Mechanisms regulating DMTF1β/γ expression and their functional interplay with DMTF1α

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Abstract. The cyclin D binding myb-like transcription factor 1 (*DMTF1*), a haplo-insufficient tumor suppressor gene, has 3 alternatively spliced mRNA isoforms encoding DMTF1α, β and γ proteins. Previous studies have indicated a tumor suppressive role of DMTF1α and the oncogenic activity of DMTF1β, while the function of DMTF1γ remains largely undetermined. In the present study, the mechanisms regulating DMTF1 isoform expression were investigated and the functional interplay of DMTF1β and γ with DMTF1α was characterized. It was found that specific regions of DMTF1β and γ transcripts can impair their mRNA integrity or stability, and thus reduce protein expression levels. Additionally, DMTF1β and γ proteins exhibited a reduced stability compared to DMTF1α and all 3 DMTF1 isoforms were localized in the nuclei. Two basic residues, K52 and R53, in the DMTF1 isoforms determined their nuclear localization. Importantly, both DMTF1β and γ could associate with DMTF1α and antagonize its transactivation of the ARF promoter. Consistently, the ratios of both DMTF1β/α and γ/α were significantly associated with a poor prognoses of breast cancer patients, suggesting oncogenic roles of DMTF1β and γ isoforms in breast cancer development.

Introduction

The cyclin D binding myb-like transcription factor 1 (DMTF1) was first identified as a cyclin D2 binding protein in a yeast two-hybrid interactive screen study and was thus named cyclin D-interacting myb-like protein (*DMP1*) *DMTF1* (1). Moreover, the affinity of DMTF1 in binding consensus DNA sequences CCCG(G/T)ATGT and its activity of promoting gene expression were also revealed. The ectopic expression of DMTF1α can cause cell cycle arrest by blocking the S phase entry. DMTF1α can also bind other D-type cyclins, such as cyclins D1 and D3. All 3 cyclins can antagonize the function of DMTF1α in activating gene expression and suppressing cell cycle progression (2). The investigation of the molecular mechanisms of DMTF1α-mediated cell cycle arrest has revealed a canonical DMTF1α recognition site in the ARF promoter and the transactivation of the ARF gene by DMTF1α (3). The tumor suppressive role of DMTF1α was reinforced by the finding that the disruption of DMTF1 can enhance cell immortalization, RAS transformation and spontaneous tumorigenesis in mice (4). In a previous study, MYC-induced lymphomas were significantly accelerated, but did not exhibit any differences between cohorts with either one or both DMTF1 alleles being deleted, suggesting that DMTF1α is a haplo-insufficient tumor suppressor (5). The authors have previously demonstrated that the mammary-specific expression of DMTF1α in transgenic mice leads to poorly developed mammary glands and reduced HER2/neu-driven oncogenic transformation (6). The authors have also revealed that the DMTF1 heterozygous status can significantly accelerate mouse mammary carcinomas with decreased apoptosis and increased metastasis at a transgenic background of cyclin D1 or cyclin D1(T286A) (7). In addition, microRNA (miRNA/miR)-155 and -675 have been reported to target DMTF1 mRNA, leading to the enhanced growth of bladder and colorectal cancer cells, respectively (8,9). All these studies strongly suggest a tumor suppressive role of DMTF1α during oncogenic transformation.

Pre-mRNA splicing is an essential step for the transcript maturation of multi-exon genes. Importantly, it allows one gene to encode multiple different isoforms that may have distinct biological functions, which greatly expands the genomic capacity of eukaryotes (10). The DMTF1 pre-mRNA consists of 18 exons with its start codon ATG present in exon 3 and stop codon in exon 18. The alternative splicing of the DMTF1 pre-mRNA was first reported by Tschan *et al* who discovered two alternative acceptor sites (or 3' splice sites) in intron 9 that led to the formation of 2 new isoforms, designated as

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DMTF1β and DMTF1γ, respectively (11). Thus, DMTF1 with tumor suppressive activity reported prior to the present study should be named as DMTF1α. The read-frames of DMTF1β and γ transcripts after the splicing are coincidently the same, while they encounter a stop codon 'UAA' inside intron 9. As a result, the β and γ isoforms are translated into 2 proteins [272 and 285 amino acids (aa), respectively], much shorter than DMTF1α (760 aa) (10). DMTF1β and γ share the first 273 amino acids with DMTF1α, which embrace the transactivation domain (TAD) and cyclin D binding site (CBS), but contain just a small part of the myb-homology region (MHR). Thus, these two short isoforms lack binding affinity to the consensus DNA element for DMTF1α. DMTF1β is weakly expressed in a number of cell lines, but exhibits a high expression in quiescent $CD34^+$ cells and peripheral blood leukocytes, while DMTF1γ is ubiquitously expressed at low levels (11). Since the specific regions for these DMTF1 proteins are very limited, it is difficult to determine their relative expression levels, particularly between DMTF1β and γ.

The functions of DMTF1β and γ had remained elusive for over a decade, since DMTF1 pre-mRNA alternative splicing was initially discovered in 2003; however, they have begun to be unraveled in recent years. It has been demonstrated that DMTF1β can stimulate mammary cell proliferation and promote mammary oncogenesis using a transgenic mouse model (12). It has also been revealed that DMTF1β is increasingly expressed in human breast cancer based on immunohistochemical studies of clinical samples and the analyses of a breast cancer RNA-seq dataset. In addition, DMTF1β levels are positively associated with the poor prognosis of breast cancer patients (12). Consistently, another group also reported that DMTF1β inhibited the transactivation of the ARF promoter (13). In addition, increased DMTF1β levels can desensitize breast cancer cells to cisplatin treatment (14).

In the present study, the factors that regulate DMTF1 expression were investigated. The functional interplay of DMTF1β and γ with DMTF1α was also explored. The data suggest that both DMTF1β and γ possess oncogenic activity by antagonizing DMTF1α-mediated ARF transactivation.

Materials and methods

Antibodies, DNA and vectors. The antibodies used herein with their catalog numbers and vendors include the following: GAPDH (10R-G109A, Fitzgerald Industries International), Flag (M2; cat. no. F1804, Sigma-Aldrich; Merck GmbH) and HA (32-6700, Invitrogen; Thermo Fisher Scientific, Inc.). RAD, a DMTF1 antibody against all 3 isoforms, was generated in our laboratory as previously reported (15). Oligonucleotides for PCR and DNA sequencing were synthesized by Genewiz. The pGL4 luciferase vector used in constructing the ARF promoter reporter was purchased from Promega Corporation. As collected by the Ensembl Project Database, the DMTF1 gene can encode 38 splice variants, while only DMTF1α, β and γ (annotated as ENST00000394703.9, ENST00000579677.5 and ENST00000447863.5, respectively) have been functionally verified. The DMTF1α and β coding sequences were obtained as previously described (12). The DMTF1γ coding sequence was obtained by reverse transcription as described below using total RNA extracted from MCF-10A cells, (purchased from ATCC) followed by PCR amplifications using the primers GTAGGGATCCAGCACAGTGGAAGAGGA TTCTG and CCTGGAATTCTTATTCTTCATTCTTCTTCT TCCC (forward and reverse, respectively, with *Bam*HI and *Eco*RI restriction sites underlined).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA samples were extracted from the cultured cells using the Tripure™ reagent (Roche Applied Science) according to the manufacturer's protocol. Reverse transcription was carried out using the One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech Co., Ltd.). In this process, 1 μg RNA of each sample was reverse transcribed by a poly(dT) or random primer at 42˚C for 30 min in a total volume of 20 μl. For quantitative PCR (qPCR), cDNA of each sample was amplified in triplicate using the LightCycler® 480 SYBR-Green I Master on LightCycler® 480II Real-Time PCR System (Roche Diagnostics) with a two-step protocol at 95˚C (30 sec) and 60˚C (30 sec) for 40 cycles. Expression was quantified according to the comparative threshold method using the $2^{-\Delta\Delta Cq}$ method (16). The primer sequences for qPCR of DMTF1, ARF and GAPDH are listed in Fig. S1.

Cell culture, transfection, lentiviral production and infection. MCF-10A, HeLa, MDA-MB-231 and MCF-7 cells were purchased from ATCC and cultured according to the suppliers' protocols. DMEM was purchased from Corning, Inc. and fetal bovine serum (FBS) was from ExCell Bio. Generally, transfection was carried out using the Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the procedure provided by the manufacturer. The cells were assayed after 48 h of transfection. Lentiviral production and infection followed a procedure described by us previously (17). Following 48 h of infection, the cells were selected using 1.0 μl/ml of puromycin for 48 h prior to use in functional assays.

