Journal of the Endocrine Society, 2021, Vol. 5, No. 8, 1–14
doi:10.1210/jendso/bvab102
Research Article

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Thyroid Hormone Receptor Beta Inhibits PI3K-Akt-mTOR Signaling Axis in Anaplastic Thyroid Cancer via Genomic Mechanisms

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Abbreviations: Akt, protein kinase B; ATC, anaplastic thyroid cancer; AXL, tyrosine-protein kinase receptor UFO; ELISA, enzyme-linked immunosorbent assay; EV, empty vector; FGFR3/4/L1, fibroblast growth factor receptor 3/4/like 1; FTC, follicular thyroid cancer; GEO, Gene Expression Omnibus; GSK3β, glycogen synthase kinase 3 beta; GYS1, glycogen synthase 1; HER2, receptor tyrosine-protein kinase erbB-2; HER3, receptor tyrosine-protein kinase erbB-3; INPP4B, inositol polyphosphate 4-phosphatase type II; INPP5J, phosphatidylinositol 4,5-bisphosphate 5-phosphatase A; JAK1, Janus kinase 1; mTORC1/2, mechanistic target of rapamycin complex 1/2; p70S6K, ribosomal protein S6 kinase beta-1 PDPK1, 3-phosphoinositide dependent protein kinase 1; PBS, phosphate-buffered saline; PDTC, poorly differentiated thyroid cancer; PEKHA2, tandem-PH-domain-containing protein-2; PHLPP1, PH domain and leucine-rich repeat-protein phosphatase 1; PI(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphoinositide 3 kinase; PIP_3, phosphatidylinositol 3,4,5 trisphosphate; PPP2R5B, protein phosphatase 2R5B; PTC, papillary thyroid cancer; PTEN, phosphatase and tensin homolog; PTPN13, protein-tyrosine-phosphatase-like protein-1; RNA-seq, RNA-sequencing; ROR1, neurotrophic tyrosine kinase receptor-related 1; RTK, receptor tyrosine kinase; RUNX2, runt-related transcription factor 2; STAT1, signal transducer and activator of transcription 1; T_3, 3,5,3′-triiodothyronine; TRβ, thyroid hormone receptor beta.

Received: 16 December 2020; Editorial Decision: 26 May 2021; First Published Online: 1 June 2021; Corrected and Typeset: 9 July 2021.

Abstract

Thyroid cancer is the most common endocrine malignancy, and the global incidence has increased rapidly over the past few decades. Anaplastic thyroid cancer (ATC) is highly aggressive, dedifferentiated, and patients have a median survival of fewer than 6 months. Oncogenic alterations in ATC include aberrant phosphoinositide 3 kinase (PI3K) signaling through receptor tyrosine kinase (RTK) amplification, loss of phosphoinositide phosphatase expression and function, and protein kinase B (Akt) amplification. Furthermore, the loss of expression of the tumor suppressor thyroid hormone receptor beta (TRβ) is strongly associated with ATC. TRβ is known to suppress PI3K in follicular thyroid cancer and breast cancer by binding to the PI3K regulatory subunit p85α. However, the role of TRβ in suppressing PI3K signaling in ATC is not completely delineated. Here we report that TRβ indeed suppresses PI3K signaling in ATC cell lines through unreported genomic

ISSN 2472-1972

mechanisms, including a decrease in RTK expression and an increase in phosphoinositide and Akt phosphatase expression. Furthermore, the reintroduction and activation of TRβ in ATC cell lines enables an increase in the efficacy of the competitive PI3K inhibitors LY294002 and buparlisib on cell viability, migration, and suppression of PI3K signaling. These findings not only uncover additional tumor suppressor mechanisms of TRβ but shed light on the implication of TRβ status and activation on inhibitor efficacy in ATC tumors.

Key Words: anaplastic thyroid cancer, PI3K, PI3K inhibitor, thyroid hormone receptor, receptor tyrosine kinase, PI3K phosphatase

Thyroid cancer is the most common endocrine malignancy, and the incidence has been rapidly increasing the past few decades [1]. While the overall prognosis for thyroid cancer is generally favorable, patients with the most aggressive and dedifferentiated subtype, anaplastic thyroid cancer (ATC), have a median survival of 3 to 5 months [2]. The current most effective treatments for ATC patients increase median survival time to only 11 (sorafenib) or 12 (dabrafenib with trametinib) months as drug resistance and tumor recurrence often develop [3, 4]. Therefore, there is an unmet need for more precise understanding of the molecular etiology of ATC tumorigenesis and new strategies for improving patient outcome.

Phosphoinositide 3 kinase (PI3K) signaling is a prominent molecular driver for aggressive and poorly differentiated thyroid cancers (PDTCs) such as ATC [5]. PI3K is recruited to the plasma membrane by phosphorylated, ligand-bound receptor tyrosine kinases (RTKs). PI3K phosphorylates the membrane lipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to PIP_3, which recruits 3-phosphoinositide dependent protein kinase 1 (PDPK1) and the mechanistic target of rapamycin complex 2 (mTORC2) to the plasma membrane to phosphorylate protein kinase B (Akt) on thr308 and ser473, respectively. Phosphatase and tensin homolog (PTEN) is a tumor suppressor that dephosphorylates PIP_3 back to $PI(4,5)P_2$. Akt phosphorylates a myriad of targets that are involved in cell cycle progression and survival signaling. In addition, Akt leads to the activation of mTORC1, which phosphorylates targets such as p70S6K that ultimately lead to the activation and assembly of translation factors for protein synthesis and cell growth [6, 7]. Multiple genes are either mutated or amplified within the PI3K pathway in ATC. Frequent alterations include amplification of RTKs such as epidermal growth factor, amplification or gain-of-function mutations in *PIK3CA* (PI3K), loss-of-function mutations or decreased expression of *PTEN*, and amplification of *AKT1* [8-11].

In addition to the canonical mechanisms of PI3K regulation, there are a multitude of other factors that regulate the pathway. These factors include members of the nuclear hormone receptor family including estrogen, androgen, and thyroid hormone receptors [12-14]. Our work has demonstrated that thyroid hormone receptor beta (TRβ) acts as a tumor suppressor in ATC cells through the repression of several pathways important for tumor growth [15, 16]. However, the potential for TRβ to exhibit tumor suppression in ATC by suppressing PI3K is not fully understood. Multiple groups have reported the potential for TRβ to bind to the regulatory subunit of PI3K, p85α, to inhibit phosphorylation of $PI(4,5)P_2$ to PIP_3 [17-19]. While these mechanisms help explain the potential for TRβ to inhibit PI3K in certain cancer models, there may be other mechanisms of TRβ-mediated suppression. While TRβ has been shown to inhibit PI3K via nongenomic mechanisms in breast [20] and follicular thyroid [21, 22] cancer (FTC), it is unknown if this mechanism or unexplored genomic mechanisms occur in ATC. Therefore, we sought to better understand the mechanism of TRβ-mediated suppression of PI3K signaling in ATC cell lines using a TRβ-expression model. Moreover, we tested the efficacy of the PI3K inhibitors LY294002 and buparlisib in cells with or without TRβ expression. These findings present previously unexplored mechanisms of the tumor suppression by TRβ, the role of TRβ in ATC cells, as well as the implication of TRβ expression status in response to PI3K-targeted therapeutic intervention.

Materials and Methods

Culture of Thyroid Cell Lines

Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 growth media with L-glutamine (300 mg/L), sodium pyruvate, and nonessential amino acids (1%) (Corning Inc), supplemented with 10% fetal bovine serum (Peak Serum) and penicillin-streptomycin (200 IU/L) (Corning) at 37 °C, 5% CO_2, and 100% humidity. The final concentration of 3,5,3′-triiodothyronine (T_3) in the media was 170 pM. Lentivirally modified SW1736 cells were generated as described [16, 23] with either an empty vector (SW-EV) or to overexpress TRβ (SW-TRβ). SW-EV and SW-TRβ

were grown in the aforementioned conditions with the addition of 2-µg/mL puromycin (Gold Bio). All the ATC cell lines used in this work come from females and present the BRAFV600E mutation [24]. All data were generated from cell lines within 1 to 5 passages from acquisition or transduction. SW1736 and KTC-2 were authenticated by the Vermont Integrative Genomics Resource at the University of Vermont (Burlington, Vermont) using short tandem repeat profiles and Promega GenePrint10 System (SW1736, May 2019; KTC-2, October 2019). 8505C, OCUT-2, and CUTC60 were authenticated by the University of Colorado by short tandem repeat profiles (8505C, June 2013; OCUT-2, June 2018; CUTC60, November 2018).

