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TRAP1-dependent regulation of p70S6K is involved in the attenuation of protein synthesis and cell migration: Relevance in human colorectal tumors

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ABSTRACT

TNF receptor-associated protein 1 (TRAP1) is an HSP90 chaperone involved in stress protection and apoptosis in mitochondrial and extramitochondrial compartments. Remarkably, aberrant deregulation of TRAP1 function has been observed in several cancer types with potential new opportunities for therapeutic intervention in humans. Although previous studies by our group identified novel roles of TRAP1 in quality control of mitochondria-destined proteins through the attenuation of protein synthesis, molecular mechanisms are still largely unknown. To shed further light on the signaling pathways regulated by TRAP1 in the attenuation of protein synthesis, this study demonstrates that the entire pathway of cap-mediated translation is activated in cells following TRAP1 interference: consistently, expression and consequent phosphorylation of p70S6K and RSK1, two translation activating kinases, are increased upon TRAP1 silencing. Furthermore, we show that these regulatory functions affect the response to translational stress and cell migration in wound healing assays, processes involving both kinases. Notably, the regulatory mechanisms controlled by TRAP1 are conserved in colorectal cancer tissues, since an inverse correlation between TRAP1 and p70S6K expression is found in tumor tissues, thereby supporting the relevant role of TRAP1 translational regulation *in vivo*. Taken as a whole, these new findings candidate TRAP1

Abbreviations: TRAP1, TNF receptor-associated protein 1; HSP, heat shock protein; KD, knockdown; ER, endoplasmic reticulum; CRC, colorectal carcinoma; TG, thapsigargin; shRNA, short-hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHX, cycloheximide; IRES, internal ribosome entry site; S6K, S6 kinases; siRNA, small interfering RNA.

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network for new anti-cancer strategies aimed at targeting the translational/quality control machinery of tumor cells.

1. Introduction

TNF receptor-associated protein 1 (TRAP1), also known as HSP75, belongs to the family of HSP90 chaperones and is involved in stress protection and control of apoptosis (Montesano et al., 2007; Matassa et al., 2012), as well as in regulation of tumor cell metabolism (Chae et al., 2013; Sciacovelli et al., 2013). Acute silencing of TRAP1 sensitizes tumor cells to apoptosis induced by several anti-tumor agents (Landriscina et al., 2010a). It is noteworthy that aberrant deregulation of TRAP1 function has been observed in colorectal and prostate carcinomas (Costantino et al., 2009; Kang et al., 2010) while accelerated neoplastic growth by mitochondrial TRAP1 has been demonstrated in other cancer cells and tissues (Sciacovelli et al., 2013) with potential new opportunities for therapeutic intervention in humans (Kang, 2012; Landriscina et al., 2010b). Recent evidence by our groups reported subcellular localizations of this chaperone other than mitochondria (Amoroso et al., 2012), and demonstrated that TRAP1 is involved in endoplasmic reticulum (ER) stress protection (Maddalena et al., 2011; Takemoto et al., 2011). In this context, TRAP1 controls ubiquitination/degradation levels of specific mitochondria-destined proteins, whose expression is decreased in TRAP1 knockdown (KD) cells (Amoroso et al., 2012). Our previous studies provided i) a detailed characterization of sub-cellular compartments in which TRAP1 quality control occurs, ii) the identification of F1ATPase and the calcium binding protein Sorcin as two substrates of TRAP1 quality control (Amoroso et al., 2012), and iii) the characterization of homeostatic control mechanisms, including co-translational ubiquitination and attenuation of protein synthesis by this chaperone (Matassa et al., 2013). However, these studies only partially described the signaling pathways regulated by TRAP1 in these extramitochondrial functions at molecular levels. Accordingly, we have addressed this point in the present article by studying molecular pathways involved in the attenuation of protein synthesis by TRAP1 in cancer cells and tissues.

Several data of current research focus on translational control as a novel therapeutic target and promising concept in the treatment of human diseases (reviewed in Ruggero, 2013). It is becoming increasingly clear that ER chaperones have critical functions, besides simply facilitating protein folding. The starting hypothesis for our studies lies in the new concept of "translation on demand", extensively described by Brockman et al. (2007). Translational control in cancer is a complex issue: it is known that tumor cells, including a subset of human colorectal cancers (CRCs), upregulate the translational machinery to fulfill the increased demand of protein synthesis upon increase in cell proliferation (Blagden and Willis, 2011). Conversely, recent work revealed that high rates of translational elongation negatively affect both the fidelity of translation and the co-translational folding of nascent polypeptides (Sherman and Qian, 2013). Consequently, by slowing down translation, cancer cells can significantly improve protein folding and cope with stressful conditions in unfavorable environments. These findings suggest that limiting protein synthesis could be therapeutic, especially for those diseases caused by protein misfolding in the ER (Sherman and Qian, 2013).

To further complicate this already complex scenario, a sophisticate balance between cap- and IRES-dependent translation governs the synthesis of specific genes, whose function is required for cell defense. In fact, during the past decade, the concept of cellular IRES (Internal Ribosome Entry Site)-elements has become a leading explanation for the continued expression of specific proteins in eukaryotic cells under conditions when cap-dependent translation initiation is inhibited (Shatsky et al., 2010).

In this article we provide evidence that an attenuation of cap-dependent translation by TRAP1 may well represent a protective mechanism used by cancer cells to regulate selectively the synthesis of specific stress-protective proteins. Accordingly, we previously demonstrated that TRAP1 is associated to ribosomes and several translation factors and is involved in the translational control (Matassa et al., 2013). Indeed, TRAP1 attenuates global protein synthesis, thus preventing translation errors and thereby reducing the co-translational ubiquitination/degradation of specific substrates (Matassa et al., 2013). Furthermore, we previously suggested that this process is mediated by the GCN2/PERK-eIF2α pathway, resulting in increased phosphorylation of eIF2α in cells expressing TRAP1 when compared to TRAP1 KD cells.

In order to shed further light on these novel TRAP1 properties, this study demonstrates that the whole pathway of cap-mediated translation is activated in sh-TRAP1 cells and, consistently, two translation activating kinases, p70S6K and RSK1, are hyperphosphorylated and/or hyper-expressed in TRAP1 KD cells. Moreover, TRAP1 involvement in protein synthesis affects response to translational stress and cell migration, processes in which both kinases are involved. It is worth noting that we demonstrate that TRAP1 regulatory mechanisms are conserved in colon cancer tissues, since an inverse correlation between TRAP1 and p70S6K expression is observed in human CRCs.

2. Materials and methods

2.1. Cell culture

Human HCT116 colon carcinoma cells and HEK293 embryonic kidney cells were purchased from American Type Culture

Collection (ATCC) and cultured in DMEM containing 10% fetal bovine serum, 1.5 mmol/L glutamine, penicillin and streptomycin. Cell lines are routinely monitored in our laboratory by microscopic morphology check. The authenticity of the cell lines was verified before starting this study by STR profiling, in accordance with ATCC product description. TRAP1-stable interfered cells were obtained as described previously (Amoroso et al., 2012).

2.2. Plasmid generation and transfection procedures

Full-length TRAP1 and mutant Δ1–59-Myc were obtained as described in Amoroso et al. (2012). The eEF1A1-GFP construct was obtained as described in Matassa et al. (2013). pLPL Cap-Renilla-IRES-Luciferase bicistronic dual reporter vector was kindly donated by Prof. R. Karni, Hebrew University-Hadassah Medical School, Jerusalem, Israel and obtained as described in Gerlitz et al. (2002). Transient transfection of DNA plasmids was performed with the Polyfect Transfection Reagent (Qiagen) according to the manufacturer's protocol, with siRNAs of TRAP1 purchased from Qiagen (cat. no. SI00115150). For control experiments, cells were transfected with a similar amount of scrambled siRNA (Qiagen; cat. no. SI03650318). Transient transfections of siRNAs were performed using HiPerFect Transfection Reagent (Qiagen) according to manufacturer's protocol.

2.3. WB/Immunoprecipitation analysis

Equal amounts of protein from cell lysates and tumor specimens were subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). WB analyses were performed as described in Landriscina et al. (2005). Protein immunoprecipitations were carried out on 1 mg of total extracts. Lysates were pre-cleared by incubating with protein A/G-Agarose (Santa Cruz Biotechnologies) for 1 h at 4 °C and then incubated in agitation for 18 h at 4 °C with the antibodies. Subsequently, samples were further incubated for 1 h at 4 °C with fresh beads. Beads were then collected by centrifugation and washed twice in lysis buffer. Where indicated, protein levels were quantified by densitometric analysis using the software ImageJ (Schneider et al., 2012). The following antibodies were used for WB, immunoprecipitation and immunofluorescence: anti-TRAP1 (sc-13557), anti-cMyc (sc-40), anti RSK1 (sc-231), anti p70S6K (sc-230), anti-TBP7 (PSMC4 sc-166003), anti-ERK1 (sc-94), anti-β-Actin (sc-69879), anti-eIF2α (sc-133132), anti-GFP (sc-81045), anti-GAPDH (sc-69778) from Santa Cruz Biotechnology; anti-phospho eIF2α (p Ser51, #9271), anti-eIF4G (#2469), anti-eIF4B (#3592), anti-eIF4E (#2067), anti-phospho eIF4G (#2441), anti-phospho eIF4B (#3591), anti-phospho eIF4E (#9741), anti phospho-p70S6K (#9205) from Cell Signaling Technology, anti-phosphoSerine (37430) from Qiagen. anti-rpL11 and anti-rpS19 antibodies have been prepared as described in Šulić et al. (2005) and in Chiocchetti et al. (2005), respectively.

