

1 **Functional Toll-like Receptor 4 Overexpression in Papillary Thyroid Cancer by** 2 **MAPK/ERK-induced ETS1 Transcriptional Activity**

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22 **ABBREVIATED TITLE:** TLR4 Overexpression in Thyroid Cancer

23 **ABBREVIATION LIST:** Toll-like receptors (TLRs), Lipopolysaccharide (LPS), Nuclear factor- κ B (NF- κ B),
24 Mitogen-activated protein kinase (MAPK), Extracellular signal-regulated kinase (ERK), E26 transformation-
25 specific (ETS), The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO).

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32 **ABSTRACT**

33 Emerging evidence suggests that unregulated Toll-like receptors (TLRs) signaling promotes tumor survival
34 signals, thus favoring tumor progression. Here, the mechanism underlying TLR4 overexpression in papillary
35 thyroid carcinomas (PTCs) mainly harboring the BRAF^{V600E} mutation was studied. TLR4 was overexpressed in
36 PTCs compared to non-neoplastic thyroid tissue. Moreover, paired clinical specimens of primary PTC and its
37 lymph node metastasis showed a significant upregulation of TLR4 levels in the metastatic tissues. In
38 agreement, conditional BRAF^{V600E} expression in normal rat thyroid cells and mouse thyroid tissue
39 upregulated TLR4 expression levels. Furthermore, functional TLR4 expression was demonstrated in PTC
40 cells by increased NF-κB transcriptional activity in response to the exogenous TLR4-agonist
41 lipopolysaccharide (LPS). Of note, The Cancer Genome Atlas (TCGA) data analysis revealed that BRAF^{V600E}-
42 positive tumors with high TLR4 expression were associated with shorter disease-free survival.
43 Transcriptomic data analysis indicated a positive correlation between TLR4 expression levels and
44 MAPK/ERK signaling activation. Consistently, chemical blockade of MAPK/ERK signaling abrogated
45 BRAF^{V600E}-induced TLR4 expression. A detailed study of the TLR4 promoter revealed a critical MAPK/ERK-
46 sensitive Ets binding-site involved in BRAF^{V600E} responsiveness. Subsequent investigation revealed that the
47 Ets-binding factor ETS1 is critical for BRAF^{V600E}-induced MAPK/ERK signaling-dependent TLR4 gene
48 expression. Together, these data indicate that functional TLR4 overexpression in PTCs is a consequence of
49 thyroid tumor-oncogenic driver dysregulation of MAPK/ERK/ETS1 signaling.

50 **IMPLICATIONS:** Considering the participation of aberrant NF-κB signaling activation in the promotion of
51 thyroid tumor growth and the association of high TLR4 expression with more aggressive tumors, this study
52 suggests a pro-oncogenic potential of TLR4 downstream signaling in thyroid tumorigenesis.

53 **KEYWORDS:** Papillary thyroid carcinoma, BRAF^{V600E}, Toll-like receptor 4, MAPK/ERK signaling pathway, ETS-
54 domain transcription factor family.

55 INTRODUCTION

56 The emerging role of Toll-like receptors (TLRs) in the maintenance of tissue homeostasis as critical
57 regulators of inflammatory processes and tissue regeneration response under physiological conditions has
58 led to the identification of aberrant functions of these receptors in different diseases, including
59 inflammatory and infectious disorders, autoimmunity and cancer (1). Particularly, unregulated TLR signaling
60 has been described in several neoplastic processes associated with exacerbated production of pro-
61 inflammatory cytokines involved in tumor progression (1,2). Indeed, TLR signaling-deficient mice are
62 resistant to tumor development in different experimental models (3,4). Likewise, transgenic mice
63 overexpressing constitutively active TLR4 under the intestine-specific villin promoter are highly susceptible
64 to colitis-associated cancer (5). The close link between dysregulated TLRs function and cancer progression
65 has set the molecular basis for the development of potential therapeutics targeting TLRs or TLRs-triggered
66 intracellular signal pathways (6).

67 Chronic activation of the immune system and tissue response might generate an inadequate negative
68 regulation of TLRs signal pathways leading to an excessive pro-inflammatory microenvironment that
69 facilitates the promotion of neoplastic processes (7). Different TLRs polymorphisms have been associated
70 with increased susceptibility or severity of infections (8). Of note, the polymorphism rs4986790 in TLR4
71 gene was identified as risk factor for the development of gastric cancer in *Helicobacter pylori*-infected
72 patients (9).

73 Functional TLRs expression in tumor cells has been linked to tumor survival and progression, evasion of
74 immune system and resistance to apoptosis. TLR4 engagement in lung cancer cells induces the expression
75 of immunosuppressive and pro-angiogenic cytokines (10). Further studies in prostate cancer cells showed
76 that TLR4 activation promotes the secretion of pro-angiogenic cytokines in response to NF- κ B signaling (11).
77 However, discrepancies regarding tumor cell survival in response to TLR4 activation in different cell models
78 have been observed. Particularly, TLR4 signaling improves survival in ovarian cancer cells (12) and promotes
79 the invasive ability of colon cancer cells (13). Conversely, studies in pituitary tumors showed that TLR4
80 activation inhibited tumor growth (14).

81 In the thyroid tissue, functional TLR3 expression was reported in normal thyrocytes. McCall *et al.* (15)
82 demonstrated high constitutive TLR3 signaling in papillary thyroid carcinomas (PTCs) compared to normal
83 thyroid tissue and follicular thyroid carcinomas (FTCs). Moreover, phenylmethimazole—a small molecule
84 that decreases TLR3 expression and signaling—inhibited PTC cells growth and migration (15). In addition,
85 we demonstrated functional TLR4 expression in normal murine thyrocytes (16). Using the exogenous TLR4-
86 agonist lipopolysaccharide (LPS), we demonstrated that NF- κ B signaling plays a central role in the
87 regulation of TLR4-induced thyroid differentiation markers gene expression (17,18). Interestingly, Hagström
88 *et al.* (19) reported a significant correlation between high TLR4 protein levels and metastatic potential in
89 FTCs. Moreover, Dang *et al.* (20) demonstrated that TLR4 is abundantly expressed in PTCs and stimulation
90 of the PTC cell line W3 with the endogenous TLR4-agonist low molecular weight hyaluronic acid promotes
91 cell proliferation and migration.

92 Here, we investigated the molecular events downstream thyroid cancer-driving oncogenes involved in the
93 upregulation of TLR4 gene expression in differentiated thyroid carcinomas. As previously described, we
94 show that TLR4 is aberrantly overexpressed in PTCs and FTCs. Moreover, TLR4 expression is functional as its
95 exogenous agonist LPS increased NF- κ B transcriptional activity in PTC cell models. Consistent with
96 transcriptomic data of PTC showing a significant correlation between TLR4 mRNA expression levels and ERK
97 activation score, chemical blockage of MAPK/ERK signaling abrogated BRAF^{V600E}-induced TLR4 expression.

98 MAPK/ERK signaling-dependent BRAF^{V600E}-induced TLR4 gene expression relies on a distal Ets binding-site
99 identified in the TLR4 promoter. Moreover, we show that the Ets-binding factor ETS1 is critical for
100 BRAF^{V600E}-induced MAPK/ERK signaling-mediated TLR4 gene expression in thyroid carcinomas. In sum, high
101 TLR4 levels in PTC cells is the consequence of dysregulated MAPK/ERK signaling as result of thyroid tumors-
102 drivers such as BRAF^{V600E}. Altogether, our evidence suggests a pro-oncogenic potential of TLR4 signaling in
103 thyroid tumorigenesis, further considering the oncogenic potential of NF-κB signaling in the promotion of
104 thyroid cancer (21).

105 MATERIAL AND METHODS

106 **Immunohistochemistry.** TLR4 and ETS1 expression in clinical thyroid samples was studied using low and
107 high-density human thyroid tissue arrays (US Biomax - Rockville, MD, USA). Endogenous peroxidase activity
108 was blocked by treatment with H₂O₂ in methanol for 15 min. Thyroid sections were then incubated for 30
109 min in 5% normal rabbit serum to block non-specific binding, followed by overnight incubation with 1 µg/ml
110 goat polyclonal anti-TLR4 antibody (sc-16240, Santa Cruz Biotechnology - Santa Cruz, CA, USA), 5 µg/ml
111 mouse monoclonal anti-TLR4 antibody (ab-22048, Abcam - Cambridge, MA, USA) or 3.3 µg/ml rabbit
112 polyclonal anti-ETS1 antibody (sc-350, Santa Cruz Biotechnology) at 4 °C in a humidified chamber. For
113 negative controls, the primary antibody was replaced by the corresponding purified non-reactive IgG in a
114 separate slide containing paraffin-embedded thyroid carcinoma tissue samples. Afterward, thyroid sections
115 were incubated with biotinylated secondary antibody (Santa Cruz Biotechnology) and ABC complex (Vector
116 Laboratories - Burlingame, CA, USA). 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich, St Louis, MO, USA) was
117 used as the chromogenic substrate for 10 min at RT, and the sections were rinsed in running water and
118 counterstained with Harris hematoxylin. Digital images were captured at 400x magnification using a Nikon
119 Eclipse TE2000-U light microscope (Nikon Instruments, Japan). Quantification of TLR4 and ETS-1 protein
120 levels was performed by assessing DAB staining intensity relative to the number of cells in the histological
121 sections in order to correct differences in tissue architecture. DAB staining intensity and the number of
122 nuclei seen in one randomly selected area of each tissue section were quantified using ImageJ software
123 (National Institute of Health - Bethesda, MD, USA).

124 **Plasmids.** The expression vectors encoding human BRAF^{V600E} and dominant-negative human Δ152–296
125 MyD88 were as reported (22,23). pCMV-mFlagEts1 (plasmid #86099) was from Addgene (Cambridge, MA,
126 USA). The NF-κB reporter vector containing five κB consensus sites linked to the luciferase coding sequence
127 (5x κB-Luc) and the expression vector encoding dominant-negative mouse Δ670-835 TLR4 were from
128 Clontech (Palo Alto, CA, USA) and InvivoGen (San Diego, CA, USA), respectively. Mouse TLR4 promoter
129 constructs +52/+223, -104/+223, -336/+223 and -608/+223 were as reported (24). Disruption of the distal
130 Ets binding-site in the mouse TLR4 promoter was performed by PCR with the oligonucleotide 5'-
131 TGGGTTTTAATCTCTAGCATTGTGAGAAAATATGTAGTTCTAGTCTGAAACATCCA carrying the desire mutation
132 (underlined) using KOD Hot Start DNA polymerase (EMD Millipore - Temecula, CA, USA) as described (25).
133 The artificial ETS reporter 3x Ets^d-Luc contains the luciferase gene under the control of a promoter
134 containing three copies in tandem of the flanking region of the distal Ets binding-site
135 (AAAATATGTTCTCTAGTCTGA) of mouse TLR4 promoter upstream the adenovirus E1B core promoter. The
136 normalization reporter pCMV-β-galactosidase was obtained from Promega (Madison, WI, USA). All
137 constructs were sequenced to verify nucleotide sequence (Instituto Nacional de Tecnología Agropecuaria,
138 Buenos Aires, Argentina).

