

1 **Thyroid hormone receptor beta inhibits the PI3K-Akt-mTOR signaling axis in anaplastic**
2 **thyroid cancer via genomic mechanisms**

3 Authors: Cole D. Davidson ^{1,2}, Eric L. Bolf ^{1,2}, Noelle E. Gillis ^{1,2}, Lauren M. Cozzens ¹, Jennifer A.
4 Tomczak ¹, and Frances E. Carr ^{1,2,*}

5 ¹ *Department of Pharmacology, Larner College of Medicine, Burlington, Vermont.*

6 ² *University of Vermont Cancer Center, Burlington, Vermont.*

7 * *Corresponding author, Email: Frances.Carr@med.uvm.edu*

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12 preparation, Visualization. **Eric L. Bolf:** Conceptualization, Formal analysis, Investigation,
13 Writing – Review & editing. **Noelle E. Gillis:** Formal analysis, Investigation, Writing – Review &
14 editing. **Lauren M. Cozzens:** Formal analysis, Investigation. **Jennifer A. Tomczak:** Investigation,
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23 **Abstract:**

24 Thyroid cancer is the most common endocrine malignancy, and the global incidence has
25 increased rapidly over the past few decades. Anaplastic thyroid cancer (ATC) is highly
26 aggressive, dedifferentiated, and patients have a median survival of fewer than six months.
27 Oncogenic alterations in ATC include aberrant PI3K signaling through receptor tyrosine kinase
28 (RTK) amplification, loss of phosphoinositide phosphatase expression and function, and Akt
29 amplification. Furthermore, the loss of expression of the tumor suppressor thyroid hormone
30 receptor beta (TR β) is strongly associated with ATC. TR β is known to suppress PI3K in follicular
31 thyroid cancer and breast cancer by binding to the PI3K regulatory subunit p85 α . However, the
32 role of TR β in suppressing PI3K signaling in ATC is not completely delineated. Here we report
33 that TR β indeed suppresses PI3K signaling in ATC through unreported genomic mechanisms
34 including a decrease in RTK expression and increase in phosphoinositide and Akt phosphatase
35 expression. Furthermore, the reintroduction and activation of TR β in ATC enables an increase in
36 the efficacy of the competitive PI3K inhibitors LY294002 and buparlisib on cell viability,
37 migration, and suppression of PI3K signaling. These findings not only uncover additional tumor
38 suppressor mechanisms of TR β but shed light into the implication of TR β status and activation
39 on inhibitor efficacy in ATC tumors.

40

41 **Abbreviations:**

42 Akt, protein kinase B; ATC, anaplastic thyroid cancer; AXL, tyrosine-protein kinase receptor
43 UFO; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay;
44 FGFR3/4/L1, fibroblast growth factor receptor 3/4/like 1; FTC, follicular thyroid cancer; GSK3 β ,

45 glycogen synthase kinase 3 beta; GYS1, glycogen synthase 1; HER2, receptor tyrosine-protein
46 kinase erbB-2; HER3, receptor tyrosine-protein kinase erbB-3; INPP4B, inositol polyphosphate
47 4-phosphatase type II; INPP5J, phosphatidylinositol 4,5-bisphosphate 5-phosphatase A; JAK1,
48 janus kinase 1; mTORC1/2, mammalian target of rapamycin complex 1/2; p70S6K, ribosomal
49 protein S6 kinase beta-1 PDK1, 3-phosphoinositide dependent protein kinase 1; PDTC, poorly
50 differentiated thyroid cancer; PEKHA2, tandem-PH-domain-containing protein-2; PHLPP1, PH
51 domain and leucine-rich repeat-protein phosphatase 1; PI(3,4)P₂, phosphatidylinositol 3,4-
52 bisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; PI(4,5)P₂, phosphatidylinositol 4,5-
53 bisphosphate; PI3K, phosphoinositide 3 kinase; PIP₃, phosphatidylinositol 3,4,5 trisphosphate;
54 PPP2R5B, protein phosphatase 2R5B; PTC, papillary thyroid cancer; PTEN, phosphatase and
55 tensin homolog; PTPN13, protein-tyrosine-phosphatase-like protein-1; ROR1, neurotrophic
56 tyrosine kinase receptor-related 1; RT-qPCR, reverse transcriptase quantitative polymerase
57 chain reaction; RTK, receptor tyrosine kinase; RUNX2, runt-related transcription factor 2;
58 STAT1, signal transducer and activator of transcription 1; T₃, triiodothyronine; TR β , thyroid
59 hormone receptor beta

60

61 **1. Introduction**

62 Thyroid cancer is the most common endocrine malignancy, and the incidence has been rapidly
63 increasing the past few decades (1). While the overall prognosis for thyroid cancer is generally
64 favorable, patients with the most aggressive and dedifferentiated subtype, anaplastic thyroid
65 cancer (ATC), have a median survival of three to five months (2). The current most effective
66 treatments for ATC patients increase survival time by only a few months as drug resistance and

67 tumor recurrence often develop (3). Therefore, there is an unmet need for more precise
68 understanding of the molecular etiology of ATC tumorigenesis and new strategies for improving
69 patient outcome.

70 Phosphoinositide 3 kinase (PI3K) signaling is a prominent molecular driver for aggressive and
71 poorly differentiated thyroid cancers such as ATC (4). PI3K is recruited to the plasma membrane
72 by phosphorylated, ligand-bound receptor tyrosine kinases (RTKs). PI3K phosphorylates the
73 membrane lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) to PIP₃, which recruits 3-
74 phosphoinositide dependent protein kinase 1 (PDK1) and the mammalian target of rapamycin
75 complex 2 (mTORC2) to the plasma membrane to phosphorylate Akt on thr308 and ser473,
76 respectively. Phosphatase and tensin homolog (PTEN) is a tumor suppressor that
77 dephosphorylates PIP₃ back to PI(4,5)P₂. Akt phosphorylates a myriad of targets that are involved
78 in cell cycle progression and survival signaling. In addition, Akt leads to the activation of mTORC1,
79 which phosphorylates targets such as p70S6K that ultimately lead to the activation and assembly
80 of translation factors for protein synthesis and cell growth (5,6). Multiple genes are either
81 mutated or amplified within the PI3K pathway in ATC. Frequent alterations include amplification
82 of RTKs such as epidermal growth factor (EGFR), amplification or gain-of-function mutations in
83 *PIK3CA* (PI3K), loss-of-function mutations or decreased expression of *PTEN*, and amplification of
84 *AKT1* (7-10).

85 In addition to the canonical mechanisms of PI3K regulation, there are a multitude of other factors
86 that regulate the pathway. These factors include members of the nuclear hormone receptor
87 family including estrogen, androgen, and thyroid hormone receptors (11-13). Our work has
88 demonstrated that thyroid hormone receptor beta (TR β) acts as a tumor suppressor in ATC

89 through repression of several pathways important for tumor growth (14,15). However, the
90 potential for TR β to exhibit tumor suppression in ATC by suppressing PI3K is not fully understood.
91 Multiple groups have reported the potential for TR β to bind to the regulatory subunit of PI3K,
92 p85 α , to inhibit phosphorylation of PI(4,5)P₂ to PIP₃ (16-18). While these mechanisms help
93 explain the potential for TR β to inhibit PI3K in certain cancer models, there may be other
94 mechanisms of TR β -mediated suppression. While TR β has been shown to inhibit PI3K via
95 nongenomic mechanisms in breast (19) and follicular thyroid (20,21) cancer, it is unknown if this
96 mechanism or unexplored genomic mechanisms occur in ATC. Therefore, we sought to better
97 understand the mechanism of TR β -mediated suppression of PI3K signaling in ATC using a TR β -
98 expression model. Moreover, we tested the efficacy of the PI3K inhibitors LY294002 and
99 buparlisib in cells with or without TR β expression. These findings present previously unexplored
100 mechanisms of the tumor suppression by TR β , the role of TR β in ATC, as well as the implication
101 of TR β expression status in response to PI3K-targeted therapeutic intervention.

102

103 **2. Materials and methods:**

104 *2.1. Culture of thyroid cell lines*

105 Cells were cultured in RPMI 1640 growth media with L-glutamine (300 mg/L), sodium pyruvate
106 and nonessential amino acids (1%) (Corning Inc, Corning, NY, USA), supplemented with 10% fetal
107 bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and penicillin-streptomycin (200
108 IU/L) (Corning) at 37°C, 5% CO₂, and 100% humidity. Lentivirally modified SW1736 cells were
109 generated as described (15,22) with either an empty vector (SW-EV) or to overexpress TR β (SW-
110 TR β). SW-EV and SW-TR β were grown in the above conditions with the addition of 2 μ g/ml

111 puromycin (Gold Bio, St Louis, MO, USA). SW1736 and KTC-2 were authenticated by the Vermont
112 Integrative Genomics Resource at the University of Vermont (Burlington, Vermont) using short
113 tandem repeat profiles and Promega GenePrint10 System (SW1736, May 2019; KTC-2, October
114 2019). 8505C, OCUT-2, and CUTC60 were authenticated by University of Colorado by short
115 tandem repeat profiles (8505C, June 2013; OCUT-2, June 2018; CUTC60, November 2018).