Flow cytometry. HeLa cells in 6-cm dishes were individually transfected with 0.5 μg of pSL2-3xFlag-DMTF1α, β and γ together with 0.6 μg of pSL3-RFP plasmid. The pSL2 vectors, generated in our laboratory, uses the CMV promoter to drive an inserted coding sequence and also expresses the ZsGreen located downstream of an internal ribosomal entry sequence (IRES). A pSL3-RFP vector that employs the CMV promoter was also generated to drive the expression of the red fluorescent protein (RFP). After being transfected for 48 h, the cells were trypsinized, washed twice with PBS, and then directly analyzed using a flow cytometer (Accuri C6, BD Biosciences).

Generation of the reporter construct and reporter assay. The following oligonucleotides were designed to amplify the -1,000 to -1 region of the human ARF promoter using the genomic DNA extracted from HeLa cells as a template: CAG TGGATCCAGGCGGCGGGATCAAGGGGAGTC and CCT GGAATTCGCGCCCGCCCCCCACCTTCAC. The 2 underlined sequences are *Bam*HI and *Eco*RI sites, respectively. The PCR fragment was inserted between the *Bam*HI and *Eco*RI sites upstream of the Firefly luciferase (Fluc) coding sequence in the pGL4 luciferase vector. The ARF promoter region in the reporter construct was sequenced to confirm its consistency with the data in the NCBI database. According

to the authors' experience, if 2 proteins bind at a 1:1 stoichiometric ratio, they should have at least similar molecular levels to make it possible for one protein to competitively interfere with the activities of the other protein (data not shown). The pcDNA3 vector could express 3 DMTF1 isoforms at comparable protein levels, but DMTF1α has a molecular weight 2-3-fold higher than that of DMTF1β or γ. Based on this stoichiometry, the same amount of 3 DMTF1 isoform plasmids should produce an approximate 1:2:2 molecular ratio of α/β/γ proteins. In reporter assays, HeLa cells were transfected with 0.3 μg of pcDNA3-3xFlag-DMTF1α, 0.15 or 0.3 μg of pcDNA3-3xFlag-DMTF1β or γ, together with 0.2 μg of ARF promoter reporter construct and 0.1 μg of CMV-SEAP (secreted alkaline phosphatase, cat# 24595, Addgene Inc.). The activities of Fluc and SEAP were measured as previously described (18). For each sample, the Fluc reading was normalized against the corresponding SEAP activity. Each condition was tested in triplicate and at least repeated 3 times.

Western blot analysis. Whole cell lysates of transfected HeLa cells prepared in ice-cold lysis buffer (5 mM EDTA, 0.1% NP-40, 150 mM NaCl, and 50 mM Tris·HCl, pH 7.5) were assayed by Bradford solution (0.05% Coomassie Brilliant Blue G-250, 23.5% methanol, 50% phosphoric acid) for protein concentrations. The same amount of proteins (10-20 μg) of each sample was mixed with an equal volume of 2X SDS loading buffer, heated at 100˚C for 5 min, and then resolved in a 10% SDS-PAGE gel, followed by a transfer to a polyvinylidene difluoride (PVDF) membrane at a constant current of 200 mA for 2 h. The membrane was then blocked by 5% skim milk at room temperature for 1 h, and incubated with a primary antibody overnight at 4˚C. Following extensive washing with PBS, the membrane was incubated with a secondary antibody [diluted 1:10,000, HRP-conjugated goat anti-mouse or anti-rabbit IgG (H+L); cat nos. G-21040 and 31460, respectively, Thermo Fisher Scientific, Inc.] at room temperature for 1 h. After 3 times of wash by PBS, the membrane was exposed using an ECL kit (Vazyme Biotech Co., Ltd.) and the image was captured by ImageQuant LAS500 (GE Health Life Sciences). The primary antibodies and their corresponding dilutions were as follows: Flag (1:2,000), HA (1:1,000), RAD (1:300) and GAPDH (1:2,000, as a protein loading control).

Protein stability assay. The half-lives of the 3 DMTF1 isoforms were determined following a previously published procedure (17). Briefly, pcDNA3-3xFlag-DMTF1α, β and γ constructs were individually transfected into HeLa cells. After 24 h, the cells in each dish were trypsinized and replated into 6 dishes (6-cm dishes). Following a further 24 h of growth, one dish for each DMTF1 isoform was harvested at 'time zero' and cycloheximide was added to a final concentration of 25 μg/ml to the remaining dishes. Cells from the dishes were collected at different time points (1, 2, 4, 8 and 12 h). The collected cells from each time point were washed and the cell lysates were normalized followed by western blot analyses using the Flag antibody. Blotting with the GAPDH antibody was used as the loading control. The densitometric density of the bands was quantified using Quantity One software (Bio-Rad Laboratories, Inc.). and the data were graphed to determine the half-lives of DMTF1 isoform proteins using linear regression.

Co-immunoprecipitation. A total of 2 μg of pcDNA3-HA-DMTF1α, β and γ were individually transfected with 2 μg of pcDNA3-3xFlag-DMTF1α into HeLa cells cultured in 6-cm dishes. After being transfected for 48 h, the cells were collected and lysed in lysis buffer containing 5 mM EDTA, 0.1% NP-40, 150 mM NaCl and 50 mM Tris·HCl, pH 7.5. Approximately 300 μg of cell lysates were incubated with 15 μl of Flag antibody-conjugated magnetic beads (Cat. no. M8823, Sigma-Aldrich; Merck GmbH) in the lysis buffer and the tubes were rotated at 4˚C overnight. The beads were then washed 8-10 times using the lysis buffer. During each step of the washing, each tube was supplemented with 1 ml of the buffer, inverted 5 times to resuspend the beads, loaded into the magnetic Particle Concentrator (cat# 123.21D, Invitrogen; Thermo Fisher Scientific, Inc.) for 30 sec, and aspirated to remove the buffer. The immunoprecipitated samples were mixed with 20 μl of SDS loading buffer, heated at 100˚C for 5 min, and then examined by western blot analysis.

Immunostaining assay. MCF-7 and MDA-MB-231 cells cultured on sterilized glass coverslips in 24-well plates overnight were individually transfected with 500 ng of pcDNA3-3xFlag-DMTF1α, β and γ together with 200 ng of an EGFP expression vector. After 2 days, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and then blocked with 1% bovine serum albumin (catalog no. 001-000-161; Jackson ImmunoResearch Laboratory). The cells were then incubated with the Flag antibody (1:500, Sigma-Aldrich; Merck GmbH) at 4˚C overnight followed by incubation with a secondary antibody (1:200, Alexa Fluor® 594 goat anti-mouse IgG; cat. no. A11032, Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Subsequently, the coverslips were individually stained by 200 μl of DAPI (1:20,000; cat. no. C1006, Beyotime Institute of Biotechnology, Inc.) for 10 min at room temperature. Images were captured with the DeltaVision Elite imaging system (GE Healthcare).

Electrophoretic mobility shift assay (EMSA). A Cy3-labeled oligonucleotide (5'-GTCAGGTGACGGATGTAGCTAGG-3') containing the DMTF1 binding motif on the human ARF promoter was synthesized, and then annealed with its complementary oligonucleotide to generate a double-stranded probe. In a binding reaction, 0.5 pmol of the labeled double-stranded probe and 1 μg of purified Hisx6-DMTF1α protein were mixed in a binding buffer (250 mM HEPES, 500 mM KCl, 20 mM $MgSO_4$, 10 mM DTT, pH 8.0) and incubated on ice for 30 min. For competitive binding, unlabeled double-stranded probe was added to a binding reaction with a mole ratio of 16:1 to the labeled probe. To evaluate the effects of DMTF1β and γ on the DNA binding affinity of DMTF1a, increasing amounts of purified Hisx6-DMTF1β and γ proteins were added to a binding reaction of DMTF1a with the probe. The samples were analyzed by 8% native polyacrylamide gel electrophoresis at 100 V for 100 min at 4˚C in a running buffer of 0.5X TBE (with final concentrations of 45 mM for Tris-Borate and 1 mM for EDTA, pH 8.0). The fluorescent intensity of the bands was determined using Typhoon FLA7000 (GE Healthcare).

Analyses of association between DMTF1 transcripts and clinical outcomes. A TCGA dataset of invasive breast carcinoma containing RNA-seq data (ID: TCGA.BRCA.sampleMap/HiSeqV2) and matched clinical outcome information (ID: TCGA.BRCA.sampleMap/BRCA_clinicalMatrix) of breast cancer patients was downloaded from the UCSC Cancer Browser (https://genome-cancer.ucsc.edu). The information of DMTF1β/α and DMTF1γ/α ratios was extracted from the TCGA Spliceseq browser (http://bioinformatics.mdanderson.org/TCGASpliceSeq). Patients were placed into the 'high' and 'low' groups based on their DMTF1a, β, γ, DMTF1β/α or DMTF1γ/α ratios higher or lower than the means, respectively. Kaplan-Meier graphs for the survival of the breast cancer patients against DMTF1β/α or DMTF1γ/α ratios in the corresponding groups were then analyzed using MedCalc software 19.5. The long- and short-term survival outcomes of the patients were analyzed using the log-rank and Gehan Breslow-Wilcoxon tests, respectively.