139 **Cell culture.** The Fischer rat thyroid cell line PCCI3 was kindly provided by Dr. Roberto Di Lauro (Università
140 degli Studi di Napoli Federico II, Naples, Italy). PCCI3 thyroid cells engineered to obtain doxycycline-
141 inducible expression of the oncogenes BRAF^{V600E} (PC/BRAF^{V600E}), RET/PTC3 (PC/PTC3) and HRas^{G12V}
142 (PC/HRas^{G12V}) were generously provided by Dr. James Fagin (Memorial Sloan-Kettering Cancer Center, New
143 York, USA). PCCI3 and PCCI3-derived cells were cultured in DMEM/Ham F-12 medium supplemented with
144 5% calf serum (Thermo-Fisher Scientific - Waltham, MA, USA), 1 mIU/ml bovine TSH (National Hormone and
145 Peptide Program, Harbor-UCLA Medical Center - Torrance, CA, USA), 10 µg/ml bovine insulin and 5 µg/ml
146 bovine transferrin (Sigma-Aldrich) and treated with 1 µg/ml of doxycycline (Sigma-Aldrich) to induce
147 oncogene expression (26). Non-tumoral human thyroid cell line Nthy-ori 3-1 was obtained from the

148 European Collection of Authenticated Cell Cultures (Salisbury, United Kingdom) and human PTC-derived cell
149 line BCPAP—harboring the BRAF^{V600E} mutation—was provided by Dr. Massimo Santoro (Università degli
150 Studi di Napoli Federico II, Naples, Italy). Human thyroid cells were cultured in DMEM medium
151 supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria). Human cell lines were
152 authenticated using short tandem repeat genotyping using PowerPlex Fusion System (Promega) at Centro
153 de Excelencia en Procesos y Productos de Córdoba (Córdoba, Argentina). Cells were also regularly tested
154 for mycoplasma contamination using in-house PCR assays. Chemical inhibitors were obtained from the
155 following suppliers and used at the indicated concentrations: PLX4032 (Selleck Chemicals - Houston, TX,
156 USA), BAY 11-7082 (Sigma-Aldrich) and U0126 (Cell Signaling Technology - Danvers, MA, USA). The
157 endotoxin lipopolysaccharide from *E. coli* 055:B5 was purchased from Sigma-Aldrich.

158 **Transgenic Mice.** FVB/N mice carrying thyroid-specific human BRAF^{V600E} gene expression under the
159 regulation of the bovine thyroglobulin promoter (Tg-BRAF^{V600E}) were provided by Dr. James Fagin (27). All
160 procedures were approved by the Institute of Biomedical Sciences, University of São Paulo (São Paulo,
161 Brazil) Ethical Committee for Animal Research.

162 **RNA isolation and Real-time PCR.** Total RNA purification, cDNA synthesis, and quantitative PCR (qPCR)
163 were performed as described (28). Gene specific primer sets were as follow: mouse/rat TLR4 (180 bp) 5'-
164 CCAAGAACCTRGAYCTGAGC (forward) and 5'-TCTTGATAGGGTTTCCTGTC (reverse), human TLR4 (180 bp) 5'-
165 CCAAGAACCTGGACCTGAGC (forward) and 5'-TCTGGATGGGGTTTCCTGTC (reverse), rat IL-6 (109 bp) 5'-
166 GACAAAGCCAGAGTCATTCAGAGC (forward) and 5'-GAGCATTGGAAGTTGGGGTAGG (reverse), rat iNOS (137
167 bp) 5'-CTTCCGAAGTTTCTGGCAGCAG (forward) and 5'-GGACCATCTCCTGCATTTCTTCC (reverse), human IL-6
168 (290 bp) 5'-GGATGCTTCCAATCTGGATTCAATGAG (forward) and 5'-CGCAGAATGAGATGAGTTGTCATGTCC
169 (reverse), human iNOS (104 bp) 5'- TGGCAGCATCAGAGGGGACC (forward) and 5'-
170 GCAGGACAGGGGACCACATCGAA (reverse), human β -actin (109 bp) 5'-ACTCTTCCAGCCTTCCTTCC (forward)
171 and 5'- GTTGGCGTACAGGTCTTTGC (reverse), rat β -actin (223 bp) 5'- CTACAATGAGCTGCGTGTGG (forward)
172 and 5'- GGGCACAGTGTGGGTGAC (reverse), mouse β -actin (157 bp) 5'- CAGCTTCTTTCAGCTCCTT (forward)
173 and 5'- CACGATGGAGGGGAATACAG (reverse). Relative changes in gene expression were calculated using
174 the $2^{-\Delta\Delta Ct}$ method normalized against the housekeeping gene β -actin. For each pair of primers a dissociation
175 plot resulted in a single peak, indicating that only one cDNA species was amplified. The amplification
176 efficiency for each pair of primers was calculated using standard curves generated by serial dilutions of
177 cDNA generated from PCC13 or BCPAP cells. All amplification efficiencies ranged between 97-105% in
178 different assays.

179 **Immunoblotting.** SDS-PAGE, electrotransference to nitrocellulose membranes, and immunoblotting were
180 as described (29). Membranes were incubated with 0.2 μ g/ml mouse monoclonal anti-TLR4 (ab-22048,
181 Abcam), 0.2 μ g/ml rabbit polyclonal anti-BRAF (sc-166, Santa Cruz Biotechnology), 0.2 μ g/ml mouse
182 monoclonal anti-p-ERK (sc-7383, Santa Cruz Biotechnology), 0.2 μ g/ml rabbit polyclonal anti-ERK (sc-94,
183 Santa Cruz Biotechnology), 0.2 μ g/ml rabbit polyclonal anti-ETS1 (sc-350, Santa Cruz Biotechnology), 0.2
184 μ g/ml rabbit polyclonal anti-PARP-1 (sc-7150, Santa Cruz Biotechnology), 0.4 μ g/ml mouse monoclonal
185 anti- α -Tubulin (T6074, Sigma-Aldrich) antibodies. After washing, membranes were further incubated with
186 IRDye 680 RD or IRDye 800 CW-linked secondary anti-rabbit and anti-mouse antibodies (LI-COR
187 Biotechnology - Lincoln, NE, USA), protected from light. Membranes were visualized and quantified by
188 Odyssey Infrared Imaging System (LI-COR Biotechnology). Equal loading was assessed by stripping and
189 reprobing the same blot with 0.1 μ g/ml rabbit polyclonal anti-GAPDH (sc-25778, Santa Cruz Biotechnology).

190 **Immunofluorescence.** Cells seeded onto glass coverslips were fixed in 2% paraformaldehyde (30). Cells
191 were immunostained with 10 µg/ml mouse monoclonal anti-TLR4 (ab-22048, Abcam) or 4 µg/ml rabbit
192 polyclonal anti-ETS1 (sc-350, Santa Cruz Biotechnology) in PBS containing 0.2% BSA. For analysis under
193 permeabilized conditions an additional 0.1% Triton was added. Secondary staining proceeded with 50 nM
194 anti-mouse Alexa-594 antibody (Thermo-Fisher Scientific). Nuclear DNA was stained with 4',6-diamidino-2-
195 phenylindole (DAPI). Coverslips were mounted with FluorSave Reagent (EMD Millipore) and images were
196 acquired on a Leica DMI8 epifluorescence microscope (Leica Microsystems - Buffalo Grove, IL, USA). The
197 number of nuclei and the intensity of TLR4 or ETS1 immunostaining were analyzed using ImageJ software.

198 **Transient transfection and reporter gene assay.** Cells were transiently co-transfected with 0.64 µg/well of
199 luciferase reporter vectors or 0.21 µg/well of luciferase reporter vectors plus 0.42 µg/well of expression
200 vectors and 0.16 µg/well of the normalization reporter β-galactosidase in 24-well plates using
201 Lipofectamine 2000 (Thermo-Fisher Scientific) (29). The luciferase activity was measured using a Luciferase
202 Assay System (Promega) according to the manufacturer's instructions and normalized relative to that of β-
203 galactosidase.

204 **Small interference RNA.** Negative control siRNA pool (D-001206-13) and rat siRNA specific for ETS1 (sc-
205 156062) were from Dharmacon (Lafayette, CO, USA) and Santa Cruz Biotechnology, respectively.
206 PC/BRAF^{V600E} cells were seeded at a density of 5x10⁵ cells/well onto 6-well plates and transfected with 10
207 nM of each siRNA pool using Lipofectamine RNAiMAX reagent (Thermo-Fisher Scientific) according to the
208 manufacturer's protocol. ETS1 protein expression was monitored by immunoblotting 48 h after
209 transfection.

210 **Chromatin Immunoprecipitation (ChIP).** Cells were crosslinked in culture media containing 1%
211 formaldehyde and nuclei were purified and lysed in 50 mM Tris-HCl (pH 8), 10 mM EDTA and 1% sodium
212 dodecyl sulfate (31). Genomic DNA was broken by sonication and 10-fold diluted in IP Dilution Buffer [50
213 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 0.5% Nonidet P-40].
214 Immunoprecipitation was performed with 2 µg non-specific mouse IgG or rabbit polyclonal anti-ETS1
215 antibody (sc-350, Santa Cruz Biotechnology) (32). Immune complexes were purified with Protein A/G PLUS-
216 Agarose (Santa Cruz Biotechnology) pre-blocked with sonicated salmon sperm DNA (Sigma-Aldrich).
217 Immunoprecipitates were washed four times with IP Dilution Buffer containing 0.1% SDS; twice with High
218 Salt IP Wash Buffer [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM EDTA, 0.1% SDS and 1% Triton X-100],
219 and once with TE [10 mM Tris-HCl (pH 8), 1 mM EDTA]. DNA was purified using Chelex-100 (Bio-Rad –
220 Hercules, CA, USA). Immunoprecipitated DNA was quantified by qPCR using the primer set 5'-
221 GAGAGAGGCTATTGCCCATG (forward) and 5'-AGCGTTTGCTGACCAGCTTC (reverse) as described. Relative
222 fold of increase were calculated according to the equation: $2^{-[(Ct.input - Ct.target) - (Ct.input - Ct.mock)]}$.