116

117 *2.2. Cell culture reagents*

118 Triiodothyronine (T_3) was purchased from Sigma (St. Louis, MO, USA) and dissolved in 1 N NaOH
119 and diluted to 10 nM in cell culture medium at the time of each application. LY294002 and
120 buparlisib were purchased from MedChemExpress (Monmouth Junction, NJ, USA). LY294002 was
121 dissolved in 100% ethanol and buparlisib was dissolved in 100% DMSO prior to indicated dilutions
122 for cell culture experiments.

123

124 *2.3. Immunoblot analysis*

125 Proteins were isolated from whole cells in lysis buffer (20mM Tris-HCl (pH 8), 137mM NaCl, 10%
126 glycerol, 1% Triton X-100, and 2mM EDTA) containing Protease Inhibitor Cocktail (Catalogue
127 Number 78410) (Thermo Fisher Scientific), 1 mM Na_3VO_4 , and 1 mM PMSF (Sigma). Proteins were
128 quantified via Pierce Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific), and 25 μ g of
129 protein per sample were resolved by polyacrylamide gel electrophoresis on 10% Tris-Glycine gels
130 (Catalogue Number XP00105BOX) (Thermo Fisher Scientific) and immobilized onto nitrocellulose
131 membranes (GE Healthcare, Chicago, IL, USA) by electroblot (Bio-Rad Laboratories, Hercules, CA,
132 USA). Membranes were blocked with 5% w/v BSA in TBS and 0.1% v/v Tween20 (Gold Bio, St

133 Louis, MO, USA) for one hour at room temperature and incubated with primary antibodies
134 overnight (Supplementary Table 1); immunoreactive proteins were detected by enhanced
135 chemiluminescence (Thermo Scientific) on a ChemiDoc XRS+ (Bio-Rad Laboratories).
136 Densitometry analysis in Figure 4B was performed using ImageJ (NIH, Bethesda, MD, USA).

137

138 *2.4. Measurement of Akt phosphorylation by ELISA*

139 Akt serine 473 phosphorylation was measured using Pathscan phospho-Akt1 sandwich ELISA kit,
140 according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, USA).
141 Samples were prepared from cells treated with 10 nM T₃ for 24 hours and then 1 hour incubation
142 in the presence or absence of 1 or 10 μM LY294002. 100 μl of samples containing equal amount
143 of protein were applied to each well.

144

145 *2.5. Measurement of PI3K activity by ELISA*

146 PI3K activity was determined using a commercially available PI3K ELISA kit (Echelon Biosciences
147 Inc., Salt Lake City, UT) according to the manufacturer's instructions. Briefly, after drug treatment,
148 cells were washed in ice-cold PBS and lysed in 500 μl ice-cold lysis buffer (137 mM NaCl, 20 mM
149 Tris-HCl (pH 7.4), 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₃VO₄, 1% NP-40 and 1 mM PMSF). PI3K was
150 then immunoprecipitated with 5 μl of antibody (anti-p85α, MilliporeSigma, Burlington, MA, USA)
151 and 60 μl of Pierce Protein A/G magnetic beads (Thermo Scientific). PI3K activity in
152 the immunoprecipitates was then assayed by PI3K ELISA according to the manufacturer's
153 instructions. The spectrophotometric data were obtained using a Synergy 2 Multi-Detection
154 Microplate Reader (Agilent Technologies, Santa Clara, CA, USA) at a wavelength of 450 nm. The

155 protein concentrations of cellular lysates were determined by Bradford assay as described above.

156 The activity of PI3K was corrected for protein content.

157

158 *2.6. RNA-seq data analysis of PI3K pathway intermediates*

159 Previously published RNA-seq data was used to determine expression levels of genes within the

160 PI3K pathway (15). Construction of the PI3K signaling genes of interest for our study was based

161 on the curated IPA pathway gene set. Additional genes of interest were added based on literature

162 search and cancer relevance. Normalized transcript counts generated with DESeq2 were used to

163 calculate fold change compared to the control condition (SW-EV -T₃). Raw and processed

164 expression data can be found in the Gene Expression Omnibus (GEO) database under accession

165 number GSE150364.

166

167 *2.7. Analysis of thyroid cancer patient sample data*

168 Publicly available microarray expression data, deposited in the GEO Database (GSE76039,

169 GSE3467, GSE82208), were analyzed using GEOR2 (www.ncbi.nlm.nih.gov/gds) to reveal

170 differential expression of genes relevant to PI3K signaling across the spectrum of thyroid cancers.

171

172 *2.8. RNA extraction and quantitative Real-Time PCR (qRT-PCR)*

173 Total RNA was extracted using RNeasy Plus Kit (Qiagen) according to manufacturer's protocol.

174 cDNA was then generated using the 5X RT Mastermix (ABM, Vancouver, Canada). Gene

175 expression to validate RNA-seq analysis was quantified by qRT-PCR using BrightGreen 2X qPCR

176 MasterMix (ABM, Vancouver, Canada) on a QuantStudio 3 real-time PCR system (Thermo Fisher

177 Scientific). Fold change in gene expression compared to endogenous controls was calculated
178 using the ddCT method. Primer sequences are indicated in Supplemental Table 2.

179

180 *2.9. In vitro cell viability assay*

181 The cell viability assay was performed by plating 1.0×10^4 SW-EV or SW-TR β cells into 12 well
182 (22.1 mm) tissue culture dishes. After adhering overnight, the cells were treated with 10 nM T₃
183 and LY294002, buparlisib, or vehicle at the indicated concentrations. Every day after treatment
184 for four days, the medium was removed, cells were washed with PBS and lifted with trypsin
185 (Thermo Scientific), and the number of surviving cells was counted with a hemocytometer.

186

187 *2.10. Migration assay*

188 Cell migration was determined by wound healing assay. Cells were plated and allowed to grow
189 to 100% confluency. Two hours prior to scratching, cells were treated with 10 μ g/ml Mitomycin
190 C (Sigma, MO, USA) dissolved in H₂O. A scratch was performed with a P1000 pipette tip and debris
191 was washed away with PBS. Migration media was supplemented with 10 nM T₃ and LY294002 or
192 vehicle. Images were obtained using a Canon digital camera connected to an Axiovert inverted
193 microscope (Carl Zeiss, Oberkochen, Germany) at 0, 24, 48, and 72 hours. Wound closure was
194 measured using ImageJ macro "Wound Healing Tool" ([http://dev.mri.cnrs.fr/projects/imagej-](http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool)
195 [macros/wiki/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%
196 closure.

197

198 *2.11. Statistics*

199 All statistical analyses were performed using GraphPad Prism software. Paired comparisons were
200 conducted by Student's t-test. Group comparisons were made by one-way ANOVA followed by
201 Dunnett's or Tukey's multiple comparison test as appropriate. Two-way ANOVA followed by a
202 Tukey's multiple comparison test was conducted for multigroup analysis. Data are represented as
203 mean \pm standard deviation. Area under the curve (AUC) at the 95th confidence interval was used
204 to evaluate statistical differences in growth and migration assays.

205

206 **3. Results**

207 *3.1 Rapid thyroid hormone receptor action fails to suppress PI3K in ATC cells*

208 TR β is a known suppressor of the PI3K signaling pathway in breast and follicular thyroid cancer.
209 This has been previously described as a nongenomic mechanism in which TR β binds to the
210 regulatory subunit of PI3K, p85 α , preventing recruitment to ligand-bound RTKs (13). This action
211 is rapid, and the addition of the thyroid hormone triiodothyronine (T₃) modulates the response
212 within 15-30 minutes (23). Therefore, we sought to evaluate the impact of short term T₃
213 treatment in the SW1736 cell line with restored stable expression of TR β or an empty vector (EV)
214 control. Analysis of pAkt and pmTOR by Western blot surprisingly revealed a minimal impact of
215 TR β with or without T₃ on PI3K suppression (Fig. 1A). To validate these results, we performed a
216 PI3K immunoprecipitation followed by ELISA to test the ability of PI3K to catalyze the
217 phosphorylation of PI(4,5)P₂ to PIP₃ following T₃ treatment. Again, we observed a modest but
218 insignificant decrease in PIP₃ production in the presence of TR β and T₃ (Fig. 1B).