Cell Culture Reagents

T_3 was purchased from Sigma and dissolved in 1-N NaOH and diluted to 10 nM in cell culture medium at the time of each application. LY294002 and buparlisib were purchased from MedChemExpress. LY294002 was dissolved in 100% ethanol and buparlisib was dissolved in 100% dimethyl sulfoxide prior to indicated dilutions for cell culture experiments.

Immunoblot Analysis

Proteins were isolated from whole cells in lysis buffer (20-mM Tris-HCl [pH 8], 137-mM NaCl, 10% glycerol, 1% Triton X-100, and 2-mM EDTA) containing Protease Inhibitor Cocktail (catalog No. 78410; Thermo Fisher Scientific), 1-mM Na_3VO_4, and 1-mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Proteins were quantified via Pierce Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific), and 25 µg of protein per sample were resolved by polyacrylamide gel electrophoresis on 10% Tris-Glycine gels (catalog No. XP00105BOX) (Thermo Fisher Scientific) and immobilized onto nitrocellulose membranes (GE Healthcare) by electroblot (Bio-Rad Laboratories). Membranes were blocked with 5% w/v bovine serum albumin in Tris-buffered saline and 0.1% v/v Tween20 (Gold Bio) for 1 hour at room temperature and incubated with primary antibodies overnight (Table 1); immunoreactive proteins were detected by enhanced chemiluminescence (Thermo Scientific) on a ChemiDoc XRS+ (Bio-Rad Laboratories). Research Resource Identifiers (RRIDs) for each antibody included the following: β-actin (AB_10979409), pAkt (T308) (AB_2629447), pAkt (S473) (AB_2315049), Akt (pan) (AB_1147620), pmTOR (S2448) (AB_330970), pmTOR (S2481) (AB_2262884), mTOR (AB_1904056), pp70S6K (AB_2269803), pGYS1 (AB_568824), GYS1 (AB_732660), TRβ (AB_10807563), mouse immunoglobulin G (AB_330924), and rabbit immunoglobulin G (AB_2099233). Densitometry analysis was performed using ImageJ (NIH). The antibodies were validated by orthogonal methods and were confirmed via immunoblots using purified TRβ protein and lysate from cells overexpressing TRβ as positive controls and/or small interfering RNA knockdown as a negative control.

Measurement of Protein Kinase B Phosphorylation by Enzyme-Linked Immunosorbent Assay

Akt serine 473 phosphorylation was measured using Pathscan phospho-Akt1 sandwich ELISA kit, according to the manufacturer's instructions (Cell Signaling Technology). Samples were prepared from cells treated with 10-nM T_3 for 24 hours and then 1 hour incubation in the presence or absence of 1- or 10-µM LY294002. A total of 100 µL of samples containing equal amount of protein were applied to each well.

Measurement of Phosphoinositide 3 Kinase Activity by Enzyme-Linked Immunosorbent Assay

PI3K activity was determined using a commercially available PI3K ELISA kit (Echelon Biosciences Inc) according to the manufacturer's instructions. Briefly, after drug treatment, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 500-µL ice-cold lysis buffer (137-mM NaCl, 20-mM Tris–HCl [pH 7.4], 1-mM $CaCl_2$, 1-mM $MgCl_2$, 1-mM Na_3VO_4, 1% NP-40, and 1-mM PMSF). PI3K was then immunoprecipitated with 5 µL of antibody (anti-p85α, RRID: AB_2714180) and 60 µL of Pierce Protein A/G magnetic beads (Thermo Scientific). PI3K activity in the immunoprecipitates was then assayed by PI3K ELISA according to the manufacturer's instructions. The spectrophotometric data were obtained using a Synergy 2 Multi-Detection Microplate Reader (Agilent Technologies) at a wavelength of 450 nm. The protein concentrations of cellular lysates were determined by Bradford assay as described earlier. The activity of PI3K was corrected for protein content.

RNA-Sequencing Data Analysis of Phosphoinositide 3 Kinase Pathway Intermediates

Previously published RNA-sequencing (RNA-seq) data were used to determine expression levels of genes within the PI3K pathway [16]. Construction of the PI3K signaling genes of interest for our study was based on

Table 1. Antibodies

Antigen	Manufacturer; catalog No.; RRID	Species	Dilution
β-actin	Thermo Fisher Scientific; MA5-15739; AB_10979409	Mouse	1:5000
pAkt, T308	Cell Signaling Technology; 13038; AB_2629447	Rabbit	1:1000
pAkt, S473	Cell Signaling Technology; 4060; AB_2315049	Rabbit	1:1000
Akt, pan	Cell Signaling Technology; 2920; AB_1147620	Mouse	1:1000
pmTOR, S2448	Cell Signaling Technology; 2971; AB_330970	Rabbit	1:1000
pmTOR, S2481	Cell Signaling Technology; 2974; AB_2262884	Rabbit	1:1000
mTOR	Cell Signaling Technology; 4517; AB_1904056	Mouse	1:1000
pp70S6K	Cell Signaling Technology; 9234; AB_2269803	Rabbit	1:1000
pGYS1	Millipore Sigma; 07-817; AB_568824	Rabbit	1:1000
GYS1	Abcam; 40810; AB_732660	Rabbit	1:1000
TRβ	Millipore Sigma; ABN25; AB_10807563	Rabbit	1:1000
p85α	Millipore Sigma; ABS234; AB_2714180	Rabbit	1:100
Mouse IgG	Cell Signaling Technology; 7076; AB_330924	Horse	1:10000
Rabbit IgG	Cell Signaling Technology; 7074; AB_2099233	Goat	1:10000

Abbreviations: Akt, protein kinase B; GYS1, glycogen synthase 1; IgG, immunoglobulin; mTOR, mechanistic target of rapamycin; RRID, Research Resource Identifiers; TRβ, thyroid hormone receptor beta.

the curated IPA pathway gene set. Additional genes of interest were added based on literature search and cancer relevance. Normalized transcript counts generated with DESeq2 were used to calculate fold change compared to the control condition (SW-EV-T_3). Raw and processed expression data can be found in the Gene Expression Omnibus (GEO) database under accession number GSE150364.

Analysis of Thyroid Cancer Patient Sample Data

Publicly available microarray expression data, deposited in the GEO Database (GSE76039, GSE3467, GSE82208 [25-27]), were analyzed using GEOR2 (www.ncbi.nlm.nih.gov/gds) to reveal differential expression of genes relevant to PI3K signaling across the spectrum of thyroid cancers. Data were background-adjusted and normalized using Robust Multichip Average, and the same chip platform (Affymetrix Human Genome U133 Plus 2.0 Array, GLP570) was used in all experiments. All oligonucleotides were mapped to the latest NetAffx annotation file version.

RNA Extraction and Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was extracted using the RNeasy Plus Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA was then generated using the 5X RT MasterMix (ABM). Gene expression to validate RNA-seq analysis was quantified by quantitative reverse transcriptase–polymerase chain reaction using BrightGreen 2X qPCR MasterMix (ABM) on a QuantStudio 3 reverse-transcriptase PCR system (Thermo Fisher Scientific). Fold change in gene expression compared to endogenous controls was calculated using the ddCT method. Primer sequences are indicated in Supplementary Table 1 [28].

In Vitro Cell Viability Assay

The cell viability assay was performed by plating 1.0×10^4 SW-EV or SW-TRβ cells into 12-well (22.1-mm) tissue culture dishes. After adhering overnight, the cells were treated with 10-nM T_3 and LY294002, buparlisib, or vehicle at the indicated concentrations. Every day after treatment for 4 days, the medium was removed, cells were washed with PBS and lifted with trypsin (Thermo Scientific), and the number of surviving cells was counted with a hemocytometer.