2.4. Confocal microscopy

HEK293 cells were fixed with 0.1 M phosphate buffer containing 4% (w/v) paraformaldehyde for 15 min, then blocked and permeabilized with 5% (w/v) BSA, 0.1% (v/v) Triton X-100 and 10% (v/v) FBS in PBS for 5 min at RT and finally stained by 2 h incubation with rabbit anti-eIF4G mAb 1:30 in PBS 1x and washed with PBS 1x. Coverslips were then incubated with goat anti-rabbit Alexa Fluor 568 antibody (Invitrogen cod. A11011) 1:500 in PBS 1x. The coverslips were mounted in Mowiol 4-88 (Calbiochem, CA) on glass slides. Immunofluorescence studies were performed by using a Carl Zeiss LSM700 confocal laser-scanning microscope. Fluorescence images were acquired by using a laser line at 555 nm and Plan-NeoFluar 63x oil objective.

2.5. RNA extraction and real-time RT-PCR analysis

RNA extraction procedures were performed as described in Amoroso et al. (2012). The following primers were used for PCR analysis. TRAP1: forward: 5'-GACGCACCGCTCAACAT-3', reverse: 5'-CACATCAAACATGGACGGTTT-3'; GAPDH: forward: 5'-AGGCTGAGAACGGGAAGC-3', reverse: 5'-CCATGGTGGTGAAGACGC-3'; p70S6K: forward: 5'-ACTTCTGGCTCGAAAGGTGG-3', reverse: 5'-TTGAGTCATCTGGGCTGTCG-3'; RSK1: forward: 5'-CTCATGGAGCTAGTGCCTCT-3', reverse: 5'-TCCCCTGAGGTCTGTCCATT-3'; 18S rRNA: forward: 5'-GGCGCCCCCTCGATGCTCTTA-3', reverse: 5'-GCTCGGGCCTGCTTTGAACAC-3'; *in vitro* synthetized M7 RNA: forward: 5'-GGCGAATTGGGCCCGACGTC-3', reverse: 5'-TGGGCTTCACGATCTTGGCG-3'. Primers were designed to be intron-spanning. The reaction conditions were 95 °C for 5 min followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. GAPDH was chosen as the internal control.

2.6. Dual luciferase reporter assay

HCT116 cells were transfected using Polyfect transfection reagent (Qiagen) with the dual reporter vector pLPL Cap-Renilla-IRES-Luciferase (Ben-Hur et al., 2013). Cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (Firefly luciferase activity) were measured with the Promega Stop and Glo assay kit according to the manufacturer's instructions.

2.7. Ribosome analysis

In order to separate cytoplasmic extracts, HCT116 or HEK293 cells were collected by scraping and then resuspended in lysis buffer (10 mM Tris–HCl pH 7.5 10 mM NaCl and 10 mM MgCl2, 0.5% NP-40, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatinA and 100 mg/mL PMSF). After incubation in ice for 10 min, the extract was centrifuged for 10 min in a microcentrifuge at a maximum speed of 4 °C with the supernatant (cytoplasmic extract) loaded onto 15–50% linear sucrose gradient containing 30 mM Tris–HCl (pH 7.5), 100 mM NaCl and 10 mM MgCl2. Gradients were centrifuged in a Beckman SW 41 rotor for 110 min at 37000 rpm, then collected while monitoring the absorbance at 260 nm. In the case of protein analysis (experiment shown in Figure 1A) 1 mL 70% sucrose cushion was added to the bottom of the gradient and collected as the first of 12 fractions. All fractions were then precipitated with trichloroacetic acid, resuspended in loading buffer and analyzed by western blot. The percentage of polysomes has

been calculated by quantifying the amount of 18S rRNA by qPCR (experiments shown in Figure 1B) or on the basis of the intensities of RPS19 signals (experiment shown in Supplementary Figure 1). In order to normalize 18S rRNA quantification, a known amount of an M7 *in vitro* synthesized RNA has been added to each fraction at the time of collection of sucrose gradients and used as a control in qPCR experiments.

2.8. Patients

Tumor and normal, non-infiltrated peritumoral mucosa were obtained from 34 patients with CRC during surgical removal of the neoplasm (Amoroso et al., 2012). Samples were divided into 125 mm^3 pieces: one specimen was fixed in formalin and used for the histopathological diagnosis, while the others were immediately frozen in liquid nitrogen and stored at −80 °C for immunoblot analysis. Express written informed consent to use biological specimens for investigational procedures was obtained from all patients. In order to compare levels of TRAP1, p70S6K and phospho-p70S6K in different tumor specimens, protein levels were quantified by densitometric analysis using the Quantity One 4.5.0 software (BioRad Laboratories GmbH, Segrate, Italy) and expressed as time increase/decrease in tumors compared to the levels in the respective peritumoral non-infiltrated mucosa (Supplementary Table 1). TRAP1 expression levels were regarded as being up-regulated if they had increased at least threefold in comparison to the corresponding non-infiltrated peritumoral mucosa, whereas p70S6K were regarded as down-regulated if they had reduced at least ≤0.5 times compared to the corresponding non-infiltrated peritumoral mucosa.

2.9. Statistical analysis

The χ^2-Test was used to establish statistical correlation between levels of TRAP1 and p70S6K or phospho-p70S6K in human CRCs. The paired Student *T* test was used to establish the statistical significance between different levels of gene expression and relative Luciferase activity in sh-TRAP1 cells compared with related scramble controls.

2.10. Time-lapse microscopy and image acquisition

Images from different samples have been acquired by using the Zeiss Cell Observer system, as reported in Sepe et al. (2013). In brief, the system is equipped with phase contrast optics and provides an incubator chamber to control the temperature (maintained at 37 °C) and CO_2 percentage (maintained at 5%) for extended observation of living cells. Within this work, digital frames were acquired as 16 bit images of 650 × 514 pixels.

2.11. Wound healing assay

In order to study the dynamics of wound closure, cells were seeded in monolayer by plating in 12-well plates 200,000 cells/well in complete medium; 24 h after plating the cell layer was scratched with sterile pipette tip. The wound healing process was followed for 24 h by acquiring digital frames at 10 min intervals with an objective 10x (scale 0.767 pixel/μ). Ribavirin (100 μg/mL) or 4EGI-1 (25 μM), were used to pre-treat cells for 16 or 1 h respectively. Quantitative analysis of wound invasion by cell populations located at the border was performed by measuring the gap area at 2 h intervals for 16 h. The gap area was defined by using the wand tool in ImageJ (National Institute of Health, USA) and manually refining the selection in the presence of gross errors. Linear edge advancement was evaluated as the ratio between the variation in the a-cellular area within a time unit (2 h) and the length of the wound edge.

3. Results

3.1. TRAP1 silencing increases the rate of protein synthesis

Our previous data demonstrated that TRAP1 silencing concomitantly increases mRNA translation rate and co-translational ubiquitination/degradation of nascent proteins. Consistently, we demonstrated for the first time that TRAP1 is associated to ribosomes (Matassa et al., 2013). To further characterize the association of TRAP1 with ribosomes, we separated cytoplasmic extracts from HCT116 cells by ultracentrifugation on sucrose gradients. Fractions from the gradient were collected and analyzed by western blot (Figure 1A). Results show that part of TRAP1 co-sediments with translationally active polyribosomal particles, thus supporting the role of TRAP1 in mRNA translation. Further evidence for the involvement of TRAP1 in protein synthesis was obtained by the analysis of polysome profiles after depletion of TRAP1 by RNA interference (sh-TRAP1). As shown in Figure 1B, inhibition of TRAP1 expression in both HCT116 and HEK293 cells causes an increase in the amount of active polysomes in the cell, thereby indicating that the rate of global protein synthesis is inversely correlated to TRAP1 expression. Moreover, western blot analysis of fractions collected by HCT116 cells expressing control (scramble) or TRAP1-specific sh-RNA shows that the residual TRAP1 protein present in the cell is still associated to active polysomes and that the distribution of a ribosomal associated factor, such as poly(A)-binding protein 1 (PABP1), does not change upon TRAP1 silencing (Supplementary Figure 1).