219

220 *3.2 Long-term T₃ treatment suppresses PI3K signaling in ATC cells*

221 Since short-term T_3 treatment did not suppress PI3K activity in our cell line model, we
222 hypothesized that long-term T_3 treatment may enable TR β -mediated suppression of PI3K
223 signaling. Therefore, we treated our EV and TR β cells with T_3 for 24 hours then measured pAkt,
224 pmTOR, and pp70S6K levels by Western blot (Fig. 2A). The SW-TR β cells treated with T_3 exhibited
225 a marked decrease in pAkt on serine 473 but not threonine 308. Serine 473 phosphorylation
226 induces a substantial increase in Akt activity following growth factor stimulation and plays a role
227 in regulating substrate specificity (24). Additionally, there was a modest reduction in pP70S6K, a
228 kinase further downstream of Akt. To validate the observed Akt ser473 dephosphorylation, we
229 conducted a sandwich ELISA with our EV and TR β cells following 24-hour T_3 treatment (Fig. 2B);
230 pAkt (ser473) was again reduced with liganded-TR β .

231 In order to test the impact of long-term T_3 exposure and heightened TR β expression we measured
232 a well-studied downstream effector, glycogen synthase kinase 3 beta (GSK3 β) and its substrate
233 glycogen synthase 1 (GYS1). GSK3 β is a multi-substrate kinase and, importantly, is implicated in
234 the progression of numerous cancers including ATC (25,26). We observed a modest increase in
235 phosphorylated, and thus inactivated, GYS1 in the presence of T_3 and a marked increase of pGYS1
236 in the presence of both TR β and T_3 (Fig. 2C). Since TR β - T_3 treatment suppressed both Akt-mTOR-
237 P70S6K and Akt-GSK3 β -GYS1 pathways, these data are suggestive of a requirement for both
238 thyroid hormone and TR β to achieve robust inactivation of PI3K signaling as observed through
239 two separate pathways downstream of Akt.

240

241 *3.3 Liganded-TR β transcriptionally remodels the PI3K signaling landscape*

242 Since a long-term T₃ treatment was needed for robust suppression of PI3K signaling in our cell
243 line model, we hypothesized that TRβ may be regulating the expression of key components of
244 this pathway. In order to better understand the extent to which TRβ suppresses PI3K signaling
245 through genomic mechanisms, we leveraged our RNA-sequencing data performed on our EV and
246 TRβ cell lines following 24 hours of T₃ treatment (15). Numerous genes involved in the PI3K
247 pathway were determined to be differentially expressed in the presence of both T₃ and TRβ (Fig.
248 3A-C).

249 Receptor tyrosine kinase gene expression was reduced in the TRβ-T₃ group, including HER3
250 (*ERBB3*), fibroblast growth factor receptor isoforms (*FGFR3*, *FGFR4*, *FGFRL1*), neurotrophic
251 tyrosine kinase receptor-related 1 (*ROR1*), and tyrosine-protein kinase receptor UFO (*AXL*).

252 While *PTEN* expression was not increased, other membrane-bound phosphoinositide
253 phosphatases did increase including phosphatidylinositol 4,5-bisphosphate 5-phosphatase A
254 (*INPP5J*), and inositol polyphosphate 4-phosphatase type II (*INPP4B*), which dephosphorylate
255 PIP₃ to PI(3,4)P₂ and PI(3,4)P₂ to PI(3)P, respectively (27). There was also an increase in expression
256 of cytosolic phosphatases that dephosphorylate Akt primarily on ser473, including tandem-PH-
257 domain-containing protein-2 and protein-tyrosine-phosphatase-like protein-1 (*PLEKHA2* and
258 *PTPN13*), the R5B localization subunit of protein phosphatase 2 (*PPP2R5B*), and PH domain and
259 leucine-rich repeat-protein phosphatase 1 (*PHLPP1*) (28-30). In addition to these genes that serve
260 to regulate PI3K activation and subsequent Akt phosphorylation, genes involved in PI3K
261 recruitment and stabilization, mTORC regulation, and translation factors were found to be
262 differentially regulated in the TRβ-T₃ group (Supplemental Fig. 1A-D and Table 3). The fold change

263 of a subset of the differentially expressed genes were validated via RT-qPCR (Supplemental Fig.
264 2).

265

266 *3.4 Endogenous TR β expression in ATC cells correlates with low pAkt ser 473 and high PI3K-Akt*
267 *signaling phosphatase expression*

268 Following our RNA-sequencing findings in transduced SW1736 cells, we next questioned if level
269 of endogenous TR β expression correlates with reduced Akt phosphorylation and increased PI3K-
270 Akt phosphatase expression. We demonstrated a significant inverse correlation between TR β
271 expression and Akt phosphorylation as shown by immunoblot (Fig. 4A and B). Furthermore,
272 8505C with the highest level of endogenous TR β also had the highest expression of the
273 phosphoinositide phosphatases *INPP4B* and *INPP5J* as well as the pAkt ser473 phosphatase
274 *PHLLP1* (Fig. 4C). These findings illustrate the trend between TR β and expression of tumor
275 suppressive genes in PI3K signaling in a genetically diverse set of ATC cell lines (Supplemental
276 Table 4).

277

278 *3.5 PI3K signaling genes regulated by TR β in SW1736 cells are aberrantly expressed in patient*
279 *thyroid cancer samples*

280 Next, we used expression data from matched normal tissue and papillary, follicular, poorly
281 differentiated, and anaplastic thyroid carcinomas to determine if any of the PI3K regulators we
282 revealed to be altered by TR β -T₃ treatment exhibited differential expression in different thyroid
283 cancer subtypes (31-33). The patient microarray data revealed that the RTKs ERBB3 (HER3),
284 ROR1, and AXL expression levels correlate with TC subtype where expression is highest in the

285 more aggressive tumors (Fig. 5A). Conversely, phosphatase expression is highest in matched
286 normal tissue and differentiated thyroid tumors (Fig. 5B). Interestingly, expression of FGFR4 and
287 FGFR1 were lowest in the more aggressive PDTC and ATC populations. We next analyzed gene
288 expression data for TR β and markers of differentiated thyroid cells to demonstrate the
289 connection between TR β -T₃ presence with increased RTK and decreased phosphatase
290 expression. *THRB* (TR β) and genes encoding enzymes and transporters for thyroid hormone
291 synthesis were coordinately lost in FTC, PDTC, and ATC patient samples (Fig. 5C). As
292 demonstrated previously, TR β protein is significantly reduced in FTC, PDTC, and ATC patients (14),
293 a finding that may contribute to the gene expression data presented here.

294

295 *3.6 TR β and T₃ inhibit cell viability and migration in serum-activated cells*

296 PI3K signaling fuels cancer progression by stimulating cell survival, proliferation, and migration.
297 We previously demonstrated that TR β inhibits SW1736 proliferation in charcoal-stripped media,
298 an observation that was dependent upon T₃ stimulation (15). To achieve phenotypic confirmation
299 of our sequencing results, we challenged our engineered cells in full serum (10%) media with or
300 without additional 10 nM T₃ to measure a functional consequence of PI3K inhibition. Even in the
301 presence of full serum and activated RTKs, the TR β group rendered the SW1736 cells less viable
302 (Fig. 6A and B). In accordance with the RNA-sequencing data, T₃ is necessary for maximum
303 inhibition of cell viability by TR β . Additionally, we challenged our cells to migrate in the presence
304 of liganded-TR β . TR β cells were unable to effectively migrate to close the wound compared to
305 the EV cells (Fig. 6C-E). These findings are similar to what we observed previously in experiments

306 using charcoal-stripped, growth-deprived media, thus indicating the absolute requirement of TR β
307 to inhibit cell viability and migration.

308

309 *3.7 TR β improves PI3K inhibitor efficacy*

310 In order to further test our hypothesis that TR β suppresses PI3K signaling in ATC cells, we
311 established the efficacy of small molecule competitive PI3K inhibitors on cell cytotoxicity. Both
312 LY294002 and buparlisib compete for the ATP-binding site of PI3K, preventing PIP₂
313 phosphorylation. It should be noted that buparlisib is 27 times more potent than LY294002 (IC₅₀
314 of 52 nM and 1.4 μ M, respectively (34)). The TR β cells showed an improved response to
315 LY294002, with an EC₅₀ value nearly 5-fold less than the EV cells (Fig. 7A). In addition, the TR β
316 cells were nearly 25-fold more sensitive to the PI3K inhibitor buparlisib than the EV cells
317 (Supplemental Fig. 3). In concordance with the RNA sequencing and cell viability data, TR β
318 requires T₃ to fully exert the tumor suppressive profile; LY294002 efficacy was not increased in
319 SW-TR β cells lacking 10 nM T₃ (Supplemental Fig. 4). LY294002 efficacy was also enhanced in the
320 TR β cells as measured by migration assay at both 1 and 10 μ M (Fig. 7B-D).

