suggest that compensatory mechanisms by ETS and other regulators likely exist and advocate for the need of a more comprehensive understanding of the mechanisms of *TERT* deregulation in thyroid tumors before eventually exploring TPM-specific therapeutic strategies.

Graphical Abstract



Introduction

Mutations in the proximal promoter of *TERT* (telomerase reverse transcriptase) are the first genomic lesions in a gene regulatory region and display a high prevalence across multiple cancer types (1). They were first identified in cutaneous melanoma (2, 3), and subsequently reported in numerous malignancies (4), including thyroid carcinomas (5, 6). *TERT* promoter mutations (TPMs) are observed with increasing frequency according to the severity of thyroid cancer (7, 8). Thus, only 9% of the papillary thyroid cancers (PTC), a predominant subtype with overwhelmingly good prognosis, harbor TPMs, and these are typically subclonal within the tumor (9). In contrast, we reported that TPM prevalence is much higher in advanced thyroid tumors, which account for most of the morbidity and mortality of the disease: they occur in 40% of poorly differentiated (PDTC) and 73% of anaplastic thyroid cancers (ATC) (8). Within each thyroid tumor subtype, TPMs associate with more aggressive features, particularly in combination with constitutive activation of the MAPK (“mitogen-activated protein kinase”) pathway, usually via BRAF^{V600E} driver mutation (8, 10, 11). Overall, this stepwise increase in frequency implies that clonal TPMs confer a strong selective advantage as PTC progress to PDTC/ATC.

TPMs occur at either c. -124 C>T or c.-146 C>T in a mutually exclusive fashion. These single nucleotide substitutions generate an identical 11-nucleotide stretch creating *de novo* binding

sites for the ETS (“E26 transforming sequence”) family of transcription factors, which comprises 27 members with diverse functions and tissue-specific roles (12). As a result, *TERT* transcription, which is typically silenced in adult tissues, is reactivated in cells carrying TPMs. The seminal papers reporting TPMs in melanoma pointed at an ETS-mediated role on *TERT* mutant promoter control (2, 3), but the identification of the specific factors involved in this regulation, particularly in thyroid cancers, has proven to be not straightforward. Nevertheless, distinguishing which ETS (and other) factors are important in the reactivation of *TERT* in aggressive thyroid tumors is a necessary first step towards enabling new methods of blocking TERT expression, and ultimately inhibiting cell growth, in a cancer-specific manner.

Several studies in *TERT*-mutant glioblastomas demonstrated that GABPA, an ETS factor acting as a tetrameric protein along with its GABPB isoform, drives *TERT*-mutant transcription in this tumor lineage, opening the door to targeting the GABPA/GABPB axis to achieve TERT downregulation in that context (13-15). The former prompted the *a priori* attractive idea of GABPA also being the key player in *TERT*-mutant regulation across TPM-harboring thyroid cancers (16). However, several other ETS members, including ELK1 and ETV1/4/5, have been proposed to control *TERT* transcription in thyroid specimens (11, 17, 18), suggesting that promiscuity and/or compensatory mechanisms among ETS, and possibly other proteins, exist. A recent study showed that GABPA has tumor suppressor properties in thyroid tumors, rendering this factor an undesirable target for inhibition in this lineage (19). In addition, some ETS proteins, as well as other MAPK-mediated factors such as Sp1 and c-Myc, are also able to bind and control *TERT* wildtype promoter (20-23).

So far, most thyroid cancer studies aimed at identifying mechanistic underpinnings of *TERT* mutant control have either focused on single ETS factors in several cell lines or screened multiple factors in a limited number of lines. Here we adopted an agnostic approach, screening all ETS factors that are expressed in thyroid tumors in numerous, fully characterized, thyroid cancer cell lines. Our results point to a greater complexity of TPM-mediated regulation of *TERT* transcription from what was previously reported, and caution against adopting what has been demonstrated in other cancer types carrying TPMs into thyroid tumors. Furthermore, we observed that MAPK blockade was more efficient at suppressing TERT transcription in cells without TPMs, suggesting that a switch in *TERT*-mutant control might operate in thyroid

cancers carrying TPMs. Overall, we believe that only those observations that are generalizable to all thyroid cancers harboring TPMs will help exploiting them as potential targets to implement tumor-specific epigenetic inhibition of this *bona fide* cancer gene.

Materials and Methods

Analysis of public expression datasets

Normalized transcriptomic data from The Cancer Genome Atlas (TCGA) evaluation of PTC (9) was either downloaded from publicly available repositories (http://firebrowse.org/?cohort=THCA&download_dialog=true) or retrieved from our published studies on PDTC, ATC and thyroid cancer cell lines (8, 24). Expression data for the specific 27 factors from the ETS family was recovered from these datasets and plotted accordingly.

Cell culture

The following authenticated thyroid cancer cell lines, which we recently characterized, were used in this study (24). Cell lines were cultured in RPMI (Cal62, MDAT41, CUTC5, BCPAP, SW1736, T238) or DMEM (TCO-1, Hth7, K1 and U251) medium, supplemented with 10% FBS, penicillin, streptomycin and L-glutamine. BHT101 cells were cultured in MEM with 20% FBS, penicillin and streptomycin. All cell lines were passaged at approximately 80% confluency and maintained in a 37°C humidified incubator with 5% CO₂.

