TRAP1 Regulates Proliferation, Mitochondrial Function, and Has Prognostic Significance in NSCLC

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Abstract

The TNF receptor-associated protein 1 (TRAP1) is a mitochondrial HSP that has been related to drug resistance and protection from apoptosis in colorectal and prostate cancer. Here, the effect of TRAP1 ablation on cell proliferation, survival, apoptosis, and mitochondrial function was determined in non–small cell lung cancer (NSCLC). In addition, the prognostic value of TRAP1 was evaluated in patients with NSCLC. These results demonstrate that TRAP1 knockdown reduces cell growth and clonogenic cell survival. Moreover, TRAP1 downregulation impairs mitochondrial functions such as ATP production and mitochondrial membrane potential as measured by TMRM (tetramethylrhodamine methylster) uptake, but it does not affect mitochondrial density or mitochondrial morphology. The effect of TRAP1 silencing on apoptosis, analyzed by flow cytometry and immunoblot expression (cleaved PARP, caspase-9, and caspase-3) was cell line and context dependent. Finally, the prognostic potential of TRAP1 expression in NSCLC was ascertained via immunohistochemical analysis which revealed that high TRAP1 expression was associated with increased risk of disease recurrence (univariate analysis, \( P = 0.008 \); multivariate analysis, HR: 2.554; 95% confidence interval, 1.085–6.012; \( P = 0.03 \)). In conclusion, these results demonstrate that TRAP1 impacts the viability of NSCLC cells, and that its expression is prognostic in NSCLC.

Implications: TRAP1 controls NSCLC proliferation, apoptosis, and mitochondrial function, and its status has prognostic potential in NSCLC. Mol Cancer Res; 12(5); 660–9. ©2014 AACR.

Introduction

Lung cancer is the leading cause of cancer death worldwide (1). Non–small cell lung cancer (NSCLC), the most common type of lung cancer, can be subdivided into two main histologic subtypes: adenocarcinoma and squamous cell carcinoma (SCC), accounting for 50% and 30% of all NSCLC cases, respectively (2). Despite the development of targeted therapies in lung cancer, there has been little improvement in 5-year survival rates. In this context, improved knowledge of the molecular biology of lung cancer, together with biomarkers that predict tumor development and prognosis, is needed.

TNF receptor-associated protein 1 (TRAP1) is a mitochondrial protein that belongs to the Hsp90 family, first identified as interacting with the intracellular domain of the type I TNF receptor (3). Later sequence analysis revealed that TRAP1 was identical to Hsp75 (4). TRAP1 is mainly localized in the cytosol, endoplasmic reticulum, and nucleus (7–9). TRAP1 interacts with several proteins such as retinoblastoma (10), the ATPase TBP7, a component of the 19S proteasome regulatory subunit (11), the Ca\(^{2+}\)-binding protein sorcin localized in the mitochondria (12), the mitochondrial protein cyclophilin D (5), and the tumor suppressors EXT1 and EXT2, proteins involved in hereditary multiple exostoses (13). Moreover, TRAP1 has been reported to protect against apoptosis (5, 14, 15) and oxidative stress (15, 17). Interestingly, it has been proposed that TRAP1 may be involved in chemoresistance by blocking drug-induced apoptosis in a variety of tumors such as prostate cancer (18), osteosarcoma.
TRAP1 Regulates NSCLC Proliferation and Mitochondrial Function

Materials and Methods

Cells
Human NSCLC cell lines NCI-A549 and NCI-H1299 were obtained from Clare Hall Laboratories and grown in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and penicillin-streptomycin at 100 U/mL. Cell cultures were incubated at 37°C in a humidified 5% CO₂ incubator.