321

322 *3.8 TR β enhances PI3K inhibitor inactivation of the PI3K-Akt-mTOR axis*

323 To further confirm that TR β is specifically inhibiting the PI3K signaling pathway, we challenged
324 our cells with LY294002 and buparlisib and measured pAkt, pmTOR, and pp70S6K levels following
325 24 hours of T₃ treatment. LY294002 and buparlisib were able to blunt Akt thr308 phosphorylation
326 in both cell lines, but there was a marked decrease in levels of pAkt (ser473), pmTOR (ser2448
327 and ser2481), and pp70S6K (Fig. 8A and Supplemental Fig. 5A). We validated these findings with

328 a pAkt (ser473) sandwich ELISA and detected lower levels of pAkt in the LY294002-treated TR β
329 cells compared to the EV control (Figure 8B).

330 In addition to the Akt-mTOR-p70S6K axis, we also observed an increase in pGYS1 following both
331 T₃ and LY294002 or buparlisib treatment in our cells, suggesting a robust Akt suppression due to
332 TR β activation in SW1736 cells (Fig. 8C and Supplemental Fig. 5B). Finally, these results appeared
333 to be dependent on long-term T₃ treatment, as short-term T₃ exposure failed to significantly
334 enhance suppression of PI3K with TR β or LY294002 (Fig. 9A and B).

335

336 4. Discussion

337 TR β has shown to be a potent tumor suppressor in several types of cancer including breast and
338 thyroid cancer (15,16,35,36). However, there are only a few tumor suppressive mechanisms
339 delineated in the literature, which include TR β -mediated JAK1/STAT1 activation and binding to
340 p85 α (15,20,35). Multiple groups have shown the potential for TR β to bind p85 α in the cytoplasm,
341 inhibiting PI3K-mediated PIP₂ to PIP₃ catalysis. While TR β and T₃ failed to rapidly inhibit PI3K
342 activity in these ATC cells, transcriptomic data revealed novel genomic mechanisms of TR β -
343 mediated PI3K suppression. Intriguingly, negatively regulated genes included RTKs such as FGFR
344 isoforms and HER3, both of which are implicated in advanced thyroid carcinomas (37-39). TR β -
345 mediated downregulation of HER3 is particularly interesting, as HER3 is the most potent
346 activating binding partner of HER2 and was found to promote PI3K inhibitor resistance (40,41).
347 In addition to regulating expression of upstream regulators of PI3K, TR β increased expression of
348 membrane-associated phosphatases, including *INPP5J* and *INPP4B*. *INPP4B* has shown to exhibit
349 remarkable tumor suppression in an *in vivo* model of thyroid cancer by regulating PI3K signaling

350 (42). Furthermore, TR β also increased expression of the phosphatases *PTPL1*, *PHLPP1*, and the
351 R5B subunit of PPP2. These phosphatases preferentially dephosphorylate ser473 on Akt, and
352 INPP4B and INPP5J modulate ser473 phosphorylation levels (28-30). Increased expression of
353 these phosphatases would account for the robust decrease in ser473 phosphorylation but not
354 thr308 and further supports the notion that TR β -mediated suppression of the PI3K pathway in
355 these cells is primarily driven by transcriptional regulation. Importantly, both Akt residues must
356 be phosphorylated for maximum activity, as thr308 phosphorylation accounts for only 10% of Akt
357 activity (43). Therefore, the robust pAkt ser473 dephosphorylation may be sufficient to inhibit
358 Akt activity, accounting for the growth and migration inhibition observed in this study.
359 Downstream consequences of TR β -mediated dephosphorylation of Akt was observed in three
360 well-established downstream targets of Akt, including GYS1, mTOR, and p70S6K.
361 In addition to the previously documented mechanisms of TR β tumor suppression, genomic
362 regulation of PI3K regulators likely drives a reduction in cell viability and migration. TR β enhanced
363 the effect of PI3K inhibitors LY294002 and buparlisib as shown by decreased cell viability,
364 migration, and cell signaling. Importantly, in a phase II trial of ATC patients, buparlisib was able
365 to reduce tumor burden and modestly improve patient survival (44). As we have observed an
366 enhanced response to PI3K inhibitors in the presence of TR β and hormone, it would be
367 worthwhile to determine if expression levels of TR β in patient tumors were correlated with
368 patient outcomes.
369 In summary, our results demonstrate that TR β suppresses PI3K signaling in SW1736 cells by
370 genomic regulation of RTKs and phosphatases. Although the potential for TR β to suppress PI3K
371 signaling by binding to the p85 α subunit in the cytoplasm is well known, this is the first report to

372 highlight genomic mechanisms by which TR β suppresses the PI3K-Akt-mTOR axis. The presence
373 and activation of TR β in ATC cells may be a promising therapeutic target to constrain tumor
374 progression and resistance to chemotherapeutics.

375

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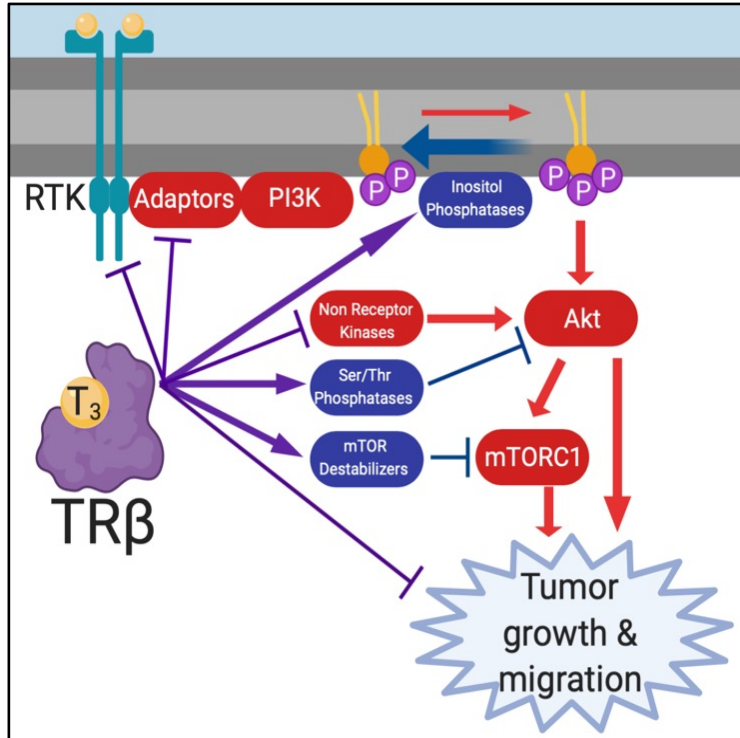
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Graphical abstract

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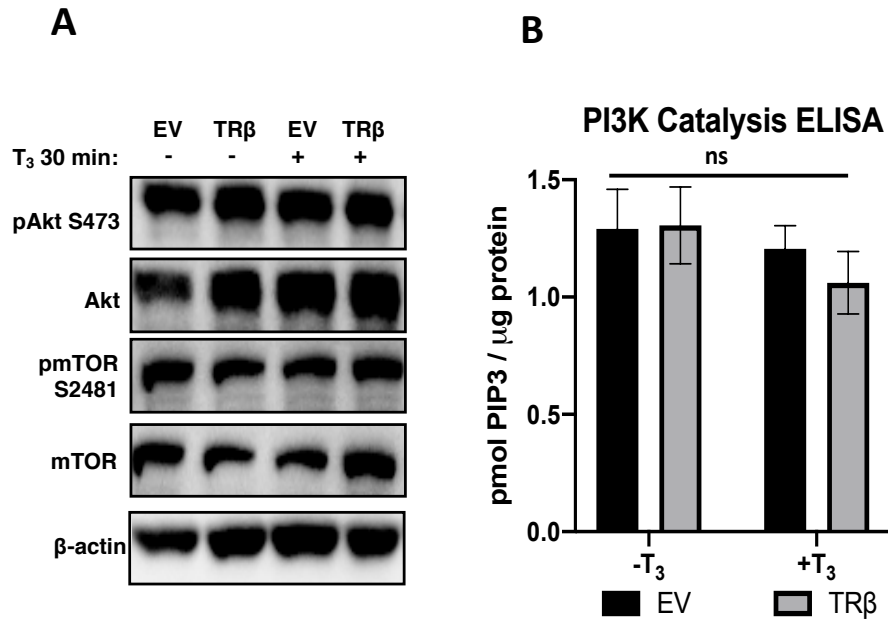


Figure 1. Short-term or no exposure to T₃ is insufficient for TR β -mediated PI3K suppression. SW1736-EV (EV) and SW1736-TR β (TR β) cells were treated with 10 nM T₃ or vehicle (1 N NaOH) for 30 min before protein levels were determined by immunoblot (**A**) or incubated with anti-p85 α antibody for PI3K catalysis ELISA (**B**). ELISA signal in **B** was standardized to protein concentration as determined by a Bradford assay. Significance in **B** was calculated by two-way ANOVA followed by Tukey multiple comparisons test. ns = no significance ($p \geq 0.05$) across treatment groups.