Gene silencing

We evaluated the effect of ETS silencing on TERT transcription via stable gene silencing. We individually silenced each of the 20 ETS factors which are expressed in thyroid cancers. To this end, we used the TRC collection of hairpins from the Genetic Perturbation Platform at the Broad Institute (<https://portals.broadinstitute.org/gpp/public/>). Short hairpin RNA (shRNA) clones were obtained from the MSKCC Gene Editing and Screening core in either the pLKO.1 or pLKO_TRCN005 plasmid backbones. Specific shRNA clones and target sequences used in our study are listed in Supplementary Table S1. Briefly, bacterial glycerol stocks were grown and purified using EndoFree Plasmid Kits (Qiagen). Lentiviral production was performed co-transfecting 293-FT packaging cells with individual pLKO_shETS plasmids, psPAX2 (packaging plasmid) and pMD2.G (envelope plasmid) using FuGENE (Promega) or Lipofectamine3000

(ThermoFisher Scientific) and following standard protocols. Target cells were subsequently infected with these lentiviruses and selected under 1 μ g/ml puromycin for at least one week. A total of 22 shRNAs against ETS factors were used: one for each of the 20 ETS factors, with the exception of ETS2 and GABPA, for which a mixture of equal amounts of two hairpins targeting different regions of the same gene achieved better silencing than individual hairpins (see Supplementary Table S1).

Real time quantitative PCR

Cells were lysed using TRIzol reagent and RNA was isolated with chloroform then precipitated from the aqueous phase using isopropanol. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative PCR was performed on a Roche LightCycler® 480 II. SYBR™ Select Master Mix (Applied Biosystems) for measuring Actin and ETS factors. TaqMan™ Gene Expression Master Mix (Applied Biosystems) and TERT TaqMan Assay (Applied Biosystems, Hs00972650_m1) were used to measure TERT expression. Cells infected with pLKO_shScramble constructs were used as reference for each experiment. Specific forward and reverse primers used for these reactions are available in Supplementary Table S2. Expression of ETS and TERT was normalized to beta-actin (*ACTB*) housekeeping gene using the delta-delta Ct method.

MAPK inhibition experiments

We ran MAPK inhibition experiments using the FDA-approved MEK inhibitor trametinib and RAF inhibitor dabrafenib (both obtained from Selleckchem). We calculated IC₅₀ values for each cell line to account for inter-specimen variability to MAPK inhibition and make our comparisons accurate. For IC₅₀ calculations, 50,000 cells were seeded in 6 well-plates, six conditions per treatment in triplicates (trametinib: 0nM (DMSO), 1nM, 2nM, 5nM, 10nM, 30nM; dabrafenib: 0nM, 5nM, 10nM, 20nM, 40nM, 80nM.). Cells were counted after incubation for 72 hours and IC₅₀ values were calculated using linear regression. For MAPK inhibition treatment experiments, cells were seeded using media with 1% FBS into 4cm dishes at a density of 4 x 10⁵ cells per plate. After 24 hours, the cells were treated with trametinib only (Cal62, KRAS^{G12D}-mutant) or trametinib plus dabrafenib (MDAT41, BCPAP and BHT101; all three BRAF^{V600E}-mutant) at the IC₅₀ dose determined for each cell line. After 6 hours, cells were harvested in total lysis buffer

with protease and phosphatase inhibitors (TRIS pH 7.4 10mM, NaCl 50mM, MgCl₂ 2mM, 1% SDS, Sigma P5726, P0044, P8340) for protein analysis, and in TRIzol for RNA isolation.

Western blotting

Total protein concentrations were measured by Pierce BCA (ThermoFisher Scientific). Equal amounts of proteins were run on NuPAGE 4 to 12%, Bis-Tris protein gels (ThermoFisher Scientific) and subsequently transferred onto PVDF membranes (ThermoFisher Scientific) by wet electroblotting. Transfer efficiency was checked with Ponceau staining. MAPK inhibition was evaluated via phospho-ERK antibody (Cell Signaling Technology, #4292). Equal protein loading was controlled with p85 antibody (Cell Signaling Technology, #4370). Antibodies were diluted 1:1000 in 5% BSA in Tris-buffered saline with 0.1% Tween20, incubated overnight at 4°C, and then probed for one hour at room temperature with a 1:3000 dilution of anti-rabbit (Cell Signaling Technology, #7074) IgG HRP-linked antibody and developed using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific).

Statistical analysis

All statistical analyses were performed using GraphPad Prism, version 9.2.0 (GraphPad Software, Inc.). Expression data for selected ETS factors is presented as the mean ± standard deviation from at least three independent experiments. Statistical differences between cell lines with ETS factor silencing against scrambled control lines and between DMSO and drug-treated samples, were analyzed by unpaired students t-tests. Schematic figures were generated using BioRender software and images were exported under a paid subscription.

Results

ETS factors show a wide range of expression in thyroid cancer specimens but remain comparable across thyroid cancer types

To assess which ETS factors are relevant in the control of *TERT* promoter in thyroid cancer specimens, we adopted an unbiased approach, evaluating their reported binding preferences and expression patterns in thyroid tumors and cell lines (Figure 1). We first compared the known consensus binding sequences for each of the 27 factors in the ETS family (25) against the

de novo sites created by TPMs in the gene promoter (5'-CCGGAA-3'; underlined adenines are created by G>A mutations (reverse strand) at TERT -124 and -146). ETS factors from classes III and IV showed binding preferences (5'-GGA-3' and 5'-GGAT-3', respectively) that likely render them less dependent to *de novo* sites created by TPMs (Figure 1, top).

We then leveraged transcriptomic data from The Cancer Genome Atlas (TCGA) analysis of PTC (9), as well as our own evaluation of PDTC, ATC and thyroid cancer cell lines (8, 20). As shown in Figure 1, seven ETS factors, including FEV, ETV2, ELF5 and all from classes III (SPI1, SPIB, SPIC) and IV (SPDEF), were not expressed in thyroid specimens, ruling them out from having any role in *TERT* promoter control in thyroid cells. Conversely, factors such as ETS1 and ETV5 showed high and consistent expression across thyroid cancer types, whereas most ETS proteins, including GABPA, were expressed at intermediate levels. Overall, these analyses narrowed the

list of candidate ETS factors regulating *TERT* transcription in thyroid tumors down to 20 members.

