

# TRAP1 regulates cell cycle and apoptosis in thyroid carcinoma cells

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## Abstract

Tumor necrosis factor receptor-associated protein 1 (TRAP1) is a heat shock protein 90 (HSP90) molecular chaperone upregulated in several human malignancies and involved in protection from apoptosis and drug resistance, cell cycle progression, cell metabolism and quality control of specific client proteins. TRAP1 role in thyroid carcinoma (TC), still unaddressed at present, was investigated by analyzing its expression in a cohort of 86 human TCs and evaluating its involvement in cancer cell survival and proliferation *in vitro*. Indeed, TRAP1 levels progressively increased from normal peritumoral thyroid gland, to papillary TCs (PTCs), follicular variants of PTCs (FV-PTCs) and poorly differentiated TCs (PDTCs). By contrast, anaplastic thyroid tumors exhibited a dual pattern, the majority being characterized by high TRAP1 levels, while a small subgroup completely negative. Consistently with a potential involvement of TRAP1 in thyroid carcinogenesis, TRAP1 silencing resulted in increased sensitivity to paclitaxel-induced apoptosis, inhibition of cell cycle progression and attenuation of ERK signaling. Noteworthy, the inhibition of TRAP1 ATPase activity by pharmacological agents resulted in attenuation of cell proliferation, inhibition of ERK signaling and reversion of drug resistance. These data suggest that TRAP1 inhibition may be regarded as potential strategy to target specific features of human TCs, i.e., cell proliferation and resistance to apoptosis.

## Key Words

- ▶ TRAP1
- ▶ thyroid carcinoma
- ▶ apoptosis
- ▶ cell cycle
- ▶ HSP90

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## Introduction

The vast majority of thyroid carcinomas (TCs) are well-differentiated tumors, usually curable by the combination of surgery, radioiodine ablation and

long-term thyroid-stimulating hormone (TSH) suppressive therapy (Mazzaferrri & Massoll 2002). A small subset of thyroid tumors are *de novo* poorly

differentiated carcinomas or arises from the (de) differentiation of initially well-differentiated carcinomas and are characterized by a more aggressive behavior, earlier metastatic spread, and loss of TSH signaling, cell differentiation and iodine retention capacity (Regalbuto et al. 2012).

The efficacy of radioiodine therapy relies on the capacity of TC cells to uptake and retain intracellular radioiodine and this is exclusively dependent on the expression of the sodium–iodide symporter (NIS) (Kogai & Brent 2012). NIS is a basolateral membrane protein primarily regulated at transcriptional level by TSH, through the TSH receptor (TSHR) signaling, and by TSH-independent mechanisms (Kogai & Brent 2012, Mu et al. 2012). In such a context, radioiodine-refractory thyroid tumors are poorly differentiated carcinomas, characterized by poor or absent NIS expression, unresponsiveness to radioiodine therapy and other cytotoxics, increased metastatic spread and, thus, worst prognosis and limited therapeutic options (Regalbuto et al. 2012). Indeed, the activation of RAF/ERK signaling pathway is a major mechanism responsible for progression toward radioiodine-refractory and apoptosis-resistant phenotypes (Nikiforov et al. 2011). Therefore, several attempts have been made to study the molecular mechanisms responsible for the more aggressive phenotype of radioiodine-refractory thyroid tumors with the aim to find molecular targets for novel, specific and more effective therapies (Haugen 2004, Alonso-Gordoa et al. 2015).

Tumor necrosis factor receptor-associated protein 1 (TRAP1) is a member of the heat shock protein 90 (HSP90) family of molecular chaperones, involved in prosurvival signaling of cancer cells, protection of mitochondria against oxidative stress, drug resistance, cell cycle regulation, metabolic reprogramming and regulation of protein homeostasis through the translational quality control of specific client proteins (Amoroso et al. 2014). Noteworthy, recent evidence suggests that TRAP1 modulates ERK signaling, likely through the regulation of BRAF synthesis/ubiquitination (Condelli et al. 2014). TRAP1 is upregulated in various tumor types, including colorectal, breast, lung, and prostate cancers (Costantino et al. 2009, Leav et al. 2010, Maddalena et al. 2013, Agorreta et al. 2014) and in some of these malignancies, such as prostate or colorectal carcinomas, TRAP1 levels correlate with poor clinical outcome, higher grading and/or advanced stages (Leav et al. 2010, Han et al. 2014), thus supporting a role of TRAP1 in cancer promotion and progression (Amoroso et al. 2014, Rasola et al. 2014). Conversely, other studies questioned the oncogenic role

of TRAP1, suggesting that TRAP1 levels correlate inversely with malignant progression and tumor grading in specific human malignancies, i.e., ovary, cervical, bladder and clear cell renal cancers (Aust et al. 2012, Yoshida et al. 2013, Rasola et al. 2014, Matassa et al. 2016). Herein, the expression of TRAP1 was evaluated for the first time in a series of human thyroid tumors ranging from well-differentiated to poorly differentiated and anaplastic carcinomas. In addition, the effect of TRAP1 knockdown on cell cycle control and protection from apoptosis *in vitro* was evaluated in this tumor type.