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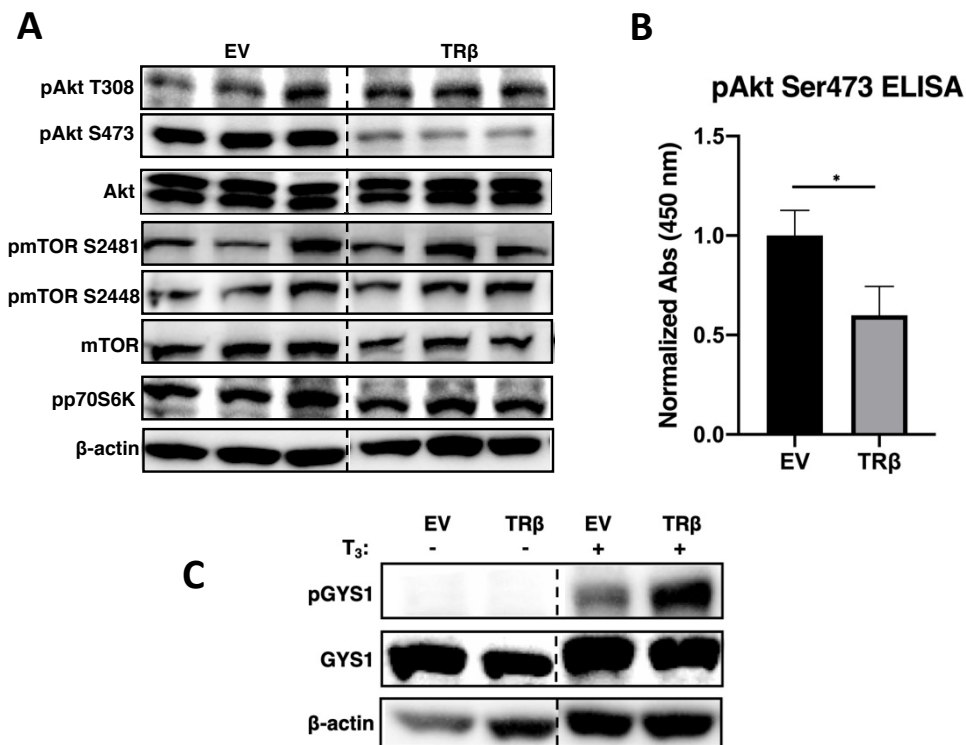


Figure 2: Long-term T₃ with TRβ inhibits the PI3K-Akt-mTOR pathway in SW1736 cells. SW1736-EV (EV) and SW1736-TRβ (TRβ) cells were treated with 10 nM T₃ for 24 hours before protein levels were determined by immunoblot (**A** and **C**) or subjugated to a pAkt Ser473 sandwich ELISA (**B**). The samples in **A** are replicates. Dashed lines in immunoblots indicate gap between two 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 Student's t-test. (*p < 0.05).

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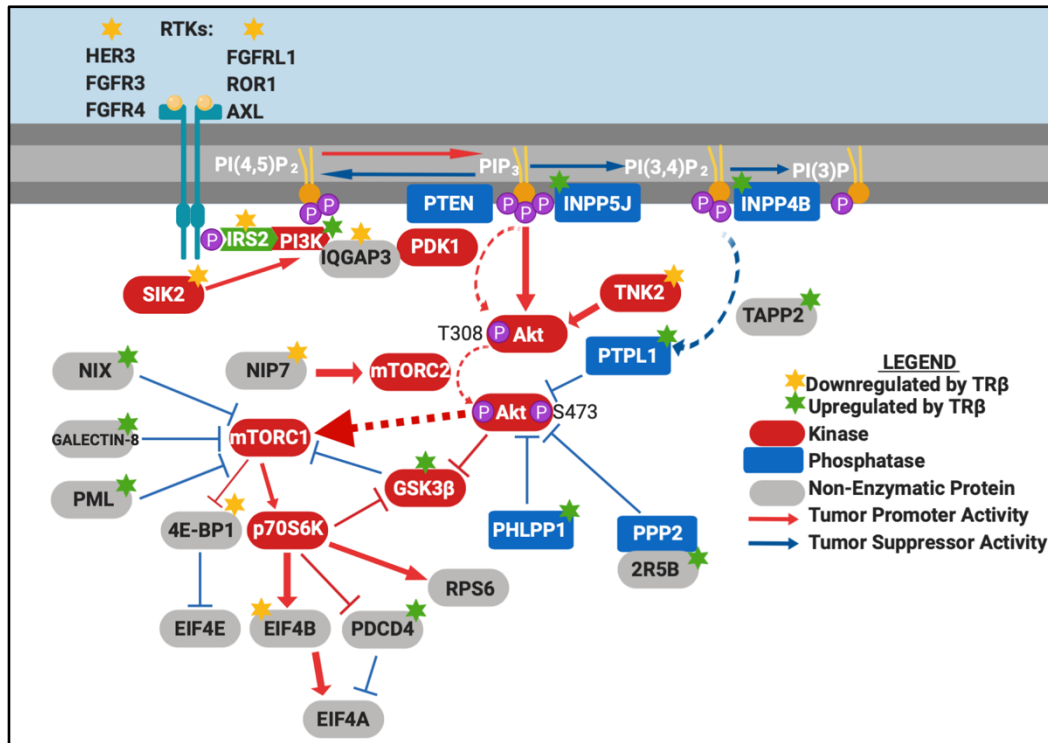
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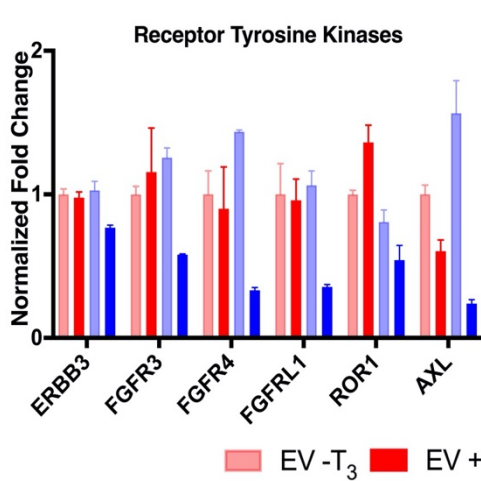
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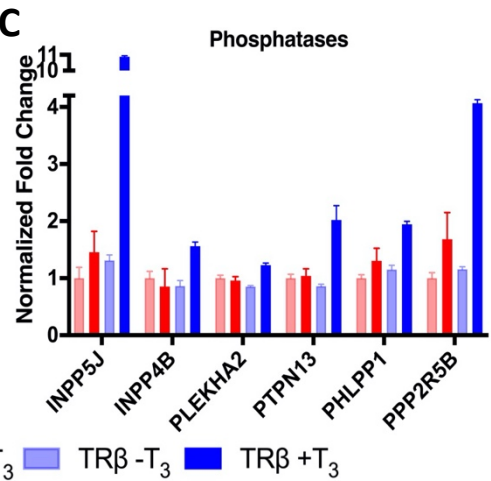
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576 **Figure 3: Liganded-TRβ decreases expression of oncogenic genes and increases tumor**
 577 **suppressible genes in the PI3K-Akt-mTOR signaling axis. A.** RTKs dimerize in response to ligand
 578 to allow for IRS2 to dock and recruit PI3K (45). PI3K phosphorylates PI(4,5)P₂ to PIP₃. PTEN and
 579 INPP5J dephosphorylate PIP₃ to PI(4,5)P₂ and PI(3,4)P₂ respectively. INPP4B dephosphorylates
 580 PI(3,4)P₂ to PI(3)P. PIP₃ recruits PDK1 (not shown) and Akt to the plasma membrane for PDK1
 581 phosphorylation of Akt T308. NIP7-activated mTORC2 phosphorylates Akt S473, which is
 582 dephosphorylated by PHLPP1 (46). ING5 has been shown to dephosphorylate Akt in hormone-
 583 dependent cancers (47). The 2R5B subunit of PPP2 directs the complex to dephosphorylate Akt
 584 T308 and S473 (30). TNK2 phosphorylates Akt Y176 to enhance plasma membrane recruitment