223 **Statistical analysis.** Results are reported as the mean ± SEM from at least three independent experiments.
224 Multiple group analysis was conducted by one-way ANOVA with Newman-Keuls multiple comparisons post-
225 test. Comparisons between two groups were performed using unpaired Student's t test or nonparametric
226 Mann-Whitney test. Immunohistochemistry quantifications were analyzed by the nonparametric Kruskal-
227 Wallis test with Dunn's multiple comparison post hoc tests. Correlation and disease-free survival analysis
228 using molecular data derived from The Cancer Genome Atlas were analyzed using Spearman's correlation
229 coefficient and Mantel-Cox test, respectively. Statistical analyses were performed using GraphPad Prism
230 (GraphPad Software - La Jolla, CA, USA). Differences were considered statistically significant at $P < 0.05$.

231

232 RESULTS

233 **TLR4 is overexpressed in differentiated thyroid carcinomas.** We studied TLR4 expression in normal (n=13)
234 and malignant thyroid sections, including PTC (n=50) and FTC (n=32) by immunohistochemistry. In
235 agreement with previous observations (16,19,20), TLR4 expression was detected on normal thyrocytes (Fig.
236 1A). Interestingly, TLR4 protein levels were significantly higher in differentiated—follicular and papillary—
237 thyroid carcinomas compared to normal thyroid tissue (Fig. 1A), although no statistical differences were
238 evidenced between papillary and follicular thyroid tumors (Fig. 1A). Moreover, similar changes in TLR4
239 protein expression were evidenced in independent assays using a different anti-TLR4 antibody (Fig. S1). Of
240 note, comparison of TLR4 expression levels between matched-samples of tumor adjacent normal thyroid
241 tissue and PTCs (n=4) showed a significant upregulation of TLR4 levels in the neoplastic tissue (Fig. 1B). No
242 significant correlation was evidenced between TLR4 protein immunostaining and tumor, node and
243 metastasis (TNM) staging in PTCs or FTCs (Fig. S2). Significantly, TLR4 protein expression was also detected
244 in lymph node metastasis derived from PTCs (Fig. 1C). Comparison of TLR4 expression levels between
245 matched-samples of primary PTCs and its lymph node metastasis (n=8) showed a significant upregulation of
246 TLR4 levels in the metastatic tissue (Fig. 1C).

247 Thereafter, we used molecular data derived from The Cancer Genome Atlas (TCGA) study of PTC (33) to
248 perform combined analysis of TLR4 mRNA expression as a function of the most frequent driver oncogenes
249 identified in PTCs, including point mutations and gene fusions (33). Although TLR4 mRNA levels were found
250 unaltered in PTCs harboring different tumor driving oncogenes, we noticed a highly heterogeneous TLR4
251 expression among PTCs harboring the same oncogene (Fig. 1D). However, a significant positive correlation
252 was evidenced between TLR4 mRNA expression levels and ERK activation score ($r_s=0.1280$, $P=0.0189$), an
253 evaluation of the activation status of MAPK/ERK signaling (33), suggesting that oncogene-stimulated
254 MAPK/ERK signaling is an important event driving TLR4 gene expression in PTCs (Fig. 1E). In agreement, the
255 Gene Expression Omnibus (GEO) dataset GSE33630 analysis also showed a significant positive correlation
256 between TLR4 mRNA expression levels and ERK activation score in PTC (Fig. S3).

257 Thereafter, we focused our analysis on PTCs harboring the oncogene BRAF^{V600E} as this mutation has been
258 reported to occur in 30-80% of PTCs (34). We evidenced a significant positive correlation between TLR4
259 mRNA expression and the thyroid differentiation score ($r_s=0.1436$, $P=0.0302$), an evaluation of the
260 dedifferentiation status of the tumor (33), supporting that TLR4 gene expression is higher in more
261 differentiated PTCs (Fig. 1F). Moreover, we observed a non-significant positive association between TLR4
262 mRNA expression and MACIS score ($r_s=0.1039$, $P=0.1548$), an outcome predictor in patients with PTC (Fig.
263 1G). The modest association may be due in part to the characteristics of the patients, as most of them fall
264 into the low-risk category (MACIS score <6). Thereafter, patient samples harboring the oncogene BRAF^{V600E}
265 (wild-type TERT status) were divided into the high or low cohort based on the median expression of TLR4
266 mRNA levels within the group. Interestingly, patients whose tumors showed high TLR4 expression, based on
267 TLR4 expression above the median, had a lower median disease-free survival than those who showed TLR4
268 expression below the median (TLR4^{high}=26.63 months vs. TLR4^{low}=31.24 months, $P=0.0071$) (Fig. 1H).
269 Together, these data suggest that TLR4 mRNA expression constitutes a potential marker of PTC
270 aggressiveness.

271

272 **PTC-driving oncogenes induce TLR4 expression.** In order to study oncogene-driving TLR4 gene expression,
273 we analyzed TLR4 mRNA expression in PCC13 thyroid cell conditionally expressing the oncogenes BRAF^{V600E},
274 HRAS^{G12V}, or RET/PTC3 in response to doxycycline. In PCC13 cells, doxycycline treatment did not up-regulate

275 TLR4 mRNA expression. Conversely, in PC/BRAF^{V600E}, PC/HRas^{G12V} and PC/PTC3 cells doxycycline-induced
276 oncogene expression was associated with a significant increase of TLR4 mRNA expression (Fig. 2A).
277 Additionally, we assessed TLR4 protein expression in PC/BRAF^{V600E} cells in response to doxycycline.
278 BRAF^{V600E} expression significantly increased TLR4 protein levels in PC/BRAF^{V600E} cells (Fig. 2B). Furthermore,
279 transient transfection of BRAF^{V600E} into non-tumoral Nthy-ori 3-1 thyroid cells significantly increased TLR4
280 protein expression levels compared to those of empty vector-transfected cells (Fig. 2C). Analysis of ERK
281 phosphorylation was used to assess BRAF^{V600E} oncogenic activity. Complementary, immunofluorescence
282 performed under non-permeabilized conditions showed increased TLR4 protein levels at the plasma
283 membrane in response to BRAF^{V600E} expression (Fig. 2D). Similar observations were done in PCCI3 cells
284 conditionally expressing the oncogenes HRas^{G12V} and RET/PTC3 (Fig. S4).

285 In order to further confirm our findings, TLR4 mRNA expression was studied in five-week old transgenic
286 mice conditionally expressing the oncogene BRAF^{V600E} in the thyroid follicular cell (27). The thyroid tumors
287 of transgenic mice showed significantly higher TLR4 mRNA expression levels compared to that of non-
288 transgenic littermates controls (Fig. 2E). In addition, the expression of TLR4 protein levels in paraffin-
289 embedded normal and malignant mice thyroid tissues was assessed by immunohistochemistry.
290 Accordingly, TLR4 immunostaining was significantly increased in the thyroid tumor tissue of BRAF^{V600E}-
291 expressing mice compared to littermates controls (Fig. 2F).

292

293 **Functional TLR4 protein expression in cell models of PTC.** All TLRs signaling pathways culminate in
294 activation of the transcription factor NF- κ B (35). Therefore, the functionality of TLR4 overexpression in
295 thyroid carcinomas was assessed by analyzing NF- κ B signaling activation in response to the TLR4 agonist
296 LPS. Consistently with previous observations indicating the activation of NF- κ B signaling in response to
297 BRAF^{V600E} expression (34), doxycycline-treated PC/BRAF^{V600E} cells showed a significant induction of the NF-
298 κ B reporter 5x κ B-Luc (Fig. 3A). Interestingly, LPS treatment induced a significant upregulation of NF- κ B
299 transcriptional activity in doxycycline-treated PC/BRAF^{V600E} cells (Fig. 3A). The NF- κ B inhibitor BAY 11-7082
300 was used to investigate the specific activation of NF- κ B pathway (Fig. 3A). Moreover, consistently with the
301 activation of NF- κ B signaling in response to TLR4 activation, we observed a significant BAY 11-7082-
302 sensitive increase in the mRNA expression of the well-known TLR4-induced NF- κ B targets interleukin-6 (IL-
303 6) and inducible nitric oxide synthase (iNOS) in response to LPS stimulation in doxycycline-treated
304 PC/BRAF^{V600E} cells (Fig. 3B and C).

305 In order to reinforce our observations, we further studied the activation of NF- κ B signaling in response to
306 the TLR4-agonist LPS in BCPAP cells harboring the BRAF^{V600E} mutation. LPS stimulation of NF- κ B reporter-
307 transiently transfected BCPAP cells showed a significant induction of NF- κ B transcriptional activity (Fig. 3D).
308 As mentioned, assay specificity was verified by inhibiting the activation of NF- κ B signaling using BAY 11-
309 7082 (Fig. 3D). Additionally, we evidenced that BCPAP cells transiently expressing a non-signaling TLR4
310 mutant missing the carboxy-terminus or a non-signaling MyD88—intracellular TLR4 signaling adaptor—
311 mutant missing the TLR4-interacting domain were not responsive to LPS stimulation (Fig. 3D). Consistently,
312 LPS-induced TLR4-dependent signaling increased IL-6 and iNOS mRNA expression in BCPAP cells (Fig. 3E and
313 F). Together, this evidence suggests that TLR4/MyD88/NF- κ B signaling is functionally conserved in the PTC
314 cell line BCPAP.

315

316 **BRAF^{V600E}-induced TLR4 gene expression involves MEK/ERK signal pathway.** The oncogene BRAF^{V600E} leads
317 to constitutive activation of MAPK/ERK pathway by directly phosphorylating MEK, resulting in thyroid
318 follicular cell transformation (23,34). To uncover the signal pathway involved in the regulation of TLR4 gene
319 expression in response to BRAF^{V600E} activity, we explored the effect of the specific BRAF^{V600E} inhibitor
320 PLX4032 and the MEK1/2 inhibitor U0126 on TLR4 expression in PC/BRAF^{V600E} cells under doxycycline
321 treatment. As expected, BRAF^{V600E} inhibition reduced the upregulated TLR4 expression in response to
322 doxycycline treatment (Fig. 4A). In line with the observed positive correlation between TLR4 expression and
323 MAPK/ERK activation score, chemical inhibition of MAPK/ERK signaling activation reduced TLR4 expression
324 in response to BRAF^{V600E} induction (Fig. 4A). We analyzed ERK phosphorylation status by immunoblot to
325 assess the inhibition of BRAF^{V600E}-induced MAPK/ERK signaling in response to PLX4032 or U0126 treatment
326 (Fig. 4A). Complementary, immunofluorescence performed under non-permeabilized conditions showed
327 that chemical inhibition of MAPK/ERK signaling abrogated BRAF^{V600E}-induced cell surface TLR4 protein
328 expression levels (Fig. S5). Moreover, chemical inhibition of MAPK/ERK signaling activation reduced TLR4
329 mRNA expression in response to BRAF^{V600E} induction (Fig. 4B). To confirm this finding, we studied BRAF^{V600E}-
330 regulated TLR4 gene expression in BCPAP cells. Interestingly, BCPAP cells treated with the chemical
331 inhibitors PLX4032 or U0126 showed a significant reduction of TLR4 protein and mRNA expression levels
332 (Fig. 4C and D). Altogether, these observations reinforce the involvement of MAPK/ERK signaling in the
333 BRAF^{V600E}-regulated TLR4 gene expression.