Screening of 20 ETS in cell lines shows variable effects on *TERT* expression

Taking the list of 20 ETS factors expressed in thyroid cell lines and tumors, we employed short-hairpin RNAs (shRNA) targeting each factor individually and generated knocked-down stable cell lines (Figure 2A and Table 1). Initially, four thyroid cancer cell lines were employed: TCO1, SW1736 and C643, which all harbor *TERT* c.-124 C>T mutation, and Cal62, which was used as a control for a wild-type *TERT* promoter. In these cell lines, stable knocking down of specific ETS

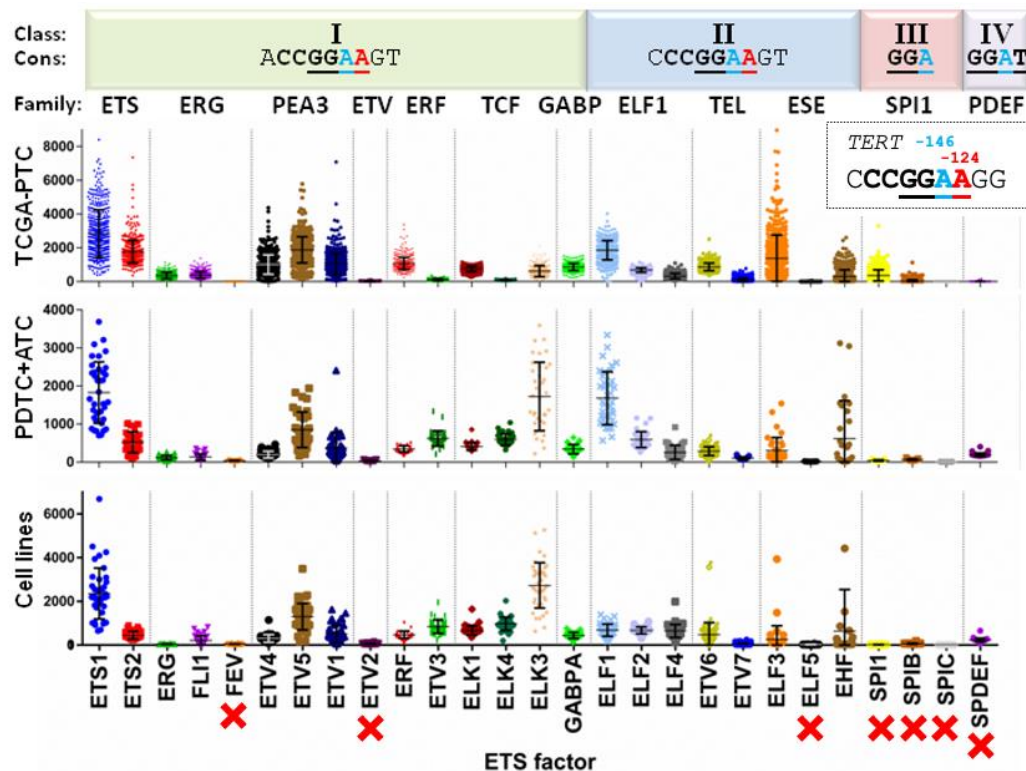


Figure 1 General classification and expression patterns of ETS family of transcription factors in thyroid cancer specimens. ETS factors are divided in four (I-IV) classes (top row) based on their binding sequence preferences (second row). Consensus (“Cons”) sites are displayed, with core sequences shown in bold and the *de novo* ETS binding sites created by *TERT* promoter mutations at -124 and -146 highlighted in red and blue font, respectively (both C>T transitions, shown in the reverse strand in the dotted line box). The third row lists the names of the 12 ETS families, based on the presence of other protein domains. Graphs show the normalized expression levels from published thyroid cancer datasets, including PTC from the TCGA study (top panel, (9)), and our data on PDTC+ATC (middle, (8)) and thyroid cancer cell lines (bottom, (20)). The seven ETS factors not expressed in thyroid cancers are indicated with red crosses. Abbreviations: ETS= E26 transforming sequence; TCGA= The Cancer Genome Atlas; PTC= Papillary Thyroid Cancer; PDTC= Poorly Differentiated Thyroid Cancer; ATC= Anaplastic Thyroid Cancer.

factors had a variable effect on *TERT* expression when compared to Scrambled shRNA cells (Figure 2B, left panel, and Supplementary Figure S1). We reasoned that ETS factors that are

specifically recruited to the *de novo* ETS site formed by the -124 C>T mutation would modify TERT expression in TCO1, SW1736 and C643 cells while Cal62 cells would remain unchanged. However, this pattern was not observed. Indeed, multiple factors exerted an effect on TERT transcription in a manner that was not always evidently TPM-mediated. Indeed, ELK1, ELK3, ELK4, ELF3, ELF4, ERF and ETV1 knockdown resulted in significant reductions in TERT expression in at least 2 out of 3 *TERT*-mutant cell lines. Of those, only ELK4, ELF4 and ETV1 did not affect TERT expression in the Cal62 cell line (*TERT* wildtype), whereas the other four did so. To further study the effects of these factors in an extended panel of cell lines, we selected the aforementioned seven ETS proteins, along with EHF and ETS2, which showed marginally significant reductions of TERT expression, and GABPA, which had been reported in other cancer lineages. Interestingly, in our experiments, GABPA knockdown produced more variable results, and did not consistently result in a reduction in TERT levels as previously described upon short-term disruption to GABPA expression.

Table 1 Name, genetic background and other features of the cancer cell lines used in this study.