585 (48). Akt leads to the activation of mTORC1 by TSC 1/2-Rheb (not shown). Nix and PML destabilize
 586 Rheb-mTORC1 binding (49). Galectin-8 inhibits and delocalizes mTORC1 (50). GSK3 β is inhibited
 587 by Akt and inhibits multiple substrates relevant to glucose and glycogen metabolism, survival
 588 signaling, and cell cycle progression. mTORC1 phosphorylates and inhibits 4E-BP1 which inhibits
 589 EIF4E. MTORC1 also activates P70S6K which inhibits GSK3 β and PDCD4 and activates EIF4B and
 590 RPS6. EIF4B and PDCD4 regulate EIF4A (49). **B.** Ligand-bound TR β decreased expression of
 591 receptor tyrosine kinases. **C.** Ligand-bound TR β increased expression of PI3K-Akt phosphatases.
 592 Significance and fold change values between EV+T₃ and TR β +T₃ are located in Supplementary
 593 Table 1.
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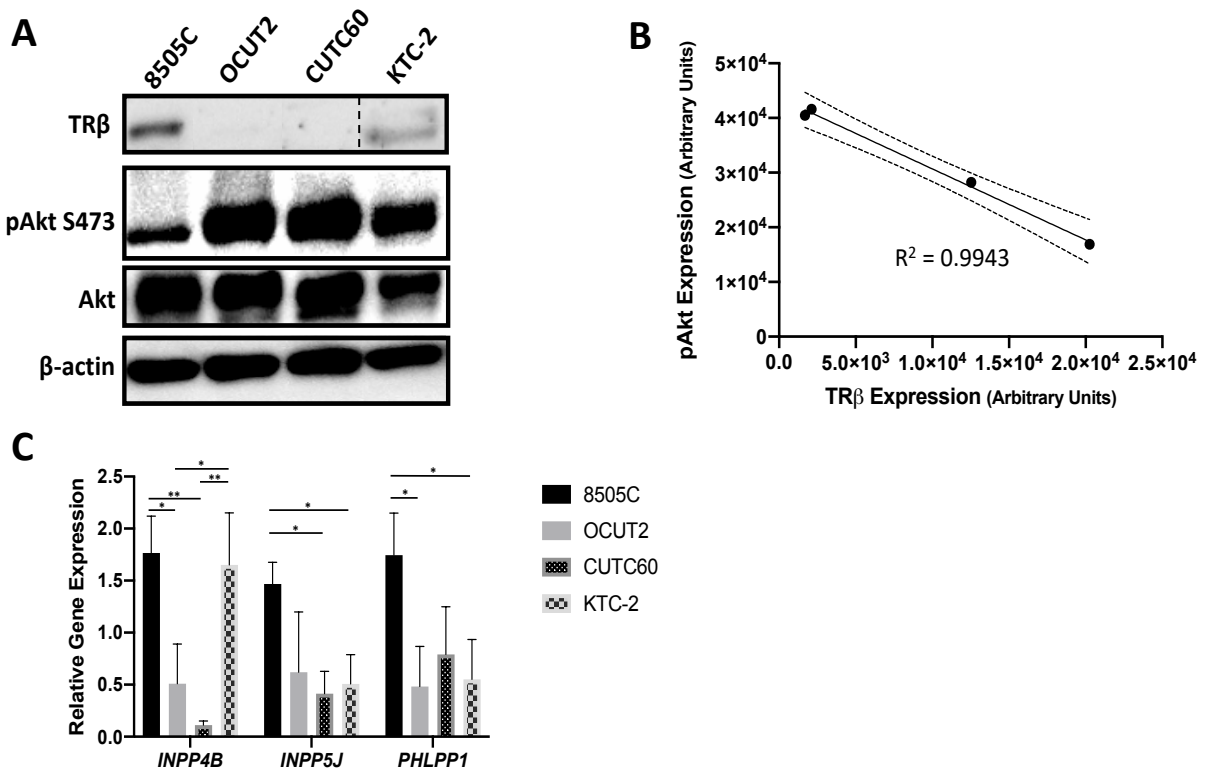
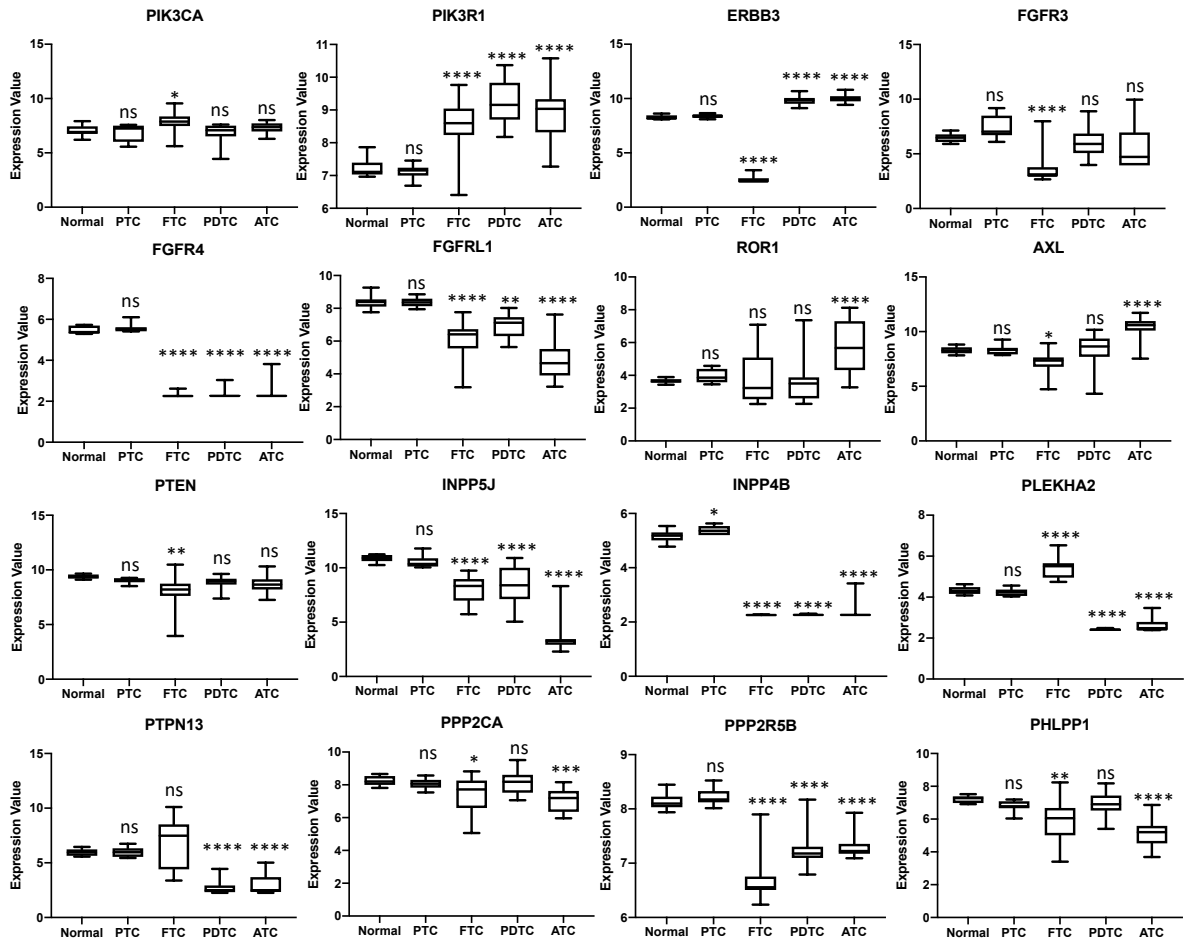


Figure 4: TR β expression correlates with low Akt ser473 phosphorylation and high phosphoinositide and Akt phosphatase expression. ATC cell lines were analyzed for TR β and pAkt ser473 expression (**A and B**) and phosphoinositide and Akt phosphatases (**C**). Dashed line in immunoblot indicates gap between two sets of lanes on the same membrane. Significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$).

