UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN BIOCHIMICA
E BIOLOGIA CELLULARE E MOLECOLARE
XXVIII CICLO

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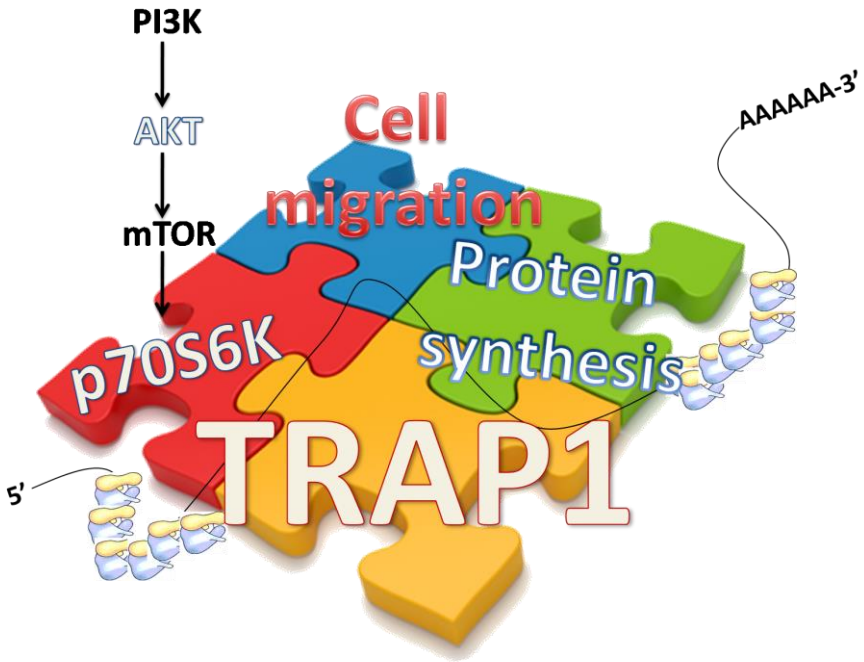

Anno Accademico 2014/2015

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Role of TRAP1 in stress adaptive response of cancer cells

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Riassunto

Nello sviluppo dei tumori si assiste ad una deregolazione quantitativa e/o qualitativa della sintesi proteica. L'espressione delle proteine è modulata da diversi processi, come la regolazione di pathway di segnalazione ed il controllo di qualità su proteine neo-sintetizzate realizzato da chaperoni associati all'apparato traduzionale. In questo scenario si inserisce la nostra ricerca focalizzata sullo studio di TRAP1, uno chaperone appartenente alla famiglia delle heat shock protein 90 e coinvolto nella regolazione della sintesi proteica e nel controllo di qualità. Recentemente abbiamo dimostrato che sul reticolo endoplasmatico TRAP1 è in grado di legare il proteasoma, i ribosomi e i fattori di inizio ed elongazione traduzionale. L'interazione con i macchinari di sintesi e degradazione consente allo chaperone di modulare co-traduzionalmente l'espressione di due proteine mitocondriali codificate dal genoma nucleare che sono più ubiquitinate e meno espresse in cellule tumorali silenziate per TRAP1. Partendo da queste osservazioni, l'obiettivo della mia tesi di dottorato è stato:

1. caratterizzare il ruolo di TRAP1 nella regolazione della sintesi proteica;
2. identificare i pathway molecolari coinvolti nella regolazione traduzionale mediata da TRAP1;
3. studiare gli effetti della regolazione della sintesi proteica mediata da TRAP1 sulla migrazione cellulare.