334

335 **A distal E26 transformation-specific (Ets)-binding site is crucial for TLR4 gene transcription in response to**
336 **constitutive BRAF^{V600E} signaling.** To uncover the molecular mechanism involved in the BRAF^{V600E}-induced
337 TLR4 gene expression, we studied the transcriptional activity of sequential mouse TLR4 promoter deletions
338 linked to luciferase in doxycycline-treated PC/BRAF^{V600E} cells. The TLR4 promoter constructs -336/+223 and
339 -608/+223 showed a significant transcriptional response to the induction of BRAF^{V600E} expression (Fig. 5A).
340 However, the promoter constructs +52/+223 and -104/+223 abrogated BRAF^{V600E}-induced TLR4
341 transcriptional activity (Fig. 5A). As reported, the sequence of the TLR4 promoter comprising nucleotides -
342 336 and -104 contains a distal Ets-binding site (Ets^d) critical for constitutive TLR4 expression in mouse
343 macrophages (24). Thereafter, we performed site-directed mutagenesis of the Ets^d DNA-binding site within
344 the -336/+223 promoter construct. Significantly, Ets^d mutagenesis reduced BRAF^{V600E}-induced TLR4
345 promoter activity (Fig. 5B). Similar observations were done in PCCI3 cells conditionally expressing the
346 oncogenes HRas^{G12V} and RET/PTC3 (Fig. S6).

347 In order to further test our observations, we constructed an artificial reporter containing three copies in
348 tandem of the flanking region of the Ets^d binding site linked to luciferase (3x Ets^d-Luc). Significantly,
349 BRAF^{V600E} expression increased the luciferase activity of PC/BRAF^{V600E} cells transiently transfected with the
350 trimeric Ets^d reporter construct (Fig. 5C). Moreover, we observed that the transcriptional activity of the
351 -336/+223 TLR4 promoter construct missing the Ets^d binding site (Ets^d mt -336/+223) was significantly
352 lower than that of the wild type promoter in BCPAP thyroid cancer cells (Fig. 5D). Together, these results
353 support the involvement of the distal Ets-binding site in the transcriptional upregulation of TLR4 in
354 response to BRAF^{V600E} expression.

355

356 **The Ets-binding protein ETS1 mediates BRAF^{V600E}-induced TLR4 gene expression.** Ets-binding proteins
357 (ETSs) are a family of transcription factors that share a conserved ~85 amino-acid sequence called Ets DNA-
358 binding domain (36). ETS transcription factors are involved in a wide variety of functions including

359 differentiation, proliferation, migration, apoptosis and angiogenesis. Significantly RAS/RAF signaling
360 increases the transcriptional activity of some, but not all, ETS transcription factors through direct
361 phosphorylation of MAPK/ERK signaling (36).

362 To uncover the downstream BRAF^{V600E}-induced ETS factors required for TLR4 gene overexpression, we used
363 transcriptomic data derived from TCGA of PTC (33) to correlate the mRNA expression of TLR4 and different
364 members of the ETS family in the subset of PTCs harboring the oncogene BRAF^{V600E}. Interestingly, although
365 a significant positive correlation with ELF1, ETV3 and GABPA, and negative correlation with ETV4 and ELK1
366 were evidenced (Fig. S7A), the ETS ETS1 showed the highest positive association with TLR4 mRNA levels
367 ($r=0.5788$, $P<0.0001$) (Fig. 6A), suggesting a potential role for ETS1 in the BRAF^{V600E}-induced TLR4 gene
368 overexpression in PTCs. In agreement, GEO dataset GSE33630 analysis also showed that the ETS family
369 member ETS1 has the highest positive correlation with TLR4 mRNA expression in PTCs (Fig. S7B).
370 Supporting these findings, ETS1 mRNA expression is up-regulated in PTCs harboring the oncogene BRAF^{V600E}
371 (37) and constitutes a critical genome-wide transcriptional effector of RAS/ERK signaling in tumor epithelial
372 cells (32).

373 We further explored ETS1 protein expression in tissue microarrays containing unmatched normal thyroid
374 tissue (n=9) and PTCs (n=24) by immunohistochemistry. Although in the normal thyroid tissue ETS1 protein
375 expression was detected at low levels, its expression was significantly upregulated in PTC (Fig. 6B). In tumor
376 tissues, assessment of high-magnification images suggested that ETS1 was localized in both the cytoplasm
377 and nucleus. Moreover, correlation analysis demonstrated a significant positive correlation between TLR4
378 and ETS1 protein levels reinforcing a potential involvement of ETS1 transcriptional activity in TLR4
379 expression levels (Fig. 6C).

380 To elucidate the involvement of ETS1 as downstream target of BRAF^{V600E} oncogenic activity, we studied
381 ETS1 protein expression in response to doxycycline treatment in PC/BRAF^{V600E} cells. Conditional BRAF^{V600E}
382 expression promoted an increase of ETS1 protein expression and its nuclear accumulation (Fig. 6D and E). In
383 addition, we evidenced the involvement of MAPK/ERK signaling in the BRAF^{V600E}-dependent ETS1 protein
384 expression in PC/BRAF^{V600E} and BCPAP cells (Fig. 6F and G).

385 Moreover, in order to analyze the involvement of ETS1 in TLR4 gene expression, we studied BRAF^{V600E}-
386 induced TLR4 mRNA expression in ETS1 knock-down PC/BRAF^{V600E} cells. Immunoblot analysis showed a
387 successful knock-down of ETS1 protein levels in ETS1 siRNA transfected cells compared to scrambled (SCR)
388 siRNA-transfected cells (Fig. 6H). Interestingly, we evidenced a significant inhibition of BRAF^{V600E}-induced
389 TLR4 mRNA expression levels in ETS1 knock-down cells compared to those of SCR siRNA-transfected cells
390 (Fig. 6I). Consistently, analysis of TLR4 promoter activity showed that ETS1 knock-down repressed the
391 BRAF^{V600E}-increased TLR4 transcriptional activity (Fig. 6J). In agreement, TLR4 protein expression (Fig. 6K)
392 and its promoter activity (Fig. 6L) showed a significant upregulation in PCCI3 normal thyroid cells transiently
393 transfected to achieve ETS1 overexpression.

394 Thereafter, we studied ETS1 binding to the TLR4 promoter in response to BRAF^{V600E} expression in
395 PC/BRAF^{V600E} cells using ChIP assays. In detail, one putative Ets-binding site located between -240 and -233
396 (relative to the transcription start site) in the rat TLR4 promoter was evidenced when its sequence was
397 analyzed for putative Ets-binding sites using MatInspector software (Genomatix AG, Munich, Germany).
398 Interestingly, ChIP assay with an anti-ETS1 antibody of doxycycline-treated PC/BRAF^{V600E} cells revealed a
399 significant enrichment in the TLR4 promoter sequence flanking the Ets-binding site (Fig. 6M). These data
400 indicated that BRAF^{V600E}-induced activation of MAPK/ERK signaling up-regulated directly ETS1 expression
401 and binding to the TLR4 promoter that, in turn, modulated TLR4 gene expression.

402

403 DISCUSSION

404 Accumulating evidence indicates the association between over-activation of TLRs signaling and cancer
405 progression (1). Indeed, dysregulated TLRs signaling has been reported in several human cancers and
406 related to an exacerbated production of a pro-inflammatory tumor microenvironment that provides the
407 necessary context for tumor progression, angiogenesis, invasion, metastasis and evasion of immune
408 surveillance (2). The biological relevance of TLRs expression in tumor cells seems complex, and the
409 regulatory mechanisms leading to differential TLRs expression in cancer cells remains unclear.

410 In the thyroid tissue, we demonstrated functional TLR4 expression in normal murine thyrocytes as TLR4
411 engagement increased the expression of differentiation markers involved in thyroid hormonogenesis (16-
412 18). Here, in agreement with previous studies (19,20), we evidenced the presence of TLR4 protein
413 overexpression in well-differentiated thyroid carcinoma compared to that of normal thyroid tissue.
414 Moreover, we observed a significant up-regulation of TLR4 protein levels in lymph node metastasis derived
415 from PTCs. Unfortunately, a limitation of our immunohistochemical study of thyroid tumor sections is the
416 unavailability of information regarding the oncogenic driver promoting thyroid tumorigenesis. Therefore,
417 we were unable to assess whether upregulated TLR4 protein expression is restricted to a particular
418 oncogenic context. However, we did not evidence different TLR4 mRNA expression patterns among PTCs
419 harboring different tumor driving oncogenes. Additionally, integrative analysis of clinical and transcriptomic
420 data derived from TCGA of PTC (33) demonstrated that patients whose BRAF^{V600E}-positive tumor showed
421 high TLR4 expression, based on TLR4 expression above the median, had a lower median disease-free
422 survival than those who showed TLR4 expression below the median. In close correlation, Dang *et al.* (20)
423 demonstrated a higher incidence of lymph node and distant metastasis in patients with PTC showing high
424 TLR4 expression levels. Mechanistically, these authors demonstrated that TLR4 engagement with low
425 molecular weight hyaluronic acid induced a significant upregulation of CXCR7, the receptor of the
426 chemokine CXCL12 involved in the promotion of tumor cell proliferation and migration (20). Although these
427 findings support a potential pro-metastatic role for TLR4-mediated signaling in thyroid tumorigenesis, its
428 participation in PTC aggressiveness and metastatic potential should be further evaluated.

429 The ETSs regulate a plethora of processes, including differentiation, proliferation, apoptosis, and
430 angiogenesis downstream MAPK/ERK signaling activation (36). Significantly, aberrant ETSs activation has
431 been reported in several solid tumors (36). Particularly, the transcriptional activity of ETS1 and ETS2 is
432 required for thyroid follicular cell transformation (38). Here, we provided novel evidence regarding the
433 mechanisms that result in functional TLR4 overexpression in PTCs. Our data indicate that TLR4 gene
434 overexpression in PTC is the consequence of dysregulated MAPK/ERK signaling due to thyroid cancer-driver
435 oncogenes such as BRAF^{V600E}. Roger *et al.* (24) carried out a detailed study of the mechanism controlling
436 TLR4 gene expression regulation in mouse macrophages evidencing a distal Ets-binding site indispensable
437 for constitutive TLR4 gene transcription which may be recognized by epithelium-specific ESE1 transcription
438 factor. In this study, we demonstrated that the same Ets-binding site in the TLR4 promoter is critical for its
439 transcriptional up-regulation in response to BRAF^{V600E} oncogenic activity. Moreover, conducting
440 bioinformatics analysis, and biochemical approaches, we revealed the direct role of ETS1 as transcriptional
441 regulator of TLR4 gene expression downstream to BRAF^{V600E}-induced MAPK-ERK signaling. In agreement, a
442 comprehensive gene expression analysis of ETS1-regulated genes in PC3 prostate cancer cells
443 demonstrated that ETS1 knock-down decreases TLR4 mRNA expression, suggesting that ETS1 constitutes a
444 positive regulator of TLR4 gene expression in cancer cells (39).