Statistical analysis. Data in reporter assays are presented as the means ± SD. Comparisons among all groups on a single parameter were conducted by one-way ANOVA, followed by Tukey's multiple comparison test. Statistical analyses were performed using SigmaStat 12.5 (Systat Software Inc.). The criterion for statistical significance was set at $P<0.05$.

Results

Generation of DMTF1 expression vectors and determination of their expression. DMTF1α, β and γ transcripts are produced by alternative splicing of the DMTF1 pre-mRNA and encode 3 proteins with different lengths (Fig. 1A). Due to the presence of a stop codon for DMTF1β and γ in intron 9, these 2 isoform proteins only retain a small portion of the Myb-like domain and thus have lost the DNA binding affinity that DMTF1α has.

The endogenous expression of DMTF1β and γ, particularly that of the latter, is relatively low, while no antibody is available to discriminate these 2 isoforms. Thus, to investigate whether any biological process in addition to alternative splicing could regulate the relative levels of DMTF1 transcripts, the present study designed 2 sets of expression vectors to express the 3 isoforms. First, the coding sequences of the 3 DMTF1 isoforms were subcloned into a lentiviral vector pSL2-3xFlag (Fig. 1B, left panel). When these plasmids were transfected into HeLa cells, well-expressed DMTF1α was detected by western blot analysis; however, no detectable expression of DMTF1β and γ was observed (Fig. 1C). Second, the 3 DMTF1 coding sequences were inserted into a pcDNA3-3xFlag vector, in which a BGH poly A site is right downstream of each coding sequence (Fig. 1B, right panel). These 3 expression vectors, pcDNA3-3xFlag-DMTF1α, β and γ, all steadily expressed DMTF1 proteins when transfected into HeLa cells (Fig. 1D).

DMTF1β and γ specific regions reduce RNA stability. The present study investigated the possible reasons that may contribute to the undetectable DMTF1β and γ expression in the bicistronic pSL2 lentiviral vector. In pSL2 vectors, the stop codon of each DMTF1 coding sequence is immediately followed by an internal ribosome entry site (IRES) and the ZsGreen coding sequence; however, in pcDNA3 vectors, a BGH poly A sequence is present right behind each DMTF1 isoform coding sequence (Fig. 1B). Thus, it was hypothesized that a poly A sequence may be essential to the mRNA stability or integrity of DMTF1β and γ. To test the cellular levels of 3 DMTF1 isoform transcripts in a form of '3xFlag-DMTF1-IRES-ZsGreen', random primer was used as a primer to reverse transcribe RNA extracted from HeLa cells that was individually transfected with pSL2-3xFlag-DMTF1α, β and γ plasmids, and several sets of primers for amplification were designed (Fig. 2A). First, semi-quantitative PCR was performed using an upstream primer in the Flag epitope tag and a downstream primer in the region shared by the 3 DMTF1 isoforms (PCR1 in Fig. 2A) and a band was amplified from each sample (compare RT-PCR lane 1 of DMTF1α, β and γ in Fig. S3B; lanes C1 to C5 are 'Plasmid PCR' controls using DMTF1 isoform plasmids as templates). Subsequently, 3 isoform-specific primers were designed using the sequences at the junction sites of DMTF1α, β and γ (Fig. S2A and B), and the specificity to their corresponding isoforms was verified by RT-qPCR (Fig. S2C). When using these isoform specific primers together with a downstream primer, ZG-94L, in ZsGreen, only a specific band from the cDNA containing DMTF1α could be amplified, but not these with DMTF1β and γ (compare lane 2 of DMTF1α, β and γ in Fig. S3B). Moreover, amplifications using 3 different upstream primers in the IRES together with primer ZG-94L could produce specific bands for all isoforms; however, the intensity of α was generally stronger than that of β and γ (compare lanes 3-5 of DMTF1α, β and γ in Fig. S3B). The quantification of relative band intensity was shown in Fig. S3C. Consistently, RT-qPCR analyses revealed comparable transcript quantities among the 3 isoforms when amplifying the region close to the 5'-ends of DMTF1 coding sequences (PCR1 in Fig. 2B), but almost undetectable β and γ transcripts when isoform-specific primers were used in qPCR (PCR2, Fig. 2B). Furthermore, the amplification of the ZsGreen region transcribed by pSL2-3xFlag-DMTF1β and γ vectors demonstrated a markedly reduced signal compared to that of the α vector (PCR5, Fig. 2B). When using RT-qPCR to quantify the transcripts of pcDNA3-3xFlag-DMTF1 isoforms, their relatively comparable levels were detected when amplifying both common and isoform-specific regions (Fig. 2C). The ZsGreen expressed by pSL2-3xFlag-DMTF1 vectors could also indirectly reflect mRNA stability. Thus, ZsGreen protein levels were evaluated in HeLa cells individually transfected with pSL2-3xFlag-DMTF1α, β and γ plasmids with a vector expressing RFP as a transfection control. With co-transfected RFP, the green fluorescent signal could only be determined in RFP-positive cells by flow cytometry, which would largely reduce the effects of transfection efficiency difference on the comparison of the green fluorescent signal among the 3 plasmids. As shown in Figs. 2D and S4, the ZsGreen signal was significantly reduced in the pSL2-3xFlag-DMTF1β- and γ-transfected cells compared to the α-transfected cells. The data presented above strongly suggested that DMTF1β and γ contain specific regions vulnerable to break if not immediately followed by a poly A. The reduced stability or integrity of the transcripts was likely the cause of undetectable DMTF1β and γ proteins when using pSL2-3xFlag vectors as an expression vector, and could also contribute to relatively low levels of downstream ZsGreen, compared to the DMTF1α vector.

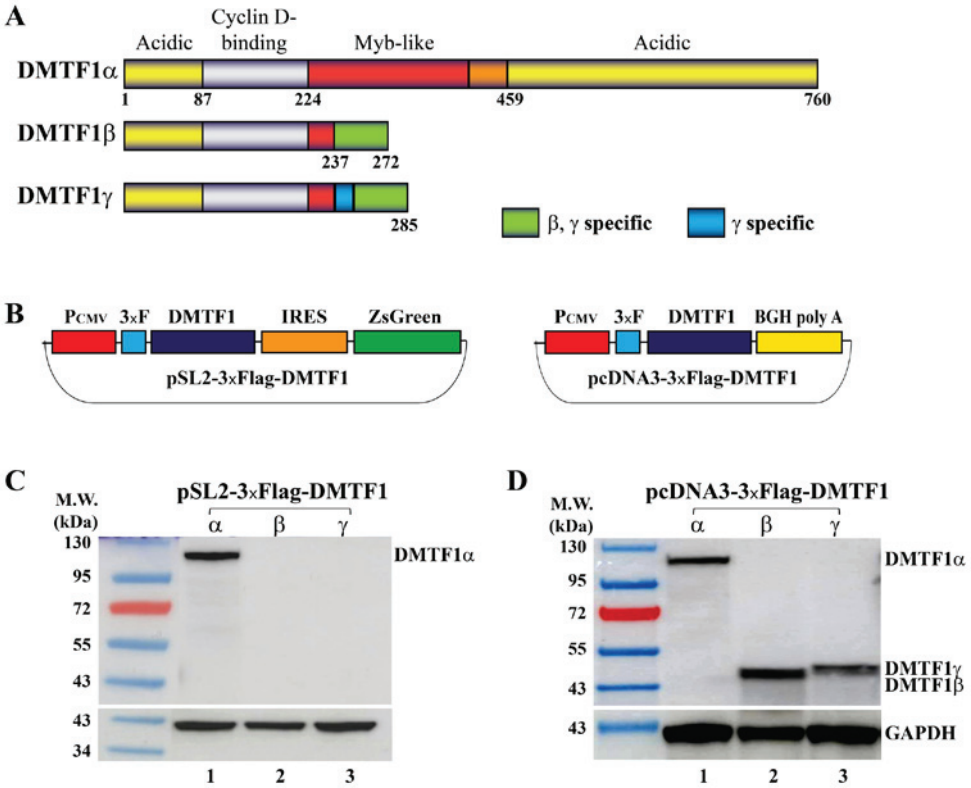

Figure 1. Construction of DMTF1 expression vectors and their expression. (A) Schematic diagram of the protein domain structures of DMTF1 isoforms. The domain structures are based on a previous study (10). (B) Schematic representation of the DMTF1 expression vectors. The name of each part is labeled on the top. pSL2 is a lentiviral vector. P_{CMV}, CMV promoter; 3xF, 3xFlag; IRES, internal ribosome entry site; DMTF1, one of the DMTF1α, β and γ isoforms. (C and D) Expression of DMTF1 vectors. pSL2- and pcDNA3-3xFlag-DMTF1α, β and γ were transiently transfected into HeLa cells. After 48 h, cells were harvested and cell lysates were quantified for protein concentrations. An equal amount of proteins for different samples was examined by western blot analysis to detect 3xFlag-DMTF1 isoforms using a Flag antibody. GAPDH was used as a loading control. DMTF1, cyclin D binding myb-like transcription factor 1.