Migration Assay

Cell migration was determined by wound healing assay. Cells were plated and allowed to grow to 100% confluency. Two hours prior to scratching, cells were treated with 10-μg/mL Mitomycin C (Sigma) dissolved in H_2O. A scratch was performed with a P1000 pipette tip and debris was washed away with PBS. Migration media was supplemented with 10-nM T_3 and LY294002 or vehicle. Images were obtained using a Canon digital camera connected to an Axiovert inverted microscope (Carl Zeiss) at 0, 24, 48, and 72 hours. Wound closure was measured using ImageJ macro "Wound Healing Tool" (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool). Values were normalized so that the initial scratch was 0% closure.

Statistics

All statistical analyses were performed using GraphPad Prism software. Paired comparisons were conducted by *t* test. Group comparisons were made by one-way analysis of variance followed by a Dunnett or Tukey multiple comparison test as appropriate. Two-way analysis of variance followed by a Tukey multiple comparison test was conducted for multigroup analysis. Data are represented as mean ± SD. Area under the curve at the 95th CI was used to evaluate statistical differences in growth and migration assays.

Results

Rapid Thyroid Hormone Receptor Action Fails to Suppress Phosphoinositide 3 Kinase in Anaplastic Thyroid Cancer Cells

TRβ is a known suppressor of the PI3K signaling pathway in breast and FTC. This has been previously described as a nongenomic mechanism by which TRβ binds to the regulatory subunit of PI3K, p85α, preventing recruitment to ligand-bound RTKs [14]. This action is rapid, and the addition of the thyroid hormone T_3 modulates the response within 15 to 30 minutes [29]. Therefore, we sought to evaluate the impact of short-term T_3 treatment in the SW1736 cell line with restored stable expression of TRβ or an EV control (Fig. 1A). Analysis of pAkt and pmTOR by Western blot surprisingly revealed a minimal impact of TRβ with or without T_3 on PI3K suppression (Fig. 1B). To validate these results, we performed a PI3K immunoprecipitation followed by ELISA to test the ability of PI3K to catalyze the phosphorylation of $PI(4,5)P_2$ to PIP_3 following T_3 treatment. Again, we observed a modest but insignificant decrease in PIP_3 production in the presence of TRβ and T_3 (Fig. 1C).

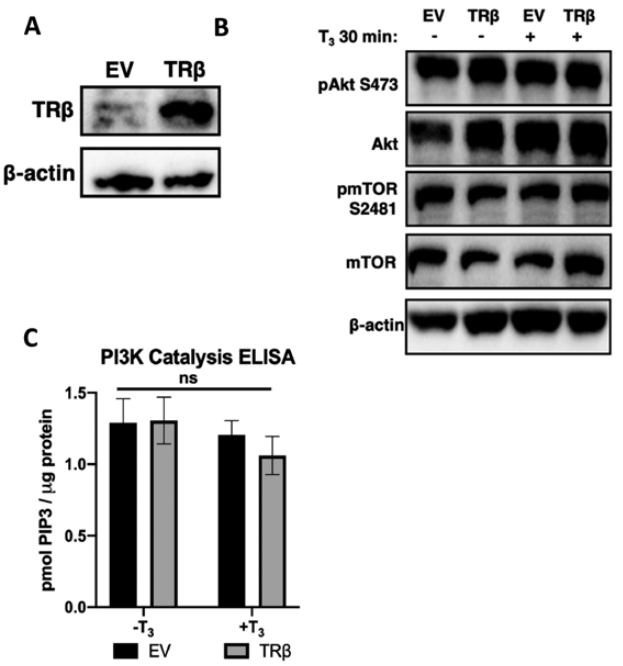

Figure 1. Short-term exposure to 3,5,3′-triiodothyronine (T_3) is insufficient to induce thyroid hormone receptor beta (TRβ)-mediated phosphoinositide 3 kinase (PI3K) suppression. A, TRβ protein was assessed in SW1736-EV (empty vector; EV) and SW1736-TRβ (TRβ) cells to ensure successful lentiviral transduction. EV and TRβ cells were treated with 10-nM T_3 or vehicle (10-µM NaOH) for 30 minutes before protein levels were determined B, by immunoblot, or C, incubated with anti-p85α antibody for PI3K catalysis enzyme-linked immunosorbent assay (ELISA). ELISA signal in C was standardized to protein concentration as determined by a Bradford assay. Significance in C was calculated by 2-way analysis of variance followed by Tukey multiple comparisons test. NS, no significance ($P \geq .05$) across treatment groups.

Long-term 3,5,3′-Triiodothyronine Treatment Suppresses Phosphoinositide 3 Kinase Signaling in Anaplastic Thyroid Cancer Cells

Since short-term T_3 treatment did not suppress PI3K activity in our cell line model, we hypothesized that long-term T_3 treatment may enable TRβ-mediated suppression of PI3K signaling. Therefore, we treated our EV and TRβ cells with T_3 for 24 hours then measured pAkt, pmTOR, pp70S6K, and pGYS1 levels by Western blot (Fig. 2A and 2C). The SW-TRβ cells treated with T_3 exhibited a marked decrease in pAkt on serine 473 but not threonine 308. Serine 473 phosphorylation induces a substantial increase in Akt activity following growth factor stimulation and plays a role in regulating substrate specificity [30]. There was no reduction in pP70S6K, a kinase further downstream of Akt. To test the impact of long-term T_3 exposure and heightened TRβ expression, we measured a well-studied downstream effector, glycogen synthase kinase 3 beta (GSK3β) and its substrate glycogen synthase 1 (GYS1). GSK3β is a multisubstrate kinase and, importantly, is implicated in the progression of numerous cancers including ATC [31, 32]. We observed a marked increase in phosphorylated, and thus inactivated, GYS1 in the presence of TRβ and T_3.

To validate the observed Akt ser473 dephosphorylation, we conducted a sandwich ELISA with our EV and TRβ cells following 24-hour T_3 treatment (Fig. 2B); pAkt (ser473) was again reduced with liganded-TRβ. Since TRβ-T_3 treatment suppressed both Akt-mTOR-P70S6K and Akt-GSK3β-GYS1 pathways, these data are suggestive of a requirement for both thyroid hormone and TRβ to achieve robust inactivation of PI3K signaling as observed through 2 separate pathways downstream of Akt.