445 Several studies indicate that single nucleotide polymorphisms in TLRs are associated with immune system
446 dysregulated and cancer development (8). Polymorphisms in TLRs genes may shift the balance between
447 pro- and anti-inflammatory cytokines, modulating the risk of infection, chronic inflammation, and cancer
448 (8). Although TLR4 has been described as a highly polymorphic gene, certain TLR4 polymorphisms have
449 been associated with a significant risk to develop epithelial cell-derived neoplasia. Particularly, the
450 polymorphisms rs10116253 and rs1927911 in TLR4 gene were associated with decreased risk to develop
451 gastric cancer (40), while the polymorphisms rs4986790, rs4986791 and rs10759932 appear to be positively
452 associated with the development of gastric cancer (41). Moreover, the polymorphism rs11536889 located
453 in the 3'-untranslated region of the TLR4 was associated with increased prostate and colon cancer risk
454 (42,43). In thyroid carcinomas, however, the presence of TLR4 polymorphisms remains to be investigated.
455 Significantly, Kim *et al.* (44) reported a case-control association study of the polymorphisms rs3804099 and
456 rs3804100 in TLR2 gene in patients with PTC and control subjects, describing a significant association
457 between polymorphisms in the TLR2 gene and bilateral thyroid tumors, a surgical criteria to perform total
458 thyroidectomy. More recently, Kim *et al.* (45) reported a significant association of the missense
459 polymorphism rs11466653 in TLR10 with a higher risk for the development of thyroid papillary
460 microcarcinomas than in the control group. Together, these findings support that abnormal TLR signaling in
461 PTC may be involved in the interplay between inflammation and tumorigenesis.

462 TLRs recognize molecules that are broadly shared by pathogens but, sometimes, indistinguishable from
463 host proteins released by stressed cells undergoing necrosis or proteolytic products derived from structural
464 components of the extracellular matrix, collectively referred to as damage-associated molecular patterns
465 (DAMPs). Here, we demonstrated that malignant thyroid follicular cells themselves are responsive to
466 exogenous TLR4 agonists. However, the question regarding the endogenous molecules that engage TLR4 in
467 the tumor environment to induce a signaling cascade leading to the NF- κ B activation remains unanswered.
468 We hypothesized that TLR4 signaling-activating DAMPs such as High mobility group box-1 (HMGB1) which is
469 usually observed at high levels in the tumor microenvironment or hyaluronan-derived proteolytic products
470 considering that hyaluronan is highly expressed in the tumor stroma of differentiated thyroid carcinomas
471 (46) are likely candidates. Moreover, regarding the importance of DAMPs in thyroid tumor progression,
472 recent evidence suggested that the multiligand receptor for advanced glycation end products (RAGE)
473 mediates the pro-migratory response of extracellular small calcium-binding protein (S100A4) in human
474 thyroid cancer cells (47).

475 TLRs-triggered signal pathways culminate in the activation of the transcription factor NF- κ B, which induces
476 the expression of pro-inflammatory cytokines, anti-apoptotic factors, and pro-angiogenic factors (35).
477 Recent evidence indicates that NF- κ B signaling, as well as the pathways that culminate in its activation, are
478 crucial players in many steps of carcinogenesis and tumor development (48). Genetic alterations leading to
479 constitutive MAPK/ERK signaling have a fundamental role in thyroid carcinogenesis (34). However,
480 additional molecular derangements are associated with tumor progression and aggressiveness. Particularly,
481 aberrant NF- κ B signaling activation is triggered by different oncogenes involved in thyroid carcinogenesis
482 associated with a papillary phenotype (49) and linked to anaplastic thyroid tumorigenesis (21). NF- κ B
483 signaling activation has been associated with tumor progression, resistance to chemotherapeutic agents-
484 induced apoptosis and invasion associated with metalloproteinases expression (21). In close relation, the
485 polymorphism rs2233406 in NFKBIA gene, an NF- κ B signaling inhibitor, was associated with a pro-
486 inflammatory tumor microenvironment resulting from the exacerbated TLR4-dependent secretion of IL-1 β
487 that could favor tumor progression (50).

488 Overall, our findings raise an intriguing question regarding the pro-oncogenic potential of TLR4 downstream
489 signaling in thyroid tumorigenesis, further considering that dysregulated NF- κ B signaling has been
490 implicated in thyroid cancer process (21). The understanding of TLR4 function in differentiated thyroid
491 tumor development and growth as well as the mechanisms involved in these processes could have great
492 clinical relevance. Further uncovering the mechanisms leading to TLR4 engagement and signaling in thyroid
493 tumors may provide novel alternatives for the establishment of future therapies for thyroid cancer, and the
494 design of new diagnostic procedures to identify a subset of potentially aggressive PTCs.

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636 **2017**;24:287-98.

637

638 FIGURES AND FIGURE LEGENDS

639 **Figure 1. TLR4 is overexpressed in differentiated thyroid carcinomas.** **A.** Representative normal and
640 malignant thyroid sections showing TLR4 protein expression levels assessed by immunohistochemistry
641 using a goat polyclonal anti-TLR4 antibody (sc-16240, Santa Cruz Biotechnology). Scale bar represents
642 20 μm . Quantification of TLR4 protein levels was expressed relative to the number of cells in the tissue
643 sections and plotted as median with interquartile range. *** $P < 0.001$ vs. normal tissue (Kruskal-Wallis test,
644 Dunn's test). **B and C.** TLR4 protein expression in match-samples of tumor adjacent normal thyroid tissue
645 and PTC, and match-samples of primary PTC and its lymph node metastasis. Scale bar represents 20 μm . *
646 $P < 0.05$ (Paired t test). **D.** Analysis of TLR4 mRNA expression as a function of the most frequent driver
647 oncogenes identified in PTCs, including point mutations of BRAF (n=234), HRAS (n=14), and NRAS (n=34)
648 genes, as well as fusions involving NTRK (n=10) and RET (n=32) tyrosine kinases, and BRAF (n=12) kinase. **E.**
649 Correlative analysis showing TLR4 mRNA expression and ERK activation score in PTCs harboring oncogenes
650 classically involved in MAPK/ERK signaling activation (n=336, Spearman's correlation test). ERK score was
651 calculated based on a 52-gene signature to assess the MAPK/ERK signaling pathway activation (33). **F.**
652 Correlative analysis showing TLR4 mRNA expression and thyroid differentiation score in PTCs harboring the
653 oncogene BRAF^{V600E} (n=234, Spearman's correlation test). Thyroid differentiation score was calculated
654 based on a 16-gene signature related to thyroid metabolism and function (33). **G.** Correlative analysis
655 showing TLR4 mRNA expression and MACIS score in PTCs harboring the oncogene BRAF^{V600E} (n=212,
656 Spearman's correlation test). **H.** Disease-free survival analysis of patients whose thyroid tumors harbored
657 the oncogene BRAF^{V600E} (wild-type TERT status) and expressed low (n=97) or high (n=88) TLR4 mRNA levels,
658 based on its expression below and above the median within the group. $P = 0.0071$ (Mantel-Cox test).

659 **Figure 2. Conditional oncogene expression induced TLR4 expression in PTC models.** **A.** RT/qPCR assessing
660 TLR4 mRNA expression levels in response to doxycycline (Dox)-induced BRAF^{V600E}, HRas^{G12V}, or RET/PTC3
661 expression at 48 h in PCCl3 cells. * $P < 0.05$ and ** $P < 0.01$ vs. vehicle-treated cells (one-way ANOVA,
662 Newman-Keuls test). **B and C.** Immunoblot assessing TLR4 protein expression levels in response to
663 conditional BRAF^{V600E} expression for 48 h in PC/BRAF cells (**B**) or transient transfection of BRAF^{V600E} into
664 non-tumoral Nthy-ori 3-1 thyroid cells (**C**). Fold change indicates relative TLR4 abundance. * $P < 0.05$ vs.
665 vehicle-treated cells or empty vector-transfected cells (unpaired Student's t test). **D.** Immunofluorescence
666 under non-permeabilized conditions analyzing TLR4 protein expression at the plasma membrane in
667 response to BRAF^{V600E} expression for 48 h in PC/BRAF cells. Scale bar represents 10 μm . *** $P < 0.001$ vs.
668 vehicle-treated cells (unpaired Student's t test). **E and F.** Analysis of TLR4 mRNA and protein expression in
669 the thyroid tissue of transgenic mice conditionally expressing BRAF^{V600E} in the thyroid follicular cell and
670 littermate control mice. Scale bar represents 20 μm . Quantification of TLR4 protein expression was
671 expressed relative to the numbers of cells in the tissue sections. * $P < 0.05$ and ** $P < 0.001$ (Mann Whitney t
672 test).

673 **Figure 3. TLR4 signaling in PTC cell models.** **A.** NF- κ B reporter activity in doxycycline (Dox)-treated
674 PC/BRAF^{V600E} cells stimulated with the exogenous TLR4-agonist LPS. The NF- κ B inhibitor BAY 11-7082 is
675 used as specificity control. *** $P < 0.001$ vs. vehicle-treated cells, # $P < 0.05$ vs. Dox-treated cells in the
676 absence of LPS, † $P < 0.01$ and ‡ $P < 0.001$ vs. Dox-treated cells (one-way ANOVA, Newman-Keuls test). **B and**
677 **C.** RT/qPCR assessing the mRNA expression of the NF- κ B targets IL-6 and iNOS in response to TLR4
678 activation for 12 h. ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle-treated cells, #### $P < 0.001$ vs. LPS-treated cells
679 (one-way ANOVA, Newman-Keuls test). **D.** NF- κ B reporter activity in empty or non-signaling TLR4 or MyD88

680 vectors-transfected BCPAP cells stimulated with LPS for 24 hs. Empty vector-transfected cells treated with
681 BAY 11-7082 serves as specificity control. ** $P < 0.01$ vs. vehicle-treated cells, ^{###} $P < 0.01$ and ^{####} $P < 0.001$ vs.
682 LPS-treated empty vector-transfected cells (one-way ANOVA, Newman-Keuls test). **E and F.** RT/qPCR
683 assessing the mRNA expression of IL-6 and iNOS in BCPAP cells stimulated with LPS for 12 hs. *** $P < 0.001$
684 vs. vehicle-treated cells, ^{####} $P < 0.001$ vs. LPS-treated cells (one-way ANOVA, Newman-Keuls test).