Patient samples
A series of 71 patients with a diagnosis of NSCLC who underwent surgical resection at Clínica Universidad de Navarra (Navarra, Spain) from 2000 through 2008 were included in this study. Clinicopathologic features of the patients are listed in Table 1. Tumor specimens were classified according to the 2004 World Health Organization criteria (25). The inclusion criteria were NSCLC histology, no neoadjuvant chemo- or radiotherapy, and absence of cancer within the 5 years previous to lung cancer surgery. The study protocol was approved by the institutional medical ethical committee. Written informed consent was obtained from each patient before participation. RFS was calculated from the date of surgery to the date of detection of recurrence or the date of the last follow-up. The median follow-up time was 42 months.

Immunohistochemistry in clinical specimens from patients with NSCLC
Formalin-fixed paraffin-embedded tissue sections were evaluated. Endogenous peroxidase activity was quenched

Table 1. Clinicopathological characteristics of the patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N = 71</th>
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<tr>
<td>Age, y (median-interquartile range)</td>
<td>63 (54–70)</td>
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<tr>
<td>Sex, n (%)</td>
<td></td>
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<tr>
<td>Male</td>
<td>60 (85%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (15%)</td>
</tr>
<tr>
<td>Stage, n (%)</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>44 (62%)</td>
</tr>
<tr>
<td>Stage II</td>
<td>15 (21%)</td>
</tr>
<tr>
<td>Stage III</td>
<td>10 (14%)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>Histology, n (%)</td>
<td></td>
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<tr>
<td>Adenocarcinoma</td>
<td>26 (37%)</td>
</tr>
<tr>
<td>SCC</td>
<td>39 (55%)</td>
</tr>
<tr>
<td>Other</td>
<td>6 (8%)</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>39 (55%)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>26 (37%)</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>6 (8%)</td>
</tr>
</tbody>
</table>

Immunostaining evaluation
Two independent, blinded observers (F. Pezzella and J. Agorte) evaluated the intensity and extensiveness of staining in all of the study samples. The evaluation of cytoplasmic TRAP1 expression was performed using the H-score system (26). Briefly, the percentage of positive cells (0%–100%) and the intensity of staining (1+, mild; 2+, moderate; and 3+, intense labeling) were scored. Disagreements were resolved by common reevaluation.

Immunoblotting
Protein and total RNA were extracted using Paris kit (Ambion-Life Technologies Ltd) according to the manufacturer’s instructions. Thirty micrograms of total protein from each lysate were boiled at 95°C for 5 minutes, separated by SDS/PAGE under reduced conditions (5% 2-
mercaptoethanol), and transferred onto a nitrocellulose membrane. The membranes were subsequently blocked in 5% defatted milk-PBS for 1 hour and incubated overnight at 4°C with a primary antibody anti TRAP1 (1:1000, Labvision) or anti β-actin (1:10000, Sigma). Blots were then incubated with a horseradish peroxidase-linked secondary antibody (1:5000; Amersham Pharmacia Biotech) and developed by chemoluminiscence with Lumilight Plus Kit (Roche diagnostics). Apoptosis detection by Western blotting was performed as described before (27).

**RNA interference**

For inhibition of TRAP1 expression, cells were seeded (1 × 10⁶ cells per well) in 10 cm dishes in antibiotic-free medium. At 24 hours, cells were transfected with 40 nmol/L of siRNA by using Oligofectamine (Invitrogen-Life Technologies) Ltd as previously described (21). Two siRNA sequences against TRAP1 were designed and synthesized by Eurogentec (TRAP1-siRNA1: 5'-AUGUUUGGAAAGUG-GAACCC-3' and 5'-ACAUCAUGAAAGGCAUGG-3'); TRAP1-siRNA2: 5'-TGCTGGTTTGAGGACTTCCACTTCCAAAC-3' and 5'-CCGTGTTTGGAACTTGGCC-CCTGACGTCAGTCAGTGGCCAAAACGTGCAGGG- TTCCACTTCCAAAC-3'). A scrambled (scr) siRNA (5'-AUGUUUGGAAAGUGGACCC-3' and 5'-UAGGU-GUACCCGAUUG-3') was used as the negative control.