## Patients and methods

### Study population

Eighty-six patients who underwent total thyroidectomy at the Universities of Foggia or Siena (Italy) were selected for this study. Thirty-eight of them were affected by papillary TC (PTC), 20 by follicular variant of PTC (FV-PTC), 12 by poorly differentiated TC (PDTC) and 16 by anaplastic TC (ATC). All of them received surgical treatment with curative intent between 2002 and 2014. Patient's characteristics, including clinical data referring to sex, age, stage, degree of differentiation, and uni/multifocality are reported in Table 1, as mean  $\pm$  s.d. for continuous variables and numbers and percentages for categorical ones. All patients gave their written informed consent to use biological specimens for investigational procedures after full explanation of the purpose and nature of the study. Demographical and clinical data were extracted from clinical records. The histopathological diagnosis was carefully reviewed at the Department of Clinical and Experimental Medicine, Section of Anatomic Pathology of the University of Foggia. Tumor extent was revised and classified according to the classification system of the American Joint Committee on Cancer (AJCC) (Wada et al. 2007).

### Immunohistochemistry

4- $\mu$ m serial sections from formalin-fixed paraffin-embedded blocks were cut and mounted on poly-L-lysine-coated glass slides. Immunohistochemical analysis was performed using a Benchmark autostainer (Ventana Medical Systems, Tucson, AZ, USA) and/or manual standard linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP), according to the best protocol for the antibody previously tested in our laboratory (Pannone et al. 2014). Sections were

**Table 1** Demographic and clinicopathological characteristics of patients.

	PTC	FV-PTC	PDTC	ATC	P value
Cases (%)	38 (44.2)	20 (23.2)	12 (14.0)	16 (18.6)	
Sex					
Male	14	8	3	6	0.632
Female	24	12	9	10	
Age (mean ± s.d.)	50.7 ± 14.5	48.7 ± 16.2	61.7 ± 18.2	75.8 ± 11.8	<0.0001
TNM stage (%)					<0.0001
I	24 (63.1)	12 (60.0)	4 (33.3)	0	
II	2 (5.2)	0	0	0	
III	8 (21.0)	5 (25.0)	2 (16.0)	0	
IV	4 (10.5)	3 (15.0)	6 (50.0)	16 (100)	
Tumor size – cm (mean ± s.d.)	1.34 ± 1.1	1.72 ± 1.2	4.9 ± 2.7	6.7 ± 2.8	<0.0001
Tumors with invasive growth (%)	24 (63.1)	11 (55.0)	4 (33.3)	4 (25.0)	0.772
Multifocal tumors (%)	7 (18.4)	6 (30.0)	2 (16.0)	3 (18.7)	0.042
Tumors with lymph node metastases (%)	9 (23.6)	8 (40.0)	3 (25.0)	4 (25.0)	0.764
Tumors with distant metastases (%)	2 (5.2)	1 (5.0)	0	0	0.305
Tumors with positive resection margins (%)	10 (26.3)	7 (35.0)	3 (25.0)	3 (18.7)	0.078
Relapses (%)	6 (15.7)	4 (20.0)	0	2 (12.5)	0.586

FV-PTC, follicular variant of papillary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma.

incubated with mouse monoclonal anti-TRAP1 antibody (1:750 dilution) (sc-73604; Santa Cruz Biotechnology). Negative controls were performed omitting primary antibody. Sections were counterstained with Gill's type 2 hematoxylin, dehydrated with ethanol and permanently coverslipped. Results of the immunohistochemical staining were evaluated separately by two observers (P B and G P), particularly trained for thyroid pathology and immunohistochemistry and completely blind to the histological diagnosis. The inter-rater reliability between the two investigators examining the immunostained sections was assessed by the Cohen's *K* test, yielding *K* values >0.70 in all instances. Normal distribution of the data was analyzed by the Kolmogorov–Smirnov test. Immune-stained cells were counted in at least 10 High Power Fields (HPF) analyzed with an optical microscope (Olympus BX53; Olympus Italia, Milan, Italy) at 40× magnification. TRAP1-positive staining was defined as cytoplasmic staining in normal peritumoral noninfiltrated and tumor tissue. Peritumoral noninfiltrated thyroid gland was classified in microfollicular and macrofollicular areas based on the prevalence of, respectively, small follicles with sparse colloid or large follicles with dense colloid (Motta 1984). The number of TRAP1-expressing tumor cells was estimated as a mean percentage of total number of cells per section and grouped according to the percentage of positive cells: 0 (no staining), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The intensity of TRAP1 staining was graded as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). A combined numeric IHC score was calculated as the product of staining intensity

and percentage of stained cells and reported as mean and s.d. (Smith *et al.* 2014).

#### Cell cultures, constructs, siRNAs and chemicals

Follicular ML1 and FTC133, papillary BCPAP, and anaplastic BHT101 and CAL62 TC cells below passage 20 were used for this study. BCPAP and CAL62 cells were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1.5 mM glutamine, and 100 U/mL penicillin and streptomycin, ML1 cells in the same medium supplemented with 1 mM sodium pyruvate, BHT101 cells in the same medium supplemented with 20% (vol/vol) FBS. FTC133 cells were cultured in DMEM:Ham's F12 (1:1) supplemented with 10% (vol/vol) FBS, 1.5 mM glutamine, and 100 U/mL penicillin and streptomycin. All media were supplemented with TSH (10 U/mL). All cell lines cells were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany) with the exception of FTC133 cells that were purchased from Sigma-Aldrich. Cell lines were routinely monitored in our laboratory by microscopic morphology, cell line authentication was verified by evaluating BRAF and KRAS mutational status by pyrosequencing. Full-length TRAP1 construct was obtained as described previously (Landriscina *et al.* 2010). Transient transfection of DNA plasmid was performed with PolyFect Transfection Reagent (Qiagen), according to the manufacturer's protocol. siRNAs of TRAP1 and TBP7 were purchased from Qiagen (Cat. No. SI00115150 for TRAP1,

Cat. No. SI00301469 for TBP7). For control experiments, cells were transfected with a similar amount of control siRNA (Qiagen, Cat. No. SI03650318). For knockdown experiments, siRNAs were diluted to a final concentration of 40 nM and transiently transfected by the HiPerFect Transfection Reagent (Qiagen), according to manufacturer's protocol. Unless otherwise specified, reagents were purchased from Sigma-Aldrich, with HSP990 kindly provided by Novartis Oncology (Origgio, VA, Italy) and gamitinib by Dr Altieri (The Wistar Institute, Philadelphia, PA, USA).