Translation control is a complicated regulatory process, which involves a sophisticated and intertwined regulation of cap/IRES-dependent translational mechanisms and modulates the expression of many proteins that are crucial in the regulation of cell physiology. In this scenario, the phosphorylation of eIF2α, known to be a key regulator of cap-dependent translation, serves to fine-tune the translation efficiency of different mRNA subsets, with the potential to allow continued translation of IRES-containing mRNAs in the presence of cellular stress that reduces cap-dependent translation (Spriggs et al., 2008). Since we have previously shown a positive correlation between TRAP1 expression and eIF2α phosphorylation (Matassa et al., 2013), in order to elucidate further the mechanisms involved in the translational

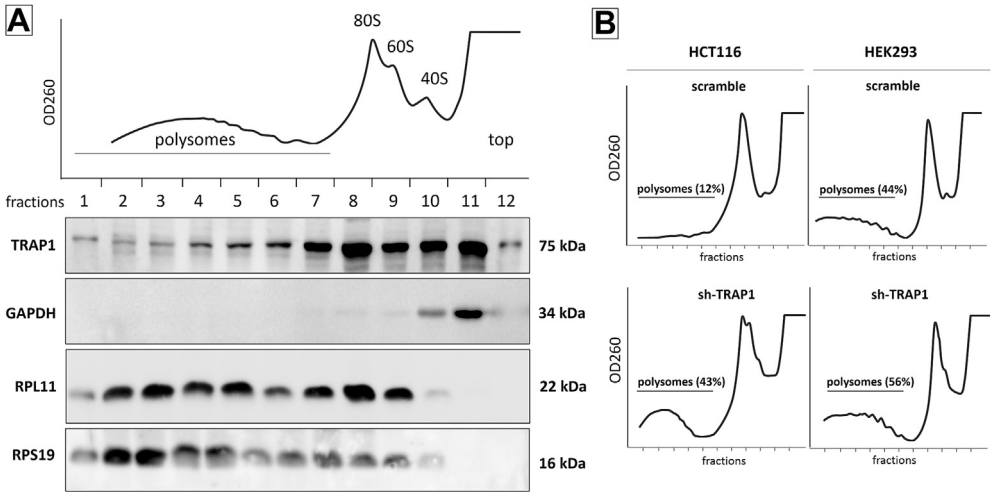

Figure 1 – TRAP1 co-sediments with polysomes and regulates protein synthesis. A) Separation of cytoplasmic extracts from HCT116 cells was performed by ultracentrifugation on sucrose gradients as described in Materials and methods. Proteins from the fractions were analyzed by western blot with the indicated antibodies. The absorbance profile in the upper panel indicates the sedimentation of the particles: fractions 1 to 7 polysomes; fractions 8 to 10 monomer (80S) and ribosomal subunits (60S, 40S); fractions 11 and 12 free cytosolic proteins or light complexes. B) Absorbance profiles, as in A, of control (scramble) and TRAP1-depleted (sh-TRAP1) HCT116 and HEK293 cells. The percentage of polysomes (indicated in the absorbance profiles) is calculated by quantifying the amount of 18S rRNA by qPCR (see Materials and methods for details).

attenuation by TRAP1, we measured the ratio between IRES- and cap-mediated translation in different experimental conditions by transfecting a dual reporter Cap-Renilla-IRES-Luciferase vector (Ben-Hur et al., 2013): two translation mechanisms from the same transcript were evaluated by assaying the luciferase activity (Figure 2A). Ratio between IRES- and cap-mediated translation in each experimental condition was calculated assuming mean level of respective control cells (scramble) equal 1. Results show that the ratio between IRES and cap-mediated translation is lower in TRAP1 KD cells, both under basal condition or upon translational stress induced by the antitumoral drug Ribavirin or the ER stress-inducer Thapsigargin. As a control, cells were treated with Cycloheximide, a known inhibitor of translation. These results clearly point to TRAP1 involvement in the attenuation of cap-dependent translation. Consistently, Figure 2B shows

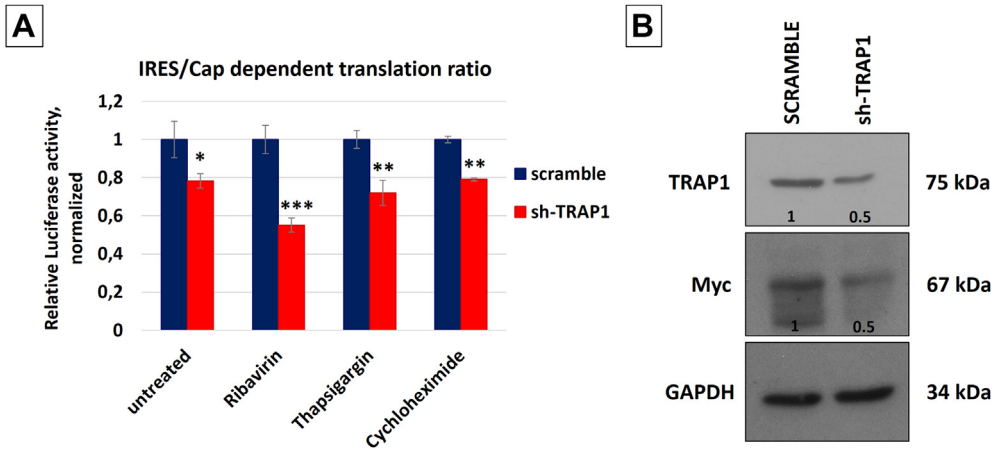

Figure 2 – TRAP1 silencing decreases ratio between IRES- and cap-dependent translation. A) HCT116 sh-TRAP1 and scramble stable clones were transfected with pLPL Cap-Renilla-IRES-Luciferase bicistronic dual reporter vector. As indicated, cells were treated with Ribavirin (100 μg/mL) for 16 h, or with Thapsigargin (1 μM) or Cycloheximide (200 μg/mL) for 6 h. Cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (Firefly luciferase activity) were measured in a dual Luciferase reporter assay (see Materials and methods for details) 24 h after transfection. Graphs represents ratio between IRES- and cap-mediated translation calculated assuming mean level of respective control cells (scramble) equal 1. All data are expressed as mean ± S.D. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. B) c-Myc expression levels verified by western blot in HCT116 stable clones. Total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective GAPDH band, which have been calculated by assuming protein levels of the control (scramble) equal 1.

that the expression levels of the c-Myc protein, an oncogene whose mRNA contains an IRES (Spriggs et al., 2009), are higher in control HCT116 cells than in the sh-TRAP1 counterpart.

3.2. *Signaling pathways regulated by TRAP1 in the attenuation of translation*

We then studied the molecular pathways modulated by TRAP1 involved in protein synthesis attenuation. RSK1 and p70S6K are translation regulatory kinases responsible for phosphorylation of rpS6 and translation initiation factors, involved in the activation of cap-mediated translation. The expression levels of both enzymes were analyzed in TRAP1 KD cells vs controls in two different cell lines: HCT116 and HEK293. As shown in Figure 3A these enzymes are, indeed, hyper-expressed in sh-TRAP1 cells compared to their scramble controls. Interestingly, and likely as a consequence of their increased expression, p70S6K and RSK1 show higher phosphorylation levels in TRAP1 KD cells compared to controls (Figure 3A–B). Remarkably, TRAP1 expression/function