I risultati ottenuti hanno confermato l'associazione di TRAP1 all'apparato traduzionale, essendo lo chaperone associato ai polisomi in attiva sintesi. Inoltre, l'incremento di questi ultimi in cellule stabilmente silenziate per TRAP1 e l'ottimizzazione della traduzione *in vitro* in seguito all'aggiunta dello chaperone nella reazione, conferma il coinvolgimento di TRAP1 nel processo di sintesi proteica. In particolare, abbiamo dimostrato che TRAP1 è in grado di favorire la sintesi IRES-mediata, mentre attenua quella cap-dipendente. Tale attenuazione è controllata indirettamente da TRAP1 attraverso la regolazione del pathway AKT/p70S6K. Infatti, sia AKT che p70S6K sono meno espresse e meno fosforilate in cellule esprimenti TRAP1 rispetto alle silenziate. Un aspetto interessante relativo alla regolazione di AKT/p70S6K è che le cellule silenziate per TRAP1 sono dipendenti dalla sitesi proteica e da questo pathway anche nella

migrazione cellulare. Infatti, saggi di migrazione dimostrano che in seguito al silenziamento di TRAP1 le cellule sono più veloci rispetto al controllo in condizioni basali, mentre rallentano notevolmente in seguito a trattamento con inibitori della sintesi proteica ed un inibitore specifico di p70S6K. Tali differenze nel comportamento migratorio non sono però da attribuire a variazioni del citoscheletro o alla riduzione dell'espressione di marcatori epiteliali. Un'analisi di espressione genica ha inoltre evidenziato la regolazione di geni coinvolti nella motilità cellulare in cellule stabilmente silenziate per TRAP1, confermando ulteriormente un ruolo per lo chaperone nel fenotipo migratorio. Infine, l'interazione di TRAP1 con il fattore di elongazione mitocondriale permette allo chaperone di regolare anche la sintesi nel suddetto organello attraverso la modulazione della fase di elongazione.

Summary

Quantitative and/or qualitative protein synthesis deregulation is a necessary event to realize and support malignant transformation. There are several molecular mechanisms that define the total amount of protein expression and assure cell homeostasis, such as signaling pathways regulation and a network of ribosome-bound chaperones that is involved in a protein quality control exerted on nascent chains. Starting from this background our research focuses on TRAP1, a member of the heat shock protein 90 family, and on his role in the regulation of protein synthesis and quality control. It has been previously demonstrated that TRAP1 interacts with a proteasomal subunit on the outer side of endoplasmic reticulum, and it has been found associated to ribosomes and to initiation/elongation translational factors. Then, this interaction with both machineries allows TRAP1 to modulate the expression of two mitochondrial proteins through a co-translational ubiquitination/degradation. Indeed, these two TRAP1 substrates are more ubiquitinated and less expressed in cancer cells upon TRAP1 silencing.

Aims of PhD thesis:

1. to characterize the role of TRAP1 in protein synthesis regulation;
2. to identify molecular pathways through which TRAP1 performs this translational modulation in cancer cells;
3. to study the effects of TRAP1 translational regulation on migratory behavior.

Firstly, we confirmed the association of TRAP1 to the translational machinery, since we found that the chaperone associates to polysomes. Moreover, an increase of the total amount of active polysomes upon TRAP1 silencing and the *in vitro* translational assays evidenced that TRAP1 is involved in protein synthesis process. Furthermore, we demonstrated that cap-dependent protein synthesis is decreased in presence of TRAP1, whereas the IRES-mediated one is enhanced. This attenuation of cap-dependent translation is achieved by TRAP1 through and indirect modulation of the expression/activity of two PI3K pathway members, AKT and p70S6K. Indeed, these two kinases result less expressed and less phosphorylated in TRAP1 expressing cells compared to TRAP1-knock down cells. A very interesting finding is that TRAP1-knock down cells are addicted to

translation and to the AKT/p70S6K axis also for other biological processes. Actually, we found that in basal condition TRAP1 silenced cells are faster than control cells in migration assays, whereas treatments with translational drugs and a p70S6K inhibitor are able to abrogate this faster migratory behavior, with scarce effects on control cells. Moreover, we excluded that the observed differences are due to a cytoskeleton reorganization and epithelial marker downregulation. The involvement of TRAP1 in migration regulation was supported by a gene expression analysis performed in colorectal cancer cells stably interfered for TRAP1, where pro-motility genes were found regulated. Finally, as supported by preliminary data/observations, TRAP1 could be involved also in mitochondrial protein synthesis regulation, where it attenuates translation elongation inhibiting the release of the mitochondrial elongation factor from the ribosome.