Cell line	Tissue derivation	MAPK driver	TERT promoter	Use
TCO1	Thyroid, ATC	BRAF V600E	c.-124C>T, HOM	1 st screen, 20 ETS
SW1736	Thyroid, ATC	BRAF V600E	c.-124C>T, HET	1 st screen, 20 ETS
C643	Thyroid, ATC	HRAS G13R	c.-124C>T, HET	1 st screen, 20 ETS
Cal62	Thyroid, ATC	KRAS G12R	Wild type	1 st screen, 20 ETS + MAPKi experiments
BCPAP	Thyroid, PTC	BRAF V600E	c.-124C>T, HET	2 nd screen, 10 ETS + MAPKi experiments
BHT101	Thyroid, ATC	BRAF V600E	c.-124C>T, HET	2 nd screen, 10 ETS + MAPKi experiments
T238	Thyroid, ATC	BRAF V600E	c.-124C>T, HET	2 nd screen, 10 ETS
MDAT41	Thyroid, PTC	BRAF V600E	Wild type	Extended screen, GABPA + MAPKi experiments
CUTC5	Thyroid, PTC	BRAF V600E	Wild type	Extended screen, GABPA
K1	Thyroid, PTC	BRAF V600E	c.-124C>T, HET	Extended screen, GABPA

Hth7	Thyroid, ATC	NRAS Q61L	c.-146C>T, HOM	Extended screen, GABPA
U251	Glioblastoma	Unknown	c.-124C>T, HOM	Extended screen, GABPA

Abbreviations: PTC= Papillary Thyroid Cancer; ATC= Anaplastic Thyroid Cancer; HET= Heterozygous; HOM= Homozygous; ETS= E26 transforming sequence; MAPK= Mitogen-activated protein kinase; MAPKi= MAPK inhibition

We subsequently screened the selected 10 ETS factors that showed considerable reduction on TERT expression in three additional cell lines: BHT101, BCPAP and T238 (Figure 2C, right panel). From these data, knockdown of ETV1, ELK1, and ERF consistently resulted in downregulation of TERT expression across all or most cell lines appraised, with varying degrees of magnitude. In many cases where knockdown of an ETS factor resulted in a downregulation of TERT expression, a correlating reduction in TERT was observed in the Cal62 control cell line. Therefore, these observations may not represent factors that specifically regulate the mutant TERT promoter.

Of note, we confirmed the functional knock down of ETS factors at the RNA level in these same specimens (Figure 2C and Supplementary Figure S1). In 85% of these stable cell lines there was at least a 50% reduction in expression of the targeted ETS factor compared to the Scrambled shRNA control. Knockdown by shRNA was notably less effective in SW1736 cell line for unknown reasons.

Overall, unlike in other lineages, the systematic knockdown of ETS proteins specifically expressed in thyroid cell lines and tumors did not point to control of the mutant *TERT* promoter by one or more specific ETS factors. The former suggests a remarkable complexity of ETS-mediated regulation of *TERT*