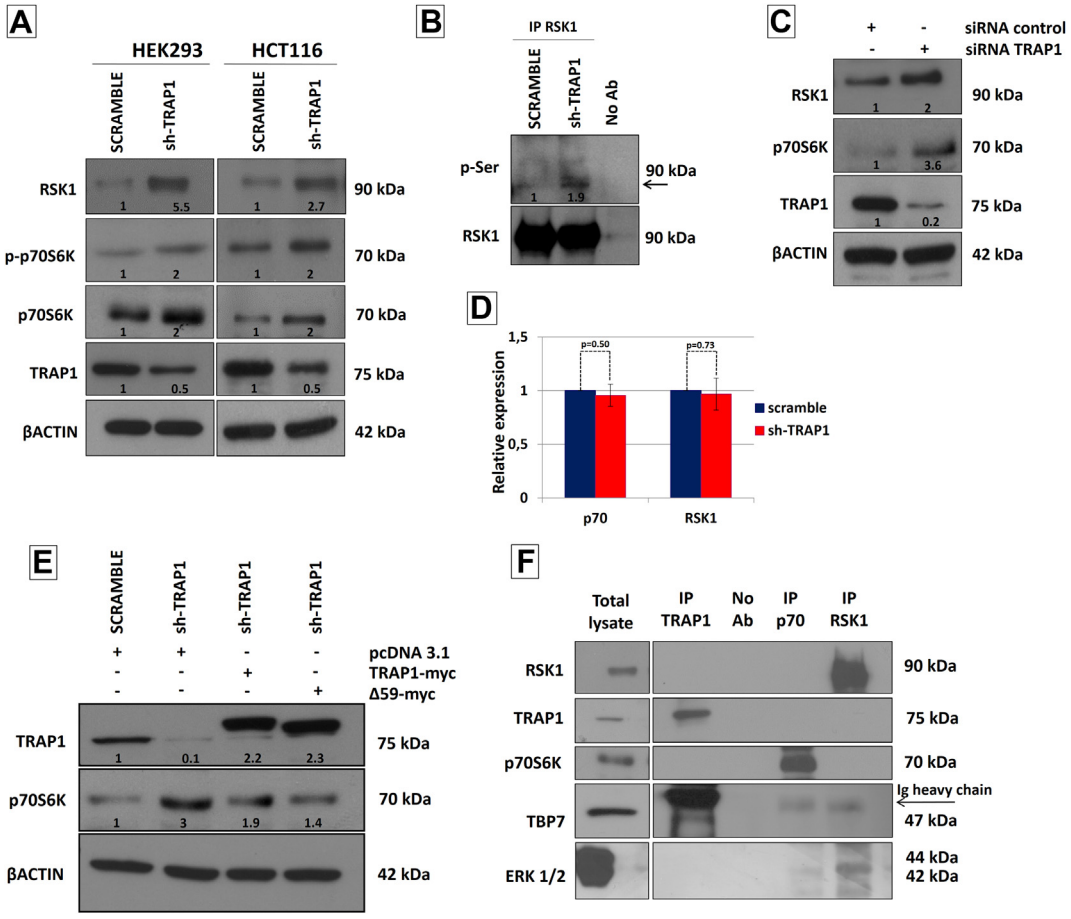

Figure 3 – TRAP1 silencing upregulates the p70S6K/RSK1 pathways. A) HCT116 and HEK293 stable clones total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective Actin band, which have been calculated by assuming protein levels of the control (scramble) equal 1. B) Scramble and sh-TRAP1 HCT116 were immunoprecipitated with anti-RSK1 and immunoblotted with anti-phosphoSerine antibodies. Numbers indicate densitometric band intensities, each normalized to the respective total RSK1 immunoprecipitated, which have been calculated by assuming protein levels of the control (scramble) equal 1. No Ab, total cellular extracts incubated with A/G plus agarose beads without antibody; IP, immunoprecipitation with the corresponding antibodies. C) HCT116 cells were transfected with non-targeted control siRNA or TRAP1-directed siRNA. 48 h after transfection, total lysates were harvested, separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective Actin band, which have been calculated by assuming protein levels of the control equal 1. D) Real-time RT-PCR analysis of p70S6K and RSK1 mRNAs expression in HCT116 sh-TRAP1 and scramble cells. All data are expressed as mean ± S.D. from three independent experiments. The *p*-values indicate the statistical significance between relative expression levels. E) sh-TRAP1 and scramble HEK293 cells were transfected with TRAP1-myc and Δ1-59-myc expression vectors (pcDNA 3.1 vector was used as control). Total cell lysates were harvested after 24 h from transfection, separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective Actin band, which have been calculated by assuming protein levels of the control (scramble) equal 1. F) Total HCT116 lysates were immunoprecipitated with anti-TRAP1, anti-p70S6K and anti-RSK1 antibodies and immunoblotted with indicated antibodies. Anti-TBP7 and anti-ERK1/2 were used as positive controls of co-immunoprecipitation. Arrow indicates immunoglobulin heavy chains. No Ab, total cellular extracts incubated with A/G plus agarose beads without antibody; IP, immunoprecipitation with the corresponding antibodies.

is important for S6 kinases (S6Ks) regulation: in fact, transient down-regulation of TRAP1 expression upon siRNA transfection yielded an increase of p70S6K and RSK1 protein levels (Figure 3C), findings that demonstrate a causal role of TRAP1 in the modulation of p70S6K/RSK1 expression. Subsequently qPCR experiments were performed to evaluate whether the different expression levels of both kinases are due to a transcriptional or post-transcriptional regulation. Results showed no differences in their mRNA levels (Figure 3D), thus allowing us to conclude that regulation of p70S6K and RSK1 expression occurs at post-transcriptional levels.

Furthermore, the transfection in sh-TRAP-1 HEK293 cells of constructs expressing either a full-length TRAP1 or TRAP1 deletion mutant (Δ1-59) (Amoroso et al., 2012), lacking the mitochondrial targeting sequence and therefore unable to enter in mitochondria, is sufficient to recapitulate p70S6K protein levels (Figure 3E). While further confirming the causal role of TRAP1 in the regulation of p70S6K protein expression/activity, these results demonstrate that regulation of protein translation by TRAP1 occurs in an extramitochondrial compartment.

Co-immunoprecipitation experiments were performed to evaluate whether this regulation is due to a direct interaction between TRAP1 and the kinases. Data in Figure 3F allow us to conclude that there is no direct binding between TRAP1 and p70S6K and/or RSK1, whereas the previously well-characterized interaction between TRAP1 and TBP7 (Amoroso et al., 2012) and between RSK1 and ERK1/2 (Roux et al., 2003), used as positive controls of these experiments, could easily be detected. This observation further supports the hypothesis that TRAP1 regulation on S6Ks depends on indirect modulation of protein translation.

Key downstream effectors of S6Ks signaling in protein synthesis regulation include several proteins involved in the regulation of cell survival upon different stimuli and some translation factors. Among others, S6Ks have been shown to impact on the initiation step of translation by phosphorylating the cap binding complex component eIF4B at serine 422 (Raught et al., 2004).

Accordingly, we analyzed phosphorylation levels of the main translation initiation factors. As represented in Figure 4A, initiation factors eIF4G, eIF4B and eIF4E show higher phosphorylation levels in HCT116 CRC cells in which TRAP1 has been down-regulated by sh-RNA stable transfection or by transient transfection of siRNAs, whereas their expression levels are unchanged.

Indeed, the fact that TRAP1 is associated to members of cell translational apparatus has already been suggested by our group, since we previously validated an interaction between TRAP1 and the eukaryotic Elongation Factor 1A (eEF1A, Matassa et al., 2013). However, this study was only at an early phase: we demonstrated, in fact, that TRAP1 regulates the rate of protein synthesis through the eIF2α pathway, favoring the activation of GCN2 and PERK kinases, with consequent phosphorylation of eIF2α and attenuation of cap-dependent translation (Matassa et al., 2013). Furthermore, specific interaction between TRAP1 and GCN2 by co-IP experiments and confocal microscopy was also identified (Matassa et al., 2013). Taking advantage of previous reports demonstrating that eEF1A is implicated in the regulation of GCN2 *in vitro* by binding its

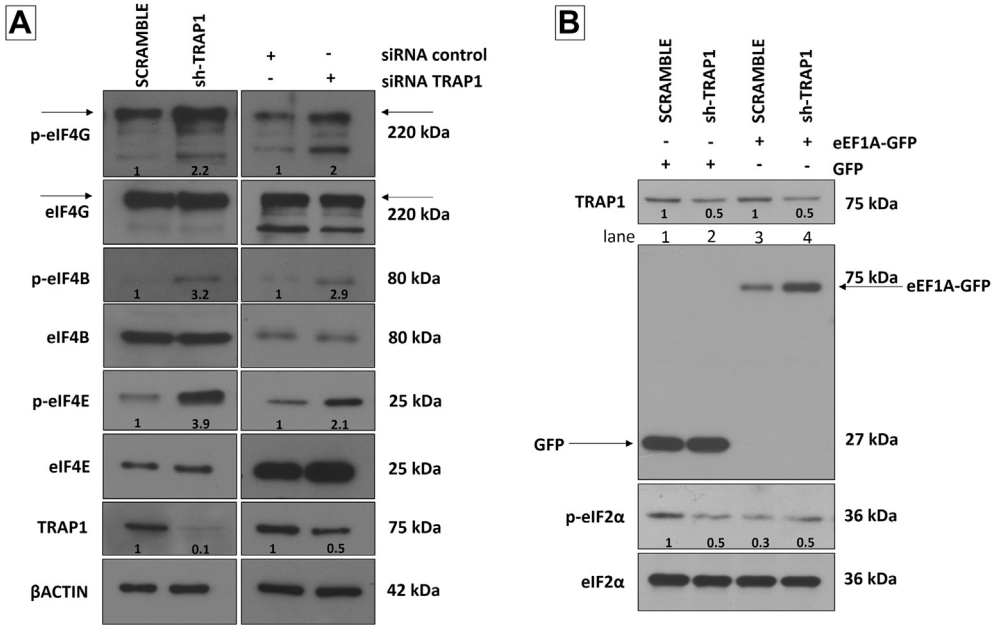

Figure 4 – TRAP1 silencing enhances translation initiation. A) Total extracts were obtained from scramble and sh-TRAP1 HCT116 stable clones and from HCT116 cells transfected with non-targeted control siRNA or TRAP1-directed siRNA for 48 h. Total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective non-phosphorylated protein band, which have been calculated by assuming protein levels of the control (scramble) equal 1. B) HCT116 sh-TRAP1 and scramble stable clones were transfected with eEF1A-GFP or GFP expression vectors. 24 h after transfection, total lysate were harvested and immunoblotted with indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective eIF2α band, which have been calculated by assuming protein levels of the control (GFP-transfected scramble) equal 1.