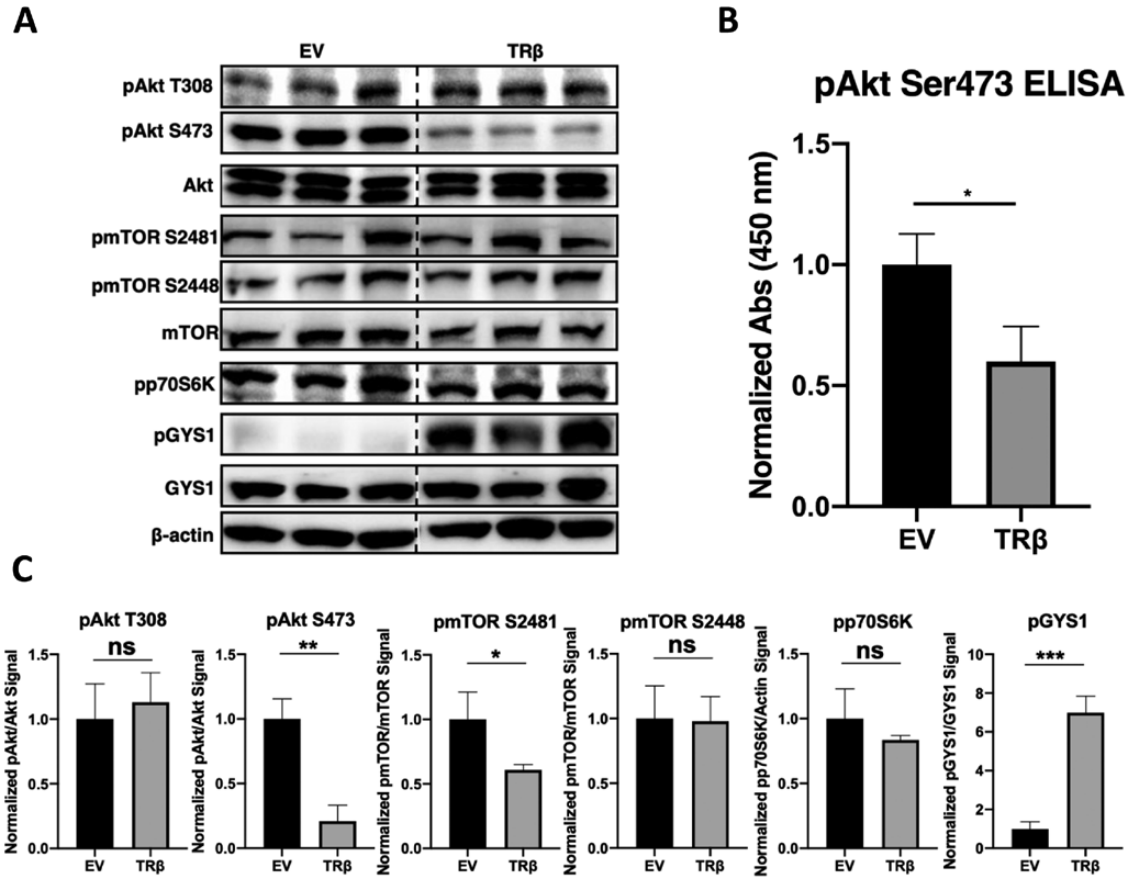

Figure 2. Long-term exposure to 3,5,3′-triiodothyronine (T_3) is required to induce thyroid hormone receptor beta (TRβ)-mediated suppression of the phosphoinositide 3 kinase–protein kinase B–mechanistic target of rapamycin (PI3K-Akt-mTOR) pathway. SW1736-EV (empty vector; EV) and SW1736-TRβ (TRβ) cells were treated with 10-nMT_3 for 24 hours before protein levels were determined by immunoblot (A and quantified in C) or B, subjugated to a pAkt Ser473 sandwich enzyme-linked immunosorbent assay (ELISA). The samples in A are biological replicates. Dashed lines in immunoblots indicate gap between 2 sets of lanes on the same membrane. ELISA signal in B was standardized to protein concentration as determined by a Bradford assay. Significance in B and C was calculated by *t* test. NS, no significance ($P \geq .05$, $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$).

Liganded Thyroid Hormone Receptor Beta Transcriptionally Remodels the Phosphoinositide 3 Kinase Signaling Landscape

Since a long-term T_3 treatment was needed for robust suppression of PI3K signaling in our cell line model, we hypothesized that TRβ may be regulating the expression of key components of this pathway. To better understand the extent to which TRβ suppresses PI3K signaling through genomic mechanisms, we leveraged our RNA-seq data performed on our EV and TRβ cell lines following 24 hours of T_3 treatment [16]. Numerous genes involved in the PI3K pathway were determined to be differentially expressed in the presence of both T_3 and TRβ (Fig. 3A-3C).

RTL gene expression was reduced in the TRβ-T_3 group, including HER3 (*ERBB3*), fibroblast growth factor receptor isoforms (*FGFR3*, *FGFR4*, *FGFRL1*), neurotrophic tyrosine kinase receptor-related 1 (*ROR1*), and tyrosine-protein kinase receptor UFO (*AXL*).

Although *PTEN* expression was not increased, other membrane-bound phosphoinositide phosphatases did increase, including phosphatidylinositol 4,5-bisphosphate 5-phosphatase A (*INPP5J*), and inositol polyphosphate 4-phosphatase type II (*INPP4B*), which dephosphorylate PIP_3 to $PI(3,4)P_2$ and $PI(3,4)P_2$ to PI(3)P, respectively [40]. There was also an increase in expression of cytosolic phosphatases that dephosphorylate Akt primarily on ser473, including tandem-PH-domain-containing protein-2 and protein-tyrosine-phosphatase-like protein-1 (*PLEKHA2* and *PTPN13*), the R5B localization subunit of protein phosphatase 2 (*PPP2R5B*), and PH domain and leucine-rich repeat-protein phosphatase 1 (*PHLPP1*) [36,41,42]. In addition to these genes that serve to regulate PI3K activation and subsequent Akt phosphorylation, genes involved in PI3K recruitment and stabilization, mTORC regulation, and translation factors were found to be differentially regulated in the TRβ-T_3 group (Supplementary Fig. 1A-1D and Table 2 [28]). The

