# Therapeutic targeting of FOS in mutant *TERT* cancers through removing TERT suppression of apoptosis via regulating *survivin* and *TRAIL-R2*

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**The telomerase reverse transcriptase (TERT) has long been pursued as a direct therapeutic target in human cancer, which is currently hindered by the lack of effective specific inhibitors of TERT. The FOS/GABPB/(mutant) *TERT* cascade plays a critical role in the regulation of mutant *TERT*, in which FOS acts as a transcriptional factor for *GABPB* to up-regulate the expression of GABPB, which in turn activates mutant but not wild-type *TERT* promoter, driving TERT-promoted oncogenesis. In the present study, we demonstrated that inhibiting this cascade by targeting FOS using FOS inhibitor T-5224 suppressed mutant *TERT* cancer cells and tumors by inducing robust cell apoptosis; these did not occur in wild-type *TERT* cells and tumors. Mechanistically, among 35 apoptotic cascade-related proteins tested, the apoptosis induced in this process specifically involved the transcriptional activation of tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (*TRAIL-R2*) and inactivation of *survivin,* two key players in the apoptotic cascade, which normally initiate and suppress the apoptotic cascade, respectively. These findings with suppression of FOS were reproduced by direct knockdown of TERT and prevented by prior knockdown of TRAIL-R2. Further experiments demonstrated that TERT acted as a direct transcriptional factor of *survivin*, up-regulating its expression. Thus, this study identifies a therapeutic strategy for *TERT* promoter mutation-driven cancers by targeting FOS in the FOS/GABPB/(mutant) *TERT* cascade, circumventing the current challenge in pharmacologically directly targeting TERT itself. This study also uncovers a mechanism through which TERT controls cell apoptosis by transcriptionally regulating two key players in the apoptotic cascade.**

FOS | TERT promoter mutation | apoptosis | survivin | TRAIL-R2

The canonical function of telomerase, a ribonucleoprotein polymerase complex, is to maintain telomeres at the ends of human chromosomes by adding TTAGGG nucleotide repeats to protect chromosomal integrity and genome stability (1). The critical catalytic subunit of telomerase is telomerase reverse transcriptase (TERT), which is usually inactive in normal cells but is widely reactivated in cancers (2, 3). It has been increasingly recognized that TERT also exerts important telomere-independent functions on various hallmarks of cancer cells, such as increased proliferation, apoptosis resistance, and invasion (4). Therefore, targeting telomerase, especially TERT, has become an attractive potential therapeutic strategy for cancer. There has been little progress in this regard, however, as the field is severely hindered by the lack of a potent and specific TERT inhibitor or an effective strategy to target TERT and selectively kill telomerase-overactive cancer cells (5).

Regulation of TERT in cancer cells can occur at various molecular levels (6). *TERT* promoter mutation is a powerful genetic mechanism for the reactivation of *TERT* widely seen in cancers, such as thyroid cancer, melanoma, glioma, hepatocellular carcinoma, and bladder cancer (7–10). There are two main recurrent TERT promoter mutations in human cancers located at hotspots of chr5, 1,295,228 C > T and 1,295,250 C > T, corresponding to the positions 124 bp and 146 bp, respectively, upstream of the TERT translation start site. *TERT* promoter mutations play an aggressive oncogenic role and are associated with poor clinical outcomes in many cancers, such as thyroid cancer, melanoma, glioma, and bladder cancer (11–14).

Mechanistically, *TERT* promoter mutations uniquely create specific binding sites for the tetrameric GABPA/GABPB transcription factor complex to up-regulate *TERT* expression and telomerase activities (15–18). GABPB plays a particularly important dictating role in this process as it selectively binds and activates the mutant but not the wild-type (WT) *TERT* promoter; its depletion induces growth defects, telomere loss, and cell death exclusively in mutant *TERT* cells (19). We have identified and characterized a regulatory mechanism for mutant *TERT* promoter in which FOS acts as a powerful transcriptional factor for *GABPB*, robustly up-regulating GABPB, which in turn binds and activates the mutant *TERT* promoter (20). Based on this, here we tested our hypothesis that targeting FOS in the FOS/GABP/(mutant) *TERT* cascade might be an effective therapeutic strategy for human cancers harboring *TERT* promoter mutations. We also explored how TERT controls apoptosis as a molecular mechanism, underpinning the effectiveness of this therapeutic strategy.

## Significance

**Given that oncogenic hotspot mutations in the promoter of the gene for telomerase reverse transcriptase (TERT) are common, it has been attractive to develop therapeutic strategies targeting TERT in mutant *TERT* human cancers. No direct TERT inhibitor is currently available, however, hindering this pursuit. By targeting FOS in the FOS/GABPB/(mutant) *TERT* cascade using a FOS inhibitor to inhibit mutant *TERT* cancer cells and tumors through induction of apoptosis by relieving TERT suppression, this study identifies a therapeutic strategy of targeting the TERT cascade for cancers. This study also uncovers a molecular mechanism in which TERT controls apoptosis by specifically regulating two key players among many in the apoptotic cascade, answering a long-time question of how TERT suppresses apoptosis.**

Author contributions: M.X. conceived and supervised the project; R.L. and M.X. designed research; R.L., J.T., X.S., K.J., C.W., G.Z., and M.X. performed research; R.L., X.S., and M.X. analyzed data; and R.L. and M.X. wrote the paper.

The authors declare no competing interest.