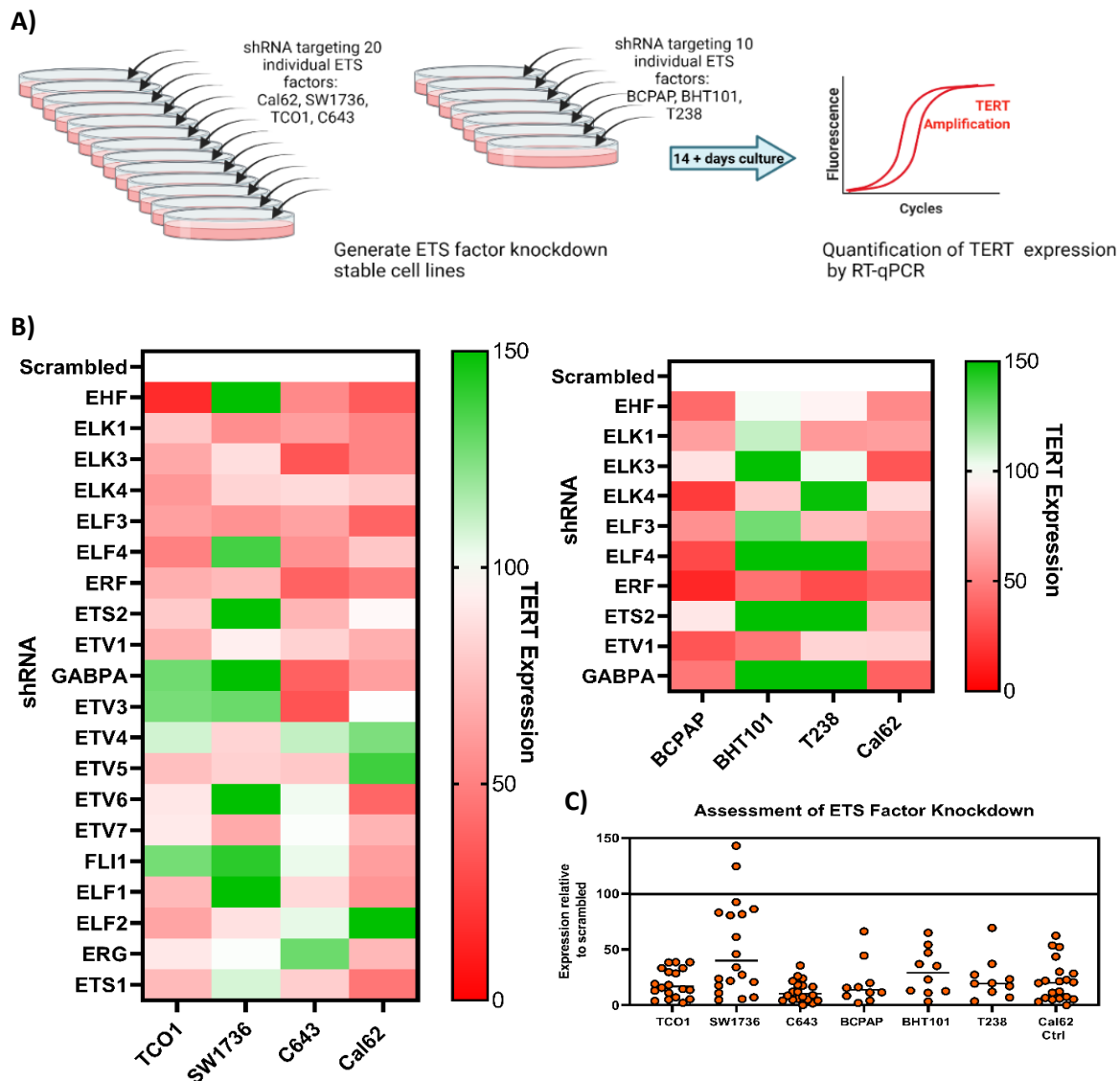


Figure 2 (A) The experimental design was setup such that twenty ETS factors were targeted by shRNA in four cell lines: TCO1, SW1736, C643 and Cal62. Ten ETS factors were targeted by shRNA in three further cell lines: BCPAP, BHT101 and T238. TERT expression was quantified by TaqMan qPCR. **(B)** Heatmap demonstrating relative changes in TERT expression compared to cells with shRNA targeting scrambled control in all cell lines. **(C)** Targeting of individual ETS factors by shRNA resulted in 85% cell lines with more than 50% reduction in expression of the targeted ETS factor.

promoter and points to potential mechanisms of transcription factor promiscuity and/or compensation beyond those already reported.

GABPA knockdown does not affect TERT expression across thyroid cancer cell lines carrying TPMs.

Given the prior body of evidence that pointed towards GABPA being a key regulator of mutant *TERT* promoter in glioblastoma, the effect of GABPA knockdown on *TERT* expression was further tested across a comprehensive range of thyroid cell lines harboring wild-type *TERT* promoter sequences, and heterozygous or homozygous TPMs. In this analysis the U251 glioblastoma cell line was also included as a positive control. GABPA was silenced using short-hairpin RNAs and cells were cultured for at least two weeks in selection media to observe the effect of sustained knockdown. Overall, GABPA knockdown did not show a consistent genotype-dependent effect on *TERT* expression across cell lines. The wild-type *TERT* promoter cell line Cal62 demonstrated a significant decrease in *TERT* expression upon GABPA knockdown against the paired scrambled shRNA control, whereas MDAT41 and CUTC5, also wild-type for TPMs, did not (Figure 3A). In comparison, the cell lines that are heterozygous for a *TERT* promoter mutation did not show a significant reduction in *TERT* expression (Figure 3B). In TCO-1 and Hth7 cells, which are homozygous for the *TERT* promoter mutation -124 C>T and -146 C>T, respectively, and which

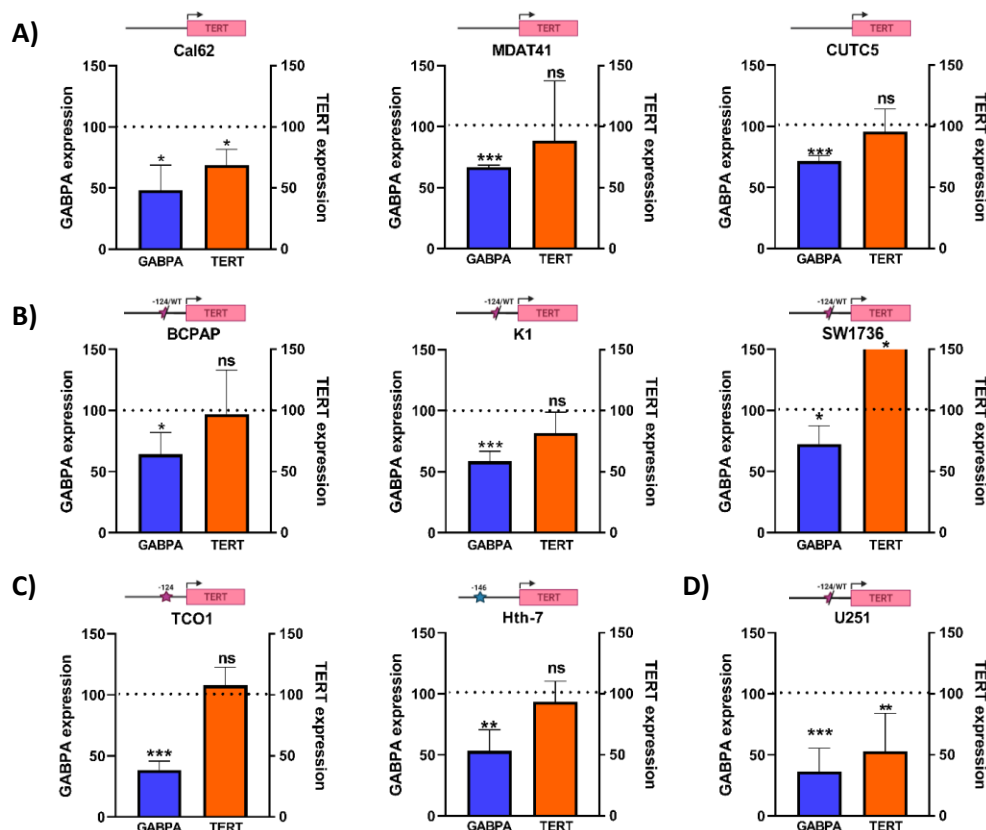


Figure 3 Knockdown of ETS factor GABPA did not result in consistent changes to *TERT* expression across multiple thyroid cancer cell lines. Stable knockdown of GABPA was generated in a total of eight thyroid cancer cell lines and one glioblastoma cell line (U251) and *TERT* expression was analyzed by RT-qPCR then normalized to Actin expression. Cal62, which acted as a non-*TERT* promoter mutation control, and U251 glioblastoma cells demonstrated a significant reduction in *TERT* expression. All other cell lines did not exhibit a significant reduction in *TERT* expression. Cells were cultured for at least two weeks prior to RNA extraction to appraise the longer-term effects of GABPA knockdown on *TERT* expression. All results are derived from at least triplicate experiments; ns = non-significant; * = $p < 0.05$; ** = $p < 0.005$, *** = $p < 0.001$.