C-terminus region and inhibiting its ability to phosphorylate its substrate eIF2α (Visweswaraiah et al., 2011), we hypothesized that TRAP1 control on GCN2-mediated eIF2α phosphorylation might involve eEF1A-GCN2 interaction. To this aim, when an eEF1A expression vector was transfected in scramble and TRAP1 KD cells, we consistently found that eIF2α phosphorylation is decreased upon eEF1A transfection only in control cells (compare lanes 1 and 3 in Figure 4B), in which eEF1A-mediated inhibition of GCN2 is normally attenuated by TRAP1. This result demonstrates that cells expressing high levels of TRAP1 are able to modulate the inhibitory effect of eEF1A towards GCN2. A model to comment these data is shown in Supplementary Figure 2.

3.3. *TRAP1 involvement in protein synthesis affects response to translational stress and cell migration*

The role of TRAP1 in the protection against several stress types has been extensively described (reviewed in Matassa et al., 2012). However, few data are available on the role of TRAP1 in the protection against the translational stress. To this aim, we treated cells with the ER-stress inducer Thapsigargin to survey stress granules (SGs) formation in scramble vs sh-TRAP1 cells. As shown in Figure 5, Thapsigargin treatment induces SGs in sh-TRAP1 cells.

Few studies suggest an involvement of TRAP1 in the regulation of the motile behavior of cancer cells (reviewed in Rasola et al., 2014). We analyzed the migratory potential of scramble and sh-TRAP1 HEK293 cells in the presence/absence of Ribavirin (Kentsis et al., 2004) and 4EGI-1 (Moerke et al., 2007), two well-known inhibitors of cap-mediated translation, in a wound healing assay. Cells were followed in time lapse experiments for 24 h after wound injury; snapshots were taken at 10 min intervals. Figure 6A includes a selection of significant frames of scramble and sh-TRAP1 HEK293, respectively treated with Ribavirin (100 μg/mL). Edge progression in TRAP1 KD cells at 12 and 16 h is less pronounced and wound closure at 24 h is not as good as in control cells. Quantitative analysis is shown in panel C as linear progression (left) and rate of advancement (right) of the wound edge during time. Linear progression, as expected, increases in time as long as the wound is open and is higher for scramble cells than sh-TRAP1 ones; the rate of edge advancement of sh-TRAP-1 HEK293 is consistently lower than scramble cells for most of the observation time, and drops at the end, when the effect of wound closure becomes predominant. The same effect is observed when the analysis is done by using 4EGI-1 (25 μM) (Panel D). The described pro-migratory effect of TRAP1 on cell migration is dependent on the addition of translation inhibitory drugs and is not easily observable in untreated cultures. In fact, in the reported experiments carried out on untreated cells, TRAP1 interfered cells are as fast as or even faster (panel B) than controls and completely fill the gap within 16 h; edge advancement becomes higher than controls after the scratch and stays higher for several hours, until it is reduced when the wound starts to close (panel E). The faster movement of TRAP1 interfered cells might be related to higher p70S6K levels in TRAP1 KD than in control cells, as reported in 3.2. Taken together, the data reveal a role of TRAP1 in counteracting the anti-migratory effect of translation inhibitory drugs.

3.4. *The role of TRAP1 in protein synthesis is relevant in cancer*

Finally, we evaluated whether TRAP1 involvement in the regulation of the protein synthesis activation pathway may be relevant in human CRCs. To this aim, we analyzed our tissue

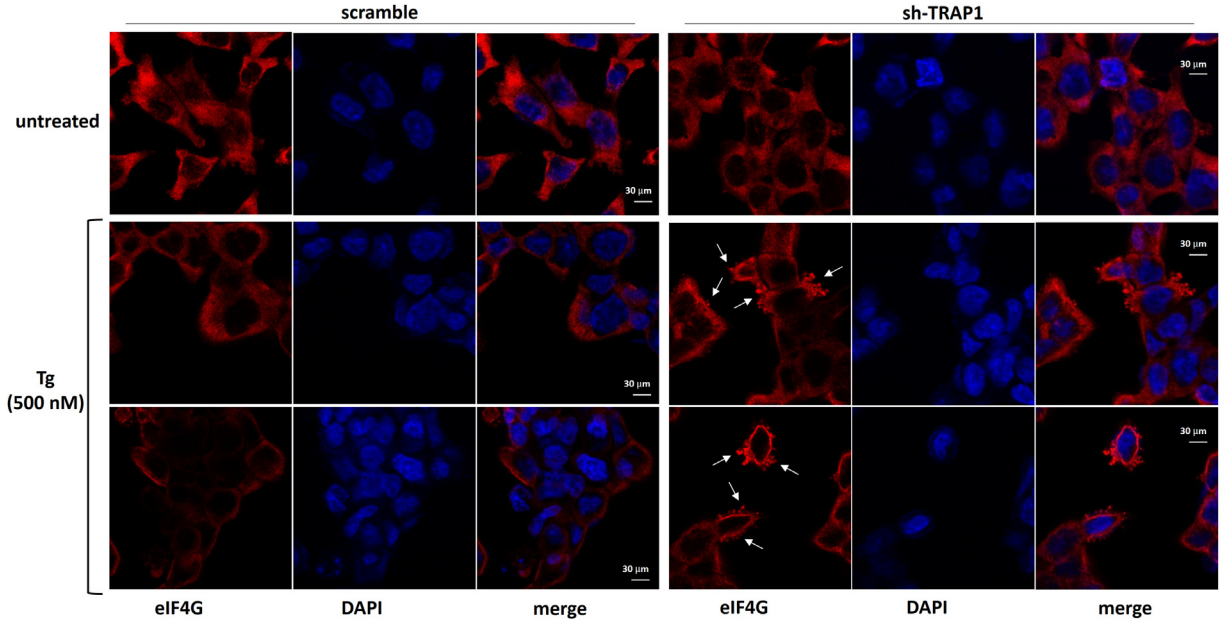

Figure 5 – TRAP1 silencing sensitizes cells to translational stress. Scrambled and sh-TRAP1 HEK293 cells were treated with Thapsigargin (500 nM) for 50 min. Stress granules were analyzed using rabbit monoclonal anti-eIF4G antibody and goat anti-rabbit Alexa Fluor 568. DAPI staining is also shown to detect nuclei.

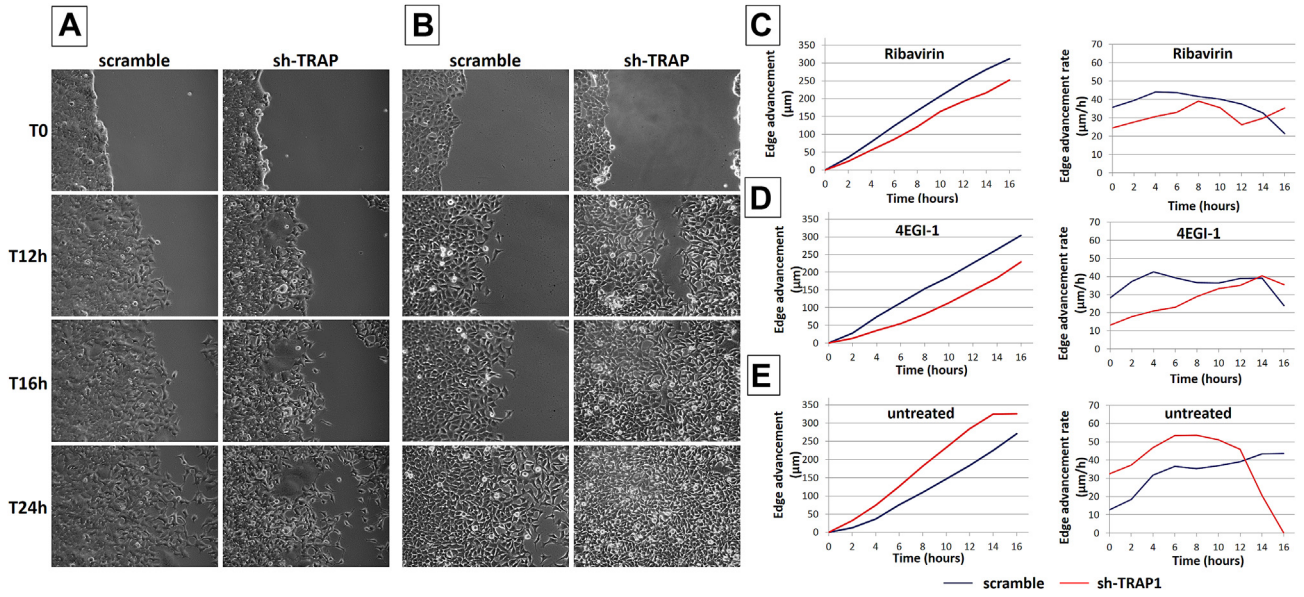

Figure 6 – TRAP1 affects cell migration. Wound healing assay with scrambled and sh-TRAP1 HEK293 cells. A, B) Time-lapse acquisition of Ribavirin-treated (A) and untreated (B) cells immediately after the wound (T0), and after 12, 16 and 24 h (T12 h, T16 h and T24 h). C, D, E) Wound closure, expressed as linear progression (left) and rate of advancement (right) during time (see Materials and methods), of scramble (blue) and sh-TRAP1 (red) HEK293 cells upon treatment with 100 µg/mL Ribavirin (C), 25 µM 4EGI-1 (D) or under control conditions (E).