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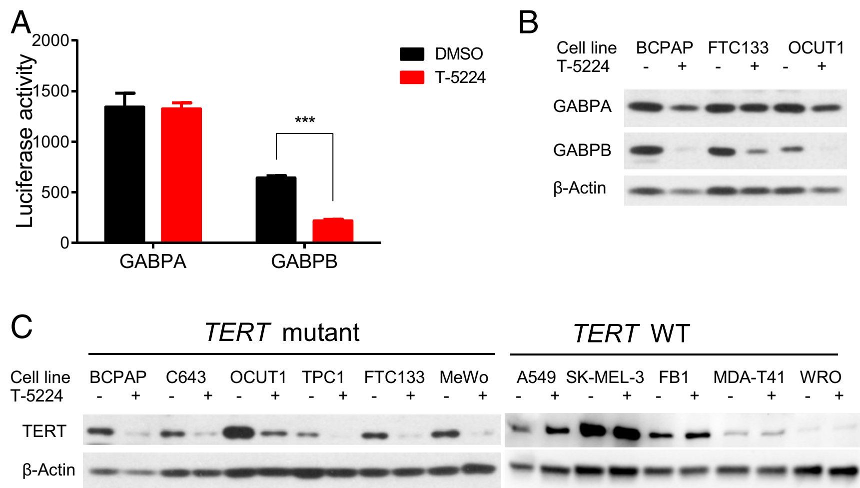


**Fig. 1.** The FOS inhibitor T-5224 selectively suppresses *GABPB* but not *GABPA* and suppresses mutant *TERT* but not WT *TERT*. (*A*) Luciferase reporter activities of *GABPA* and *GABPB* promoters in FTC133 cells transfected with the corresponding constructs. Cells were treated with DMSO or T-5224 at 10 μM for 40 h in the presence of 0.5% FBS, followed by the measurement of luciferase activity. (*B*) Western blot analysis of GABPA and GABPB in cells treated with DMSO or T-5224 at 20 μM for 48 h in cell culture medium containing 0.5% FBS. (*C*) Western blot analysis of TERT in cells treated with DMSO or 20 μM T-5224 for 2 d. ****P* < 0.001.

## Results

**FOS Inhibitor T-5224 Selectively Inhibited *TERT* Expression in Mutant *TERT* Cancer Cells but Not in Wild-Type *TERT* Cells.** To test the foregoing hypothesis, we first examined the effect of the FOS inhibitor T-5224 on the recently identified FOS/*GABPB*/(mutant) *TERT* cascade (20). We found that in a luciferase reporter assay, T-5224 treatment decreased the promoter activity of *GABPB* but not of *GABPA* (Fig. 1*A*). Western blot analysis showed that GABPB expression was inhibited after T-5224 treatment, while GABPA expression was not affected (Fig. 1*B*).

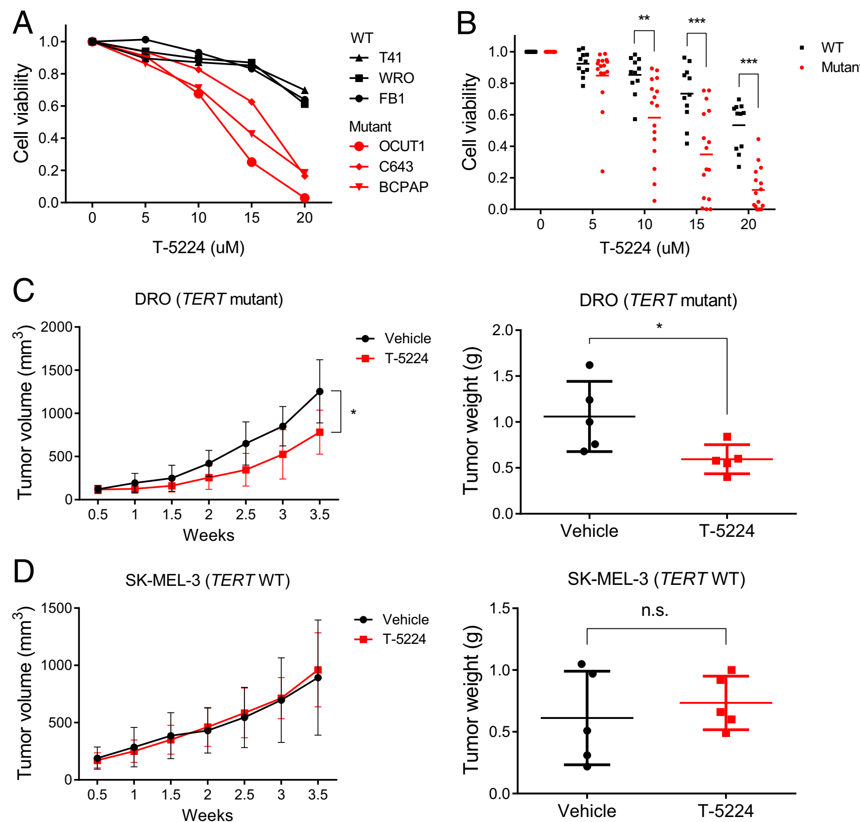


**Fig. 2.** *TERT* promoter mutation confers cells sensitivity to T-5224. (*A*) Mutant *TERT* (red) and WT *TERT* thyroid cancer cell lines (black) were profiled against T-5224 in cell viability assay. (*B*) A total of 16 mutant *TERT* and 11 WT *TERT* cell lines were profiled for sensitivity to T-5224 in a cell viability assay. (*C* and *D*) DRO (mutant *TERT*) and SK-MEL-3 (WT *TERT*) xenograft tumor models were used to test the effect of T-5224 on tumor growth in vivo. All error bars represent SDs. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s.: *P* > 0.05.