685 **Figure 4. MAPK/ERK signaling regulates TLR4 gene expression in response to BRAF^{V600E}.** **A and B.** TLR4
686 protein and mRNA expression in doxycycline-treated PC/BRAF^{V600E} cells in the presence of the chemical
687 inhibitors PLX4032 or U0126. Fold change indicates relative TLR4 abundance. * $P < 0.05$ and ** $P < 0.01$ vs.
688 vehicle-treated cells, [#] $P < 0.05$ vs. same condition in absence of inhibitor (one-way ANOVA, Newman-Keuls
689 test). **C and D.** TLR4 protein and mRNA expression in BCPAP cells treated in the presence of the chemical
690 inhibitors PLX4032 or U0126. Fold change indicates relative TLR4 abundance. * $P < 0.05$ and ** $P < 0.01$ vs.
691 vehicle-treated cells (one-way ANOVA, Newman-Keuls test).

692 **Figure 5. Ets^d binding site is crucial for TLR4 gene transcription in response to constitutive BRAF^{V600E}**
693 **signaling.** **A.** Transcriptional activity of TLR4 promoter deletion constructs in response to BRAF^{V600E}
694 expression in PC/BRAF^{V600E} cells. * $P < 0.05$ and *** $P < 0.001$ vs. vehicle-treated cells (unpaired Student's t
695 test). **B.** Transcriptional activity of the Ets^d binding-site mutated TLR4 promoter construct -336/+223 in
696 PC/BRAF^{V600E} cells. *** $P < 0.001$ vs. vehicle-treated cells, ^{####} $P < 0.001$ vs. -336/+223-transfected cells (one-
697 way ANOVA, Newman-Keuls test). **C.** Transcriptional activity of the trimeric Ets^d reporter construct 3x Ets^d-
698 Luc in PC/BRAF^{V600E} cells in response to doxycycline (Dox) treatment. * $P < 0.05$ (unpaired Student's t test). **D.**
699 Transcriptional activity of the TLR4 promoter construct -336/+223 and Ets^d binding-site mutated TLR4
700 promoter construct -336/+223 in BCPAP cells. ** $P < 0.01$ vs. -336/+223-transfected cells (unpaired Student's
701 t test).

702 **Figure 6. ETS1 mediates BRAF^{V600E}-induced TLR4 gene expression.** **A.** Correlation analysis of TLR4 mRNA
703 expression as a function of ETS1 mRNA expression in the subset of PTC harboring the oncogene BRAF^{V600E}
704 (n=234, Spearman's correlation test). **B.** Representative unmatched sections of normal thyroid tissue and
705 PTC showing ETS1 protein expression levels assessed by immunohistochemistry using a rabbit polyclonal
706 anti-ETS1 antibody (sc-350, Santa Cruz Biotechnology). Scale bar represents 20 μ m. Quantification of ETS1
707 protein levels was expressed relative to the number of cells in the tissue sections and plotted as median
708 with interquartile range. *** $P < 0.001$ vs. normal tissue (Mann Whitney test). **C.** Correlation analysis of TLR4
709 and ETS1 protein expression assessed by immunohistochemistry in equivalent PTC tissues (n=24,
710 Spearman's correlation test). **D.** Immunoblot analysis assessing nuclear ETS1 protein levels in response to
711 BRAF^{V600E} expression in PC/BRAF^{V600E} cells. PARP-1 and α -Tubulin were used as nuclear and cytoplasmic
712 loading markers, respectively. Fold change indicates relative ETS1 abundance. * $P < 0.05$ (unpaired Student's
713 t test). **E.** Immunofluorescence analysis under permeabilized conditions showing ETS1 protein expression in
714 response to doxycycline (Dox) treatment in PC/BRAF^{V600E} cells. Scale bar represents 10 μ m. * $P < 0.05$
715 (unpaired Student's t test). **F and G.** MAPK/ERK-dependent ETS1 protein expression in response to
716 BRAF^{V600E} oncogenic activity in PC/BRAF^{V600E} and BCPAP cells. Fold change indicates relative ETS1
717 abundance. * $P < 0.05$ vs. vehicle-treated cells (one-way ANOVA, Newman-Keuls test). **H.** Immunoblot
718 analysis showing ETS1 protein expression in scramble (SCR) or ETS1 siRNA transfected PC/BRAF^{V600E} cells.
719 Fold change indicates relative ETS1 abundance. * $P < 0.05$ (unpaired Student's t test). **I.** TLR4 mRNA
720 expression in doxycycline (Dox)-treated scramble (SCR) or ETS1 siRNA-transfected PC/BRAF^{V600E} cells. **

721 $P < 0.01$ vs. vehicle-treated cells, $^{###} P < 0.01$ vs siSCR-transfected cells (one-way ANOVA, Newman-Keuls test).
722 **J.** Transcriptional activity of the TLR4 promoter construct -336/+223 in doxycycline (Dox)-treated scramble
723 (SCR) or ETS1 siRNA-transfected PC/BRAF^{V600E} cells. * $P < 0.05$ (one-way ANOVA, Newman-Keuls test). **K.**
724 Immunoblot analysis showing TLR4 protein expression in empty or ETS1 expressing vector-transfected
725 normal PCCI3 cells. Fold change indicates relative TLR4 abundance. * $P < 0.05$ (unpaired Student's t test). **L.**
726 Transcriptional activity of the TLR4 promoter construct -336/+223 in empty or ETS1 expressing vector-
727 transfected normal PCCI3 cells. ** $P < 0.01$ (unpaired Student's t test). **M.** ChIP analysis assessing ETS1
728 binding to TLR4 promoter region -365 to -24 (relative to the transcription start site) in response to BRAF^{V600E}
729 induction in PC/BRAF^{V600E} cells. ** $P < 0.01$ vs. vehicle-treated cells (unpaired Student's t test).

FIGURE 1

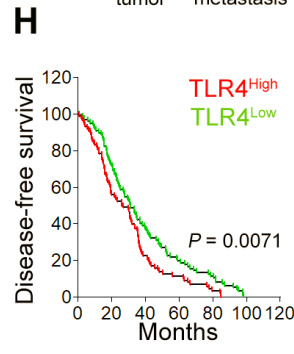
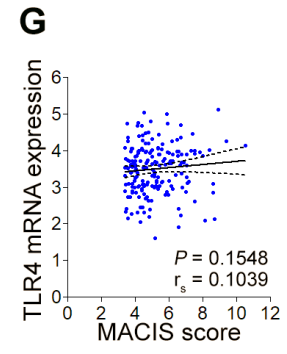
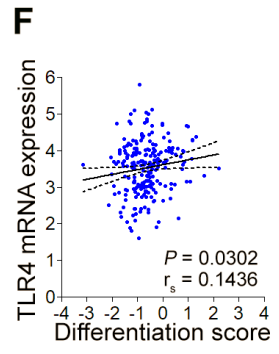
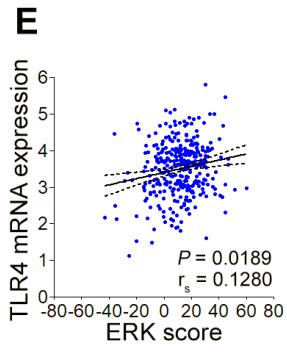
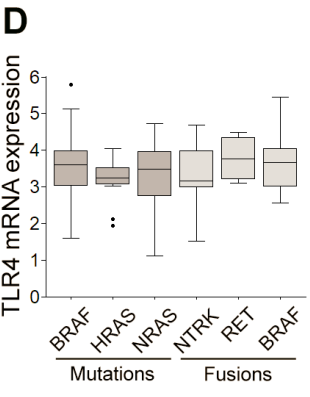
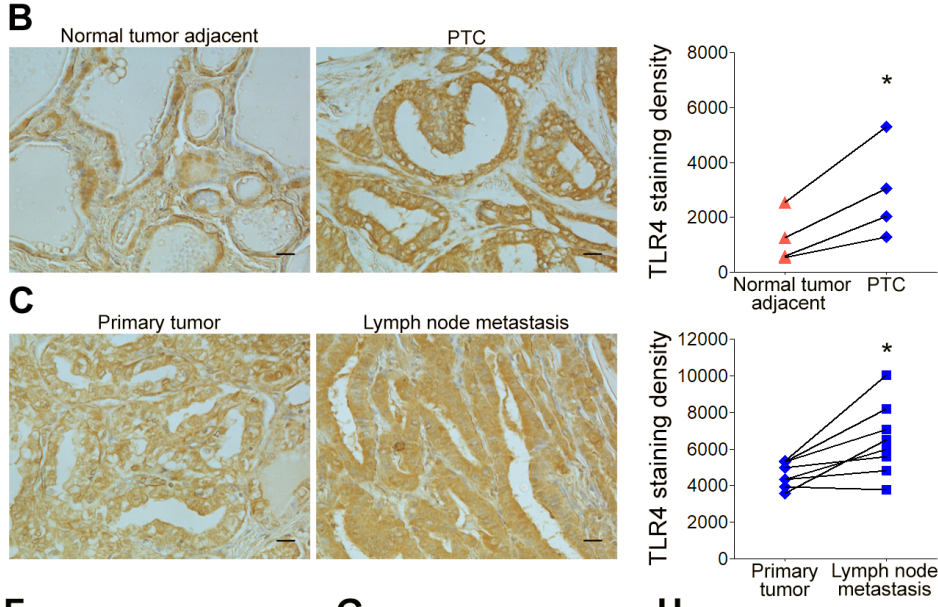
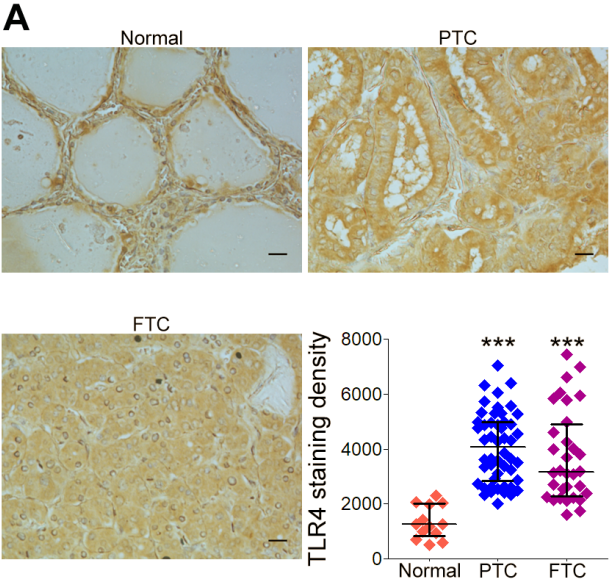