collection of CRCs, previously characterized for the co-expression of TRAP1 and several of its client proteins (Amoroso et al., 2012; Matassa et al., 2013). We analyzed 34 human colon malignancies for TRAP1 and p70S6K expression by immunoblot analysis, with 17 TRAP1-upregulated and 17 TRAP1-non upregulated. Figure 7 reports the immunoblot analysis of these proteins in 8 tumor samples representative of our tumor analysis, with detailed results of the densitometric analysis reported in Supplementary Table 1. Remarkably, the majority of TRAP1-upregulated tumors exhibited the downregulation of p70S6K (12/17 cases), as confirmed by the Chi-square test ($p = 0.04$). Phosphorylation levels were also assessed, with similar results, as reported in Figure 7 and Supplementary Table 1. By contrast, tumors with non upregulated TRAP1 levels showed stable or up-regulated levels of p70S6K and phospho-p70S6K. Taken together, these observations suggest that TRAP1-dependent regulation of p70S6K and, likely, its downstream pathway is conserved in human colorectal tumors with high TRAP1 expression. It is likely that different and TRAP1-independent molecular mechanisms may contribute to p70S6K regulation in colorectal tumors with TRAP1 levels undistinguishable from or above peritumoral mucosa.

4. Discussion

The translational control is a key regulatory principle, modulating the expression of many proteins that are crucial in cell physiology. In fact, several data of current research focus on this process as a novel therapeutic target and promising concept in the treatment of human diseases, including cancer (reviewed in Ruggero, 2013).

In the present article, some molecular pathways involved in the attenuation of protein synthesis by TRAP1 in cancer cells and tissues are identified and characterized. Remarkably, aberrant deregulation of TRAP1 function has been demonstrated in several cancer types (Landriscina et al., 2010b; Leav et al., 2010). Previous studies by our group identified novel roles of TRAP1 in protection from several stresses and apoptosis, both in mitochondrial and extramitochondrial compartments, and in the quality control of mitochondria-destined proteins through an attenuation of protein synthesis (Matassa et al., 2013; Maddalena et al., 2013).

Translation “on demand” is a new concept proposed in several recent studies to characterize the responses of tumor cells in different biological phenotypes (Brockmann et al., 2007). This scenario involves, among others, a sophisticate and intertwined regulation of cap/IRES-dependent translational control, allowing for continued translation in the presence of cellular stresses that reduce cap-dependent translation (Spriggs et al., 2008). In the present study, we show that TRAP1 is involved in the attenuation of cap-dependent translation, suggesting that this translational control mechanism would provide a survival advantage to cancer cells, expanding indefinitely their growth even under unfavorable conditions. Indeed, we show a change in the balance between cap and IRES dependent translation in the presence of TRAP1, leading to an attenuation of cap-dependent translation, favoring IRES-dependent one. This mechanism is relevant in cancer development, because among 70 experimentally verified cellular IRES elements (Mokrejs et al., 2010), a large number are found in cancer-related genes (Holcík, 2004). Interestingly, we show that in low TRAP1 background decreased expression of some IRES-containing proteins/oncogenes, such as c-Myc, is observed,

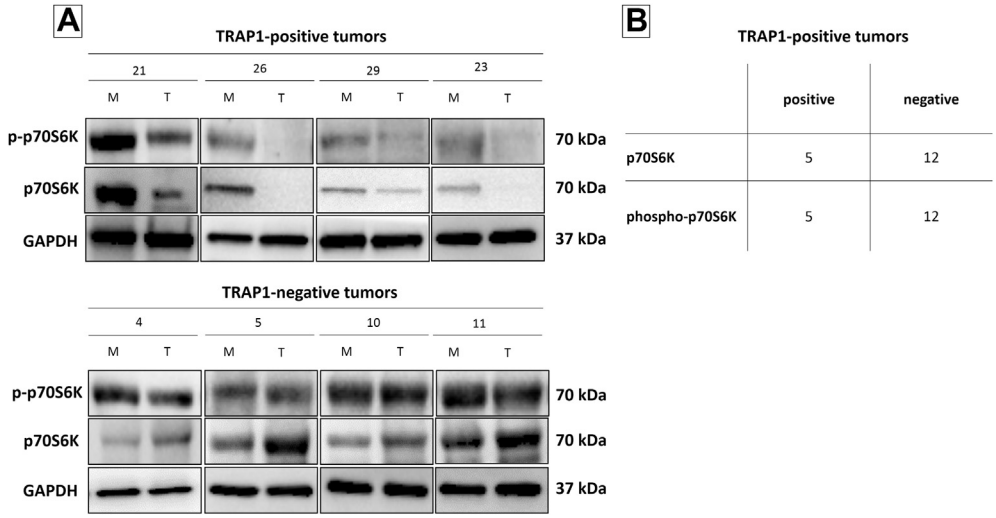

Figure 7 – TRAP1-dependent regulation of p70S6K is conserved in human CRCs. A) Total cell lysates from 8 human CRCs (T) and the respective non-infiltrated peritumoral mucosa (M) (4 TRAP1-upregulated and 4 TRAP1-non upregulated tumors) were separated by SDS-PAGE and immunoblotted using indicated antibodies. B) Distribution of CRCs according to TRAP1, p70S6K and phospho-p70S6K levels.

findings that suggest a further potential mechanism of TRAP1-driven tumorigenesis beyond those previously described (Sciacovelli et al., 2013).

The efficient biogenesis and maturation of functional proteins is critical for cell viability. Indeed, eukaryotic cells have evolved a complex machinery of ribosome-bound chaperones that interacts with and facilitates the folding of nascent polypeptides (Hartl et al., 2011), suggesting that the ribosome serves as a hub for co-translational folding, thus playing a major role in protein homeostasis. Remarkably, we recently demonstrated that TRAP1 is associated to ribosomes (Matassa et al., 2013) and in the present study we further characterize its function within the ribosomal compartments. Recent studies have revealed that high rates of translation elongation negatively affect both the fidelity of translation and the co-translational folding of nascent polypeptides. In fact, by slowing down translation, cancer cells can efficiently improve the correct folding of proteins relevant for tumorigenesis (Sherman and Qian, 2013). Consistently with the TRAP1 role in proteostasis, polysomal profiling of TRAP1 KD HCT116 cells shows a higher ratio of active polysomes than control cells, thus indicating that the rate of global protein synthesis is inversely correlated to TRAP1 expression. Therefore, TRAP1 induction may likely represent a protective mechanism used by cancer cells to regulate selectively the synthesis of specific stress-protective proteins and facilitate their correct folding.

Starting from these findings, we became interested in identifying and characterizing new regulatory pathways of protein synthesis in cancer systems controlled by TRAP1, which incidentally is considered a novel biomarker in several cancer types (Landriscina et al., 2010a, 2010b; Kang et al., 2010). Our previous studies on TRAP1 translational control only partially led to a molecular characterization of the signaling cascades regulated by TRAP1 in extramitochondrial compartments. The data in this study show that expression and consequent phosphorylation of p70S6K and RSK1, two translation activating kinases, are increased in TRAP1 KD cells and that the regulation of p70S6K and RSK1 expression occurs at post-transcriptional levels. Several mechanisms can be responsible for S6Ks regulation of expression at translation level: the presence of a 5'-terminal oligopyrimidine tract (Meyuhas, 2000) has been found in mRNAs of some members of translational apparatus, controlling their selective translation. Moreover, microRNAs (miRNAs) inhibit protein synthesis by actively repressing translation (reviewed in Fabian et al., 2010). Interestingly, it has been recently shown that such translational inhibition depends on miRNAs impairing the function of the eIF4F initiation complex (Meijer et al., 2013), whose components are TRAP1 partners (Matassa et al., 2013). Future experiments are aimed at testing the involvement of such mechanisms in p70S6K and RSK1 regulation. Notably, to our knowledge, this is the first report of a role of TRAP1 in the modulation of S6Ks activity in the attenuation of translation.