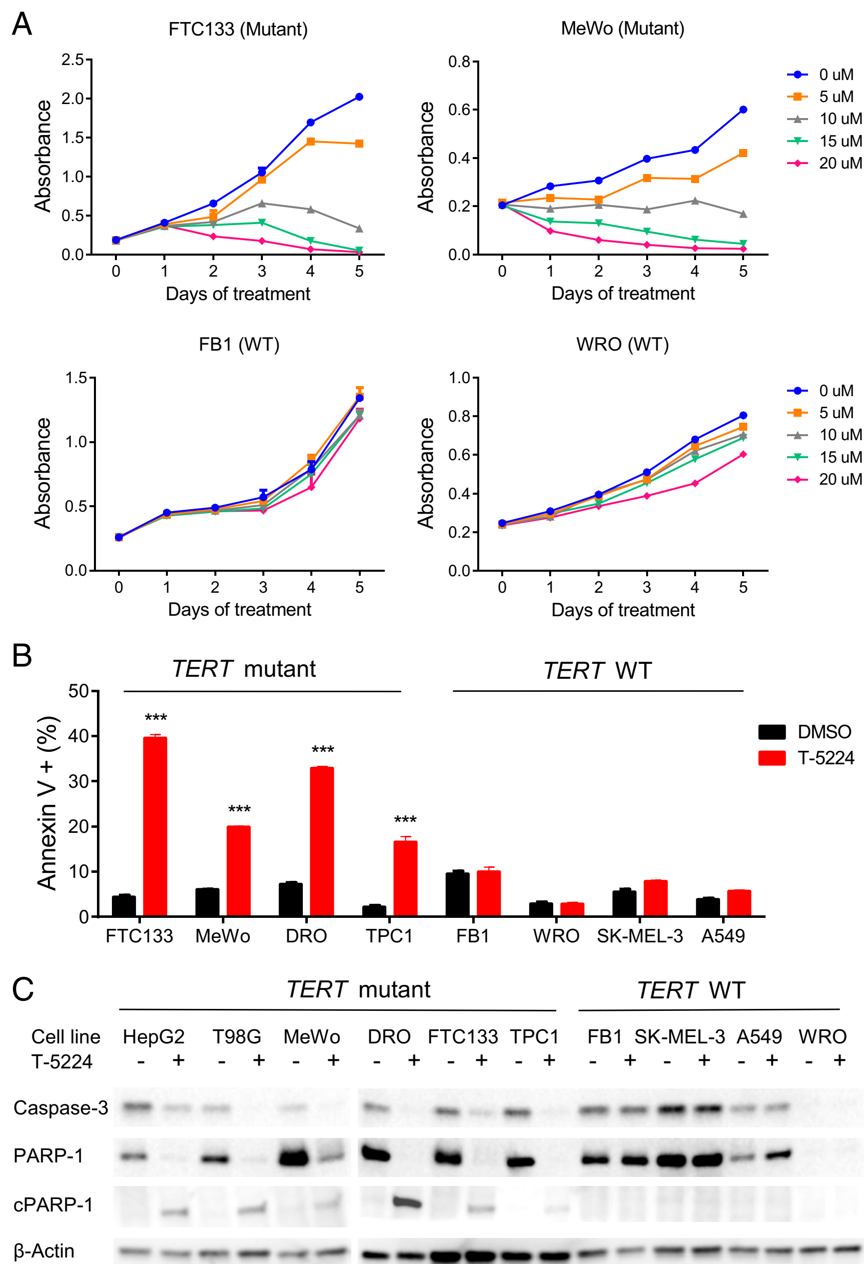


**Fig. 3.** T-5224 selectively induces apoptosis in mutant *TERT* cancer cell lines. (*A*) Growth curves for cell lines harboring *TERT* promoter mutation (FTC133 and MeWo) and cell lines harboring the WT *TERT* promoter (FB1 and WRO). (*B*) The percentage of annexin V- positive cells after treatment with T-5224 at 20 μM for 40 h in the indicated cells. (*C*) Western blot analysis of apoptosis-related proteins caspase-3, PARP-1, and cleaved PARP-1 after the treatment of the indicated cells with T-5224 at 20 μM for 2 d. ***$P < 0.001$.

Consistent with the previous finding that GABP selectively regulates *TERT* expression in mutant *TERT* cells (15, 16), T-5224 selectively and nearly completely suppressed *TERT* expression in cancer cells harboring *TERT* promoter mutations, but not in cells harboring WT *TERT* (Fig. 1*C*). These data demonstrate that by targeting FOS, T-5224 inhibits the FOS/GABPB/(mutant) *TERT* cascade, suppressing the mutant *TERT*.

***TERT* Promoter Mutation Conferred Cancer Cell Sensitivity to T-5224.** To test the effect of T-5224 on cancer cell viability, we initially treated six thyroid cancer cells with T-5224 and found that mutant *TERT* cells exhibited greater sensitivity than WT *TERT* cells (Fig. 2*A*). We then expanded to a larger panel of cells of various cancer types (*SI Appendix*, Fig. S1 and Table S1) and found that cell viability was also preferentially suppressed in mutant *TERT* cells compared with WT *TERT* cells at 10 μM or higher concentrations of T-5224 (Fig. 2*B*). Also, T-5224 treatment nearly completely inhibited the colony formation of mutant *TERT* cells with no effect in WT *TERT* cells (*SI Appendix*, Fig. S2).