FIGURE 2

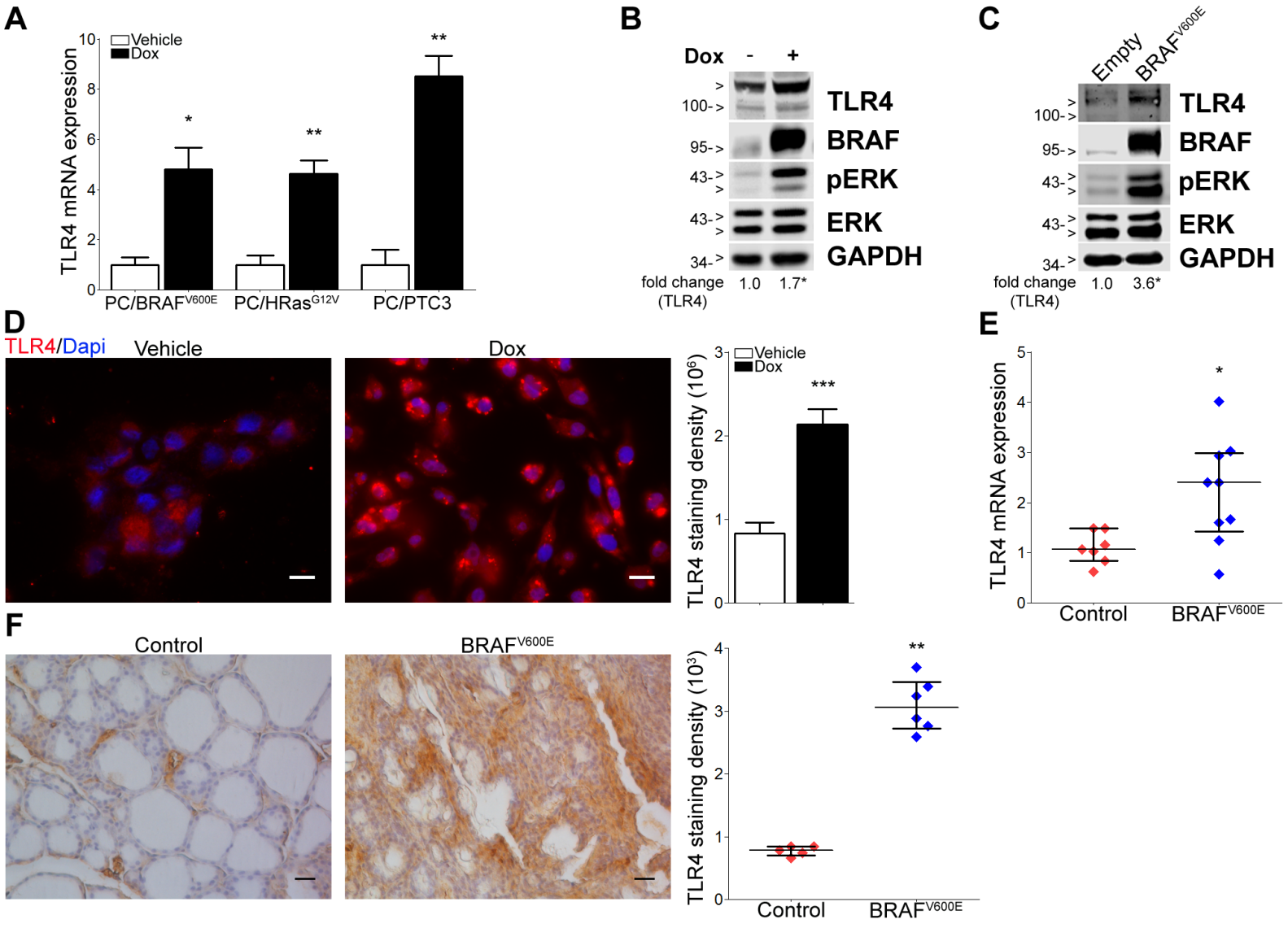


FIGURE 3

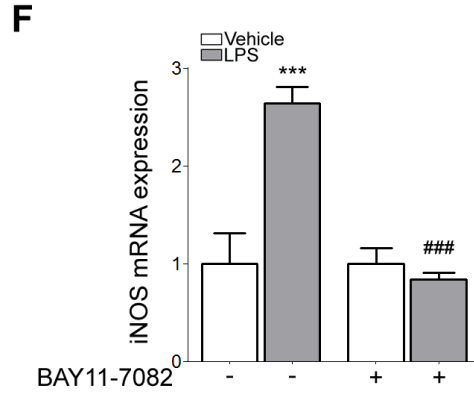
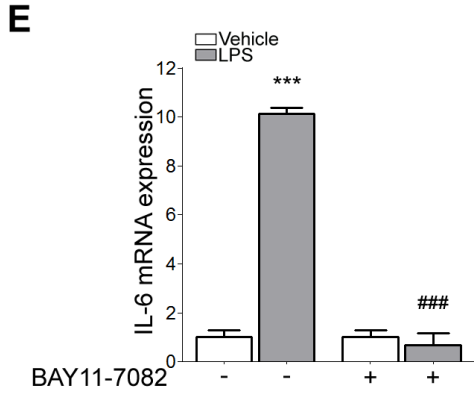
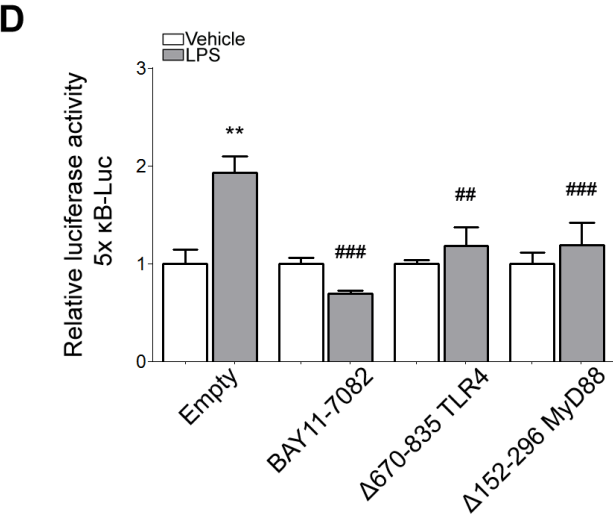
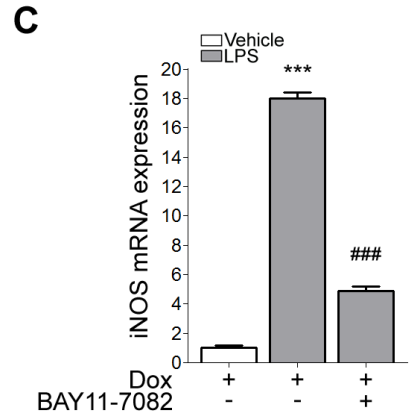
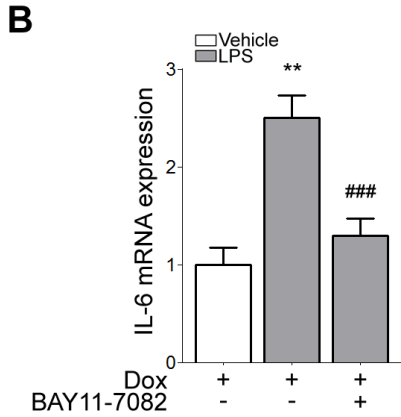
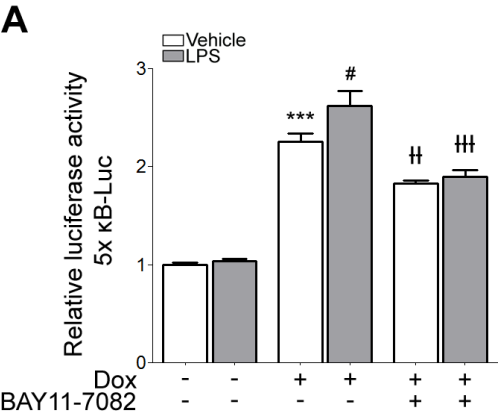