### Immunoblot analysis

Total cell lysates were obtained by homogenization of cell pellets and tissue samples in cold lysis buffer (20 mmol/L Tris (pH 7.5) containing 300 mmol/L sucrose, 60 mmol/L KCl, 15 mmol/L NaCl, 5% (vol/vol) glycerol, 2 mmol/L EDTA, 1% (vol/vol) Triton X100, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 0.2% (wt/vol) deoxycholate) for 1 min at 4°C and further sonication for an additional 30 s at 4°C. Protein concentration was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories), according to the manufacturer's procedures. Samples were resolved on SDS-PAGE, transferred on nitrocellulose membrane (Bio-Rad Laboratories), and immunoblotted with the following mouse monoclonal antibodies from Santa Cruz Biotechnology: anti-TRAP1 (sc-73604), anti-BRAF (sc-5284), anti-TBP7 (sc-166003) and antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-69778). The following antibodies were also used: mouse monoclonal antiphospho 44/42 MAPK (pERK1/2, #9106), rabbit monoclonal anti-eIF4E (#C46H6) from Cell Signaling Technology, rabbit polyclonal anti-MAPK ERK1/2 from Calbiochem (#442704). Rabbit polyclonal anti-Sorcin antibody was kindly provided by Prof E Chiancone (University of Rome 'La Sapienza'). Specific bands were revealed using the Clarity Western ECL Substrate (Bio-Rad Laboratories).

### Cell cycle analysis

Cells were incubated in a culture medium supplemented with 20 mmol/L 5-bromo-20-deoxyuridine (BrdUrd) for 20 min and harvested. Subsequent to incubation in a solution containing 3N HCl for 30 min at room temperature to obtain DNA denaturation, cell pellets were further incubated in the presence of anti-BrdUrd FITC-conjugated antibody (Becton Dickinson, Milan,

Italy) for 1 h at room temperature in the darkness. After washing with PBS, cells were further incubated with 6 mg/mL propidium iodide (PI) for 20 min and then evaluated using FACSCalibur (Becton Dickinson).

### Apoptosis assay

Apoptosis was evaluated by cytofluorimetric analysis of Annexin-V and 7-amino-actinomycin-D (7-AAD)-positive cells using the FITC-Annexin-V/7-AAD kit (Beckman Coulter, Milan, Italy). Stained cells were analyzed using the FACSCalibur (Becton Dickinson). Positive staining for Annexin-V as well as double staining for Annexin-V and 7-AAD was interpreted as signs of early and late phases of apoptosis, respectively (Maddalena et al. 2011).

### Statistical analysis

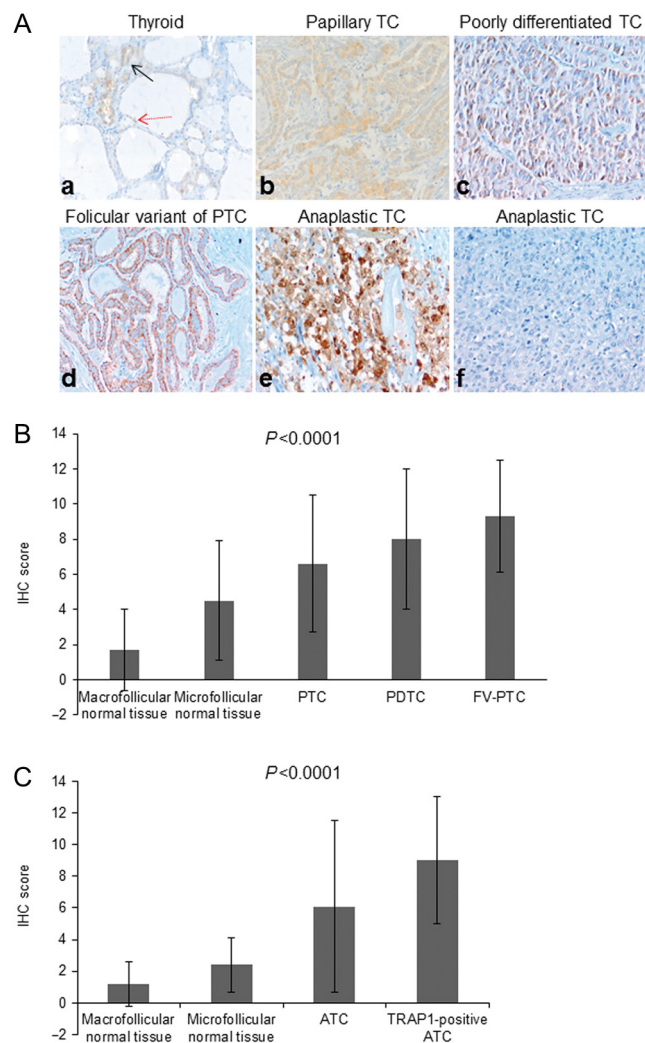
The paired Student's *t*-test was used to establish the statistical significance in apoptosis and cell cycle distribution between silenced and control cells or drug- and vehicle-treated cells. Statistically significant values ( $P < 0.05$ ) are reported in the Figure legends. All experiments were independently performed at least three times.