We next examined whether the *TERT* promoter mutation-dependent effects of T-5224 observed in vitro could be reproduced in vivo using xenograft tumor models. As shown in Fig. 2*C*, daily treatment with T-5224 significantly suppressed the growth of mutant *TERT* DRO xenograft tumors. In contrast, WT *TERT* SK-MEL-3 xenograft tumors were completely insensitive to T-5224 (Fig. 2*D*). Thus, the anticancer effects of targeting FOS were *TERT* promoter mutation-dependent in both in vitro and in vivo systems.

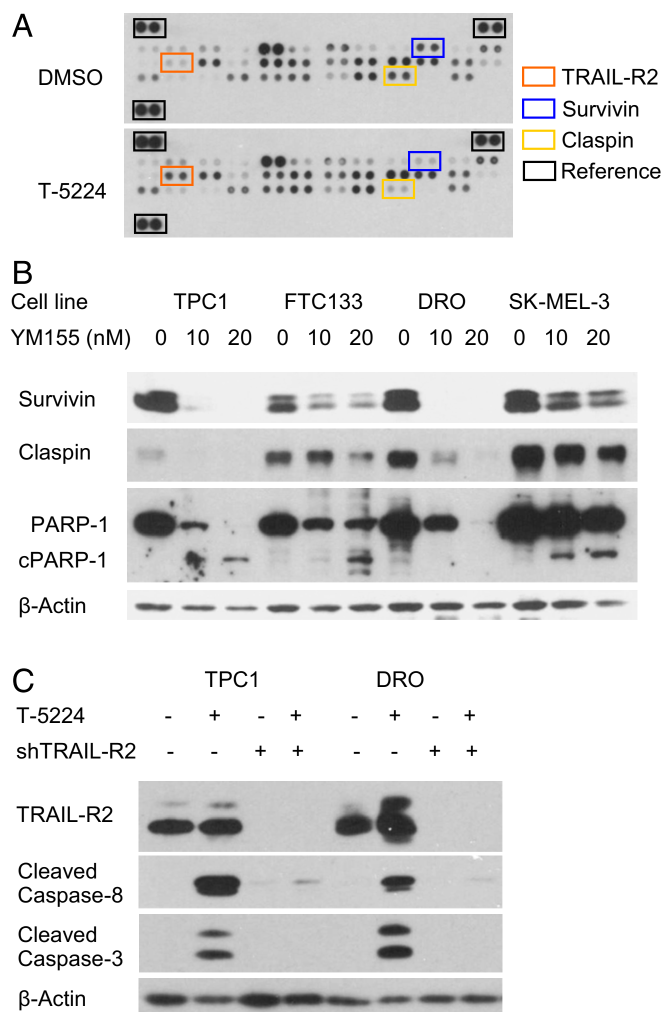


**Fig. 4.** T-5224–induced apoptosis is mediated by alterations in survivin and TRAIL-R2. (*A*) Change in the expression pattern of apoptosis-related proteins in response to the treatment of MeWo cells with T-5224 at 20 μM for 40 h. (*B*) Western blot of survivin, claspin, PARP-1, and cleaved PARP-1 in the indicated cells treated with the survivin inhibitor YM155 for 2 d. (*C*) Western blot of TRAIL-R2, cleaved caspase-8, and cleaved caspase-3 in the indicated cells after treatment with T-5224 at 20 μM for 2 d with or without stable knockdown of TRAIL-R2. Specific shRNA against TRAIL-R2 were used to knock down its expression, and scramble shRNA was used as control.

**T-5224 Selectively Induced Apoptosis in Cells Harboring *TERT* Promoter Mutation.** In mutant *TERT* cells, the inhibition of cell viability and net decreases in cell number were evident when treated with 10 μM or higher concentration of T-5224, while no net decrease in cell number occurred with T-5224 treatment in WT *TERT* cells (Fig. 3*A*). We examined whether this was due to cell apoptosis. Indeed, T-5224 treatment significantly increased the annexin V-positive populations (Fig. 3*B* and *SI Appendix*, Fig. S3) and PARP cleavage (Fig. 3*C*) in mutant *TERT* cells, while no apoptosis was observed after T-5224 treatment in WT *TERT* cells (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S3). Thus, the FOS inhibitor T-5224 selectively induced cancer cell apoptosis in a *TERT* promoter mutation-dependent manner.

**TERT Controlled Apoptosis by Regulating *survivin* and *TRAIL-R2*.** TERT is a well-known apoptosis suppressor, but how TERT suppresses apoptosis mechanistically has remained unclear. To address this important question, we analyzed key proteins involved in the apoptotic cascade using a human apoptosis array. Among the 35 apoptosis cascade-related proteins analyzed, the expression of tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2; also known as death receptor 5 [DR5]) was significantly increased, while the expression levels of survivin and claspin were decreased, after T-5224 treatment in mutant *TERT* MeWo and FTC133 cells (Fig. 4*A* and *SI Appendix*, Fig. S4). Treatment of cells with the survivin inhibitor YM155 decreased the claspin level and increased cleavage of PARP (Fig. 4*B*), consistent with the well-known role of survivin in suppressing apoptosis and supporting its role in T-5224–induced apoptosis. Knockdown of TRAIL-R2 abolished the apoptosis induced by T-5224 in mutant *TERT* cells (Fig. 4*C*), also suggesting an important role of TRAIL-R2 in T-5224–induced apoptosis. Thus, T-5224 induced apoptosis by involving two key points in the apoptotic cascade: survivin and TRAIL-R2.