**RT-PCR**

Retrotranscription was performed using RetroScript Kit (Ambion). TRAP1 and β-actin expression was analyzed by PCR using TaqMan Gene Expression Assays (Applied Biosystems). The reaction was performed on a PTC-200 thermal cycler with a Chromo 4 continuous fluorescence detector (Bio-Rad). The comparative cycle threshold (Ct) method was used to analyze the data by generating relative values of the amount of target cDNA, according to the 2⁻ΔΔCt method (28) using β-actin as endogenous gene and scramble (scr) expression as calibrator.

**Growth curves**

Cells were seeded on 6-well dishes at a density of 1 × 10⁵ cells per well in triplicate and cultured for 1 to 7 days. Subsequently, cell number was assessed with a Coulter Z2 particle count and size analyzer (Beckman Coulter). Automatically cell count was carried out with a Cell IQ microscope (Chipman Technologies).

**Clonogenic assay**

Twenty four hours after siRNA transfection, cells were harvested, seeded in triplicate (300 cells per well) in 6-well plates, and incubated at 37°C in a 5% CO₂ atmosphere. After 14 days, colonies were fixed in methanol-acetic acid (1:1), stained with crystal violet, and counted.

**Proliferation index determination**

siRNA-treated cells were seeded in 10 cm dishes and grown for 1, 3, or 5 days. Subsequently, cells were harvested and fixed overnight in 4% phosphate-buffered formalin (pH 7.0), suspended in agar, and embedded in paraffin. Antigen retrieval was carried out in 3 μm sections by pressure cooking in 10 mmol/L citrate buffer, pH 6, and immunohistochemical staining for the human Ki-67 protein was performed using the anti-MIB1 antigen antibody (Dako) at 1:50 for 30 minutes at room temperature. Sections were incubated with the Envision detection system (Dako) and developed with dianobenzidine. Immunohistochemical scoring was performed as previously described (29).

**Cell-cycle and apoptosis analysis**

Cell-cycle analyses were performed on trypsin-disaggregated cryopreserved cell suspensions containing floating and attached cells. Following thawing, cells were centrifuged to remove the cryopreservation solution (10% dimethyl sulfoxide in FBS), fixed in 70% ethanol on ice, treated with 1 μg/mL RNase, stained with 10 μg/mL propidium iodide, and examined with a FACSCalibur instrument fitted with a Cell Quest software package (BD Biosciences). About 50,000 cells per sample were analyzed. Percentages of cells in the Sub-G₁, G₁, S and G₂-M phases were determined. For apoptosis analysis, fresh trypsin-disaggregated cell suspensions containing floating and attached cells were used as previously described (30). Briefly, cells were washed and stained with 2 μL of Annexin V (BD Biosciences) and 2 μL of 10 μg/mL of propidium iodide (Sigma). Apoptosis was induced by staurosporine treatment (1 μg/mL for 4 h). Samples were analyzed on a FACSCalibur instrument and quadrant analysis was performed with FlowJo 9.3 software (Tree Star). At least three independent experiments per condition were performed.

**Mitochondrial function**

The amount of ATP was measured in lysates of 10⁵ cells using the ATP Bioluminescence Assay Kit (Roche) in accordance with the manufacturer’s instructions. This method uses the ATP dependency of the light-emitting, luciferase-catalyzed oxidation of luciferin for the measurement of ATP concentration. To analyze the mitochondrial membrane potential, TMRM (tetramethylrhodamine methylester; Invitrogen) staining was used because it is a cell-permeant, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria. MitoTracker Green staining (Molecular Probes-Life Technologies Ltd) was also used to measure mitochondrial mass regardless of mitochondrial membrane potential. Moreover, the production of reactive oxygen species (ROS) was evaluated by the MitoSOX staining (Molecular probes) as previously described (31). Fluorescence images were collected using a confocal microscope (Zeiss LSM 510 META; Carl Zeiss) and fluorescence intensity was measured with ImageJ software (NIH, Bethesda, MD).

**Electron microscopy**

Cells were fixed in 4% glutaraldehyde in 0.1 mol/L phosphate buffer and processed for routine electron
microscopy as previously described (32). Mitochondrial mass was measured with ImageJ software.