S6Ks have been shown to accelerate the initiation step of translation by phosphorylating the cap binding complex component eIF4B at serine 422 (Raught et al., 2004). Consistently, we show that phosphorylation levels of translation initiation factors, namely eIF4G, eIF4B and eIF4E, are higher in colorectal cancer cells upon TRAP1 knock down, thus indicating a condition of improved cap-dependent translation. The working hypothesis addressed in this study is summarized in the model shown in Figure 8: TRAP1 is bound to actively translating ribosomes and is involved in protein synthesis regulation through p70S6K activation pathway. Upon TRAP1 silencing, p70S6K is overexpressed and consequently phosphorylated, resulting in stronger activation of initiation factors and increased mRNA translation rate. Remarkably, we demonstrate that TRAP1 regulatory mechanisms are conserved in colorectal cancer tissues, since the majority of CRCs with TRAP1 overexpression showed attenuation of p70S6K expression/activity, thus supporting the relevant role

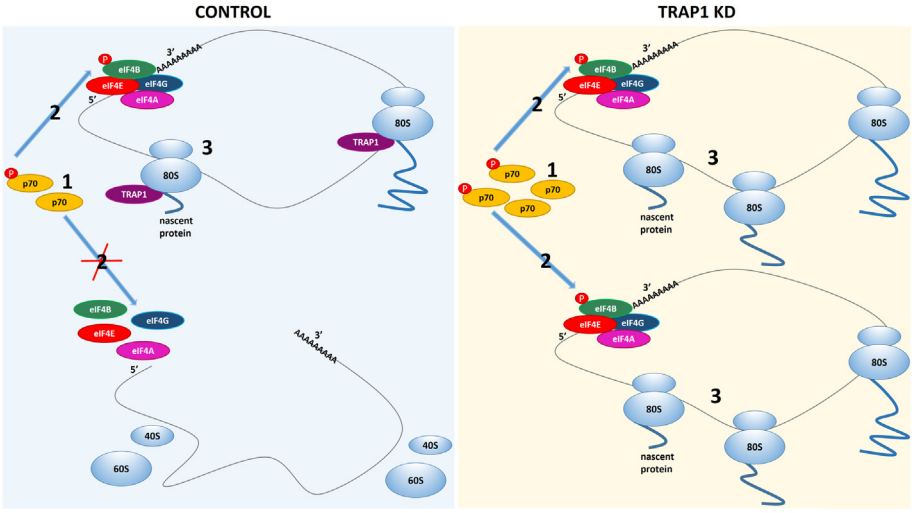

Figure 8 – TRAP1 is bound to actively translating ribosomes and is involved in protein synthesis regulation through the p70S6K activation pathway. Upon TRAP1 silencing (TRAP1 KD), p70S6K is overexpressed and consequently hyperphosphorylated (step 1). Active p70S6K phosphorylates IF4B (step 2), thus favoring translation initiation complex assembly. This results in increased mRNA translation rate (step 3). TRAP1-expressing cells (control) show lower level of p70S6K, reduced activation of translation initiation complex (step 2) and overall protein synthesis (step 3).

of TRAP1 translational regulation *in vivo*. Significantly, constitutive activation of p70S6K is found more often in malignant ovarian tumors than in normal or benign tissues, suggesting a critical role for p70S6K in ovarian tumorigenesis (Pon et al., 2008). Intriguingly, an inverse correlation in a subset of ovarian cancers was found between TRAP1 expression and overall survival. These findings, albeit in apparent contradiction, provide a further demonstration of the complexity and peculiarity of this tumor type, as well as the complexity of TRAP1-regulatory mechanisms, and confirm previous data indicating a predictive role for TRAP1 in ovarian models (Aust et al., 2012).

Finally, a new finding in this article is that TRAP1 involvement in protein synthesis affects the response to translational stress and cell migration in wound healing assays, both processes in which TRAP1-regulated S6 kinases are involved (Ip et al., 2011; Smolen et al., 2010). The different effect observed in the presence and absence of the drug could be explained by postulating that TRAP1 might influence global mRNA translation and favor the synthesis of pro-motion molecules, thus exerting a "protective" effect under conditions where cell migration is impaired. Considering also its preferential expression within cells from tumors at advanced stages, TRAP1 could therefore become a good candidate as a marker of drug sensitivity and a potential target for enhancing the activity of anti-tumor agents, particularly those involved in translational control. This explanation would be coherent with a similar "protective" effect of TRAP1 on cell survival under Ribavirin or 4EGI-1 treatment, as shown in a previous work (Matassa et al., 2013). Notably, the role of TRAP1 in promotility/metastatic phenotypes is still an open issue. In fact, it should be mentioned that opposite effects on cell migration/invasion on compromising TRAP1 function have been observed, likely reflecting the altered metabolic environment found in diverse tumor types examined under distinct conditions (reviewed in Rasola et al., 2014). Although all reports agree that TRAP1 has important implications for neoplastic progression, data from the different groups only partially overlap, suggesting that TRAP1 may have complex and possibly contextual effects on tumorigenesis (Rasola et al., 2014). All these features candidate TRAP1 as intriguing tumor biomarker to be further characterized in different environments and multiple tumor types.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.06.003.

REFERENCES

Amoroso, M.R., Matassa, D.S., Laudiero, G., Egorova, A.V., Polishchuk, R.S., Maddalena, F., Piscazzi, A., Paladino, S., Sarnataro, D., Garbi, C., Landriscina, M., Esposito, F., 2012.

TRAP1 and the proteasome regulatory particle TBP7/Rpt3 interact in the endoplasmic reticulum and control cellular ubiquitination of specific mitochondrial proteins. Cell Death Differ. 19 (4), 592–604.

Aust, S., Bachmayr-Heyda, A., Pateisky, P., Tong, D., Darb-Esfahani, S., Denkert, C., Chekerov, R., Sehouli, J., Mahner, S., Van Gorp, T., Vergote, I., Speiser, P., Horvat, R., Zeillinger, R., Pils, D., 2012. Role of TRAP1 and estrogen receptor alpha in patients with ovarian cancer-a study of the OVCAD consortium. Mol. Cancer 11, 69.

Ben-Hur, V., Denichenko, P., Siegfried, Z., Maimon, A., Krainer, A., Davidson, B., Karni, R., 2013. S6K1 alternative splicing modulates its oncogenic activity and regulates mTORC1. Cell Rep. 3 (1), 103–115.

Blagden, S.P., Willis, A.E., 2011. The biological and therapeutic relevance of mRNA translation in cancer. Nat. Rev. Clin. Oncol. 8, 280–291.

Brockmann, R., Beyer, A., Heinisch, J.J., Wilhelm, T., 2007. Posttranscriptional expression regulation: what determines translation rates? PLoS Comput. Biol. 3 (3), e57.

Chae, Y.C., Angelin, A., Lisanti, S., Kossenkov, A.V., Speicher, K.D., Wang, H., Powers, J.F., Tischler, A.S., Pacak, K., Fliedner, S., Michalek, R.D., Karoly, E.D., Wallace, D.C., Languino, L.R., Speicher, D.W., Altieri, D.C., 2013. Landscape of the mitochondrial Hsp90 metabolome in tumours. Nat. Commun. 4, 2139.

Chiocchetti, A., Gibello, L., Carando, A., Aspesi, A., Secco, P., Garelli, E., Loreni, F., Angelini, M., Biava, A., Dahl, N., Dianzani, U., Ramenghi, U., Santoro, C., Dianzani, I., 2005. Interactions between RPS19, mutated in Diamond-Blackfan anemia, and the PIM-1 oncoprotein. Haematologica 90 (11), 1453–1462.

Costantino, E., Maddalena, F., Calise, S., Piscazzi, A., Tirino, V., Fersini, A., Ambrosi, A., Neri, V., Esposito, F., Landriscina, M., 2009. TRAP1, a novel mitochondrial chaperone responsible for multi-drug resistance and protection from apoptotis in human colorectal carcinoma cells. Cancer Lett. 279 (1), 39–46.

Fabian, M.R., Sonenberg, N., Filipowicz, W., 2010. Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. 79, 351–379.

Gerlitz, G., Jagus, R., Elroy-Stein, O., 2002. Phosphorylation of initiation factor-2 alpha is required for activation of internal translation initiation during cell differentiation. Eur. J. Biochem. 269 (11), 2810–2819.

Hartl, F.U., Bracher, A., Hayer-Hartl, M., 2011. Molecular chaperones in protein folding and proteostasis. Nature 475, 324–332.

Holcík, M., 2004. Targeting translation for treatment of cancer–a novel role for IRES? Curr. Cancer Drug Targets 4 (3), 299–311.

Ip, C.K., Cheung, A.N., Ngan, H.Y., Wong, A.S., 2011. p70 S6 kinase in the control of actin cytoskeleton dynamics and directed migration of ovarian cancer cells. Oncogene 30 (21), 2420–2432.