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1. Introduction

Cancer cells are characterized by a series of hallmarks acquired during the complex multistep process of tumor development. These include sustained proliferation, evasion from growth suppression, acquisition of replicative immortality, resistance to cell death, induction of angiogenesis, invasion and metastasis[1]. Recently, metabolic reprogramming and escape from immune destruction have been suggested as new hallmarks to add to this previous list[1]. Among those, sustained proliferation, which is an essential part of cancer development and progression, is mediated by several mechanisms. including a deregulation of mRNAs translation, with an increase of overall protein synthesis and selective translation of proteins that positively influence cancer progression. Indeed, almost all the mentioned tumor hallmarks need a quantitative and/or qualitative translation alteration to take place and to be sustained. For example, several reports have underlined the translation involvement in cell death escape through the aberrant synthesis of "Inhibitor of Apoptosis" (IAP) proteins, a family of endogenous caspase inhibitors. Cellular stress signals, such as low-dose irradiation, anoxia, serum starvation and chemotherapeutic drugs, have been reported to favor translation of XIAP or cIAP1[2]. Moreover, Dobson and coworkers[3] have reviewed that the endothelial growth factor (VEGF), an angiogenesis inducer, belongs to the proteins upregulated in cancer cells thanks specific features present in its 5' untraslated region (5'-UTR). In agreement with these observations, Yi et al.[4] demonstrated that HIF-1α up-regulates the eukaryotic initiation factor 4E1, which, in turn, promotes VEGF translation in hypoxic breast cancer cells. Finally, recent findings revealed how protein synthesis alteration is involved in immune system escape strategies. The Programmed death 1 receptor and its ligand PD-L1 are upregulated on immune cell clusters surrounding prostate cancer lesions but not in healthy prostate or benign hyperplastic prostate[5]. Moreover, in glioma as well as in trophoblasts, signaling pathway activation correlates with recruitment of PD-L1 transcripts to polysomes, leading to increased PD-L1 translation[6].

Thus, protein synthesis regulation is emerging as a key process in cancer cell biology, whose deregulation is required for the onset and progression of malignancies. Therefore, it does not surprise that many

recent studies suggest therapeutic approaches based on the targeting of protein synthesis for the treatment of cancer.

1.1 Cap-dependent and IRES-mediated translational mechanisms sustain cancer cells proliferation

The most fundamental trait of transformed cells is the capability of sustaining chronic proliferation: unlike normal cells, in cancer cells the production and release of growth-promoting signals are deregulated, making cancer cells masters of their own destinies[7]. Interestingly, they can acquire the capability to sustain proliferative signaling in several ways. They can produce growth factor ligands themselves, which results in an autocrine proliferative stimulation[1], or sending signals to normal cells within the tumor-associated stroma, as is the case of Transforming Growth Factor β (TGFβ) and Hepatocyte growth factor, both involved in the stimulation of fibroblasts, the major cellular component of the stroma[8]. Moreover, cancer cells can also increase the levels of receptor proteins at the cancer cell surface, such as the epidermal growth factor receptor, whose overexpression along with its ligands have been correlated with poor prognosis[9]. Furthermore, a growth factor independence has also been described for cancer cells , dependent on the constitutive activation of signaling pathways operating downstream of these receptors.

It is well known that protein synthesis positively correlates with cell proliferation rate[10] and that upregulated mRNA translation is a common feature of pathological states characterized by aberrant proliferation, including malignancies. As a consequence, translational pathways deregulation is common feature in cancer cells. The mechanistic/mammalian target of rapamycin (mTOR) bolsters cell proliferation and growth stimulating anabolic processes including protein synthesis. Its hyperactivation can be elicited by several mutations recently described in cancer[10] or by hyperactivation of upstream pathways. Indeed, it is well known that the PI3K/AKT/mTOR pathway shows gain of function in numerous and several human cancers generating signals that have a positive effect on the initiation of protein synthesis[11]. In response to upstream signals, PI3K phosphorylates phosphatidylinositol producing phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate[11]; these products of PI3K are recognized by the Ser–Thr kinase PDK1 that phosphorylates and thereby activates AKT. AKT in