Statistical analysis
Statistical analysis was performed using SPSS 15.0. Data obtained from cell count, colony formation, MIB1 staining, cell cycle, and mitochondrial function experiments were analyzed by the Student t test or the Mann–Whitney U test for parametric and nonparametric variables, respectively. For survival analysis, Kaplan–Meier survival curves and the log-rank test were used to analyze differences in RFS (the median was selected as the cutoff value). Multivariate analysis was carried out using the Cox proportional hazards model. Only variables of \( P < 0.1 \) from the univariate analysis were entered in the Cox regression analysis. The proportional hazards assumption was examined by testing interactions between the covariates of the final model and time. \( P < 0.05 \) was considered statistically significant.

Results
Expression of TRAP1 is necessary for cell growth
To examine the effect of TRAP1 inhibition on cell proliferation, we carried out downregulation experiments in lung cancer cell lines. Knockdown was carried out in H1299 and A549 cells using two different siRNAs and the efficacy of TRAP1 siRNA downregulation was verified by Western blotting and real-time PCR (RT-PCR; Fig. 1A and B). TRAP1 downregulation resulted in a significant reduction in cell growth in both H1299 and A549 cell lines as confirmed by TRAP1-siRNA1 and 2 sequences (Fig. 1C). Cell growth of TRAP1-siRNA1–treated A549 cells was also significantly reduced as compared to the control cells (Fig. 1E).

Figure 1. TRAP1 knockdown inhibits cell proliferation and survival on the H1299 and A549 cell lines. Successful knockdown of TRAP1 expression by two independent TRAP1-siRNA sequences was demonstrated by RT-PCR (A) and Western blot analysis (B) in both H1299 and A549 cell lines at day 4. To determine the effect of TRAP1 siRNA knockdown on tumor cell proliferation, cells were transfected with control- (scr) or TRAP1-siRNAs and cell number was determined by a Coulter Z2 particle count and size analyzer (C) or automatically determined by a Cell IQ microscope (D). E, TRAP1 downregulation significantly reduced colony formation in the A549 and H1299 cell lines. Data, mean ± SD from at least three independent experiments.
monitored by time-lapse video microscopy for 5 days at a 35-minute interval, confirming the reduction in cell number after TRAP1 knockdown (Fig. 1D and Supplementary Movies S1 and S2). The impairment of cell survival was further confirmed by clonogenic assay in H1299 and A549 cell lines (Fig. 1E). We next investigated the effect of TRAP1 knockdown on cell proliferation by staining cell pellets of scr- and TRAP1-siRNA A549-treated cells at different time points for ki-67 protein (MIB1 antigen). We found that from day 3, there was a significant reduction of MIB1-positive cells when TRAP1 was inhibited (Fig. 2A). Cell-cycle analysis by flow cytometry showed a significant reduction in the percentage of cells in G2–M phase after TRAP1 knockdown (Fig. 2B and C), confirming the results from the immunohistochemical analysis of ki-67 expression.

TRAP1 downregulation has a variable effect on apoptosis

Quantification of apoptotic cells by Annexin V/PI assay showed that TRAP1 downregulated A549 cells had increased apoptotic rates as compared with scr-siRNA treated (Fig. 2D, top). Those effects were less evident in H1299 cell line (Supplementary Fig. S1). The induction of apoptosis in A549 cells was confirmed by the increase of activated (cleaved) caspase-3, caspase-9, and PARP (Fig. 2E, left). It should be noted that when apoptosis was induced by treating cells with staurosporine, there was a dramatic induction of apoptosis in TRAP1-siRNA–treated cells but not in scr-control cells (Fig. 2D, bottom and Fig. 2E, right). The cell line H1299 did not show clear evidence of apoptosis as silencing of TRAP produced a mild increase of PARP but a similarly mild decrease of