DMTF1β and γ proteins exhibit reduced stability compared to DMTF1α. The present study also evaluated the relative stability of the 3 DMTF1 protein isoforms. For this purpose, HeLa cells were transfected with pcDNA3-3xFlag-DMTF1 plasmids and the cells were treated with 25 μg/ml cycloheximide followed by cell harvesting at the time points of 0, 1, 2, 4, 8 and 12 h. After determining the protein concentrations of the cell lysates, the same amount of proteins was loaded on SDS-PAGE followed by western blot analysis. Apparently, DMTF1α exhibited a longer half-life than DMTF1β and γ based on the gel western blot images (Fig. 3A). The densitometric density of each band was then quantified using Quantity One software and the data were graphed as shown in Fig. 3B. The DMTF1β sample collected at 1 h was abandoned due to its obvious deviation. Based on regression lines of these samples, the half-lives of DMTF1α, β and γ proteins were approximately determined as 8.7, 3.5 and 1.9 h, respectively. Therefore, the 2 shorter DMTF1β and γ isoforms clearly exhibited reduced stability compared to DMTF1α.

All 3 DMTF1 isoforms are localized in nucleus. To predict the subcellular localization of the 3 DMTF1 isoforms, their protein sequences were first analyzed using a previously reported strategy (19) and a putative nuclear localization signal (NLS) was identified among the amino acids 47-59 (IEPPHKRLCLSSE), shared by the 3 isoforms (Fig. 4A). Consistent with this prediction, previous studies have demonstrated the nuclear localization of DMTF1α (1,2). Moreover, DMTF1 proteins were also analyzed for their nuclear export signal (NES) based on a published algorithm (20) and a weak NES sequence (IAELDV, with crucial residues underlined) was identified among the amino acids 351-356, which is located in the Myb-like domain, only present in DMTF1α (Fig. 4A). To determine the localization of DMTF1 proteins, pcDNA3-3xFlag-DMTF1α, β and γ were individually transfected into MCF-7 and MDA-MB-231 cells, and the transfected cells were immunostained using a Flag antibody. As shown in Figs. 4B and S5A, 3xFlag-DMTF1α, β and γ were all localized in the nuclei of the transfected breast cancer cells. In these images, the EGFP signal was used to visualize the regions of both nucleus and cytoplasm. To determine whether the potential NLS plays a role in nuclear localization of DMTF1 proteins, the K52 and R53 were replaced by 2 alanines to generate KR-2A mutants of DMTF1α, β and γ. The immunostaining analyses of MCF-7 and MDA-MB-231 cells transfected with these 3xFlag-tagged mutants indicated that the KR-2A mutations led to DMTF1α nuclear exclusion and DMTF1β, γ distribution in both cytoplasm and nuclei (Figs. 4C and S5B), suggesting that K52 and R53 are essential residues for the nuclear localization of DMTF1 proteins.

Both DMTF1β and γ interfere with DMTF1α-mediated transactivation. The ARF promoter contains a DMTF1α consensus binding site 'GACGGATGT' in the region of -312 to -304, if the

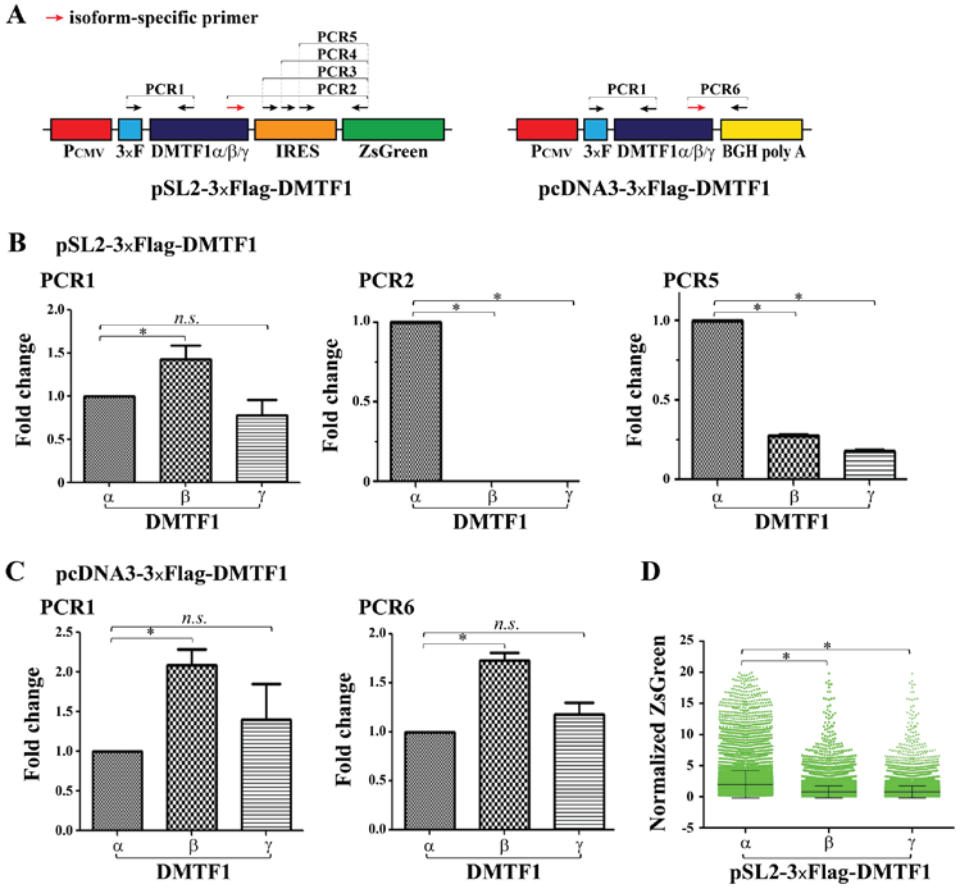

Figure 2. Evaluation of potential reasons for differential DMTF1 expression using the two vector systems. (A) Schematic diagrams of the positions of different primer sets used in determining mRNA integrity. The predicted transcripts of pSL2- and pcDNA3-3xFlag-DMTF1α, β and γ are shown at the left and right, respectively. The primers pairs are indicated and their sequences are presented in Fig. S1. (B and C) RT-qPCR using primer pairs indicated in (A). (D) ZsGreen signaling normalized against RFP in HeLa cells co-transfected with pSL2-3xFlag-DMTF1 isoforms and pCMV-RFP. *P<0.05; n.s., not significant. DMTF1, cyclin D binding myb-like transcription factor 1.

first nucleotide downstream the transcription start site (TSS) is designate as '-1' (Fig. 5A) (3). To determine the effect of DMTF1 proteins on its target gene ARF, the-1,000 to -1 region of the ARF promoter (ARFp) was amplified and inserted upstream of the Fluc coding sequence in the pGL4 vector to generate pGL4-ARFp reporter construct (Fig. 5A). The ratio of the transfected pGL4-ARFp plasmid and DMTF1α expression vector was first optimized. When 0.2 μg of the pGL4-ARFp reporter was co-transfected with increasing amounts of DMTF1α vector into HeLa cells, the Fluc activity increased accordingly, reaching its highest value at 0.5 μg of DMTF1α, and then decreased (Fig. 5B). CMV-SEAP was used as a transfection control and Fluc data were normalized against the SEAP activity. When determining the effects of DMTF1β and γ on DMTF1α-mediated transcription, 0.3 μg of DMTF1α vector was used together with 0.15 and 0.3 μg of DMTF1β and γ expression plasmids to transfect HeLa cells. As shown in the top panel of Fig. 5C, while DMTF1α stimulated ARF promoter activity, DMTF1β and γ did not have this effect (compare columns 3 and 4 to 1 and 2), consistent with their loss of the DNA binding site due to alternative splicing (10). Actually, DMTF1γ could even decrease the expression of the ARF promoter reporter. Importantly, it was observed that both DMTF1β and γ attenuated the DMTF1α-mediated transactivation (compare columns 5-8 to 2, top panel of Fig. 5C). In the samples for the reporter assay, transfected DMTF1 isoform plasmids could steadily express corresponding proteins, as determined by western blot analysis (bottom panel of Fig. 5C). To evaluate the effects of DMTF1 proteins on endogenous ARF expression, DMTF1α was co-transfected with an empty vector, DMTF1β or DMTF1γ into HeLa cells and endogenous ARF expression was determined by RT-qPCR. While DMTF1α alone significantly increased the level of endogenous ARF mRNA, DMTF1β and γ markedly antagonized this effect (Fig. 5D, top panel). Western blot analysis confirmed the expression of DMTF1 proteins in the present study (Fig. 5D, bottom panel). Thus, the effects of DMTF1 isoform proteins on the expression of endogenous ARF are consistent with the results of reporter assays. Since DMTF1β and γ reduced DMTF1α-activated ARF promoter activity, the present study wished to determine whether these two short isoforms can alter DMTF1α binding to the ARF promoter. In EMSA experiments using purified recombinant DMTF1 proteins and a probe based on the DMTF1α-binding element on the ARF promoter, slowly migrated bands for the complex of DMTF1a and Cy3-labeled probe were detected on native polyacrylamide gel electrophoresis (lanes 3 vs. 1 in Fig. 5E and F), while DMTF1β and γ did not exhibit any binding affinity to the probe (lanes 10 and 9 in Fig. E and F, respectively). The retarded bands were diminished when unlabeled probe was