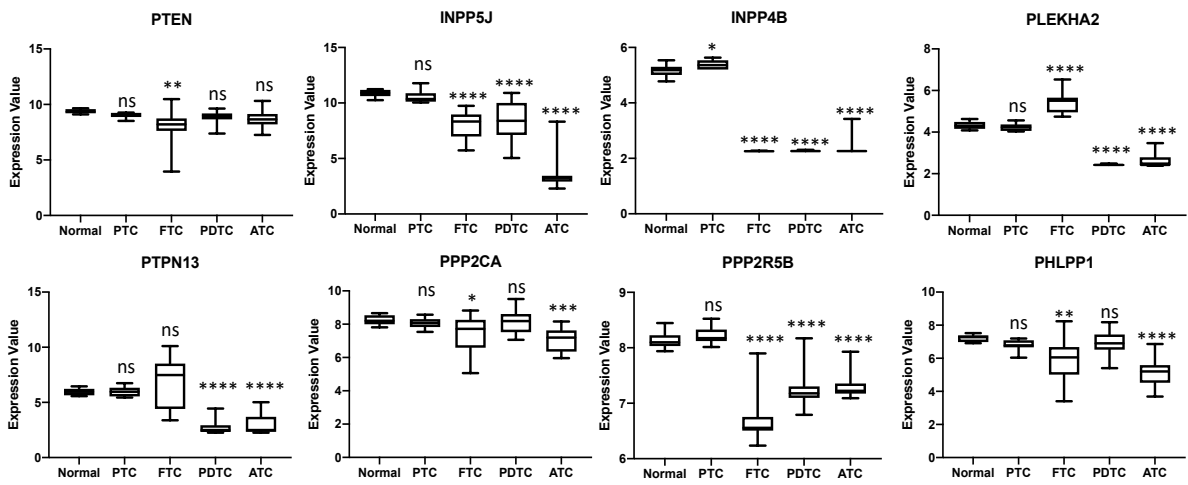
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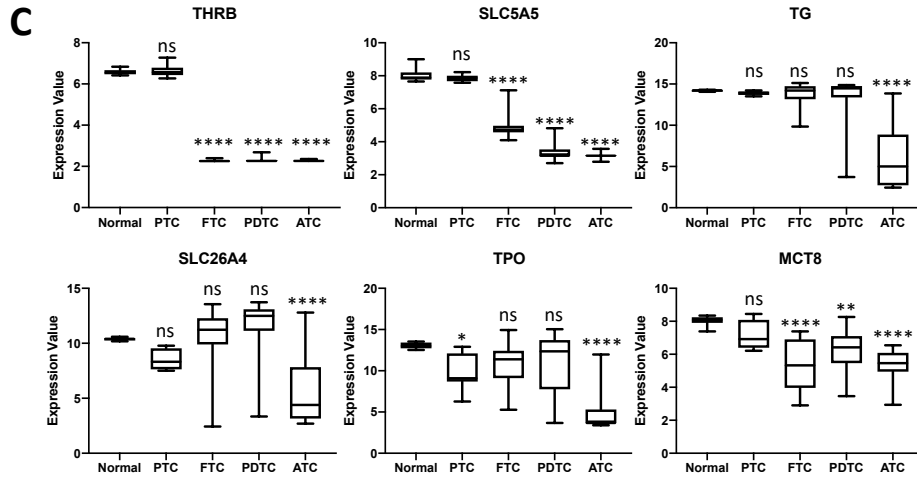


Figure 5: PI3K signaling genes regulated by TRβ in SW1736 cells are aberrantly expressed in patient thyroid cancer samples. Patient thyroid cancer microarray data (GSE76039, GSE3467, GSE82208) were analyzed for genes encoding receptor tyrosine kinases (A), phosphoinositide and Akt phosphatases (B), and TRβ, enzymes, and transporters necessary for synthesizing thyroid hormones. Significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test (ns = no significance $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Normal n = 9. PTC n = 9, FTC n = 27, PDTC n = 17, ATC n = 20

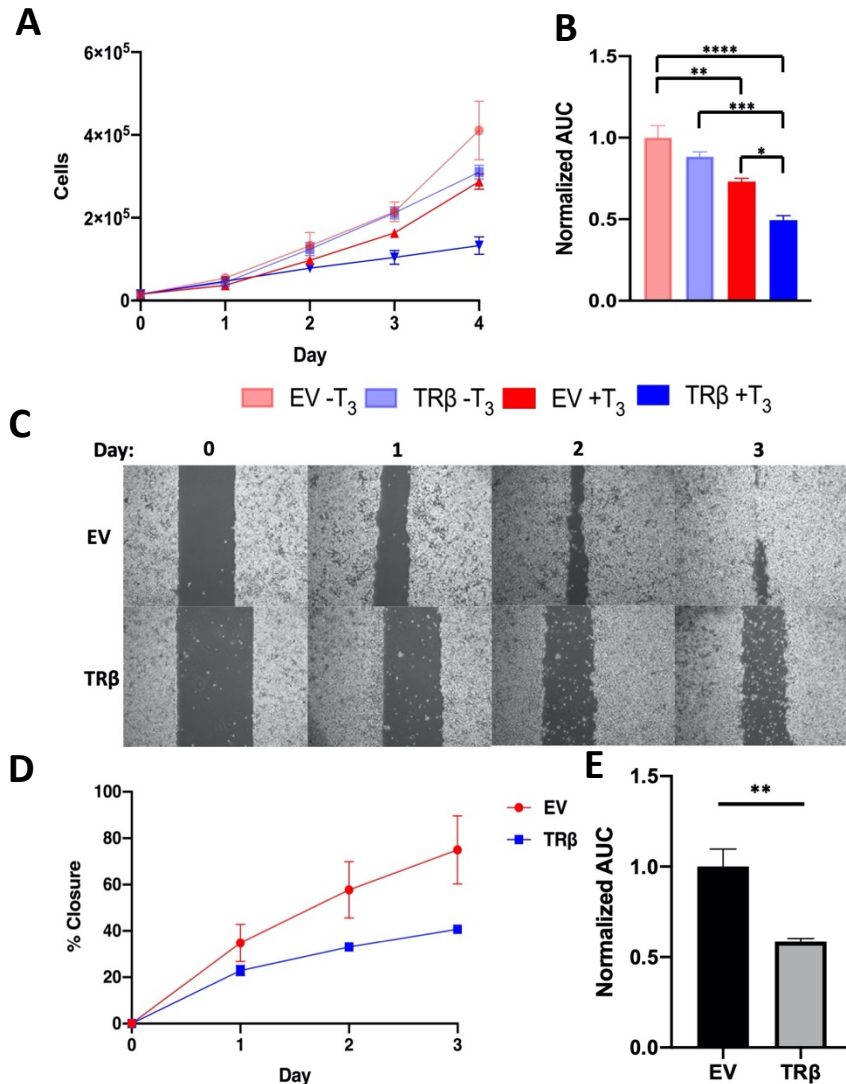


Figure 6: TRβ and T₃ reduce cell viability and migration in serum-activated SW1736 cells.

A. SW1736-EV (EV) and SW1736-TRβ (TRβ) cells were treated with 10 nM T₃ or vehicle (1 N NaOH) for four days. Each day the cells were lifted with trypsin and counted using a hemocytometer for viable cells. Area under the curve (AUC) analysis was performed (**B**) and normalized to the EV-T₃ group. Significance was calculated using one-way ANOVA followed by Tukey's multiple comparisons test (* p < 0.05, ** p < 0.01, ***p < 0.001, **** p < 0.0001). **C.** 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 PBS and treated with media containing 10 nM T₃. **D.** Wells were imaged each day and % wound closure relative to day 1 was calculated. **E.** Area under the curve (AUC) analysis was performed and normalized to the EV group. Significance was calculated using Student's t-test test. ** p < 0.01.