Mean differences in IHC score and other variables were compared by unpaired Student's *t*-test or one-way ANOVA F-tests, as appropriate. Differences between categorical variables were tested by Pearson's  $\chi^2$ . The statistical packaged SPSS version 13.0 (SPSS) was used. A *P* value  $< 0.05$  was considered to be significant.

## Results

### TRAP1 expression is progressively upregulated from normal thyroid to PDTC and FV-PTCs

TRAP1 expression was evaluated in a series of 86 thyroid tumors ranging from well-differentiated to poorly differentiated and anaplastic TCs, subdivided in 38 PTCs, 20 FV-PTCs, 12 PDTCs, and 16 ATCs. TRAP1 expression was visualized as cytoplasmic staining in normal peritumoral noninfiltrated thyroid gland and tumor cells and expressed as IHC score. Representative IHC images are reported in Fig. 1A, whereas the results of immunohistochemical staining according to histological subtypes are shown in Fig. 1B and C. TRAP1 expression was also confirmed by immunoblot analysis in selected tumors (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). TRAP1 was detectable, although at low level,



**Figure 1**

TRAP1 expression is progressively upregulated from normal thyroid gland to PTCs, PDTCs and FV-PTCs. (A) TRAP1 IHC staining in representative cases of noninfiltrated macrofollicular (red arrow) and microfollicular (black arrow) areas of thyroid gland (a), papillary thyroid carcinoma (b), poorly differentiated thyroid carcinoma (c), follicular variant of papillary thyroid carcinoma (d) and anaplastic thyroid carcinoma (e and f); automated LSAB-HRP technique, counterstaining with Gill's type 2 hematoxylin, original magnifications 100 $\times$ . (B and C) TRAP1 IHC scores in microfollicular and macrofollicular noninfiltrated normal thyroid tissue, PTCs, PDTCs and FV-PTCs (B) and in ATCs compared with macrofollicular and microfollicular normal thyroid gland (C).

in noninfiltrated peritumoral normal thyroid gland, being its expression higher in microfollicular structures compared with macrofollicular areas ( $P < 0.0001$ ; Fig. 1A, panel a and Fig. 1B). TRAP1 was also detectable in all PTCs, PDTCs, and FV-PTCs, with a statistically significant progressive increase in TRAP1 IHC score from normal thyroid to PTCs, PDTCs, and FV-PTCs ( $P < 0.0001$ ; Fig. 1A, panels b-d and Fig. 1B). This difference between PTCs, PDTCs, and FV-PTCs is statistically significant also

after excluding normal thyroid gland from the analysis ( $P = 0.04$ ; Supplementary Fig. 1B). The IHC tumor tissue score of TRAP1 was also significantly higher in PTCs, FV-PTCs, and PDTCs, each compared independently with respective macrofollicular and microfollicular peritumoral thyroid tissues ( $P < 0.0001$ ; Supplementary Fig. 1C, D and E), whereas only FV-PTCs showed significantly higher TRAP1 levels compared with PTCs ( $P = 0.012$ ). Among 16 ATCs, TRAP1 was highly expressed in 11 out of 16 cases, 5 ATCs being completely negative upon TRAP1 immunostaining (Fig. 1A, compare panels e and f). For this peculiar and unexpected pattern, ATCs were analyzed independently of other subgroups: indeed, TRAP1 IHC tissue score was significantly higher in the whole cohort of ATCs and in the 11 TRAP1-positive ATCs compared with respective macrofollicular and microfollicular peritumoral areas ( $P < 0.0001$ ; Fig. 1C). Remarkably, 5 out of 16 ATCs showed lack of TRAP1 expression, below normal thyroid gland levels (Fig. 1, panel f). These data suggest that TRAP1 expression is increased in TCs compared with normal thyroid and that there is trend for a progressive increase of TRAP1 levels from PTCs to PDTCs and FV-PTCs.

#### TRAP1 regulates survival in follicular and anaplastic TC cells

FV-PTCs are characterized by a more aggressive behavior compared with classical PTCs (Vivero et al. 2013, Yu et al. 2013), as well as PDTCs are aggressive tumors with poor prognosis and lack of response to radioiodine (Patel & Shaha 2014). Consistently, a progressive and statistically significant increase of tumor size and tumor stage was observed from PTCs to FV-PTCs, PDTCs and ATCs ( $P < 0.0001$  for both variables; Table 1) in our series of 86 TCs. Thus, to evaluate the role of TRAP1 pathway in TC progression, the correlation between TRAP1 expression and specific features of more aggressive/less differentiated TC cells was analyzed in a panel of TC cell lines: i.e., follicular ML1 and FTC133, papillary BCPAP, and anaplastic BHT101 and CAL62 carcinoma cells. Indeed, BHT101 and BCPAP cells are characterized by the BRAFV600E mutation, CAL62 cells by the KRAS G12R mutation (Piscazzi et al. 2012), whereas ML1 and FTC133 cells by a wild-type genotype for both genes. Based on the well-established role of TRAP1 in inducing cytoprotective responses by inhibiting mitochondrial apoptotic pathway (Kang et al. 2007, Montesano Gesualdi et al. 2007), TRAP1 was silenced in our panel of TC cell lines to assess protection from paclitaxel-induced apoptosis (Fig. 2A).

A significant increase of apoptotic cell death was observed in TRAP1-silenced cell lines upon exposure to paclitaxel (Fig. 2B). In concomitant experiments, apoptotic cell death was assessed upon exposure of TC cell lines to gamitrinib, a dual HSP90/TRAP1 inhibitor engineered to accumulate into mitochondria and counteract TRAP1-dependent inhibition of mitochondrial transition pore (Kang et al. 2009). Indeed, gamitrinib induced apoptosis in a dose-dependent manner (Fig. 2C) and, consistently, the pretreatment with subcytotoxic doses of gamitrinib significantly enhanced paclitaxel-induced apoptosis in TC cell lines (Fig. 2D). Different from what was observed in colon carcinoma cells (Condelli et al. 2015), gamitrinib cytotoxicity was independent of BRAF mutational status in TC cells. These data suggest that TRAP1 is responsible for enhancing the apoptotic threshold of TC cells, thus, inducing resistance to chemotherapeutics.