should theoretically facilitate GABPA binding, showed no effects on TERT expression upon GABPA knockdown (Figure 3C). This was not a result of poor knockdown efficiency as TCO-1 and Hth7 demonstrated sustained and consistent reduction of GABPA expression (average decrease of 61.5% and 46.8% respectively; Figure 3C). In contrast, continued GABPA knockdown in U251 glioblastoma cells confirmed the previously shown significant reduction in TERT expression (Figure 3D).

Taken together, these data caution against GABPA being a universal regulator of the mutant *TERT* promoter in thyroid cancer cells and suggest targeting GABPA over a sustained period is unlikely to result in a consistent reduction of TERT expression in this tumor type.

Inhibiting MAPK signaling primarily affects expression of TERT in cells with wild-type TERT promoter

Considering the known role of MAPK signaling in regulating wild-type TERT expression, we sought to explore the effect of MAPK blockade in thyroid cancer cells with or without TPMs and to assess whether it cooperates with ETS to control TERT transcription. To this end, we treated cells with FDA-approved dabrafenib (RAF inhibitor) and/or trametinib (MEK inhibitor), depending on their driver mutation (BRAF^{V600E} or oncogenic RAS). To ensure accurate comparisons across cell lines with variable MAPK outputs, IC₅₀ values for trametinib and dabrafenib were first determined, and each cell line was treated at its IC₅₀ (Figure 4A). Cell lines were transduced with short-hairpin RNAs targeting the ETS factors: ETV1, ELK1 and GABPA and cultured under puromycin selection media for at least two weeks prior to treatment to ensure sustained RNA knockdown (Figure 4A). After treatment at the IC₅₀ dose with the appropriate MAPK inhibitors, Cal62 and MDAT41 cells demonstrated a strong reduction in TERT expression across all shRNA conditions. Comparatively, BHT101 and BCPAPs were refractory to this treatment and did not show significant reductions in TERT expression at their IC₅₀ doses. Although this may point to a partial switch of TERT regulation away from MAPK towards other transcription factors such as ETS factors in TPM-harboring cells, TERT expression was not further reduced upon knockdown of either ETV1, ELK1 or GABPA in BHT101 or BCPAP cells. Functional knockdown of the MAPK pathway was confirmed after the short-term treatment as shown by diminished phospho-ERK1/2 levels (Figure 4C). Overall, our experiments did not show cooperativity between ETS- and MAPK-mediated control of TERT transcription but pointed to a shift of *TERT* mutant promoter regulation.