**TERT Functioned as a Transcriptional Factor Directly Regulating the *survivin* Gene.** We next investigated how T-5224 regulates *survivin* and *TRAIL-R2* expression. After T-5224 treatment, *survivin* promoter activity was significantly decreased, while *TRAIL-R2* promoter activity was increased (Fig. 5*A*). Moreover, T-5224 induced a decrease in *survivin* mRNA and an increase in *TRAIL-R2* mRNA (Fig. 5*B* and *SI Appendix*, Fig. S5), suggesting that T-5224 affected their expression at the transcription level. Since the effect of T-5224 occurred by affecting *TERT* expression in mutant *TERT* cells, we next directly investigated whether TERT regulated the expression of survivin and TRAIL-R2. Similar to T-5224 treatment, knockdown of TERT led to a decrease in *survivin* promoter activity and mRNA expression and an increase in *TRAIL-R2* promoter activity and mRNA level (Fig. 5 *C* and *D* and *SI Appendix*, Fig. S6). There was some evidence that TERT was capable of functioning as a transcriptional factor in regulating certain genes (21). We investigated whether TERT could function as a direct transcriptional factor of *survivin* and *TRAIL-R2*. Our chromatin immunoprecipitation (ChIP) assay showed that TERT could bind to the promoter region of *survivin*, but not *TRAIL-R2* (Fig. 5*E*). By analyzing the RNA sequencing data from the Cancer Cell Line Encyclopedia and TCGA pan-cancer database, we found a strong association between *TERT* and *survivin* expression levels in cancer cells and tumor samples (Fig. 5*F* and *SI Appendix*, Fig. S7). Taken together, these data suggest that TERT acts as a direct transcription factor of *survivin* to up-regulate it.

## Discussion

TERT has been well known to play a prominent role in oncogenesis and as such has long been pursued as a potential therapeutic target in human cancers. However, these has been no success in developing an effective and specific drug or strategy directly targeting TERT to treat human cancers, illustrating the therapeutic challenge in this field. Alternative therapeutic strategies targeting TERT-promoted oncogenesis are needed. Based on the recently established regulatory mechanism of mutant *TERT* through the FOS/GABP/(mutant) *TERT* cascade (20), in the present study we explored a therapeutic strategy for mutant *TERT* human cancers by targeting FOS in this cascade instead of directly targeting TERT.

We demonstrated that inhibiting FOS using the FOS inhibitor T-5224 could robustly suppress mutant *TERT* but not WT *TERT* cancer cells and tumors. Our complementary experimental approaches corroboratively demonstrated that T-5224 effected through suppressing the FOS/GABP/(mutant) *TERT* cascade to induce cell apoptosis. As TERT controls apoptosis by acting as its strong suppressor, cancer cells transformed with *TERT* promoter mutations may develop dependence on apoptosis suppression for survival by overactivated TERT. Thus, suppression of the FOS/GABP/(mutant) *TERT* cascade can trigger apoptotic