turn has numerous protein targets, including pro-apoptotic proteins and tumor suppressors, which are downregulated , and growth-promoting proteins, which are upregulated. Then, the activated PI3K/AKT axis activates mTOR that is a sensor of nutrient status, a regulator of transcription, and an indirect regulator of protein synthesis. This last function is obtained by mTORC1, one of the two distinct complexes formed by mTOR, through the phosphorylation of 4E-binding proteins (4E-BPs), which are negative regulators of translation[11] and p70S6 kinase (p70S6K), which has 40S S6 ribosomal protein as physiological target[12]. Through their binding to the eukaryotic initiation factor 4E (eIF4E), the cap-binding component of the translational initiation complex 4F, 4E-BPs are able to prevent eIF4E from forming this complex. Phosphorylation of 4E-BPs by mTORC1 leads to dissociation from eIF4E that can take part to the translational initiation process[11], enhancing, in particular, the translation of mRNAs with complex secondary structures in their 5'-UTR regions. Many of these messages code for growth-related proteins including growth factors, receptors, kinases, transcriptional regulators and cell cycle proteins[11]. Moreover, translation activation causes a positive loop that sustains the translation itself: in fact, mTOR-mediated activation of p70S6K enhances translation of 5′ terminal oligopyrimidine messages, which code for basic components of the translational machinery[11].

Protein synthesis triggered by mTOR signaling pathway represents the main mechanism for protein translation in eukaryotic cells. It is called cap-dependent translation, a process in which the small ribosomal subunit is recruited to the mRNA in a 5′-7-Methylguanosine cap proximal position during translation initiation, a process stimulated by factors that bind to the cap structure[13]. In stress conditions, such as hypoxia, starvation and response to DNA damage-inducing therapy[13], and during tumor growth, the cap-dependent mechanism is reduced and this event is followed by an increase of the Internal Ribosome Entry Site (IRES) translation, in which an IRES element localized in 5'-UTRs of transcripts that are efficiently translated under stress conditions, allows the ribosomal subunits recruitment without a cap[13]. Interestingly, most of the small subset of identified eukaryotic IRESs are located in mRNAs encoding, among others, oncogenes, such as c-MYC, growth factors, such as fibroblast growth factor 2, growth factor receptors, such as TrkB, pro- and anti-apoptotic factors, such as

XIAP and APAF-1, respectively, and angiogenic factors, such as VEGF[3].
Taken together, a general increased protein synthesis and overexpression of a specific subset of proteins using specific mRNA features and/or different translation mechanisms allow cancer cells to increase and sustain their proliferation rate.

1.2 Role of protein synthesis in the EMT program

A carcinoma arisen from epithelial tissues progresses to higher pathological grades of malignancy, with local invasion and metastasis, as well as alteration in the shape and in the attachment to other cells and to extracellular matrix (ECM). The molecular mechanism involved in invasion and metastasis formation is known as epithelial to mesenchimal transition (EMT). This is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. This implicates the acquisition of new features, including enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components[14]. Master regulators of EMT act on the expression of epithelial and mesenchymal markers, such as E-cadherin, a calcium-dependent cell-cell adhesion molecule, N-cadherin, a transmembrane protein, and Vimentin, a major constituent of the intermediate filament family of proteins[15]. The loss of E-cadherin by carcinoma cells represent the best characterized EMT hallmark. By forming adherens junctions with adjacent epithelial cells, E-cadherin helps to assemble epithelial cell sheets and maintain the quiescence of the cells within these sheets. An association between E-cadherin loss and invasiveness has been established for bladder, renal, endometrial, head, neck, gastric, liver, pancreatic and lung cancer[16], evidence that strong supports the role of E-cadherin as a key suppressor of this metastatic capability. Several mechanisms have been implicated in the regulation of E-cadherin expression during tumor progression, including genetic, transcriptional and epigenetic changes[17]. Genetic alterations of the E-cadherin loci are not frequent in tumors, and the majority of carcinomas with downregulated E-cadherin maintain an intact E-cadherin locus. Most transcriptional repressors of E-cadherin have