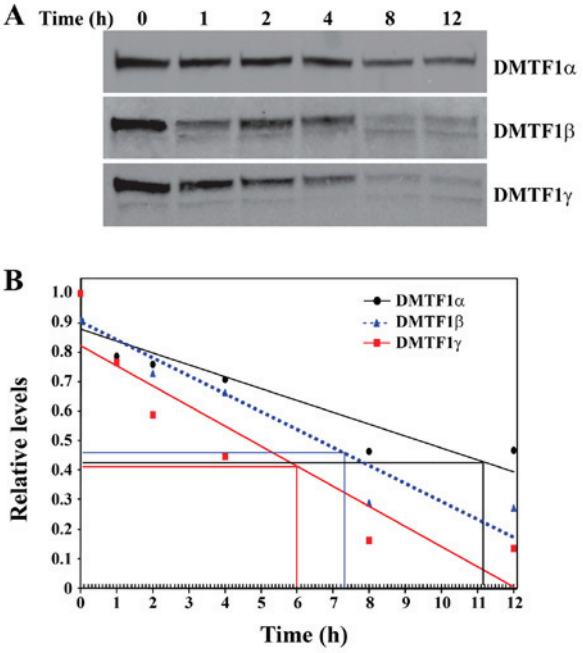

Figure 3. Relative protein stability of DMTF1 isoforms. (A) Western blot analysis of transfected DMTF1 isoforms in HeLa cells incubated in presence of cycloheximide. HeLa cells were individually transfected by pcDNA3-3xFlag-DMTF1α, β and γ vectors followed by incubation of 25 μg/ml cycloheximide. Samples were collected at the time points of 1, 2, 4, 8 and 12 h, and then examined by western blot analysis using a Flag antibody. (B) Quantification of western blots. The densitometric density of western blot analyses in (A) was quantified and the data were graphed for the time points of sample collection. Linear regression was performed for each sample and the estimated half-lives of DMTF1α, β and γ proteins were determined as 11.2, 7.3 and 6.0 h, respectively. DMTF1, cyclin D binding myb-like transcription factor 1.

excessively added (lanes 2 of Fig. 5E and F). With increasing amounts of DMTF1β (lanes 4-7 in Fig. 5E), the DMTF1a-probe complex exhibited a reduced intensity. However, the addition of DMTF1γ (lanes 4-5 in Fig. 5F) exerted a relatively modest effect. Of note, the further increase in DMTF1β and γ somehow promoted the DMTF1α-probe complex formation (lanes 7-9 in Fig. 5E and lanes 6-8 in Fig. 5F). Overall, DMTF1α activated ARF promoter activity, while DMTF1β counteracted this effect, possibly by interfering with DMTF1α-binding to the promoter. However, the effects of DMTF1γ on DNA binding affinity of DMTF1α were very marginal.

DMTF1α physically associates with both DMTF1β and γ. To explore the mechanism underlying the antagonistic effects of DMTF1β and γ on DMTF1α-mediated ARF promoter transactivation, we carried out co-immunoprecipitation experiments to determine their potential interaction. The 3xFlag-DMTF1α vector was co-transfected with pcDNA3-HA-DMTF1α, β and γ vectors, respectively, into HeLa cells, and Flag antibody-conjugated agarose beads were then used to immunoprecipitate 3xFlag-DMTF1α (Fig. 6, top labels of lanes 2, 5 and 8). When blotting the samples by an HA antibody, HA-DMTF1β and γ, but not α, we could detected, brought down together with 3xFlag-DMTF1α (compare lanes 5 and 8 with lane 2 in bottom panel of Fig. 6), suggesting that DMTF1β and γ could physically associate with DMTF1α to interfere with its mediated transactivation.

Increased DMTF1β/α and γ/α ratios are associated with a poor prognoses of breast cancer patients. Previous studies by the authors indicate a tumor suppressive role of DMTF1α in breast cancer development (6,7); thus, the activities of DMTF1β and γ in binding and antagonizing DMTF1α function suggest their oncogenic or proliferative role in cancer development. It has previously been demonstrated that DMTF1β can promote mammary oncogenesis in a transgenic mouse model (12). This led us to determine whether the ratio of DMTF1β/α or γ/α is associated with the clinical outcomes of breast cancer patients. Since no antibody was available to discriminate β and γ isoform proteins, the current experiments were based on the DMTF1 mRNA levels of breast cancer patients. In the present study, the Kaplan-Meier survival curve was employed to analyze the RNA-seq data of 1,095 breast cancer samples derived from a TCGA SpliceSeq Database (ID: TCGA.BRCA.sampleMap/HiSeqV2) (21), and the associations of the DMTF1β/α and γ/α ratios with patient survival were assessed using the log-rank and Gehan Breslow-Wilcoxon tests. In general, the log-rank test tends to be sensitive to distributional differences that are most evident later in time, while the Wilcoxon test tends to be more powerful in detecting differences early in time (22), and thus they can be used to evaluate long- and short-term survivals of a patient cohort, respectively. Consistent with the findings of a previous study (12), the survival analyses displayed significant associations between increased DMTF1β/α ratios with the poor prognosis of breast cancer patients both at long- and short-term follow-up (log-rank, P=0.0055; Breslow, P=0.0071; Fig. 7A). Importantly, it was also found that high DMTF1γ/α ratios were significantly associated with the reduced long- and short-term survival rates of the patients (log-rank, P=0.0041; Breslow, P=0.0023; Fig. 7B). These data suggested the oncogenic activities of DMTF1β and γ in breast cancer.

The three DMTF1 mRNA isoforms originate from the same pre-mRNA; thus, increased splicing of one isoform will theoretically lead to reduced levels of the others. To prove this notion, 1,095 patients from the aforementioned database were divided into 8 groups with varying combinations of high (H-) or low (L-) levels of the 3 isoforms, based on the DMTFα, β or γ expression of each sample being higher or lower than its corresponding mean value of all samples (Fig. 8A). In addition, H-α and L-α expression was deliberately combined with 4 different β and γ combinations; i.e., H-β,H-γ; H-β,L-γ; L-β,H-γ and L-β,L-γ (Fig. 8B). With H-α expression, only 9 patients had the H-β,H-γ status and the majority of the patients (n=268) exhibited L-β,L-γ. On the other hand, at the L-α condition, the majority of patients exhibited H-β,L-γ or H-β,H-γ levels (210 and 241 patients, respectively), but only 3 patients had the L-β,L-γ status (Fig. 8A and B). These data suggested the competitive splicing among the 3 DMTF1α isoforms.

To evaluate the contributions of DMTF1 isoforms to breast cancer development, statistical analyses among were performed for these 8 groups. First, patient survival according to the varied DMTF1α and β expression at the L-γ or H-γ background was assessed. At the L-γ condition, the patients in the L-α,H-β group exhibited a statistically significant decrease in long-term survival compared with the patients in the H-α,L-β group (log-rank, P=0.0485); however, their short-term survival did not differ significantly (Fig. 8C). However, at the H-γ background,