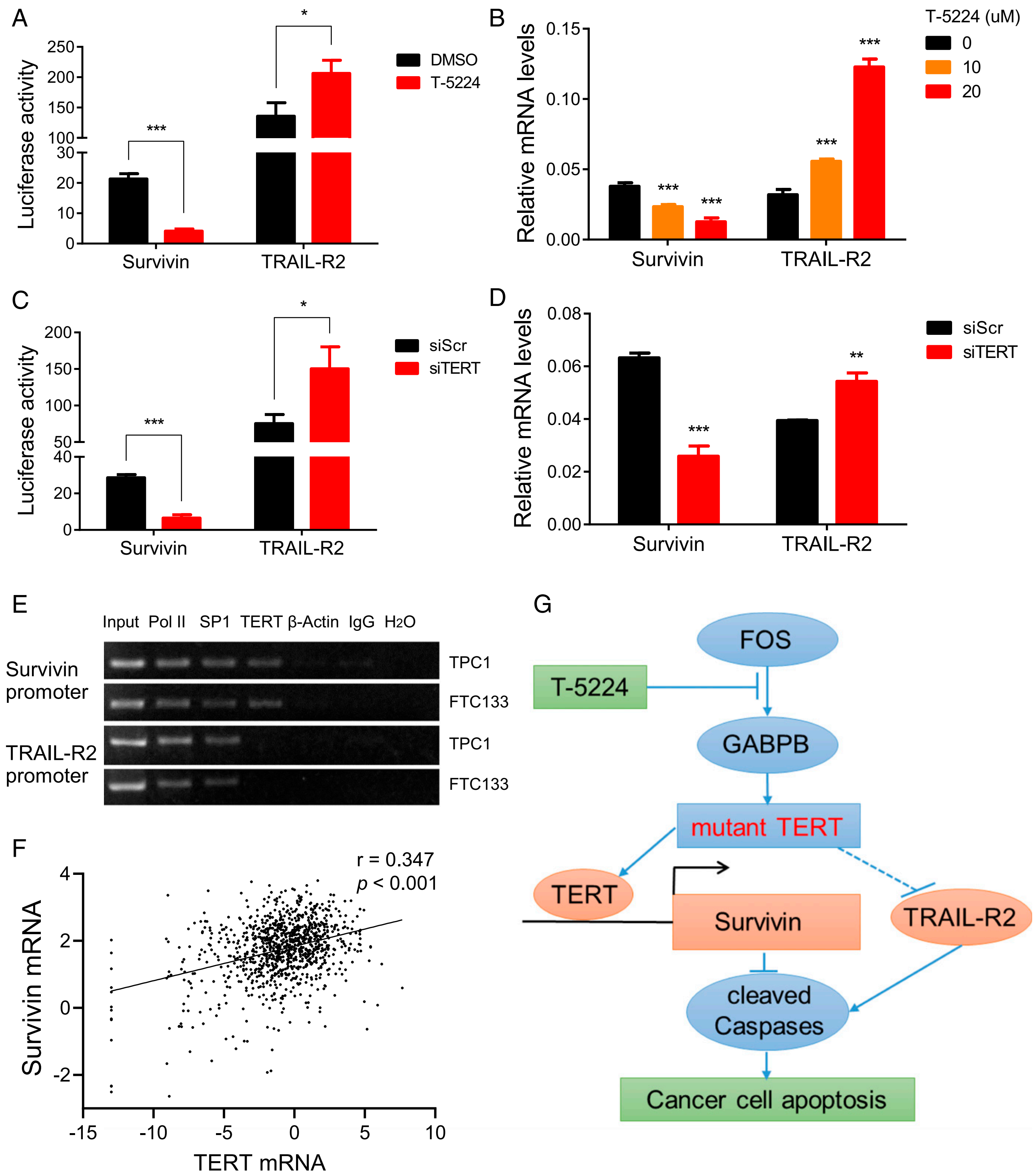


**Fig. 5.** TERT regulates transcriptionally the expression of *survivin* and *TRAIL-R2*. (*A*) Luciferase reporter assay of *survivin* and *TRAIL-R2* promoter activities in TPC1 cells treated with DMSO or T-5224 at 20 μM. (*B*) mRNA expression of *survivin* and *TRAIL-R2* in TPC1 cells treated with T-5224 at the indicated concentrations analyzed by qPCR. (*C*) *Survivin* and *TRAIL-R2* promoter-luciferase reporter assays in TPC1 cells after specific TERT (siTERT) knockdown or scramble siRNA (siScr) control treatment. (*D*) mRNA expression of *survivin* and *TRAIL-R2* in TPC1 cells after specific TERT (siTERT) knockdown or scramble siRNA (siScr) control treatment. (*E*) ChIP assay for TERT occupancy at the promoter regions of *survivin* and *TRAIL-R2*. Pol II and SP1 were used for positive controls and β-actin and IgG served as negative controls. (*F*) Positive correlation between *TERT* and *survivin* expression in 1,020 cancer cell lines in the Cancer Cell Line Encyclopedia. (*G*) Mechanistic model illustrating how inhibition of FOS targeted by inhibitor T-5224 suppresses the FOS/GABPB/(mutant) *TERT* cascade, triggering cell apoptosis by relieving the transcriptional control of *survivin* and *TRAIL-R2* by TERT. Error bars represent SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

death of mutant *TERT* cancer cells by shutting off *TERT* expression. This mechanistically explains and predicts the potential curative anticancer effectiveness of targeting FOS by inhibitor T-5224. As WT *TERT* cancer cells have not developed dependence for survival on the FOS/GABP/(mutant) *TERT* cascade, targeting FOS does not affect the survival of such cells. This is similar to the proapoptotic effects of targeting BRAF or MEK in the MAP kinase pathway in cancers harboring the genetic duet of *BRAF* V600E and *TERT* promoter mutations, which we have recently proposed to represent an "Achilles heel" susceptible to effective therapeutic targeting in human cancer (22). Yet targeting FOS would have a broader application, as it also includes mutant *TERT* cancers without *BRAF* V600E.

Although the apoptosis-suppressing function of TERT has long been recognized (23, 24), its molecular mechanism remains undefined. The present study demonstrates that TERT controls cell apoptosis by regulating *survivin* and *TRAIL-R2*, two critical players in the apoptosis cascade. Our study demonstrates that TERT up-regulates *survivin* by acting as its direct transcriptional factor. Survivin is widely expressed in human cancers, with a primary role in supporting cell survival by suppressing apoptosis through directly inhibiting caspase-3 and caspase-7 (25–27). To complement this mechanism, our study also demonstrated that TERT suppresses *TRAIL-R2* transcriptionally, which is a classical apoptosis initiator (28), through an indirect mechanism. Thus, by dually regulating two key players in the apoptosis cascade, TERT forcefully suppresses apoptosis. This should be a major mechanism with which TERT exerts its oncogenic function, constituting a molecular basis for the effective therapeutic targeting of the FOS/GABP/(mutant) *TERT* cascade in mutant *TERT* cancers.

The concentrations of T-5224 required in this study were relatively high, probably due to the relatively low aqueous solubility of this drug (29). Its differential effects between the mutant *TERT* and WT *TERT* cancer cells and tumors were clear, however. As a FOS inhibitor, T-5224 has been shown to inhibit several inflammatory diseases, such as arthritis, in a preclinical model (29). Moreover, it is possible to achieve therapeutic concentrations of T-5224 in humans, as previous clinical trials for treatment of rheumatoid arthritis demonstrated the drug's clinical effectiveness (30). Thus, it may be reasonable to propose clinical trials of T-5224 for the treatment of mutant *TERT* cancers. The development of more soluble (i.e., potent) FOS inhibitors for mutant *TERT* cancer therapy is strongly encouraged by the present study.

In summary, as illustrated in Fig. 5*G*, the present study identifies a therapeutic strategy that shows great promise in treating mutant *TERT* cancers by targeting FOS to induce cell apoptosis through suppressing the FOS/GABP/(mutant) *TERT* cascade. This circumvents the current challenge of the lack of direct TERT inhibitors. To support this strategy, this study also elucidates a mechanism underlying TERT suppression of apoptosis, which involves the transcriptional regulation of *survivin* and *TRAIL-R2*. This strategy, which merits clinical testing, will likely have an important clinical impact, as *TERT* promoter mutations are widely present in human cancers.