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**Figure 2.** Downregulation of TRAP1 arrests cell proliferation and induces apoptosis in the A549 lung cancer cell line. A, proliferative fraction given by the percentage of ki-67–positive cells was significantly reduced in TRAP1-siRNA1–treated cells. B, cell-cycle distribution of A549 cells at different days after TRAP1-siRNA1 transfection. C, differences in the percentage of cells in S and G2–M phases at day 3. D, Annexin V/propidium iodide staining was performed on A549 cells transfected with scr, TRAP1-siRNA1, and TRAP1-siRNA2 sequences (top) or transfected cells treated with 1 μg/mL staurosporine (bottom) and analyzed by flow cytometry. Percentages of intact cells (Annexin V−/PI−), early apoptotic cells (Annexin V−/PI+), and late apoptotic or necrotic cells (Annexin V+/PI+) are shown in the plot. One representative experiment is shown from three independent repetitions. E, apoptosis was also demonstrated by Western blot analysis of cleaved PARP and cleaved caspase-3 and -9 in A549 cells. Data, mean ± SD from at least three independent experiments.
TRAP1 Regulates NSCLC Proliferation and Mitochondrial Function

TRAP1 expression was analyzed by immunohistochemistry in a series of 71 patients with NSCLC. The main clinical and pathologic characteristics of these patients are summarized in Table 1. Moderate TRAP1 staining was found in normal bronchial mucosa adjacent to the tumor (Fig. 4A), although no immunoreactivity was found in alveoli (Fig. 4B). High TRAP1 expression is associated with worse prognosis in patients with NSCLC.

TRAP1 downregulation impairs mitochondrial function

We hypothesized that the effects of TRAP1 inhibition could be caused by mitochondrial dysfunction because TRAP1 is known to be mainly expressed in the mitochondria. Therefore, we analyzed a variety of mitochondrial functions after TRAP1 inhibition. First, we analyzed ATP production in scr- and TRAP1-siRNA1–treated cells, showing that TRAP1 inhibition was associated with a 30% reduction of ATP (P = 0.002; Fig. 3A). Next, we examined the effect of TRAP1 expression on mitochondrial membrane potential as measured by TMRM uptake. A significant reduction on membrane potential was shown in TRAP1-siRNA1–treated cells as compared with scr-siRNA (P < 0.001; Fig. 3B and C). However, no differences were found in the mitochondrial mass measured by MitoTracker staining (P = 0.809; Fig. 3D) or in ROS production measured by MitoSOX staining (P = 0.078; Fig. 3E). Electron microscopy also did not demonstrate changes in mitochondrial morphology or mitochondrial mass (Fig. 3F).

Figure 3. TRAP1 downregulation impairs mitochondrial function in A549 cells. A, measurement of ATP levels by a bioluminescence assay demonstrates that TRAP1 inhibition reduces ATP levels. B, representative images of TMRM uptake in scr- and TRAP1-siRNA1–treated cells. C, quantification of TMRM uptake by image analysis shows a reduction in mitochondrial membrane potential in TRAP1-siRNA1–treated A549 cells as compared with control cells. D, mitochondrial mass measured by MitoTracker staining was similar in scr and TRAP1-siRNA1–treated cells. E, no differences in ROS production, as determined by MitoSOX staining, were found. F, the ultrastructure of the mitochondria was visualized using transmission electron microscopy, and no differences in the mitochondrial morphology were observed between scr- and TRAP1-siRNA1–treated cells. Moreover, no significant differences in mitochondrial mass were found. Scale bar, 500 nm. Data, mean ± SD from three independent experiments.
4B). In tumor samples, TRAP1 staining was observed predominantly in the cytoplasm of all tumors analyzed (Fig. 4C). In some cases, TRAP1 staining was both in the nucleus and in the cytoplasm (Fig. 4D).