FIGURE 4

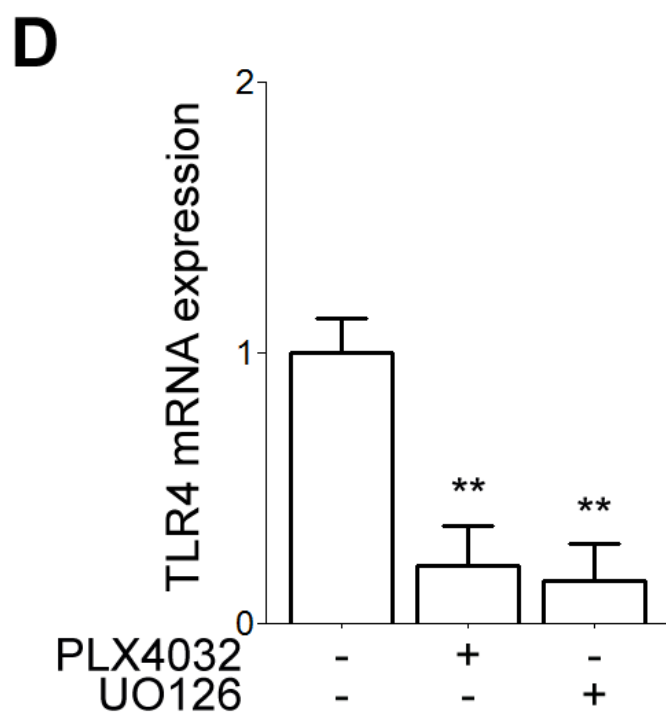
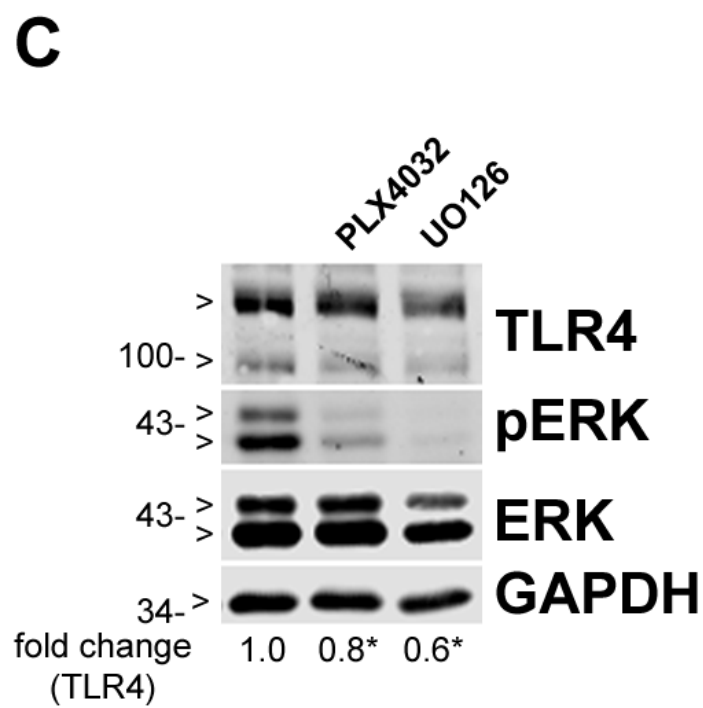
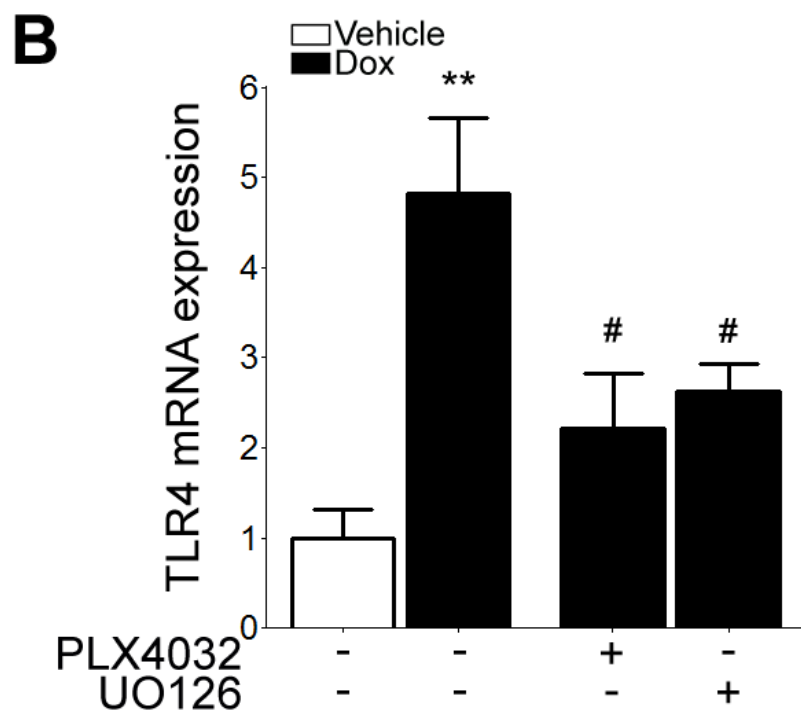
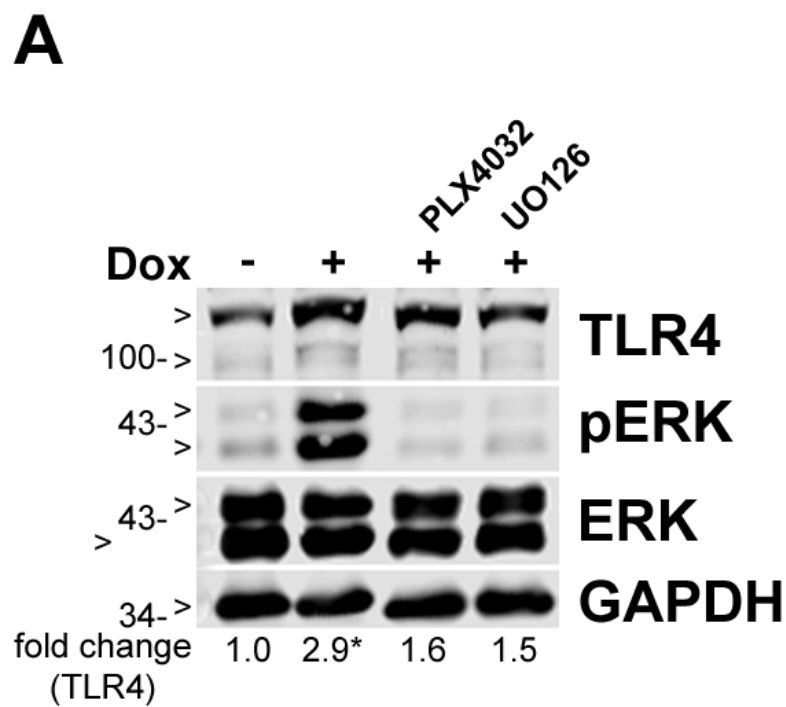


FIGURE 5

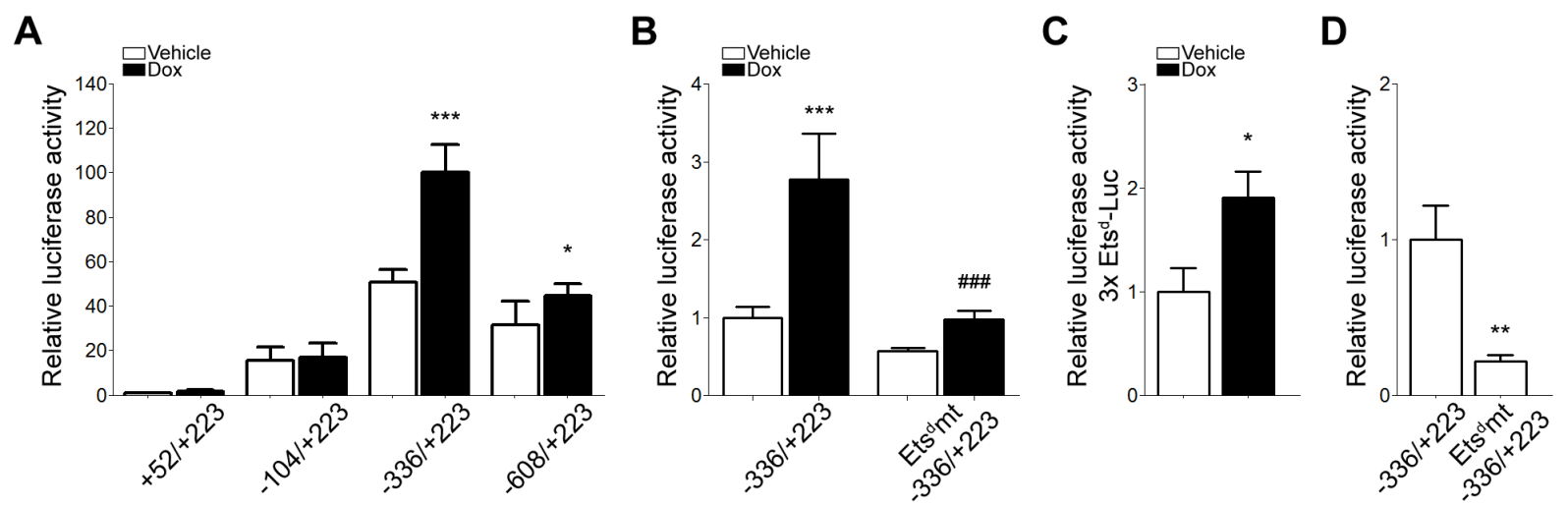
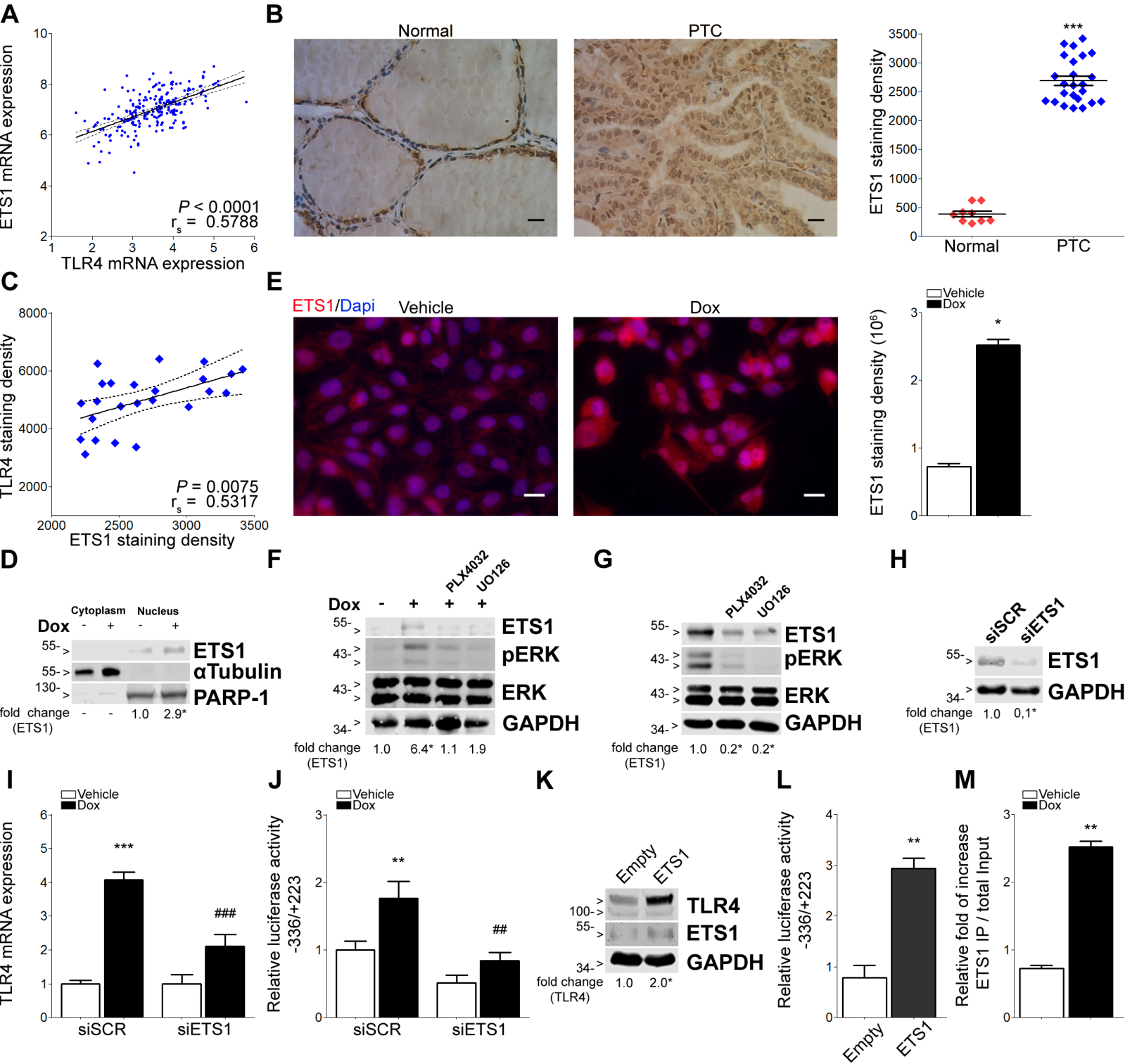


FIGURE 6



Molecular Cancer Research

Functional Toll-like Receptor 4 Overexpression in Papillary Thyroid Cancer by MAPK/ERK-induced ETS1 Transcriptional Activity

Victoria Peyret, Magalí Nazar, Mariano Martin, et al.

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