## Materials and Methods

**Cell Lines.** The following cell lines were used in this study: thyroid cancer cell lines BCPAP, FTC133, KAT18, C643, TPC1, OCUT1, ATC241, WRO, FB1, MDA-T41, T351, and Cal-62; melanoma cell lines DRO, CHL-1, M14, MeWo, SK-MEL-2, SK-MEL-3, and SK-MEL-28; glioblastoma cell line T98G; liver cancer cell lines HepG2 and SK-HEP-1; breast cancer cell line MDA-MB-231; non-small cell lung cancer cell line A549; colon cancer cell lines HT29 and HCT116; normal thyroid epithelial cell line HTORI-3; and human embryonic kidney cell line 293T. The origin and growth media of the cell lines are described elsewhere (20, 31, 32). Fetal bovine serum (FBS) was purchased from Gibco (#26140079). All cell lines were authenticated by short tandem repeat profiling and tested for the absence of *Mycoplasma*. Cell line genomic DNA was isolated by the standard phenol-chloroform extraction method. The *TERT* promoter mutation region was amplified and sequenced to analyze the mutation status for each cell line as described previously (33).

**Inhibitors.** FOS inhibitor T-5224 was purchased from ApexBio Technology (B4664), and survivin inhibitor YM155 was purchased from Selleck Chemicals (S1130). All compounds were dissolved in DMSO and then diluted to the indicated concentrations for in vitro studies. Cells were treated with YM155 at 10 or 20 nM for 2 d. Since growth factors in the serum can create strong background growth signals in cells, to minimize these signals we used a low concentration of serum (0.5%) in the cell medium in some experiments designed to study the role of mutant *TERT*.

**Cell Viability Assay.** Cells were seeded in triplicate on 96-well plates at a density of 600 to 2,500 cells per well. The seeding densities were optimized for each cell line to obtain 80 to 90% confluence at harvest. At 16 h after cell seeding, cells were treated with T-5224 at the indicated concentrations for 5 d under low serum concentration condition (0.5% FBS), and an MTT assay (M6494; Invitrogen) was carried out to evaluate cell viability. The absorbance was read at 570 nm on a microplate reader.

**Monolayer Colony Formation Assay.** Two hundred cells were plated in triplicate on 6-well plates and treated with DMSO or 20 μM T-5224 daily for the same length of up to 2 wk in the presence of 0.5% FBS. The cell colonies were stained with 0.1% crystal violet and photographed.

**RNA Isolation and Quantitative Real-Time PCR.** After treatment with T-5224 at the indicated concentrations for 40 h, total RNA was isolated from cultured cells using TRIzol reagent (15596018; Invitrogen) and reverse-transcribed to cDNA using the RevertAid First-Strand cDNA Synthesis Kit (K1622; Thermo Fisher Scientific). The resulting cDNA was diluted, and gene expression was analyzed in triplicate using PowerUp SYBR Green Master Mix (A25742; Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative expression of each gene was calculated according to the $2^{-\Delta\Delta Ct}$ method. GAPDH served as an internal control. The primers used for qRT-PCR are listed in *SI Appendix*, Table S2.

**Western Blot Analysis.** After treatment with DMSO or T-5224 at 20 μM for 48 h, cells were lysed in the RIPA buffer (sc-24948; Santa Cruz Biotechnology) supplemented with protease inhibitor (P8340; Sigma-Aldrich) and phosphatase inhibitor (P0044; Sigma-Aldrich). Cell lysates were centrifugated at 15,000 × *g* for 30 min and denatured by boiling at 95 °C for 5 min. Cell lysates were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes (10600023; GE Healthcare Life Sciences). After blocking with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) buffer at room temperature for 1 h, membranes were incubated with primary antibodies in TBST buffer with 5% nonfat milk at 4 °C overnight, washed with TBST, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. Signals were finally detected by ProtoGlow ECL (CL-300; National Diagnostics). The primary antibodies against TERT (H-231), GABPA (H-180), GABPB (E-7), Claspin ($B_6$), caspase-3 (E-8), PARP-1 (F-2), cleaved PARP-1 (194C1439), and β-actin (C-4) were purchased from Santa Cruz Biotechnology. The primary antibodies against cleaved caspase-3 (5A1E), cleaved caspase-8 (18C8), survivin (71G4B7), and TRAIL-R2 (D4E9) were purchased from Cell Signaling Technology. The HRP-linked secondary antibodies against mouse IgG (7076S) and rabbit IgG (7074S) were also purchased from Cell Signaling Technology.

**Annexin V-FITC Assay.** The annexin V-fluorescein isothiocyanate (FITC) apoptosis assay was carried out using the TACS Annexin V-FITC Apoptosis Detection Kit (4830-250-K; R&D Systems). Cells were seeded on 6-well plates and treated with DMSO or T-5224 at 20 μM for 40 h in the presence of 0.5% FBS. Cells were then collected and resuspended in binding buffer and stained with FITC annexin V and propidium iodide. After incubation at room temperature for 15 min, the samples were evaluated with a BD LSRII flow cytometer (BD Biosciences); 10,000 events per sample were acquired, with data analyzed using FlowJo software.

**Apoptosis Profile Array.** MeWo and FTC133 cells were treated with DMSO or T-5224 at 20 μM for 40 h in the presence of 0.5% FBS, and the expression profile of 35 apoptosis-related proteins was analyzed using the Proteome Profiler Human Apoptosis Array Kit (ARY009; R&D Systems) according to the

manufacturer's instructions. Arrays were incubated with 300 μg of each cell lysate.