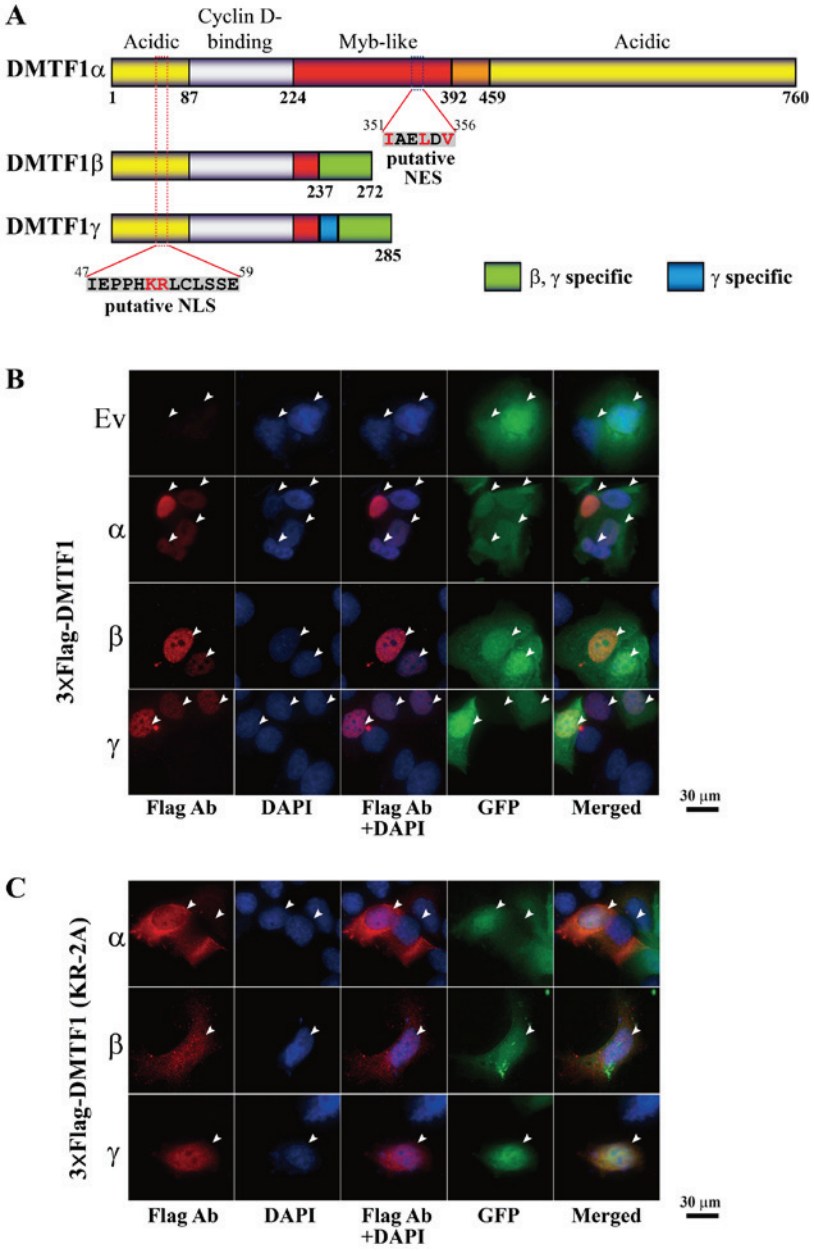

Figure 4. Subcellular localization of DMTF1 isoform proteins. (A) Schematic diagram for the predicted nuclear localization signal (NLS) and nuclear export signal (NES) of DMTF1 protein isoforms. The domain structures are based on a previous report (10). The predicted nuclear localization signal (NLS) and nuclear export signal (NES) are indicated (B and C) Immunostaining of wild-type DMTF1 isoforms (B) and their K52/R53-to-2A (i.e., KR-2A) mutants (C) in MCF-7 cells. MCF-7 cells were individually transfected with an empty vector, pcDNA3-3xFlag-DMTF1α, β and γ wild-type or KR-2A mutants together with an EGFP expression vector, and then immunostained using a Flag antibody. White arrow heads were used to indicate and align transfected cells. DMTF1, cyclin D binding myb-like transcription factor 1.

both the long- and short-term survival rates of the L-α,H-β group were significantly decreased compared to the H-α,L-β group (log-rank, P=0.0142; Breslow, P=0.0133; Fig. 8D). The data suggested that the increased expression DMTF1γ enhanced DMTF1β-mediated mammary oncogenesis. Second, the contribution of DMTFα and γ at the L-β or H-β background to patient survival was evaluated. At the L-β background, patients in the L-α,H-γ group exhibited a significantly decreased long-term (log-rank, P=0.03), but not short-term (Breslow, P=0.0634) survival comparted to the patients in the H-α,L-γ group (Fig. 8E). However, at the H-β background, significant difference were observed in the short-term (Breslow, P=0.0231), but not the long-term survival (log-rank, P=0.0553, Fig. 8F). Third, two mixed conditions were analyzed. Surprisingly, the patients with the L-α,H-β,H-γ status only exhibited a significantly decreased long-term survival compared to those with H-α,L-β,L-γ expression (log-rank, P=0.0299), while the difference of short-term survival was not significant (Breslow, P=0.0934, Fig. 8G). The 2 patient groups with L-α,L-β,L-γ and H-α,H-β,H-γ statuses did not exhibit significant differences in survival due to very few numbers of patients (Fig. 8H). Overall, the increased splicing of DMTFβ and γ isoforms versus the α isoform was associated with the poor prognosis of breast cancer patients. These data also confirmed the tumor suppressive role of DMTF1α; its low expression was always associated with the decreased survival of patients.

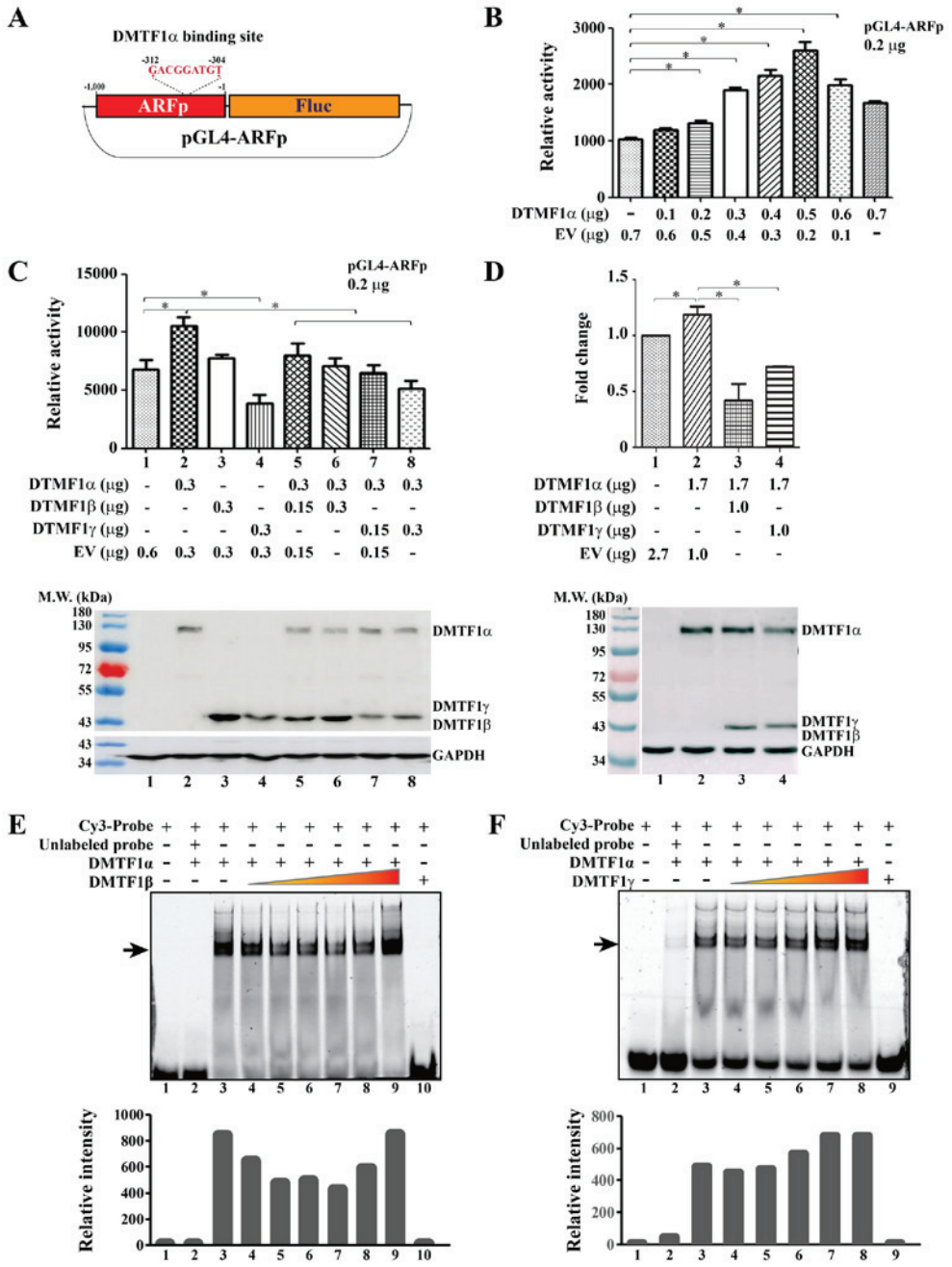

Figure 5. Evaluation of DMTF1β and γ expression on DMTF1α-mediated activity of the ARF promoter. (A) Schematic diagram of an ARF promoter reporter construct. The position of predicted DMTF1α binding site in the ARF promoter is indicated on the top. (B) Optimization of DMTF1α amounts in reporter assay. HeLa cells were transfected by indicated amounts of DMTF1α expression vector and empty vector as indicated at the bottom, together with 0.2 μg of ARF promoter reporter and 0.1 μg of CMV-SEAP. After 48 h, cells were harvested to determine the Fluc and SEAP activities using the procedure described in Materials and methods. (C) Reporter assays for the effects of DMTF1 proteins on the ARF promoter. Top panel: Reporter assays were conducted as B with DMTF1β and γ expression vectors co-transfected as shown in the middle panel. Bottom panel: Western blot analysis of samples used in the reporter assays using a Flag antibody (top) and GAPDH antibody. (D) Effects of DMTF1 proteins on endogenous ARF expression. Top panel: DMTF1 isoform expression vectors and an empty vector were transfected into HeLa cells in 6-well plates as indicated in the middle panel. Endogenous ARF mRNA levels were determined by quantitative PCR analyses and normalized by corresponding GAPDH levels. Bottom panel: Western blot analysis of samples used in the quantitative PCR studies. *P<0.05. (B-D) EV represents empty vector (E and F) EMSA studies to evaluate the effects of DMTF1β and γ on DNA binding affinity of DMTF1α. DMTF1α protein (1 μg) and Cy3-labeled probe (0.5 pmol) were incubated with increasing amounts of DMTF1β (from 0.125 to 4 μg in panel E) or γ (from 0.25 to 4 μg in panel F). DMTF1, cyclin D binding myb-like transcription factor 1.