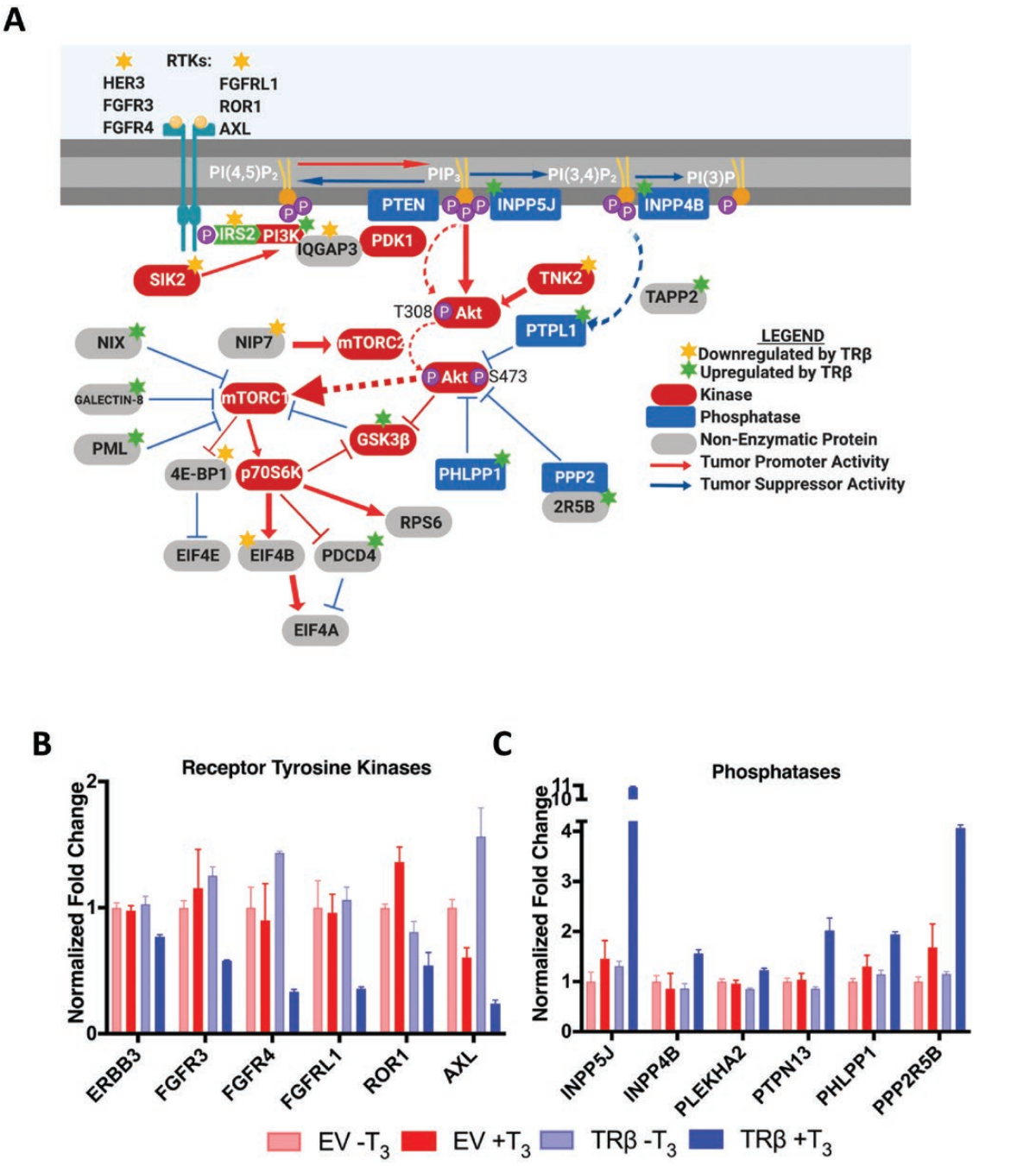

Figure 3. Liganded thyroid hormone receptor beta (TRβ) decreases expression of oncogenic genes and increases tumor-suppressive genes in the phosphoinositide 3 kinase–protein kinase B–mechanistic target of rapamycin (PI3K-Akt-mTOR) signaling axis. A, Receptor tyrosine kinases (RTKs) dimerize in response to ligands to allow for IRS2 to dock and recruit PI3K [33]. PI3K phosphorylates $PI(4,5)P_2$ to PIP_3. Phosphatase and tensin homolog (PTEN) and phosphatidylinositol 4,5-bisphosphate 5-phosphatase A (INPP5J) dephosphorylate PIP_3 to $PI(4,5)P_2$ and $PI(3,4)P_2$, respectively. Inositol polyphosphate 4-phosphatase type II (INPP4B) dephosphorylates $PI(3,4)P_2$ to PI(3)P. PIP_3 recruits PDK1 (not shown) and Akt to the plasma membrane for PDK1 phosphorylation of Akt T308. NIP7-activated mechanistic target of rapamycin complex 2 (mTORC2) phosphorylates Akt S473, which is dephosphorylated by PH domain and leucine-rich repeat-protein phosphatase 1 (PHLPP1) [34]. ING5 has been shown to dephosphorylate Akt in hormone-dependent cancers [35]. The 2R5B subunit of protein phosphatase 2 (PPP2) directs the complex to dephosphorylate Akt T308 and S473 [36]. TNK2 phosphorylates Akt Y176 to enhance plasma membrane recruitment [37]. Akt leads to the activation of mTORC1 by TSC 1/2-Rheb (not shown). Nix and PML destabilize Rheb-mTORC1 binding [38]. Galectin-8 inhibits and delocalizes mTORC1 [39]. Glycogen synthase kinase 3 beta (GSK3β) is inhibited by Akt and inhibits multiple substrates relevant to glucose and glycogen metabolism, survival signaling, and cell cycle progression. mTORC1 phosphorylates and inhibits 4E-BP1, which inhibits EIF4E. mTORC1 also activates P70S6K, which inhibits GSK3β and PDCD4 and activates EIF4B and RPS6. EIF4B and PDCD4 regulate EIF4A [38]. B, Ligand-bound TRβ decreased expression of RTKs. C, Ligand-bound TRβ increased expression of PI3K-Akt phosphatases. Significance and fold-change values between empty vector (EV) $+T_3$ and TRβ $+T_3$ are located in Supplementary Table 2. Data were previously generated via RNA-sequencing [16].

fold change of a subset of the differentially expressed genes was validated via reverse transcriptase–quantitative polymerase chain reaction (Supplementary Fig. 2 [28]).

Endogenous Thyroid Hormone Receptor Beta Expression in Anaplastic Thyroid Cancer Cells Correlates With Low Phosphorylated Protein Kinase B (pAkt) Ser473 and High Phosphoinositide 3 Kinase–Akt Signaling Phosphatase Expression

Following our RNA-seq findings in transduced SW1736 cells, we next questioned if level of endogenous TRβ expression correlates with reduced Akt phosphorylation and increased PI3K-Akt phosphatase expression. We demonstrated a significant inverse correlation between TRβ expression and Akt phosphorylation as shown by immunoblot (Fig. 4A and 4B). Furthermore, 8505C with the highest level of endogenous TRβ also had the highest expression of the phosphoinositide phosphatases *INPP4B* and *INPP5J* as well as the pAkt ser473 phosphatase *PHLLP1* (Fig. 4C). These findings illustrate the trend between TRβ and expression of tumor suppressive genes in PI3K signaling in a genetically diverse set of ATC cell lines (Supplementary Table 3 [28]).

Phosphoinositide 3 Kinase Signaling Genes Regulated by Thyroid Hormone Receptor Beta in SW1736 Cells Are Aberrantly Expressed in Patient Thyroid Cancer Samples

Next, we used expression data from matched normal tissue, follicular thyroid adenoma, and papillary, FTC, PDTC, and ATC to determine if any of the PI3K regulators we revealed to be altered by TRβ-T_3 treatment exhibited differential expression in different thyroid cancer subtypes [25–27]. The patient microarray data revealed that the RTKs *ERBB3* (HER3), *ROR1*, and *AXL* expression levels correlate with TC subtype where expression is highest in the more aggressive tumors (Fig. 5A). Conversely, phosphatase expression is highest in matched normal tissue and differentiated thyroid tumors (Fig. 5B). Interestingly, expression of *FGFR4* and *FGFRL1* were lowest in the more aggressive PDTC and ATC populations. We next analyzed gene expression data for TRβ and markers of differentiated thyroid cells to demonstrate the connection between TRβ-T_3 presence with increased RTK and decreased phosphatase expression. *THRB* (TRβ) and genes encoding enzymes and transporters for thyroid hormone synthesis were coordinately lost in FTC, PDTC, and ATC patient samples (Fig. 5C). As demonstrated previously, TRβ protein is significantly reduced in FTC, PDTC, and ATC patients [15], a finding that may contribute to the gene expression data presented here.

Thyroid Hormone Receptor Beta Improves Phosphoinositide 3 Kinase Inhibitor Efficacy

PI3K signaling fuels cancer progression by stimulating cell survival, proliferation, and migration. We previously demonstrated that TRβ inhibits SW1736 proliferation in charcoal-stripped media, an observation that was dependent on T_3 stimulation [16]. To achieve phenotypic confirmation of our sequencing results, we challenged our engineered cells in full serum (10%) media with or without additional 10-nM T_3 (background T_3: 170 pM) to measure a functional consequence of PI3K inhibition. Even in the presence of full serum and activated RTKs, the TRβ group rendered the SW1736 cells less viable (Supplementary Fig. 3A and 3B [28]). In accordance with the RNA-seq data, T_3 is necessary for maximum inhibition of cell viability by TRβ. Additionally, we challenged our cells to migrate in the presence of liganded-TRβ. TRβ cells were unable to effectively migrate to close the wound compared to the EV cells (Supplementary Fig. 3C-3E [28]). These findings are similar to what we observed previously in experiments using charcoal-stripped, growth hormone–deprived media, thus indicating the absolute requirement of TRβ with T_3 to inhibit cell viability and migration.

To further test our hypothesis that TRβ suppresses PI3K signaling in ATC cell lines, we established the efficacy of small-molecule competitive PI3K inhibitors on cell cytotoxicity. LY294002 and buparlisib both compete for the ATP-binding site of PI3K, preventing PIP_2 phosphorylation. Buparlisib is 27 times more potent than LY294002 (half maximal inhibitory concentration of 52 nM and 1.4 μM, respectively [43]). The TRβ cells showed an improved response to LY294002, with a half-maximal response value nearly 5-fold less than the EV cells (Fig. 6A). In addition, the TRβ cells were nearly 25-fold more sensitive to the PI3K inhibitor buparlisib than the EV cells (Supplementary Fig. 4 [28]). In concordance with the RNA-seq and cell viability data, TRβ requires T_3 to fully exert the tumor suppressive profile; LY294002 efficacy was not increased in SW-TRβ cells lacking 10-nM T_3 (Supplementary Fig. 5 [28]). LY294002 efficacy was also enhanced in the TRβ cells as measured by migration assay both at 1 and 10 μM (Fig. 6B-D).