### TRAP1 regulates cell cycle progression and ERK signaling in TC cell lines

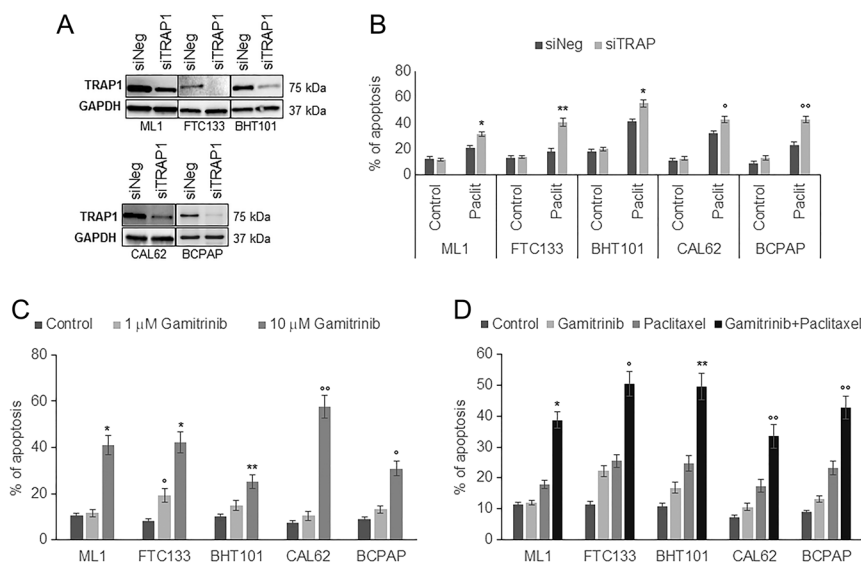
Previous evidences suggest an involvement of TRAP1 in cell cycle regulation in colon and breast carcinoma cells (Condelli et al. 2014). Therefore, the role of TRAP1 in favoring cell cycle progression was assessed in TC cell lines. Indeed, TRAP1 silencing resulted in a modest, but significant attenuation of cell proliferation (Fig. 3A) and this correlated with a parallel significant inhibition of S-phase, with accumulation of cells in G0-G1 and/or G2-M phase, depending on the specific TC cell line (Fig. 3B).

Previous evidences by our group suggest that TRAP1 is responsible for BRAF quality control and regulation of downstream ERK signaling (Condelli et al. 2014), a key

event in TC progression (Nikiforov et al. 2011). Therefore, BRAF expression levels and ERK phosphorylation were evaluated upon TRAP1 silencing in TC cells. Indeed, TRAP1 interference induced a strong downregulation of ERK signaling in all TC cell lines and this was paralleled by the expected downregulation of BRAF protein expression in ML1, FTC133, BHT101 and BCPAP cells (Fig. 4A). Surprisingly, ERK phosphorylation was significantly downregulated, independently from BRAF expression, in KRAS-mutated CAL62 TC cells (Fig. 4A). These data suggest that TRAP1 control of cell cycle progression correlates with regulation of ERK signaling.

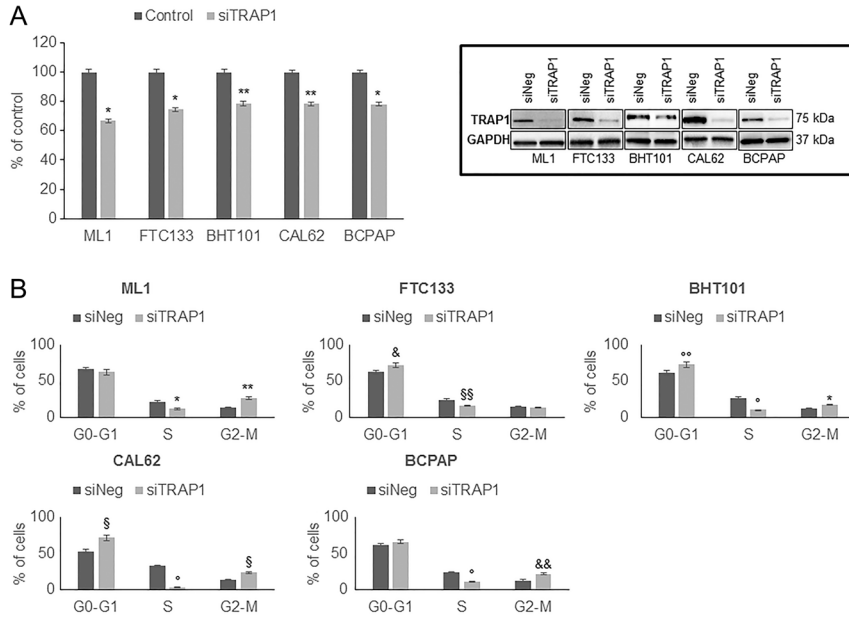
### TRAP1 targeting results in inhibition of ERK signaling and cell proliferation in TC cell lines