**Transient TERT Knockdown.** The siRNA targeting human *TERT* gene (SR322002) and negative control siRNA were purchased from Origene and transfected to cells using Lipofectamine RNAiMAX reagent (13778075; Invitrogen) according to the manufacturer's instruction.

**Stable Knockdown of TRAIL-R2.** A pLKO.1-puro based lentiviral vector expressing shRNA against TRAIL-R2 was purchased from Sigma-Aldrich (TRCN0000005933) (34), and the pLKO.1-puro vector with scramble shRNA was obtained from Addgene (plasmid 1864). Lentiviral particles were produced by cotransfecting HEK293T cells with the shRNA-expressing vector with packaging plasmid psPAX2 (12260; Addgene) and envelope plasmid pMD2.G (12259; Addgene) using Lipofectamine 3000 (Invitrogen). Cells were then exposed to lentivirus for 24 h in the presence of 8 μg $mL^{-1}$ polybrene (Millipore Sigma), and the stable transfection cell pools were selected by 2 μg $mL^{-1}$ puromycin (Sigma-Aldrich) for 1 wk and then confirmed by Western blot analysis.

**Luciferase Reporter Gene Construct and Reporter Gene Assay.** The luciferase reporters harboring *GABPA* and *GABPB* promoters were constructed as described previously (20). To construct the luciferase reporters containing the *survivin* or *TRAIL-R2* promoter, the promoter regions of *survivin* (−702 to +55 from the translation start site) and *TRAIL-R2* (−969 to −1 from the translation start site) were amplified using genomic DNA of normal human thyroid cell line HTORI-3 and then cloned into the pGL3-Basic luciferase vector (Promega), respectively. The primers used for cloning are listed in *SI Appendix*, Table S2.

For the promoter activity assay, TPC1 cells were seeded in triplicate onto a 24-well plate and then transfected with 250 ng of pGL3 plasmids containing the *GAPBA*, *GABPB*, *survivin*, or *TRAIL-R2* promoter, together with 10 ng *Renilla* luciferase (pRL-TK) plasmid as a normalizing control using jetPRIME transfection reagent (114-07; Polyplus-transfection). At 4 h after transfection, the cell culture medium was refreshed, and cells were further treated with DMSO or T-5224 at 20 μM for 40 h. Luciferase activity was finally measured using the Dual-Luciferase Reporter Assay System (Promega) on a TD20/20 luminometer (Turner Designs). The relative luciferase activity was calculated by dividing the activities of firefly luciferase by the activity of *Renilla* luciferase.

**ChIP Assay.** The ChIP assay was performed according to the fast ChIP method with minor modifications (35). In brief, cells were fixed with 1% freshly prepared formaldehyde solution for 10 min at room temperature, followed by incubation with 125 mM glycine for 5 min. Cells were then lysed and sonicated in seven rounds of 15-s pulses at 40% power output using a Branson 150D Sonifier Liquid Processor. The cross-linked protein/DNA was incubated with antibodies against Pol II (sc-899X; Santa Cruz Biotechnology), SP1 (sc-17824X; Santa Cruz Biotechnology), TERT (ab32020; Abcam), β-actin (sc-47778; Santa Cruz Biotechnology) or nonspecific IgG (12-371; Millipore Sigma) overnight at 4 °C and purified by Protein G Sepharose 4 Fast Flow (17-0618-01; GE Healthcare). The precipitated DNA fragments were isolated with Chelex 100 resin (142-1253; Bio-Rad) and subjected to PCR amplification using Q5 High-Fidelity DNA Polymerase (M0491; New England BioLabs). The primers used for amplifying *survivin* and *TRAIL-R2* promoter regions are listed in *SI Appendix*, Table S2.

**Retrieval of Gene Expression Data from The Cancer Genome Atlas Pan-Cancer Atlas and Cancer Cell Line Encyclopedia.** The mRNA expression of *TERT* and *survivin* was retrieved from The Cancer Genome Atlas (TCGA) Pan-Cancer Atlas and Cancer Cell Line Encyclopedia (CCLE) database. The expression data of the two genes is available in 10,324 tumor samples in the TCGA Pan-Cancer dataset and in 1,020 cancer cell lines in the CCLE database, respectively. The correlation between *TERT* and *survivin* mRNA expression was analyzed using the linear regression model.

**In Vivo Study.** All the in vivo experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Johns Hopkins University. The 3- to 4-wk-old female nude mice (Hsd:Athymic Nude-$Foxn1^{nu}$ mice) were obtained from Harlan Laboratories. Xenograft tumors were generated by injecting either $5 \times 10^6$ DRO or SK-MEL-3 cells subcutaneously into the right flank of nude mice. At 2 d after cell inoculation, the animals were divided randomly into 2 groups to receive T-5224 daily at a dose of 200 mg $kg^{-1}$ or vehicle only as control. T-5224 was dissolved in polyvinylpyrrolidone solution and administered by oral gavage. Tumor volumes were determined twice weekly with digital calipers using the formula ($width^2 \times length$)/2. At the end, mice were killed by $CO_2$ euthanasia, and tumors were surgically removed, photographed, and weighed.

**Statistical Analysis.** The two-sided Student's *t* test was used to determine the significance between two groups. The correlation between gene expressions was assessed by the linear regression model. $P < 0.05$ was considered significant. Analyses were performed using Stata/SE version 10 for Windows (StataCorp) and GraphPad Prism version 6 for Windows.

**Data Availability.** All data are available in the main text and *SI Appendix*.

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