We next sought to evaluate the prognostic role of TRAP1 expression in our cohort of NSCLC. Nuclear expression of TRAP1 did not correlate with prognosis (log-rank test; \( P = 0.486 \)). However, when cytoplasmic TRAP1 expression was analyzed, those patients with high TRAP1 expression (using the median as the cutoff value) showed shorter RFS than patients with low levels of TRAP1 (\( P = 0.008 \); Fig. 4E). Multivariate analysis using Cox regression model was performed to determine the independent prognostic factors. Relevant clinicopathological variables such as age, gender, stage, histology, and smoking history, as well as cytoplasmic TRAP1 expression, were analyzed in the Cox univariate model, and only those variables with a \( P \) value < 0.1 (stage and TRAP1 expression) were included in the multivariate model. The Cox regression analysis revealed that high cytoplasmic TRAP1 expression was an independent predictor of shorter RFS when patients were adjusted by stage [HR = 2.554; confidence interval (CI), 1.085–6.012; Table 2].

**Table 2.** Multivariate Cox regression analysis of RFS in patients with NSCLC

<table>
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<tr>
<th></th>
<th>HR (95% CI)</th>
<th>( P )</th>
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<tr>
<td>Cytoplasmic TRAP1 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>High</td>
<td>2.554 (1.085–6.012)</td>
<td>0.032</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
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<tr>
<td>I, II</td>
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<td>–</td>
</tr>
<tr>
<td>III, IV</td>
<td>1.720 (0.725–4.077)</td>
<td>0.218</td>
</tr>
</tbody>
</table>

![Figure 4.](image) High cytoplasmic TRAP1 staining is associated with adverse prognosis in NSCLC. Representative TRAP1 immunostaining in bronchial epithelium (A), lung parenchyma (B), lung adenocarcinoma (C), and SCC of the lung (D). E, Kaplan-Meier RFS curves for TRAP1 expression and log-rank test. Shorter RFS time was found in tumors bearing high TRAP1 expression. Scale bar, 50 μm.
These results demonstrate that TRAP1 expression correlates with poor outcome in patients with NSCLC.

Discussion

In the present study, we have demonstrated that TRAP1 has important effects on mitochondrial function and plays a key role in the regulation of proliferation, survival, and apoptosis of NSCLC cells. Moreover, we have shown that high cytoplasmic TRAP1 expression is associated with worse prognosis in patients with NSCLC.

During the last 10 years, an increasing number of studies have demonstrated the versatility of TRAP1 protein and its involvement in a number of pathways. Originally cloned because of its interaction with TNF receptor, and therefore likely to be involved in cell signaling (3, 4), it was then found that TRAP1 also acts as a chaperon to retinoblastoma, maintaining retinoblastoma protein in its active conformation (10). The importance of the role of TRAP1 as chaperon has quickly outgrown its original role in retinoblastoma as its pivotal role in cytoprotection has emerged. TRAP1 has been described to protect mitochondria from oxidative stress and ROS (reviewed in refs. 33–35). In this sense, TRAP1 blocks ROS activity (15), ROS production (36), and regulates the mitochondrial permeability transition pores (5, 37). It has been recently demonstrated that TRAP1 has an important role controlling central metabolic networks in the mitochondria of tumor cells (38, 39). All these functions are believed to be important for its role in protecting from apoptosis and inducing chemoresistance (11, 33).

In the present study, we investigated the role of TRAP1 in NSCLC cell lines growth by looking at its effects on cell proliferation, apoptosis, and mitochondrial function.

Downregulation of TRAP1 expression in NSCLC cell lines produced a significant reduction of cell proliferation and survival as assessed by cell count, clonogenic assays, ki-67 expression, and cell-cycle analysis. In agreement with these results, proliferation had been previously linked to TRAP1 by our group (24) and others (40). However, there is no general agreement in literature as some authors have failed to notice any effect on cell growth (38). Moreover, we have determined that TRAP1 knockdown is associated with a subtle and variable effect on induction of apoptosis. This induction becomes more evident when apoptosis is induced pharmacologically with staurosporine. Our data obtained from lung primary tumors suggest that TRAP1 can have according to the type of cell being investigated.