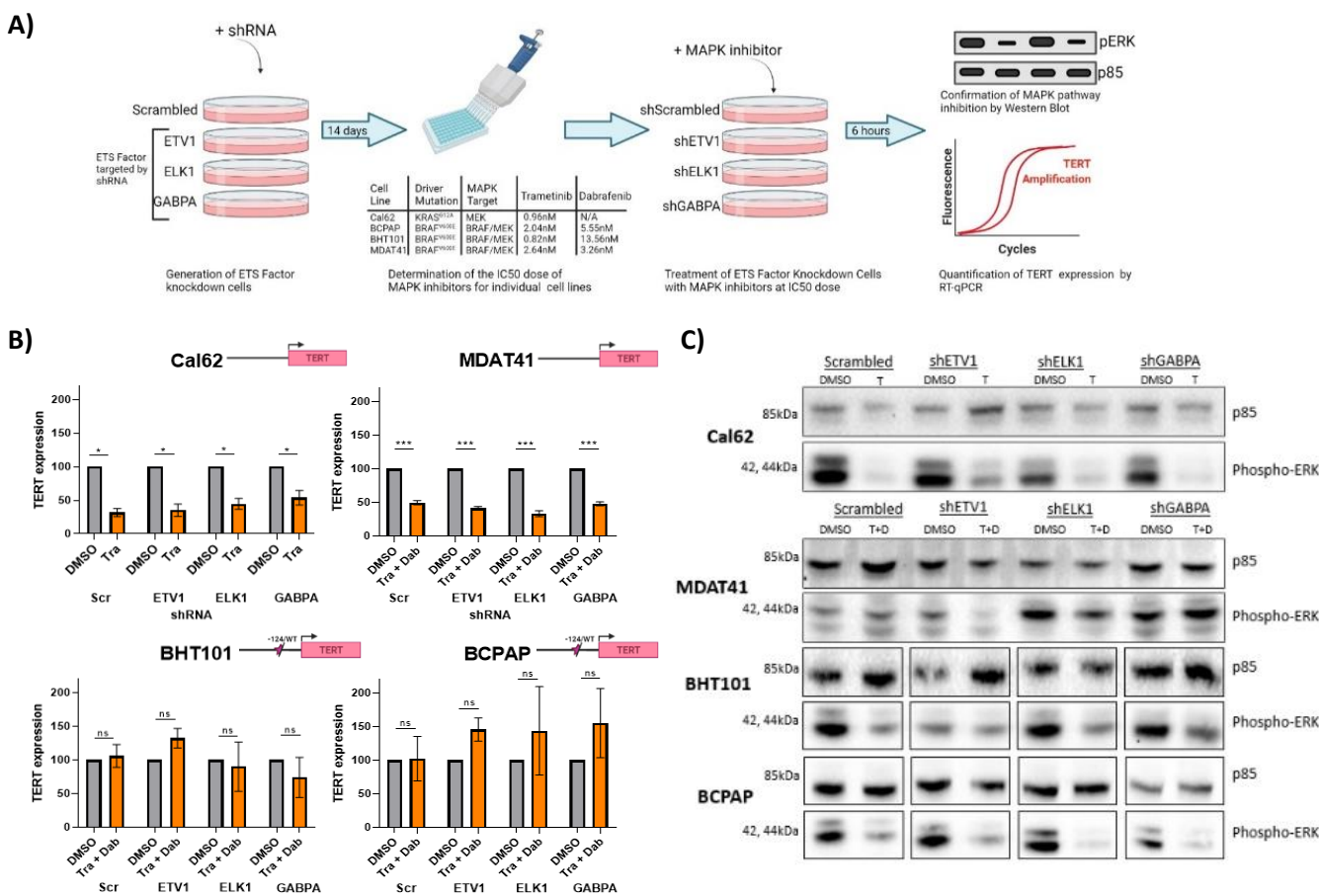


Figure 4 (A) knockdown cell lines were generated using shRNA targeting ETV1, ELK1 and GABPA. The IC₅₀ value for Trametinib (Cal62 0.96nM; BHT101 2.04nM; BCPAP 0.82nM) and Dabrafenib (BHT101 5.55nM; BCPAP 13.56nM) was calculated by cell viability assay and shRNA cell lines were treated at the IC₅₀ dose. Protein and RNA were extracted to confirm pathway inhibition and assess effect on TERT expression, respectively. Created with Biorender.com (B) RT-qPCR analysis demonstrated a significant reduction in TERT expression across all Cal62 and MDAT41 ETS knockdown cell lines. BHT101 and BCPAP did not demonstrate a reduction in TERT expression after treatment with Trametinib and Dabrafenib for six hours. (C) Western blotting confirmed functional inhibition of the MAPK pathway after treatment with Trametinib or Trametinib + Dabrafenib using phospho-ERK as a biomarker. Samples were run on one membrane per cell line and BCPAP and BHT101 images were arranged for a consistent loading order. ns = non-significant; * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.001$

Discussion

In this study, we comprehensively assessed the ability of the ETS family of transcription factors to control TERT expression in thyroid cancer cells with and without TPMs. We also evaluated the *TERT* promoter genotype-dependent role of MAPK signaling in TERT expression in these same specimens. Our findings suggest a multi-layered pattern of regulation, likely more complex than that described in glioblastoma cells carrying TPMs. In our cell systems, multiple ETS factors regulated TERT expression in a manner that is not exclusive of the presence of TPMs. We also observed that TPMs likely render thyroid cancer cells less dependent to MAPK-

mediated control of *TERT* transcription, whereas pharmacological inhibition of MAPK signaling effectively suppressed *TERT* mRNA levels in the absence of TPMs.

The discovery of hotspot mutations in the *TERT* core promoter, first in metastatic melanomas and subsequently in aggressive tumors of multiple lineages, revitalized the interest in understanding the mechanisms of telomerase reactivation in cancer. The fact that the two non-overlapping c.-124C>T and c.-146C>T mutations create *de novo* consensus binding sites for the ETS family of transcription factors made them excellent candidates to be controlling TPM-mediated *TERT* re-expression (2-4). The former prompted to the search of which ETS factors bind mutant *TERT* promoter as a first step towards exploring the idea of targeting *TERT* mutant transcription.

Several studies, typically using siRNA silencing, reporter assays and ChIP approaches, on glioblastoma cells carrying TPMs demonstrated that the ETS factor GABPA controls *TERT* mutant promoter expression. Bell and colleagues sequentially knocked-down thirteen ETS transcription factors in two glioblastoma lines and showed that GABPA selectively reduces *TERT* transcription in the presence of the c.-124C>T mutation. They extended their observations to three other TPM-carrying tumor types: melanomas, hepatocellular and urothelial carcinomas (13). This same group elegantly showed that GABPA acts as a tetramer with their beta-isoform (GABPB), and that GABPβ1L disruption reverses replicative immortality in glioblastoma cells with TPMs (15). Interestingly, another group showed that glioblastoma cells carrying the alternative hotspot mutation (*TERT* c.-146C>T) are bound by ETS factors ETS1/2, which cooperate with non-canonical NF-κB signaling via p52 to reactivate mutant *TERT* (26). They subsequently showed that GABPA is also able to upregulate mutant *TERT* in glioblastoma and melanoma cells, but it does so, at least partially, via long-range chromatin interactions (14). Overall, GABPA seems a solid candidate to explore TPM-specific epigenetic blockade of *TERT* re-expression in glioblastoma, but its role in thyroid cancers remains uncertain.

In this regard, the capacity of GABPA to bind to mutant *TERT* promoters in thyroid cancer was reported through ChIP assays on the K1 papillary thyroid carcinoma cell line (16). The authors suggested that FOS cooperates with GABPA in *TERT* mutant control. Our results show that GABPA stable silencing does not universally reduce *TERT* transcription in *TERT*-mutant thyroid cancer cells, and that it also controls *TERT* wildtype promoter (e.g., in Cal62 cells). In addition, GABP

seems an unattractive target for inhibition in thyroid cancers due to its recently reported tumor suppressor role (19).