We recently demonstrated that the dual HSP90/TRAP1 inhibitor HSP990, that in previous studies was shown to inhibit TRAP1 ATPase domain (Menezes et al. 2012), antagonizes TRAP1 chaperoning activity toward its client proteins and acts as cytostatic agent in BRAF-mutated colon carcinoma cells (Condelli et al. 2014). Hence, this agent was used to further validate TRAP1 as a potential therapeutic target in TC cells. In preliminary experiments, HSP990 was confirmed to induce the downregulation of TRAP1-specific client proteins (i.e., 18 kDa Sorcin and eIF4E) (Landriscina et al. 2010, Matassa et al. 2013) in ML1, CAL62 (Fig. 4B), and BHT101 (Supplementary Fig. 2A) TC cells, thus resembling the phenotype obtained by TRAP1 and TBP7 dual silencing (Fig. 4C). Consistently, HSP990 induced a parallel attenuation of BRAF protein expression and ERK phosphorylation (Fig. 4D), significantly inhibiting



**Figure 2**

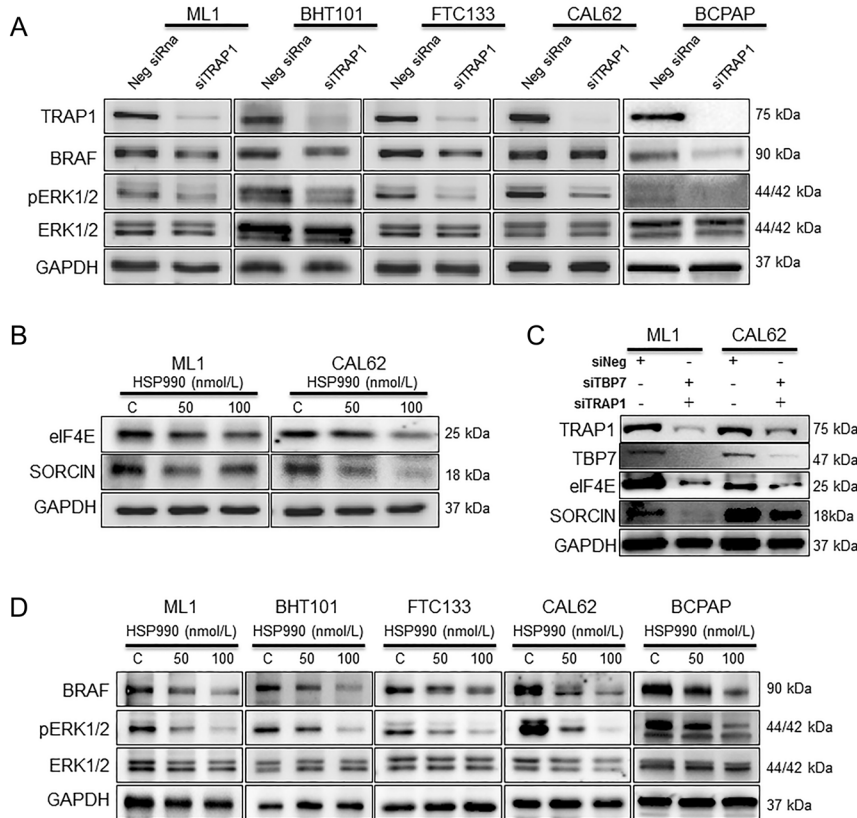
TRAP1 protects from apoptosis. (A and B) TRAP1 expression (A) and apoptotic levels (B) in ML1, FTC133, BHT101, CAL62 and BCPAP cells transfected with control and TRAP1 siRNAs and exposed to 1  $\mu$ M paclitaxel for 24 h. Statistical significance with respect to negative siRNA cells treated with paclitaxel: \* $P=0.002$ ; \*\* $P=0.0003$ ;  $^{\circ}P=0.005$ ;  $^{\circ\circ}P=0.004$ . (C) Apoptotic levels in ML1, FTC133, BHT101, CAL62, and BCPAP cells treated with 1 and 10  $\mu$ M gamitrinib for 24 h. Statistical significance with respect to vehicle-treated cells: \* $P=0.0002$ ;  $^{\circ}P=0.003$ ; \*\* $P=0.002$ ;  $^{\circ\circ}P=0.0001$ . (D) Apoptotic levels in ML1, FTC133, BHT101, CAL62, and BCPAP cells pretreated with 1  $\mu$ M gamitrinib for 6 h and subsequently exposed to 1  $\mu$ M paclitaxel for 24 h (0.25  $\mu$ M in BHT101 cells, 0.5  $\mu$ M in BCPAP cells). Statistical significance with respect to cells treated with paclitaxel as single agent: \* $P=0.0003$ ; \*\* $P=0.001$ ;  $^{\circ}P=0.0008$ ;  $^{\circ\circ}P=0.003$ .