Discussion

Alternative RNA splicing is a well-regulated process in eukaryotic cells during development and contributes to functional diversity of proteins and non-coding RNAs (ncRNAs) in various cell signaling pathways. Under dysregulated conditions, such as cancer cells, aberrantly alternative splicing can produce RNAs or proteins with distinct or opposite functions compared to cognate normal products. With the progress of the Ensembl Project, a stunning amount of genomic data and their annotations are publicly available, which include a huge number of new splicing species for different genes.

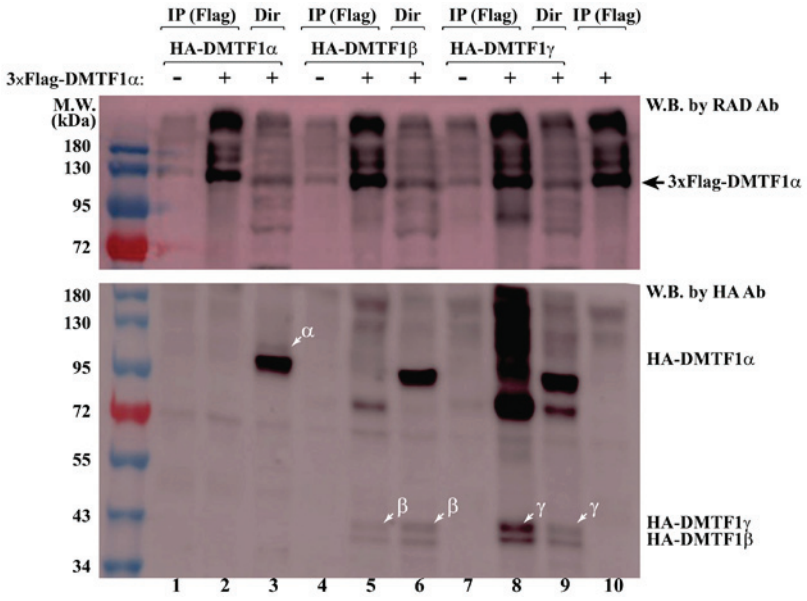

Figure 6. Co-immunoprecipitation assay to determine DMTF1 isoform interaction. pcDNA3-3xFlag-DMTF1α or empty vector was co-transfected with pcDNA3-HA-DMTF1α, β or γ, as indicated on the top section. After 48 h, cells were collected and cell lysates were immunoprecipitated (IPed) by Flag antibody-conjugated agarose. The IPed samples (IP) and direct samples (Dir) were subjected to western blot (W.B.) analysis using the DMTF1 antibody RAD (for all isoforms) and an HA antibody, as indicated. DMTF1, cyclin D binding myb-like transcription factor 1.

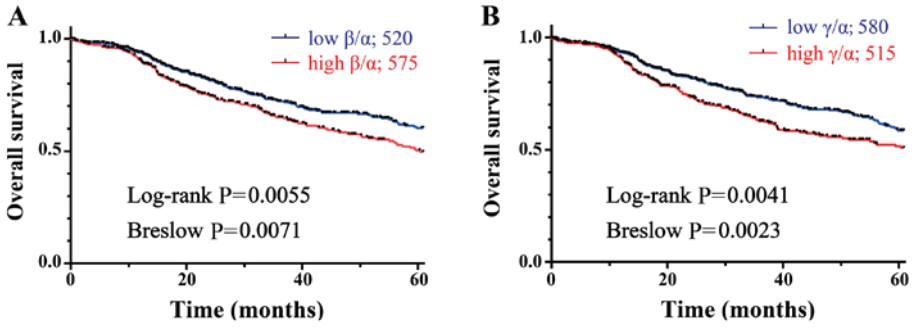

Figure 7. Survival curves of breast cancer patients based on DMTF1 isoform ratios. A breast invasive carcinoma TCGA dataset (ID: TCGA.BRCA. sampleMap/HiSeqV2) was analyzed as described in the Materials and methods. Kaplan-Meier graphs for the survival of the breast cancer patients against (A) DMTF1β/α and (B) DMTF1γ/α ratios in the corresponding groups were presented. Patient numbers were labeled behind corresponding group names. DMTF1, cyclin D binding myb-like transcription factor 1.

However, a number of transcripts in the database only have partial sequences available and lack information regarding their activity or cellular abundancy. For example, the DMTF1 gene has 38 splicing variants and 20 of them can possibly encode proteins based on the annotation in the Ensembl Project Database. Currently, only 3 DMTF1 isoforms have been reported (11). In a more recent study, this group indicated that DMTF1β, but not γ could antagonize DMTF1α-mediated ARF expression, and no immunoblot for the expression of the two short protein isoforms was presented (13). Thus, the present study aimed to characterize the expression and function of DMTF1β and γ.

Due to the relatively low expression of endogenous DMTF1γ, and lack of antibody in discriminating DMTF1β and γ isoforms, we designed two sets of vectors expressing 3 DMTF1 isoforms to indirectly evaluate the contributions of mRNA and protein stability to their relative cellular levels. The results may provide a conceptual base to understand differentially regulated DMTF1 isoform expression in cancer cells. Many factors can alter expression levels of protein coding genes. In the present study, it was observed that vectors containing DMTF1β and γ coding sequences could only express DMTF1 proteins when then were immediately followed by a poly A sequence (Fig. 1C and D). The failure of DMTF1β and γ expression when their stop codons are not adjacent to a poly A sequence is reminiscent of nonsense-mediated decay (NMD) that eukaryotes utilize to eliminates mRNA transcripts with premature stop codons (23). This mechanism can avoid the translation of aberrant mRNAs into proteins, which potentially have deleterious gain-of-function or dominant negative activity (24). Thus, it is possible that the stop codon of DMTF1β and γ in intron 9 triggers the NMD mechanism, which reduces their expression. The present study also demonstrated markedly shorter half-lives of DMTF1β and γ than α (Fig. 3A and B). In these experiments, Flag tags were added to the DMTF1 N-terminals, which shared 237 amino acids among the three isoforms (Fig. 1A). Thus, it was unlikely that Flag tag could differentially affect protein expression in the