Our study shows that multiple ETS factors are able to control *TERT* mutant transcription, suggesting that no clear (GABPA-like) candidate exists in this lineage, and that compensatory mechanisms across ETS proteins likely operate. Our work confirms some of the observations and further expands the findings reported by other thyroid cancer groups. Of note, Bullock and colleagues nicely showed that both ELK1 and ETV5 are able to bind *TERT* promoter in discrete thyroid cell lines, and that they do so in cooperation with thyroid transcription factor FOXE1 (17, 18). A separate study reported that ETV1, ETV4 and ETV5 preferentially bind *TERT* in various thyroid cell lines carrying c.-124C>T mutation (11). Taken together, our exhaustive screen of all 20 ETS proteins expressed in thyroid cells show that no unequivocal ETS factor is responsible for *TERT* mutant control. We show that sustained knockdown of specific ETS over weeks, at most, moderately impacts TERT levels. This is a key difference with other studies, which assessed TERT knockdown transiently, not allowing for the likely compensatory mechanisms that other factors exert in our cells, and probably in actual tumor specimens re-expressing telomerase.

Our work also explored the role of MAPK pathway blockade, via pharmacological inhibition by dabrafenib and/or trametinib, in TERT transcription. We observed that at low doses (low nanomolar range), MAPK inhibition was very effective at suppressing TERT mRNA levels in cells without TPMs, but not in their *TERT*-mutant counterparts. Although we acknowledge the preliminary nature of this observation, our results point to a potential partial switch of *TERT* mutant regulation, which might have been missed by other works employing MAPK inhibitors in the micromolar range, which fully suppressed this pathway (16, 18). Studying the cooperation between ETS- and MAPK-mediated regulation in TERT transcription is inherently difficult due to their roles in the absence of TPMs. This is because *TERT* wildtype promoter has native ETS sites, as well as binding motifs for Sp1 and c-Myc, both MAPK-activated factors that control TERT expression (20-23). In addition, MAPK signaling is able to activate discrete ETS factors either via transcription (27) or phosphorylation (28), which would feed into both wildtype and mutant *TERT* regulation. However, in the absence of unambiguous ETS proteins controlling *TERT* mutant expression, the TPM-specific role of MAPK input remains elusive.

Beyond the regulation by the ETS/MAPK axis, *TERT* promoter has been shown to be subjected to other regulatory inputs, some of which might be modified by the presence of TPMs, whereas

others likely operate as determinants of *TERT* baseline transcriptional repression. Although this is beyond the scope of this paper, the role of genomic insulator CTCF, which likely determines long-distance interactions (29), as well as the contribution of DNA methylation and allele-specific histone marks, which were recently characterized in thyroid cancer cell lines (18, 30, 31), are worth noting. Overall, these add to the idea of a tightly regulated, multi-faceted control of *TERT* transcription, which only becomes unchecked during cancer transformation. Incidentally, it is possible that some of the variability in ETS-binding is determined by specific co-factors and chromatin states that might respond to other signaling inputs. Other differences are likely attributable to the stable nature of the ETS silencing used here (vs. transient approaches in other studies), which likely allows cancer cells to enhance compensatory mechanisms. Overall, we believe that our observations, together with the remarkable efforts from other colleagues, have exhausted the possibilities of finding unequivocal, mutation-specific, ETS factors controlling *TERT* in thyroid cancers, and we caution against the generalization of discrete ETS findings from other tumor lineages. In the future, a less ETS-centric vision of *TERT* promoter control will be required to fully understand this process. In this regard, our ongoing research directions involve exploring ways of assessing the proteomic landscape of this genomic region in defined genetic states using isogenic models.

Conclusions

In conclusion, our data suggest that the ETS-mediated regulation of *TERT* mutant promoter in thyroid cancers likely differs from that reported in other tumors carrying TPMs in the same hotspots. Epigenetic targeting of mutant *TERT* transcription remains an attractive, yet ambitious, goal due to the high prevalence of TPMs in advanced tumors and the cancer-specific nature of this approach. However, we believe that the former will first require a detailed understanding of the transcriptional regulation of *TERT* mutant promoter specifically in thyroid specimens. To achieve this, a precise role of the mechanisms of compensation across different ETS factors, the integration with other inputs (e.g., other regulators activated by MAPK and/or other pathways) and ultimately a factor-agnostic approach, ideally in cancer isogenic models, which assesses the epigenetic reconfiguration around *TERT* mutant promoter, will be required.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1: Supplementary Figure S1: Examples of ETS and TERT silencing assessment in thyroid cancer cell lines; Supplementary Figure S2: Results from the shRNA knockdown screen of ETS factors ELK1, ETV1 and GABPA in seven thyroid cancer cell lines; Supplementary Table S1: List of short hairpin RNA (shRNA) clones; Supplementary Table S2: List of primers used for quantitative PCR.

Author Contributions: Conceptualization, I.L.; methodology, C.E.M.T., J.H., P.P.T. and I.L.; formal analysis, C.E.M.T., J.H. and I.L.; investigation, C.E.M.T., J.H., P.P.T. and I.L.; data curation, I.L.; writing—original draft preparation, C.E.M.T., J.H. and I.L.; writing—review and editing, C.E.M.T., J.H., and I.L.; supervision, I.L.; funding acquisition, I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Cancer Institute Career Transition Award, grant number 1K22CA230381–01A1 (I.L.) and the Brigham and Women’s Hospital, Department of Medicine, Innovation Evergreen Fund (I.L.).

Data Availability Statement: This manuscript includes gene expression analyses performed on openly available genomic data repositories, including the TCGA study of papillary thyroid carcinoma (http://firebrowse.org/?cohort=THCA&download_dialog=true) and the Gene Expression Omnibus dataset #GSE76039 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76039>; previously generated by us (8, 24)).

Acknowledgments: The cell lines used in this study were a generous gift from Dr. James Fagin lab at Memorial Sloan Kettering Cancer Center in New York. We also thank Dr. Fagin for the support in the early stages of this project.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results

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