We have previously demonstrated that after a short hypoxic shock, TRAP1 translocates to the nucleus and is essential to maintain retinoblastoma suppressor gene function: in its absence, the cells fail to slow down proliferation (24). However, this is a short-term effect and, in agreement with our previous results and as demonstrated here, in normoxia and in the longer term TRAP1 promotes the cell cycle. Therefore, it seems to have two opposite functions: in normoxia, TRAP1 promotes cell proliferation and protects from apoptosis; however, following a hypoxic shock it moves to the nucleus where it is essential for retinoblastoma to induce a rapid, short-term slowing down of proliferation. If hypoxia persists, TRAP1 cytoplasmic levels will decrease (unpublished results) alongside a diminution of the proliferation rate. In this respect, it can be considered to have oncogenic properties as suggested by Sciacovelli and colleagues (40) although the potential suppressor effect in malignancies, due to its chaperone role to retinoblastoma (10), remains to be elucidated.

On the other hand, TRAP1 expression has also been correlated with chemoresistance in breast, colorectal, and ovarian carcinoma (7, 19, 43, 45). Although there is consensus that TRAP1 expression is a predictive biomarker for drug resistance, because of the broad range of cellular functions influenced by TRAP1 (33), it is perhaps not surprising that its role as immunohistochemical prognostic biomarker is controversial. As a matter of fact, a role as oncogene (40), together with a role as tumor suppressor gene (44), has been proposed. Our data obtained from lung primary tumors showed both nuclear and cytoplasmic staining of TRAP1 in tumor cells, as it was previously reported in NSCLC, breast carcinoma, ovarian cancer, and other malignancies (18, 24, 45). Only a few studies have been performed looking at TRAP1 expression in tumor samples and correlations with prognosis (24, 45, 46), and the results are controversial. In colorectal carcinoma, high TRAP1 expression has been correlated with shorter RFS and overall survival (OS; ref. 46). However, in a study on ovarian carcinoma, cytoplasmic expression of TRAP1 was associated with better OS, whereas
no association was found with RFS (45). Finally, in a series of breast carcinoma, we found no correlation between cytoplasmonic TRAP1 and OS or RFS, although nuclear TRAP1 expression was instead associated with both retinoblastoma positivity and longer RFS but no association was found with OS (24). In the present study, we demonstrated that cytoplasmonic expression of TRAP1 is an independent predictor of the shortest relapse-free survival in surgically resected NSCLC. To our knowledge, this is the first study that analyzes the role of TRAP1 in the prognosis of lung cancer. Although the causes underlying diverse results in different tumor types are still unknown, we may argue that TRAP1 plays organ-specific roles in each tumor type. Indeed, it should be noted that the association between cytoplasmonic staining and worse prognosis demonstrated in NSCLC in the present study and in colorectal carcinoma (46) is fully consistent with the suggested role of TRAP1 in cell proliferation and protection to apoptosis. However, more extensive studies exploring the role of TRAP1 as a potential target or predictor of response in NSCLC are warranted.

In conclusion, the complexity of the role played by TRAP1 in the cancer cell biology is the most likely explanation for the discrepant results observed in literature; however, our data further support the role played by TRAP1 in mitochondrial function and regulation of apoptosis, but also demonstrate a role in cell growth through the regulation of the cell cycle. Furthermore, we have demonstrated that high TRAP1 expression is an adverse prognostic factor for patients with NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Agorreta, J. Hu, A.L. Harris, K. Gatter, F. Pezzella
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Agorreta, J. Hu, D.J.P. Ferguson, I. Zudaire, R. Pio, L.M. Montuenga, A.L. Harris, F. Gatter, F. Pezzella
Writing, review, and/or revision of the manuscript: J. Agorreta, J. Hu, D. Delia, H. Turley, M.J. Pajares, M. Larrazo, I. Zudaire, R. Pio, L.M. Montuenga, A.L. Harris, K. Gatter, F. Pezzella
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Agorreta, M. Larrazo, K. Gatter, F. Pezzella
Study supervision: J. Agorreta, J. Hu, A.L. Harris, F. Pezzella

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