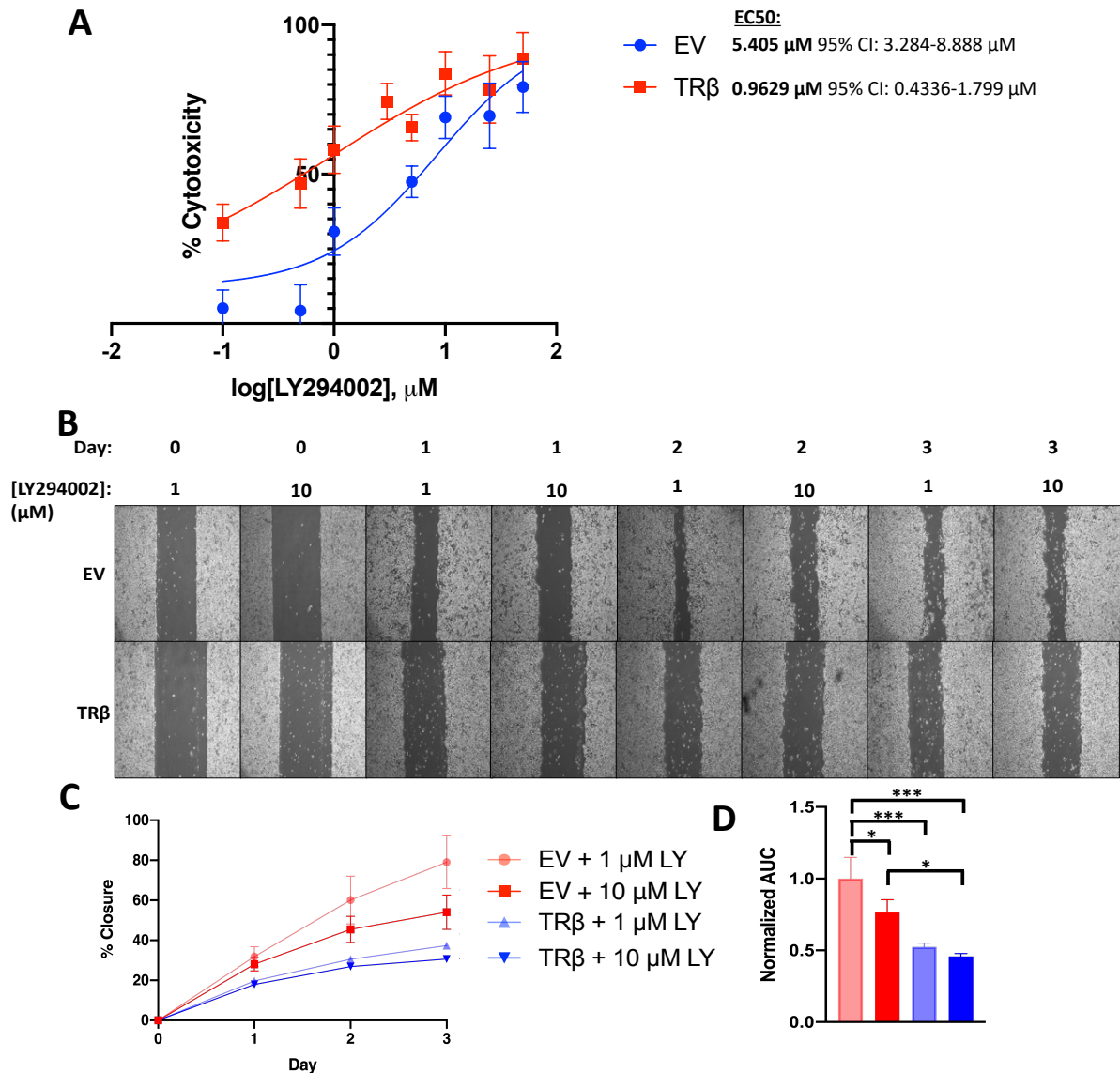


Figure 7: The effect of LY294002 on cytotoxicity and migration is enhanced in SW1736-TR β cells. **A.** EV and TR β cells were treated with 10 nM T₃ and LY294002 (0.1-50 μM) or matched-concentration vehicle (100% EtOH) for four days. Each day the cells were lifted with trypsin and counted using a hemocytometer for viable cells. Area the curve analysis was conducted for each LY294002 concentration to calculate % cytotoxicity relative to EV vehicle at each concentration of LY294002. EC50 values were calculated using GraphPad Prism's nonlinear regression package. **B.** EV and TR β cells were grown to confluency in 6 well plates before treatment with 10 $\mu\text{g}/\text{ml}$ mitomycin C for 2.5 hours. Media were aspirated, and the cells were scratched with a P1000 pipette tip before being washed with PBS and treated with media containing 10 nM T₃ and 1 or 10 μM LY294002. **C.** Wells were imaged each day and % wound closure relative to day 1 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 ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

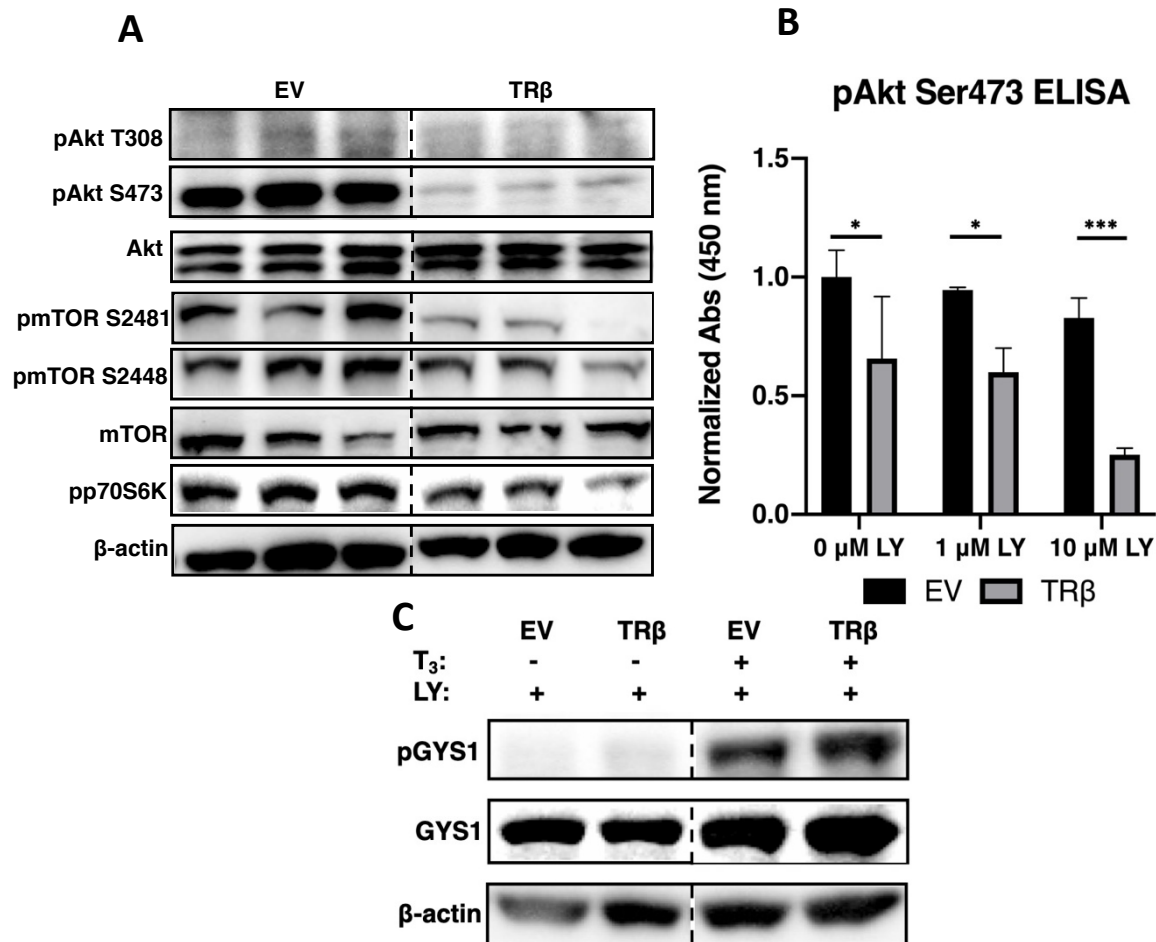


Figure 8: TRβ enhances LY294002-mediated inactivation of the PI3K-Akt-mTOR axis.

SW1736-EV (EV) and SW1736-TRβ (TRβ) cells were treated with 10 nM T₃ for 24 hours before 1 hour of LY294002 (LY) treatment (**A** and **C**: 10 μM, **B**: 0, 1, or 10 μM). Protein levels were determined by immunoblot (**A** and **C**) or subjugated to a pAkt Ser473 sandwich ELISA (**B**). Samples in **A** are replicates. Dashed lines in immunoblots indicate gap between two 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 ANOVA followed by Sidak's multiple comparison test. (*p < 0.05, ***p < 0.001).

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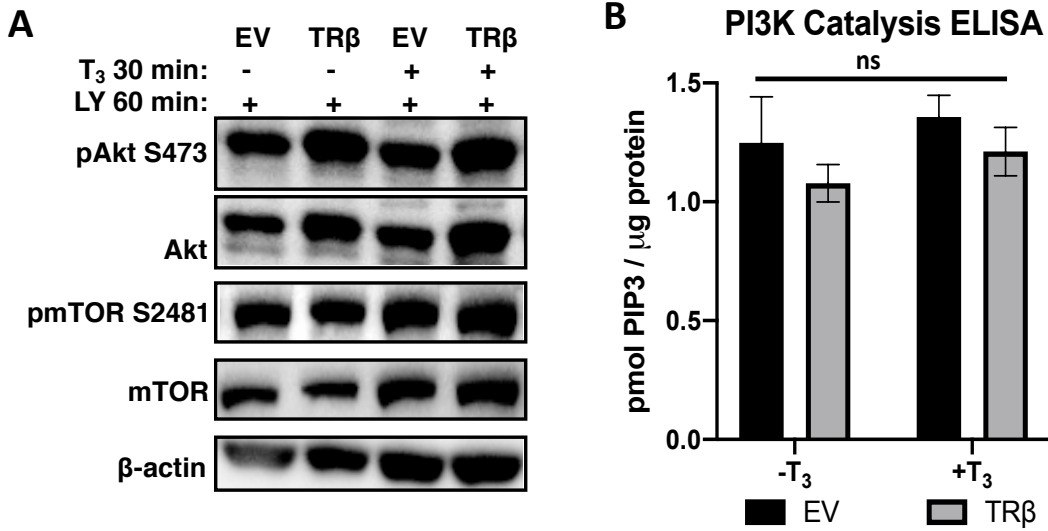


Figure 9. Short-term or no exposure to T₃ is insufficient for TRβ to enhance LY294002 suppression of PI3K.

SW1736-EV (EV) and SW1736-TRβ (TRβ) cells were treated with 10 nM T₃ or vehicle (1 N NaOH) for 30 min before 1 hour of 10 μM LY294002 (LY) treatment. Protein levels were determined by immunoblot (A) or incubated with anti-p85α antibody for PI3K catalysis ELISA (B). ELISA signal in B was standardized to protein concentration as determined by a Bradford assay. Significance in B was calculated by two-way ANOVA followed by Tukey multiple comparisons test. ns = no significance ($p \geq 0.05$) across treatment groups.

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