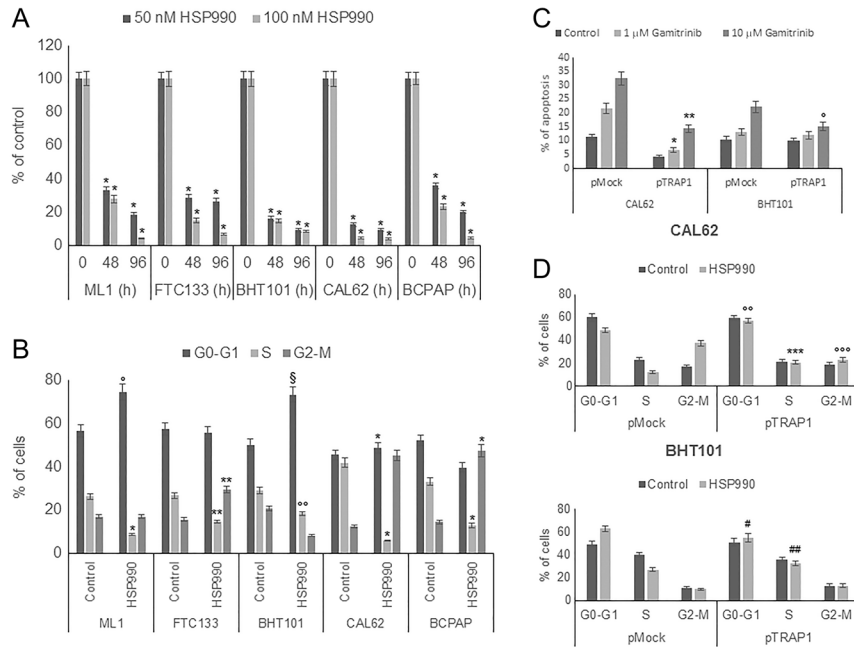


cell proliferation (Fig. 5A) and cell cycle progression (Fig. 5B) in our panel of TC cell lines. As observed for gamitrinib, HSP990 activity was independent from BRAF mutational status (Fig. 5). These data suggest that TRAP1 may represent a novel therapeutic target in PDTCs.

**Gamitrinib and HSP990 cytotoxic/cytostatic activity depends on TRAP1 inhibition**

In order to demonstrate that the observed cytotoxic/cytostatic activities showed by gamitrinib and HSP990 depend on TRAP1 targeting, both agents were tested in



**Figure 5**

HSP90/TRAP1 targeting results in attenuation of cell proliferation and cell cycle progression. (A) ML1, FTC133, BHT101, CAL62 and BCPAP cells were plated in 6-well plates, treated with 50 and 100 nM HSP990 and counted after 48 and 96 h. Statistical significance with respect to vehicle-treated cells: \* $P < 0.0001$ . (B) Cell cycle distribution in ML1, FTC133, BHT101, CAL62 and BCPAP cells treated with 100 nM HSP990 for 24 h. Statistical significance with respect to vehicle-treated cells: \* $P < 0.0001$ ; \*\* $P = 0.0001$ ; † $P = 0.002$ ; †† $P = 0.0004$ ; ††† $P = 0.0008$ . (C and D). Apoptotic levels (C) and cell cycle distribution (D) in CAL62 and BHT101 cells transfected with pMock or TRAP1 cDNA and treated with 1 or 10  $\mu\text{M}$  gamitrinib (C) or 100 nM HSP990 for 24 h. Statistical significance with respect to pMock cells: \* $P = 0.0002$ ; \*\* $P = 0.0003$ ; \*\*\* $P = 0.002$ ; † $P = 0.007$ ; †† $P = 0.01$ ; ††† $P = 0.001$ ; # $P = 0.03$ , ## $P = 0.02$ .

CAL62 and BHT101 cells upon transfection of TRAP1 cDNA (Supplementary Fig. 2B and C). Of note, either gamitrinib or HSP990 failed to induce, respectively, apoptosis (Fig. 5C and Supplementary Fig. 2B) and inhibit cell cycle (Fig. 5D and Supplementary Fig. 2C) in a high TRAP1 background. These evidences suggest that TRAP1 targeting is relevant for the cytotoxic/cytostatic activity of both HSP90 inhibitors.

## Discussion

The rationale for evaluating TRAP1 role in TC progression relies on recent evidences suggesting a key involvement of this HSP90 molecular chaperone in human carcinogenesis (Amoroso et al. 2014, Rasola et al. 2014). Indeed, TRAP1 multifaceted functions in protection from apoptosis, regulation of cell cycle, cell metabolism and protein homeostasis have been widely demonstrated in several human malignancies (Kang et al. 2007, Amoroso et al. 2012, Matassa et al. 2013, Condelli et al. 2014, Rasola et al. 2014). However, controversial data have been reported on whether TRAP1 behaves as oncogene or oncosuppressor, being its expression levels increased in the majority of human malignancies (Costantino et al. 2009, Maddalena et al. 2013, Agorreta et al. 2014, Rasola et al. 2014), but downregulated along with increased tumor grading in specific human tumors (i.e. ovarian, cervical and kidney carcinomas) (Yoshida et al. 2013, Matassa et al. 2016). This issue is relevant in human TC cells since activation of ERK signaling with parallel loss of TSHR signaling and

thyroid-specific characters correlates with worsening of clinical outcome (Menezes et al. 2012, Regalbuto et al. 2012, Patel & Shaha 2014). Interestingly, while the majority of TCs are well-differentiated tumors with excellent prognosis, a subset of TCs are characterized by a more aggressive biological and clinical behavior, unresponsiveness to radioiodine therapy and chemotherapeutics and increased metastatic dissemination (Mazzaferrri & Massoll 2002, Regalbuto et al. 2012). In this context, the validation of biomarkers for early detection of these more aggressive TCs and the development of new therapeutic strategies targeting molecular mechanisms responsible for the aggressive behavior of these thyroid malignancies are relevant objectives to improve the prognosis of these patients (Omur & Baran 2014).