Kang, B.H., Siegelin, M.D., Plescia, J., Raskett, C.M., Garlick, D.S., Dohi, T., Lian, J.B., Stein, G.S., Languino, L.R., Altieri, D.C., 2010. Preclinical characterization of mitochondria-targeted small molecule hsp90 inhibitors, gamitrinibs, in advanced prostate cancer. Clin. Cancer Res. 16 (19), 4779–4788.

Kang, B.H., 2012. TRAP1 regulation of mitochondrial life or death decision in cancer cells and mitochondria-targeted TRAP1 inhibitors. BMB Rep. 45 (1), 1–6.

Kentsis, A., Topisirovic, I., Culjkovic, B., Shao, L., Borden, K.L., 2004. Ribavirin suppresses eIF4 E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap. Proc. Natl. Acad. Sci. USA 101, 18105–18110.

Landriscina, M., Fabiano, A., Altamura, S., Bagalà, C., Piscazzi, A., Cassano, A., Spadafora, C., Giorgino, F., Barone, C., Cignarelli, M., 2005. Reverse transcriptase inhibitors down-regulate cell proliferation in vitro and in vivo and restore thyrotropin signaling and iodine uptake in human thyroid anaplastic carcinoma. J. Clin. Endocrinol. Metab. 90 (10), 5663–5671.

Landriscina, M., Laudiero, G., Maddalena, F., Amoroso, M.R., Piscazzi, A., Cozzolino, F., Monti, M., Garbi, C., Fersini, A., Pucci, P., Esposito, F., 2010a. Mitochondrial chaperone Trap1 and the calcium binding protein Sorcin interact and protect cells against apoptosis induced by antiblastic agents. Cancer Res. 70 (16), 6577–6586.

Landriscina, M., Amoroso, M.R., Piscazzi, A., Esposito, F., 2010b. Heat shock proteins, cell survival and drug resistance: the mitochondrial chaperone TRAP1, a potential novel target for ovarian cancer therapy. Gynecol. Oncol. 117 (2), 177–182.

Leav, I., Plescia, J., Goel, H.L., Li, J., Jiang, Z., Cohen, R.J., Languino, L.R., Altieri, D.C., 2010. Cytoprotective mitochondrial chaperone TRAP-1 as a novel molecular target in localized and metastatic prostate cancer. Am. J. Pathol. 176 (1), 393–401.

Maddalena, F., Laudiero, G., Piscazzi, A., Secondo, A., Scorziello, A., Lombardi, V., Matassa, D.S., Fersini, A., Neri, V., Esposito, F., Landriscina, M., 2011. Sorcin induces a drug-resistant phenotype in human colorectal cancer by modulating Ca^{2+} homeostasis. Cancer Res. 71, 7659–7669.

Maddalena, F., Sisinni, L., Lettini, G., Condelli, V., Matassa, D.S., Piscazzi, A., Amoroso, M.R., La Torre, G., Esposito, F., Landriscina, M., 2013. Resistance to paclitxel in breast carcinoma cells requires a quality control of mitochondrial antiapoptotic proteins by TRAP1. Mol. Oncol. 7 (5), 895–906.

Matassa, D.S., Amoroso, M.R., Maddalena, F., Landriscina, M., Esposito, F., 2012. New insights into TRAP1 pathway. Am. J. Cancer Res. 2 (2), 235–248.

Matassa, D.S., Amoroso, M.R., Agliarulo, I., Maddalena, F., Sisinni, L., Paladino, S., Romano, S., Romano, M.F., Sagar, V., Loreni, F., Landriscina, M., Esposito, F., 2013. Translational control in the stress adaptive response of cancer cells: a novel role for the heat shock protein TRAP1. Cell Death Dis. 4, e851. http://dx.doi.org/10.1038/cddis.2013.379.

Meijer, H.A., Kong, Y.W., Lu, W.T., Wilczynska, A., Spriggs, R.V., Robinson, S.W., Godfrey, J.D., Willis, A.E., Bushell, M., 2013. Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. Science 340 (6128), 82–85.

Meyuhas, O., 2000. Synthesis of the translational apparatus is regulated at the translational level. Eur. J. Biochem. 267 (21), 6321–6330.

Moerke, N.J., Aktas, H., Chen, H., Cantel, S., Reibarkh, M.Y., Fahmy, A., Gross, J.D., Degterev, A., Yuan, J., Chorev, M., Halperin, J.A., Wagner, G., 2007. Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G. Cell 128, 257–267.

Mokrejs, M., Masek, T., Vopálensky, V., Hlubucek, P., Delbos, P., Pospísek, M., 2010. IRESite–a tool for the examination of viral and cellular internal ribosome entry sites. Nucleic Acids Res. 38, D131–D136.

Montesano Gesualdi, N., Chirico, G., Pirozzi, G., Costantino, E., Landriscina, M., Esposito, F., 2007. Tumor necrosis factor-associated protein 1 (TRAP-1) protects cells from oxidative stress and apoptosis. Stress 10 (4), 342–350.

Pon, Y.L., Zhou, H.Y., Cheung, A.N., Ngan, H.Y., Wong, A.S., 2008. p70 S6 kinase promotes epithelial to mesenchymal transition through snail induction in ovarian cancer cells. Cancer Res. 68 (16), 6524–6532.

Rasola, A., Neckers, L., Picard, D., 2014. Mitochondrial oxidative phosphorylation TRAP(1)ped in tumor cells. Trends Cell Biol. http://dx.doi.org/10.1016/j.tcb.2014.03.005. Apr 11. pii: S0962–8924(14)00050-6, in press.

Raught, B., Peiretti, F., Gingras, A.C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., Hershey, J.W., 2004. Phosphorylation of eucaryotic translation

initiation factor 4B Ser422 is modulated by S6 kinases. EMBO J. 23 (8), 1761–1769.
Roux, P.P., Richards, S.A., Blenis, J., 2003. Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. Mol. Cell Biol. 23 (14), 4796–4804.
Ruggero, D., 2013. Translational control in cancer etiology. Cold Spring Harb. Perspect. Biol. 5 (2), a012336.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
Sciacovelli, M., Guzzo, G., Morello, V., Frezza, C., Zheng, L., Nannini, N., Calabrese, F., Laudiero, G., Esposito, F., Landriscina, M., Defilippi, P., Bernardi, P., Rasola, A., 2013. The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. Cell Metab. 17 (6), 988–999.
Sepe, L., Ferrari, M.C., Cantarella, C., Fioretti, F., Paolella, G., 2013. Ras activated ERK and PI3K pathways differentially affect directional movement of cultured fibroblasts. Cell Physiol. Biochem. 31 (1), 123–142.
Shatsky, I.N., Dmitriev, S.E., Terenin, I.M., Andreev, D.E., 2010. Cap- and IRES-independent scanning mechanism of translation initiation as an alternative to the concept of cellular IRESs. Mol. Cells 30 (4), 285–293.
Sherman, M.Y., Qian, S.B., 2013. Less is more: improving proteostasis by translation slow down. Trends Biochem. Sci. 38 (12), 585–591.
Smolen, G.A., Zhang, J., Zubrowski, M.J., Edelman, E.J., Luo, B., Yu, M., Ng, L.W., Scherber, C.M., Schott, B.J., Ramaswamy, S., Irimia, D., Root, D.E., Haber, D.A., 2010. A genome-wide RNAi screen identifies multiple RSK-dependent regulators of cell migration. Genes Dev. 24 (23), 2654–2665.
Spriggs, K.A., Stoneley, M., Bushell, M., Willis, A.E., 2008. Re-programming of translation following cell stress allows IRES-mediated translation to predominate. Biol. Cell. 100 (1), 27–38.
Spriggs, K.A., Cobbold, L.C., Jopling, C.L., Cooper, R.E., Wilson, L.A., Stoneley, M., Coldwell, M.J., Poncet, D., Shen, Y.C., Morley, S.J., Bushell, M., Willis, A.E., 2009. Canonical initiation factor requirements of the Myc family of internal ribosome entry segments. Mol. Cell Biol. 29 (6), 1565–1574.
Šulić, S., Panić, L., Barkić, M., Merćep, M., Uzelac, M., Volarević, S., 2005. Inactivation of S6 ribosomal protein gene in T lymphocytes activates a p53-dependent checkpoint response. Genes Dev. 19 (24), 3070–3082.
Takemoto, K., Miyata, S., Takamura, H., Katayama, T., Tohyama, M., 2011. Mitochondrial TRAP1 regulates the unfolded protein response in the endoplasmic reticulum. Neurochem. Int. 58, 880–887.
Visweswaraiah, J., Lageix, S., Castilho, B.A., Izotova, L., Kinzy, T.G., Hinnebusch, A.G., Sattlegger, E., 2011. Evidence that eukaryotic translation elongation factor 1A (eEF1A) binds the Gcn2 protein C terminus and inhibits Gcn2 activity. J. Biol. Chem. 286 (42), 36568–36579.