Thyroid Hormone Receptor Beta Enhances Phosphoinositide 3 Kinase (PI3K) Inhibitor Inactivation of the PI2K–Protein Kinase B–Mechanistic Target of Rapamycin Axis

To further confirm that TRβ is specifically inhibiting the PI3K signaling pathway, we challenged our cells with LY294002 and buparlisib and measured pAkt, pmTOR,

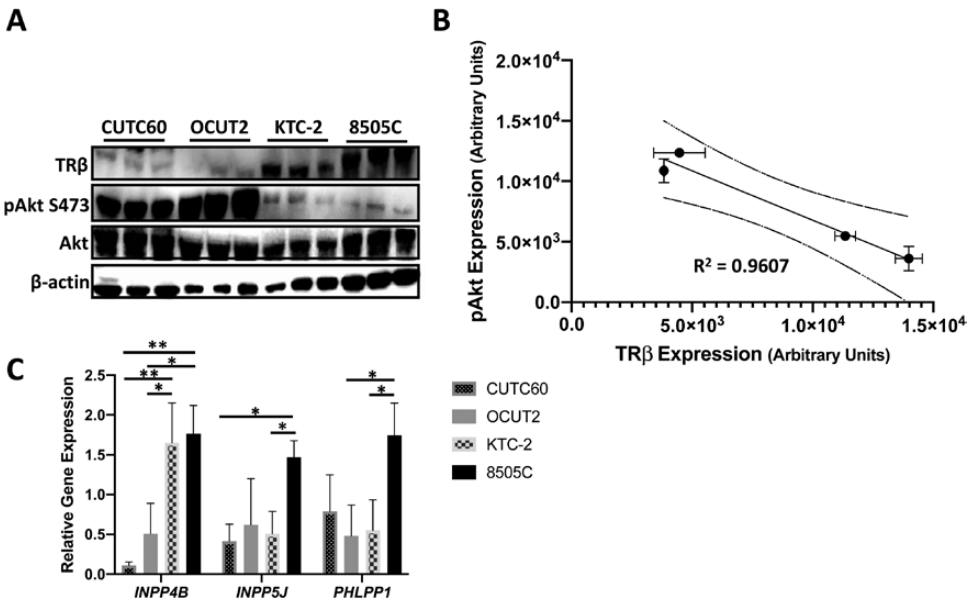

Figure 4. Thyroid hormone receptor beta (TRβ) expression correlates with low protein kinase B (Akt) ser473 phosphorylation and high phosphoinositide and Akt phosphatase expression. Anaplastic thyroid cancer (ATC) cell lines were analyzed for A and B, TRβ and pAkt ser473 expression, and C, phosphoinositide and Akt phosphatases. Significance was determined by one-way analysis of variance followed by Dunnett multiple comparisons test (**P* < .05, ***P* < .01).

pp70S6K, and pGYS1 levels following 24 hours of T_3 treatment. LY294002 and buparlisib were significantly more effective in the TRβ cells, demonstrating a decrease in levels of pAkt (thr308 and ser473), pmTOR (ser2448 and ser2481), and pp70S6K (Fig. 7A and 7C and Supplementary Fig. 6A [28]). In addition to the Akt-mTOR-p70S6K axis, we also observed an increase in pGYS1 following both T_3 and LY294002 or buparlisib treatment in our cells, suggesting robust Akt suppression due to TRβ activation in SW1736 cells (Fig. 7A and 7C and Supplementary Fig. 6B [28]). We validated these findings with a pAkt (ser473) sandwich ELISA and detected lower levels of pAkt in the LY294002-treated TRβ cells compared to the EV control (Fig. 7B).

Finally, these results appeared to be dependent on long-term T_3 treatment, as short-term T_3 exposure failed to significantly enhance suppression of PI3K with TRβ or LY294002 (Fig. 8A and 8B).

Discussion

TRβ has shown to be a potent tumor suppressor in several types of cancer, including breast and thyroid cancer [16, 17, 44, 45]. However, there are only a few tumor-suppressive mechanisms delineated in the literature, which include TRβ-mediated Janus kinase 1 (JAK1)/STAT1 activation and binding to p85α [16, 21, 44]. Multiple groups have shown the potential for TRβ to bind p85α in the cytoplasm, inhibiting PI3K-mediated PIP_2 to PIP_3 catalysis. While TRβ and T_3 failed to rapidly inhibit PI3K activity in these ATC cells, transcriptomic data revealed novel genomic mechanisms of TRβ-mediated PI3K suppression. Intriguingly, negatively regulated genes included RTKs such as FGFR isoforms and HER3, both of which are implicated in advanced thyroid carcinomas [46-48]. TRβ-mediated downregulation of HER3 is particularly interesting, as HER3 is the most potent activating binding partner of HER2 and was found to promote PI3K inhibitor resistance [49, 50].

In addition to regulating expression of upstream regulators of PI3K, TRβ increased expression of membrane-associated phosphatases, including *INPP5J* and *INPP4B*. *INPP4B* has shown to exhibit remarkable tumor suppression in an in vivo model of thyroid cancer by regulating PI3K signaling [51]. Furthermore, TRβ also increased expression of the phosphatases *PTPL1*, *PHLPP1*, and the R5B subunit of PPP2. These phosphatases preferentially dephosphorylate ser473 on Akt, and INPP4B and INPP5J modulate ser473 phosphorylation levels [36, 41, 42]. Increased expression of these phosphatases would account for the robust decrease in ser473 phosphorylation but not thr308 and further supports the notion that TRβ-mediated suppression of the PI3K pathway in these cells is primarily driven by transcriptional regulation. Importantly, both Akt residues must be phosphorylated for maximum activity, as thr308 phosphorylation accounts for only 10% of Akt activity [52]. Therefore, the robust pAkt ser473 dephosphorylation may be sufficient to inhibit Akt activity, accounting for the growth and migration inhibition observed in this study. Downstream consequences of