Thus, TRAP1 expression was analyzed in a large series of TCs with different grading and stages and its involvement in driving specific features of PDTC cells (i.e., increased cell proliferation and resistance to apoptosis) was studied *in vitro*. Remarkably, TRAP1 expression is induced along tumor (de)differentiation: (i) in microfollicular compared with macrofollicular areas of normal thyroid gland, (ii) in well-differentiated TCs compared with normal tissues, and (iii) in the transition from well-differentiated to PDTCs and in FV-PTCs, two tumor entities with worst biological and clinical phenotypes compared with well-differentiated TCs (Vivero et al. 2013, Yu et al. 2013, Patel & Shaha 2014). Consistently, *in vitro* experiments suggest that TRAP1 is involved in the regulation of cell cycle progression and the apoptotic



threshold of TC cells. In this context, it is worth noting that TRAP1 knocking down correlates with increased sensitivity to paclitaxel, reduction of S-phase fraction of cell cycle, and attenuation of ERK signaling, a pathway known to drive TC cell (de)differentiation (Nikiforov *et al.* 2011), thus suggesting that TRAP1 upregulation is likely responsible for TC progression. Conflicting with this evidence is the observation that TRAP1 expression is completely lost in a subset of high-grade ATCs, being conversely upregulated in the majority of these highly aggressive tumors (11/16 cases in our cohort). Although this observation cannot be easily argued, it is intriguing to speculate that a subset of ATCs may be characterized by a different metabolic and biologic phenotype (Ragazzi *et al.* 2014) and the loss of TRAP1 may be relevant in favoring these differences. This hypothesis is reinforced by the recent observation that TRAP1 is downregulated in selected malignancies along with increased tumor grading (Yoshida *et al.* 2013, Matassa *et al.* 2016) and that TRAP1 may enhance or inhibit oxidative phosphorylation in cancer cells based on the tumor type or the cell context (Rasola *et al.* 2014). This observation suggests that the loss of TRAP1 expression in specific tumor types/conditions may be functional to metabolic reprogramming of cancer cells. However, the limited number of ATCs in our series does not allow to rule out/confirm the hypothesis that these TRAP1-negative ATCs may represent a different biological entity, which represents a topic that deserves further investigation.

Relevant is the observation that TRAP1 silencing induces a profound attenuation of ERK phosphorylation. Indeed, a large subgroup of thyroid malignancies is characterized by the activation of the RAS/RAF/ERK pathway due to activating mutations/rearrangement of *RET/PTC*, *BRAF* or *RAS* genes, which results in more aggressive clinical phenotypes (Riesco-Eizaguirre *et al.* 2006, Gao *et al.* 2012, Omur *et al.* 2014, Yang *et al.* 2014). Furthermore, this biological evidence was translated at clinical level in the development of novel anticancer therapies targeting this signaling axis (Fallahi *et al.* 2015). Intriguingly, TRAP1 silencing induced the parallel downregulation of BRAF and ERK signaling in follicular *BRAF/RAS* wild-type ML1 and FTC133 cells and BRAFV600E BHT101 and BCPAP cells, but only the downregulation of ERK phosphorylation in *KRAS*-mutated CAL62 cells. Indeed, while this is the first observation that TRAP1 is responsible for the modulation of ERK signaling independently from its regulatory activity on BRAF synthesis (Condelli *et al.* 2014), it supports the hypothesis that TRAP1 targeting may represent a strategy to inhibit ERK pathway in TCs. However, whether the lack of correlation between BRAF

expression and ERK activation in low TRAP1 background is due to the *RAS* mutational status of CAL62 cells or to other unknown regulatory mechanisms is still an open issue.

Clinically relevant is the evidence that TRAP1 targeting results in reversion of resistance to apoptosis and inhibition of cell proliferation. Two independent agents, targeting TRAP1 in a compartmentalized manner, achieved this result. Indeed, gamitrinib, a dual HSP90/TRAP1 ATPase inhibitor, which accumulates in mitochondria favoring the opening of the mitochondrial transition pore (Kang *et al.* 2009), showed a single-agent cytotoxic activity and enhanced the cytotoxicity of paclitaxel. Consistently, HSP990, which is not engineered to enter mitochondria, significantly inhibited ERK signaling, cell cycle progression and cell proliferation. HSP990 is a TRAP1 inhibitor, as previously demonstrated in other tumor cell models (Menezes *et al.* 2012, Condelli *et al.* 2014) and confirmed in TC cells by the downregulation of specific TRAP1 client proteins. It is also an inhibitor of HSP90 and likely other HSP90 chaperones, as their ATPase domains are highly homologous. Indeed, this multiple inhibitory activity may account for some differences in cell cycle inhibition profiles observed between HSP990-treated and TRAP1-silenced TC cells and for the dramatic difference between its cytostatic activity and the effect of TRAP1 silencing on cell proliferation. Overall, these observations suggest that HSP90 chaperones may represent valuable molecular targets to develop novel therapeutic strategies in radioiodine-refractory TCs, a subset of TCs with poor prognosis and limited therapeutic options (Xing *et al.* 2013). In such a context, the MEK inhibitor selumetinib showed interesting re-differentiating activity against radioiodine-refractory advanced TCs, producing clinically meaningful increases of iodine uptake and retention (Ho *et al.* 2013) and similar observations were reported upon treatment of BRAF-V600E-mutant PTCs with the BRAF inhibitor dabrafenib (Rothenberg *et al.* 2015). Intriguingly, HSP990 downregulated BRAF in TC cells and induced a significant downregulation of cell proliferation independently from *BRAF* mutational status, thus suggesting that HSP90/TRAP1 pharmacological targeting deserves to be evaluated as a strategy to inhibit ERK signaling in radioiodine-refractory TCs. This issue is becoming relevant considering the recent development of the first mitochondria-targeted HSP90 inhibitor designed on the crystal structure of human TRAP1 (Lee *et al.* 2015).

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0063>.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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### Author contribution statement

Study concept and design, M C, F E, M L; acquisition of data, G P, G P, A P, T N, L S, A S, G S; analysis and interpretation of data, G P, G P, P T, P B, F E, M L; drafting of the manuscript, G P, G P, M L; critical revision of the manuscript, M C, F E, M L; statistical analysis, O L, G S; obtained funding, F E, M L; study supervision, M L.

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