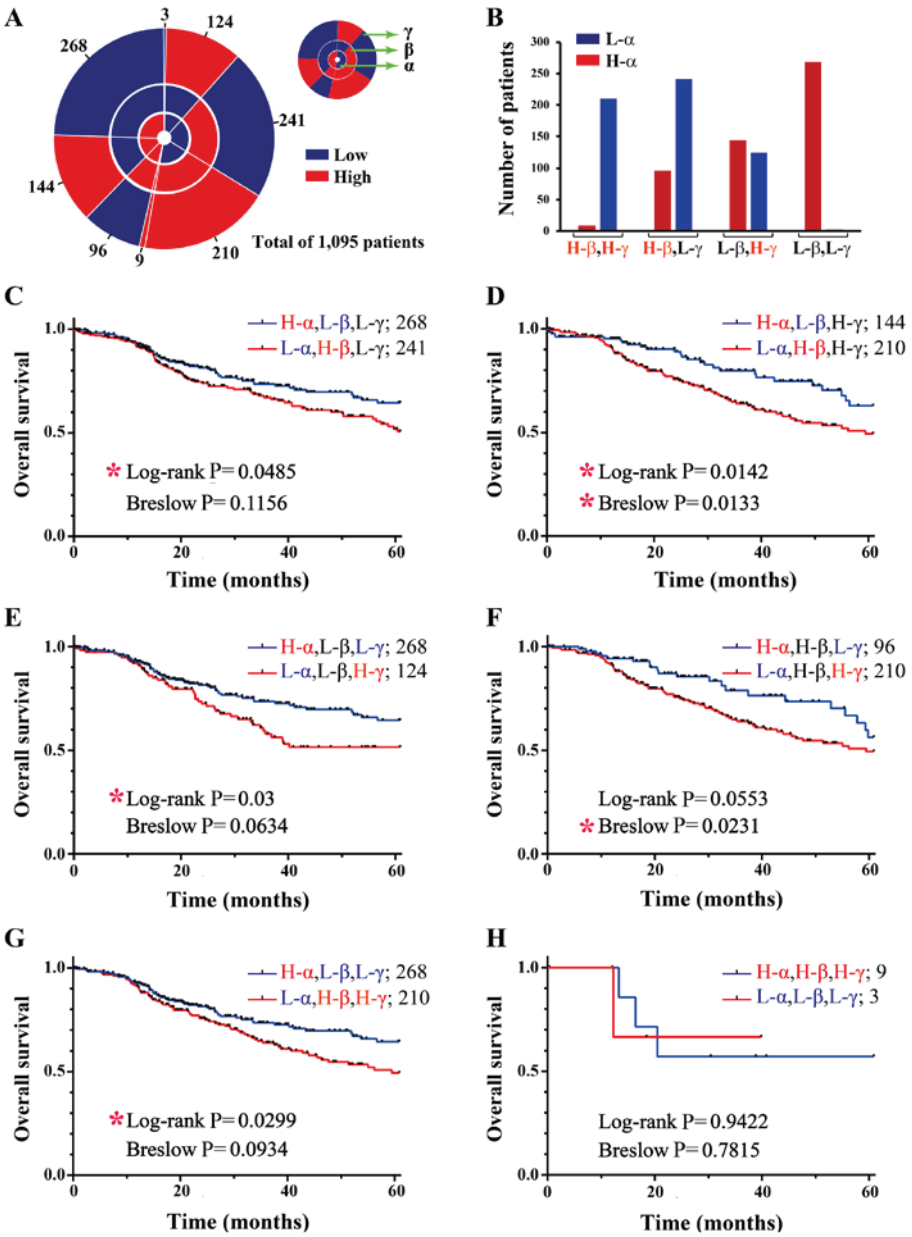

Figure 8. Survival curves of grouped breast cancer patients based on combinations of high or low expression of different DMTF1 isoforms. (A) Pie chart of grouped breast cancer patients from the TCGA dataset (ID: TCGA.BRCA.sampleMap/HiSeqV2). The 3 circles from inside to outside represent DMTF1α, β and γ. The 1,095 patients were divided into 8 groups with various combinations of high (red) or low (blue) levels of the 3 isoforms, based on the DMTFα, β or γ expression of each sample being higher or lower than its corresponding mean value of all samples. The numbers of the 8 patient groups are labeled outside of the chart. (B) Using H and L to denote high and low expression, respectively, the patients with H-α and L-α were grouped and simultaneously expressing H or L levels of β and γ to generate the bar chart, which allowed for the intuitive visualization of the patient numbers of each group. (C-H) The comparison of long- and short-term survival rates among the 8 groups of patients based on the log-rank p and Breslow P-values, respectively. Patient numbers were labeled behind corresponding group names. The red asterisk (*) denotes P<0.05, suggesting statistically significant differences. DMTF1, cyclin D binding myb-like transcription factor 1.

plasmids. These data suggest that DMTF1β and γ expression is antagonized through both enhanced degradation of their transcripts and reduced stability of their proteins to eliminate their potentially deleterious effects (Figs. 2, 3 and S3). During the malignant transformation or other biological deregulation, these mechanisms may be attenuated, thereby leading to DMTF1β and γ accumulation.

Notably, semi-quantitative RT-PCR and RT-qPCR analyses suggested that only DMTF1β- and γ-specific regions in the transcripts were disrupted and the downstream coding sequence of ZsGreen exhibited reduced levels, although the upstream regions shared by the 3 isoforms remained apparently intact (Figs. 2 and S3). This observation indicates that the 5' regions of DMTF1 coding sequences may possess a stable or protective structure resistant to further RNA degradation.

The study by Tschan *et al* indicated that DMTF1β was dominantly localized in the nucleus, but DMTF1γ was located in both the nucleus and cytoplasm, when they were expressed as fusion proteins with EGFP (13). They also observed that only DMTF1β, but not DMTF1γ, could interfere with DMTF1α-mediated ARF transcription. However, the data clearly demonstrated the nuclear localization of all

3 DMTF1 isoforms (Figs. 4B and S5A). Importantly, we also discovered an NLS sequence in a region shared by the three DMTF1 isoforms and a putative NES sequence only present in DMTF1α (Figs. 4 and S5). The discrepancies between the results in the aforementioned study and those of the present study are likely due to the difference in DMTF1 expression strategies, expression levels and employed cell lines. Tschan *et al* expressed DMTF1 isoforms as fusion proteins with EGFP (13); however, the present study merely added an N-terminal 3xFlag epitope; they expressed DMTF1γ with a markedly reduced level compared to DMTF1β, but the present study expressed all three isoforms at comparable levels; they used 293T cells, but the present study transfected both MCF-7 and MDA-MB-231 cells with 3xFlag-DMTF1 expression vectors. Thus, it is unclear whether EGFP can affect DMTF1γ localization and whether DMTF1γ subcellular localization is cell type-specific. Importantly, it was predicted the NLS location of DMTF1 in the region of I^{47}EPPHKRLCLSSE^{59} based on a published algorithm and validated this prediction using mutagenesis experiments, in which K52-R53 substitutions by alanines led to cytoplasmic translocation of all 3 DMTF1 isoforms (Figs. 4C and S5B). To the best of our knowledge, the present study is the first to map the NLS domain of DMTF proteins. Notably, the KR-2A mutations caused the nuclear exclusion of DMTF1α, but only rendered DMTFβ and γ present in both the cytoplasm and nucleus. Whether the putative NES sequence I^{351}AELDV^{356} only presents in DMTF1α (Fig. 4A) and contributes to its nuclear exclusion warrants further investigation.

In reporter assays, luciferase expression driven by the ARF promoter did not monotonically increase with the escalated DMTF1α expression (Fig. 5B). It was predicted that this phenomenon was likely caused by the quenching effects (25) of overexpressed DMTF1α that could bind and even saturate transcription co-factors in a status unassociated with the ARF promoter and thus interfere with the transcription mediated by the target promoter. In addition, the observed basal level of the ARF-promoter reporter was likely driven by endogenous DMTF1α. In the EMSA experiments, the 2 short DMTF1 isoforms, particularly DMTF1β, exhibited modest effects in attenuating DMTF1α binding to its consensus element on the ARF promoter (Fig. 5E and F). Thus, the association of DMTF1β and γ with α may attenuate DMTF1α-mediated transcription by disrupting its recruited transcriptional machinery. In recent years, phase separation caused by intrinsically disordered regions of transcription factors has been demonstrated to play a key role in regulating gene expression (26). It was noted that the N-terminal region shared by the 3 DMTF1 proteins is highly disordered (data not shown). Thus, the regulation of DMTF1α transcriptional activity by DMTF1β and γ may also employ the mechanism of the phase separation. In Fig. 5E and F, a reduced complex intensity was observed when excessive DMTF1β and γ proteins were added. It is possible that the incorporation of DMTF1β and γ into DMTF1α-containing complex formed by the phase separation mechanism could initially reduce its DNA binding affinity. However, with the further increased expression of DMTF1β and γ proteins, DMTF1α could be 'squeezed out' from the complex and then bind to the probe, which may only occur in the *in vitro* assays. Whether the interaction of DMTF1 isoforms is physiologically significant and where the binding site(s) is localized warrants further investigation (Fig. 6).

A number of studies have demonstrated positive correlations between oncogene expression and the poor clinical outcomes of breast cancer patients, such as PI3K, AKT, mTOR, MYC and HER2 (27-30). When analyzing a breast cancer database, the present study also discovered that both DMTF1β/α and DMTF1γ/α ratios were inversely associated with the survival rates of the patients (Fig. 7A and B), supporting the oncogenic activities of DMTF1β and γ demonstrated in the functional analyses. When the patients were divided into 8 groups based on their high or low DMTF1 isoform expression, it was suggested that the 3 DMTF1 isoforms could competitively splice and DMTF1a played a dominant role to suppress breast cancer development (Fig. 8). Based on the analyses of the log-rank and Breslow-Wilcoxon tests, DMTF1β and γ could mutually enhance each other to reduce the long- or short-term survival rates of breast cancer patients (Fig. 8C-H).

Overall, the data of the present study strongly support that the oncogenic activity of DMTF1β in mammary oncogenesis is through its antagonism of DMTF1α. Although DMTF1γ can negatively affect DMTF1α-mediated ARF promoter activity, it exerted marginal effects on the DNA binding affinity of DMTF1α. It is possible that DMTF1β and γ can both contribute to oncogenesis in a cell and tumor type-dependent manner, while the detailed regulatory mechanisms warrant further exploration.

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Availability of data and materials

All data generated or analyzed during this study are included either in this article or in the supplementary information files.

Authors' contributions

JL, KS and GS conceived the study, wrote the manuscript and generated the figures. JL, KS, TX, JH, TL and KC conducted the experiments. JL and GL analyzed the bioinformatics data. DL provided technical support and was involved in the conceptual design in several key experiments. KI was involved in conceiving the project and provided several important reagents and suggestions to the research plan. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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