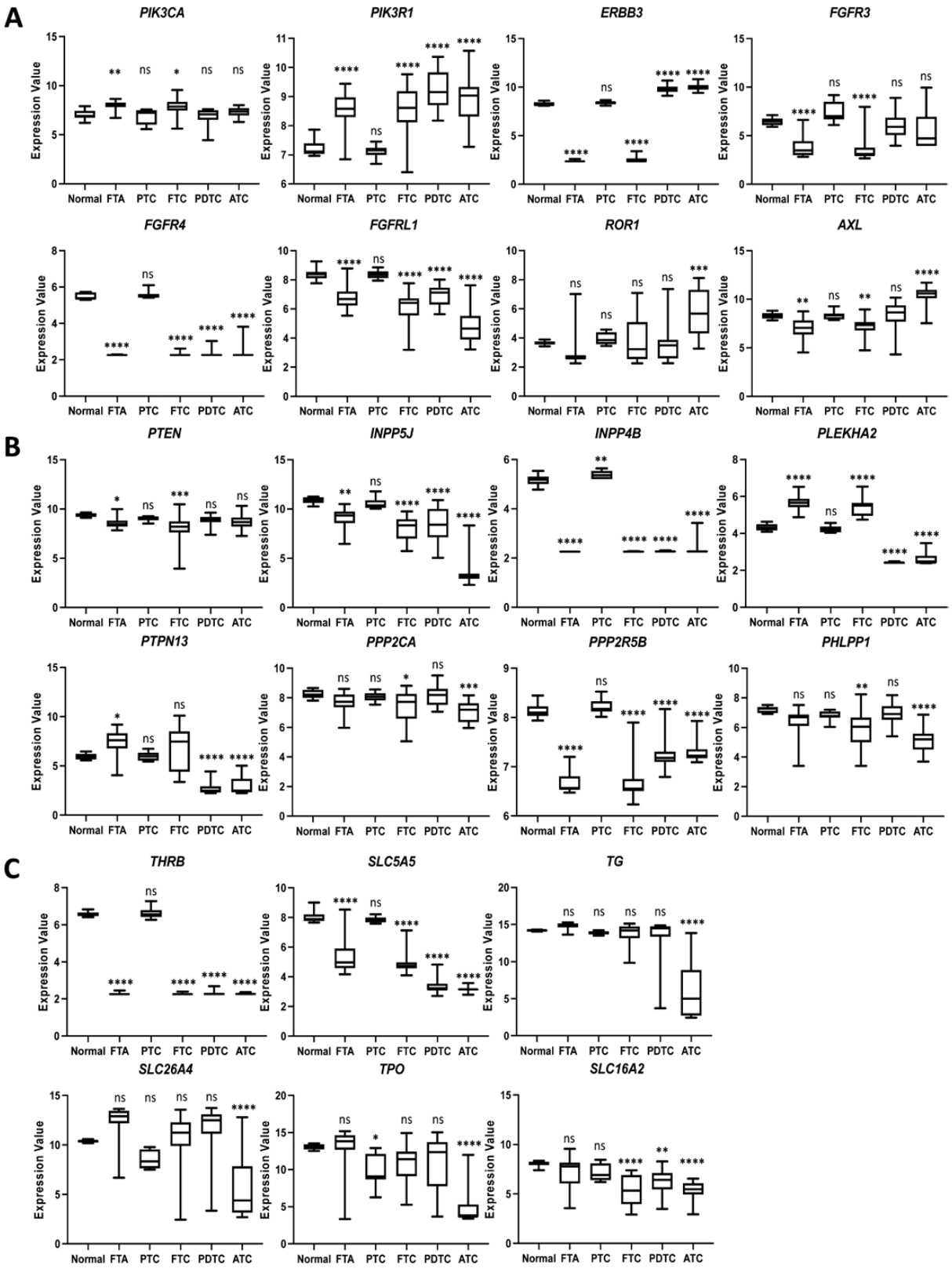

Figure 5. Phosphoinositide 3 kinase (PI3K) signaling genes regulated by thyroid hormone receptor beta (TRβ) in SW1736 cells are aberrantly expressed in patient thyroid cancer samples. Patient thyroid cancer microarray data (GSE76039, GSE3467, GSE82208) were analyzed for genes encoding A, receptor tyrosine kinases; B, phosphoinositide and protein kinase B (Akt) phosphatases; and C, TRβ, enzymes, and transporters necessary for synthesizing thyroid hormones. Significance was determined by one-way analysis of variance followed by Dunnett multiple comparisons test NS, no significance ($P \geq .05$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$). Normal n = 9. Papillary thyroid cancer (PTC) n = 9, follicular thyroid cancer (FTC) n = 27, poorly differentiated thyroid cancer (PDTC) n = 17, anaplastic thyroid cancer (ATC) n = 20.

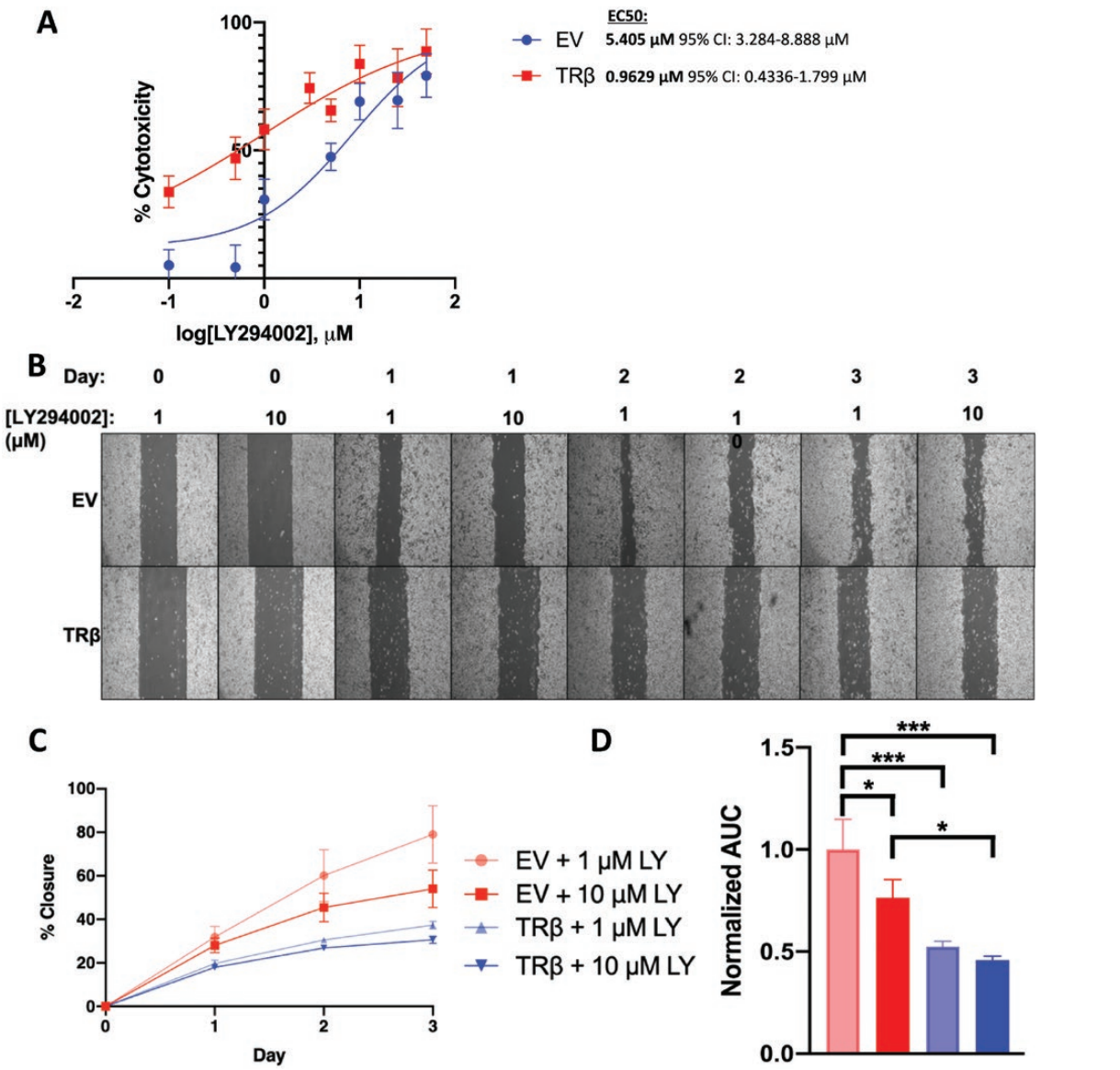

Figure 6. The effect of LY294002 on cytotoxicity and migration is enhanced in SW1736- thyroid hormone receptor beta (TRβ) cells. A, Empty vector (EV) and TRβ cells were treated with 10-nM 3,5,3′-triiodothyronine (T_3) and LY294002 (0.1-50 μM) or matched-concentration vehicle (100% EtOH) for 4 days. Each day the cells were lifted with trypsin and counted using a hemocytometer for viable cells. Area under the curve analysis was conducted for each LY294002 concentration to calculate percentage of cytotoxicity relative to EV vehicle at each concentration of LY294002. Half maximal response (EC50) values were calculated using the GraphPad Prism nonlinear regression package. B, EV and TRβ cells were grown to confluency in 6-well plates before treatment with 10-μg/mL mitomycin C for 2.5 hours. Media were aspirated, and the cells were scratched with a P1000 pipette tip before being washed with phosphate-buffered saline and treated with media containing 10-nM T_3 and 1- or 10-μM LY294002. C, Wells were imaged each day and percentage of wound closure relative to day 0 was calculated. D, Area under the curve (AUC) analysis was performed and normalized to the EV 1 μM LY294002 group. Significance was calculated using one-way analysis of variance followed by aTukey multiple comparisons test (*$P < .05$, **$P < .01$, ***$P < .001$).

TRβ-mediated dephosphorylation of Akt was observed in 3 well-established downstream targets of Akt, including mTOR, p70S6K, and GYS1.

In addition to the previously documented mechanisms of TRβ tumor suppression, genomic regulation of PI3K regulators likely drives a reduction in cell viability and migration. TRβ enhanced the effect of PI3K inhibitors LY294002 and buparlisib as shown by decreased cell viability, migration, and cell signaling. Importantly, in a phase 2 trial of ATC patients, buparlisib was able to reduce tumor burden and modestly improve patient survival [53]. As we have observed an enhanced response to PI3K inhibitors in the presence of TRβ and hormone, it would be worthwhile to determine if expression levels of TRβ in patient tumors were correlated with patient outcomes.

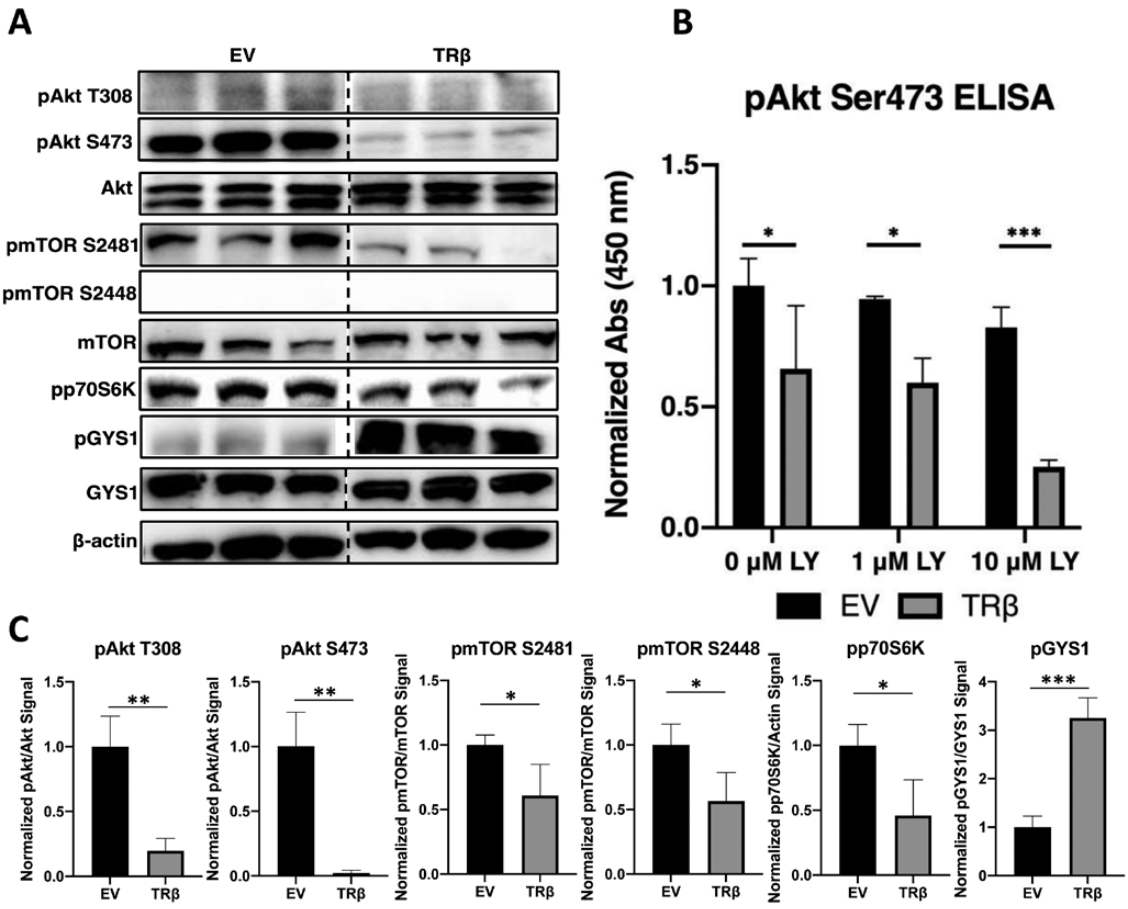

Figure 7. Thyroid hormone receptor beta (TRβ) enhances LY294002 (LY)-mediated inactivation of the phosphoinositide 3 kinase–protein kinase B–mechanistic target of rapamycin (PI3K-Akt-mTOR) axis. SW1736-EV (empty vector; EV) and SW1736-TRβ (TRβ) cells were treated with 10-nM 3,5,3′-triiodothyronine (T_3) for 24 hours before 1 hour of LY treatment (A, 10 μM; B, 0, 1, or 10 μM). Protein levels were determined by immunoblot (A and quantified in C) or B, subjugated to a pAkt Ser473 sandwich enzyme-linked immunosorbent assay (ELISA). Samples in A are biological replicates. Dashed lines in immunoblots indicate gap between 2 sets of lanes on the same membrane. ELISA signal in B was standardized to protein concentration as determined by a Bradford assay. Significance in B was calculated by one-way analysis of variance followed by a Sidak multiple comparison test. Significance in C was calculated by *t* test. NS, no significance ($P \geq .05$, $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$).

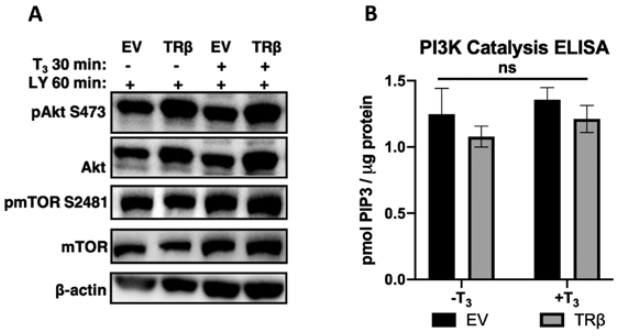

Figure 8. Short-term or no exposure to 3,5,3′-triiodothyronine (T_3) is insufficient for thyroid hormone receptor beta (TRβ) to enhance LY294002 (LY) suppression of phosphoinositide 3 kinase (PI3K). SW1736-EV (empty vector; EV) and SW1736-TRβ (TRβ) cells were treated with 10-nMT_3 or vehicle (10 μM NaOH) for 30 minutes before 1 hour of 10-μM LY treatment. Protein levels were determined by A, immunoblot or B, incubated with anti-p85α antibody for PI3K catalysis enzyme-linked immunosorbent assay (ELISA). ELISA signal in B was standardized to protein concentration as determined by a Bradford assay. Significance in B was calculated by 2-way analysis of variance followed by a Tukey multiple comparisons test. NS, no significance ($P \geq .05$) across treatment groups.

In summary, our results demonstrate that TRβ suppresses PI3K signaling in SW1736 cells by genomic regulation of RTKs and phosphatases. Although the potential for TRβ to suppress PI3K signaling by binding to the p85α subunit in the cytoplasm is well known, this is the first report to highlight genomic mechanisms by which TRβ suppresses the PI3K-Akt-mTOR axis. The presence and activation of TRβ in ATC cells may be a promising therapeutic target to constrain tumor progression and resistance to chemotherapeutics.

Acknowledgments

The SW1736 and KTC-2 cell lines were generously provided by Dr John Copland III (Mayo Clinic), and 8505C, OCUT-2, and CUTC60 cells were generously provided by Dr Rebecca Schweppe (University of Colorado). Human cell line authentication, NextGen sequencing, automated DNA sequencing, and molecular imaging were performed in the Vermont Integrative Genomics Resource supported by the University of Vermont (UVM) Cancer

Center, Lake Champlain Cancer Research Organization, and the UVM Larner College of Medicine. Additional human cell line authentication was performed by the CU Cancer Center Tissue Culture Shared Resource supported by the National Cancer Institute (P30CA046934). Lentiviral constructs were made with the assistance of Dr Jon Ramsey, UVM Cancer Translational Research Laboratory, UVM. Biorender vector graphics software was used to generate Fig. 3A. We thank Dr Jane Lian for early conceptualization and design of this project. We thank Dr Eyal Amiel for his generous donation of several antibodies and for support on the manuscript discussion.

Financial Support: This work was supported by the National Institutes of Health (grant No. U54 GM115516) for the Northern New England Clinical and Translational Research Network; the National Cancer Institute (grant No. 1F99CA245796-01); the UVM Cancer Center-Lake Champlain Cancer Research Organization (C3; grant No. 12577-21); and UVM Larner College of Medicine.

Author Contributions: Conceptualization, formal analysis, investigation, writing and original draft preparation, visualization: C.D.D.; conceptualization, formal analysis, investigation, writing, review, and editing: E.L.G.; formal analysis, investigation, writing, review, and editing: N.E.G.; formal analysis and investigation: L.M.C.; investigation, writing, review, and editing: J.A.T.; resources, writing, review, and editing, supervision, project administration, and funding acquisition: F.E.C.

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Disclosures:The authors have nothing to disclose.

Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in "References."

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