been identified, such as the zinc finger factors Snail and Slug, the two-handed zinc factors ZEB1 and ZEB2 and the bHLH factor E12/E47[17]. Mechanisms responsible for E-cadherin regulation in most carcinomas are mainly represented by epigenetic processes; however, protein synthesis also takes part to the EMT program realization. Indeed, several of the transcription factors have identified as master regulators of EMT are modulated at transcriptional, translational and protein stability level by a variety of cell-intrinsic pathways as well as extracellular cues[18]. It has been demonstrated that the activation of cap-dependent translation causes cancer epithelial cells to undergo EMT, thanks to the upregulation of the EMT inducer Snail which, in turn, represses E-cadherin expression, promoting cell migratory and invasive capabilities as well as metastasis[19.] Recent data suggests that regulation of gene expression at the posttranscriptional level plays an important role in TGFβ-mediated EMT. It has been reported that TGFβ binds a region in the 3'-UTR and inhibits the translation of two mRNAs mediating EMT, Dab2 and ILEI[20].

Whatever signaling pathways are activated, cell motility is driven ultimately by adapted cytoskeletal remodeling. PI3K/AKT/p70S6K pathway is involved in this process, also through the translation regulation of specific proteins. Indeed, it has been reported that AKT is a critical mediator of VEGF-induced endothelial cell migration through actin reorganization[21]; moreover, p70S6K colocalizes with actin stress fibers, suggesting that p70S6K activation plays a role in actin polymerization[12]. Finally, an interesting link between translation and cell migration is given by the negative effect of rapamycin on this phenotype: through the inhibition of mTORC1, rapamycin is in fact able to inhibit the synthesis of RhoA, Cdc42, and Rac1, crucial regulatory proteins for cell migration. Thus mTORC1-mediated 4E-BP1 and S6K1 pathways were essential for the expression of these small GTPases[22].

1.3 Translational machinery and its regulation in cancer cells

Translation initiation entails decoding of the AUG start codon in mRNA by Methionyl initiator transfer RNA (Met-tRNAi). This process is significantly different between eukaryotes and prokaryotes, which has profound implications for translational control[23]. In eukaryotes, translation consist of three stages, initiation, elongation and termination.

1.3.1 Initiation step

Translation initiation is the process of assembly of elongation-competent 80S ribosomes, in which the initiation codon is base-paired with the anticodon loop of Met-tRNAi in the ribosomal P-site. This process requires at least nine eukaryotic initiation factors (eIFs) and it is made up of two steps, the formation of 48S initiation complex, with established codon–anticodon base-pairing in the P-site of the 40S ribosomal subunit, and the joining of 48S complex with the 60S subunit. On most mRNAs, a 'scanning' mechanism is required to form 48S complex, through which a 43S preinitiation complex attaches to the 7-Methylguanosine cap at the 5' end of mRNA. In this first step of translation, both the eIF4F complex, containing the scaffold protein eIF4G, the DEAD-box helicase eIF4A and the cap-binding protein eIF4E, and the Polyadenylate-binding protein (PABP) play a fundamental role. PABP attaches to both the 3' poly(A) tail and eIF4G, and brings the 3' and 5' ends of the mRNA together to form a circular mRNA loop[24]. A stabilization of the complex is achieved by an interaction between eIF4B, which binds eIF4A and PABP[24]. The 40S ribosomal subunit is recruited to this complex via its interaction with eIF3, which in turn binds eIF4G. Met-tRNAi is delivered to the 40S subunit in the Ternary Complex (TC) with eIF2-GTP[23]. Then, the 40S subunit, in cooperation with the TC, eIF1, eIF1A, eIF3, and probably eIF5, forms the 43S preinitiation complex . This complex can bind the 7-Methyl-guanosine cap and scan the 5′-UTR in the 5′ to 3′ direction to the initiation codon thanks to the unwinding of the mRNA's 5′ terminal secondary structure operated by eIF4A, eIF4B and eIF4F[23]. Thus, the first AUG encountered is favored as the start codon; it enters the ribosomal P-site and base pairs with the anticodon of Met-RNA$_i$ to form the 48S complex[23-24]. After initiation codon recognition and 48S complex formation, eIF5 and eIF5B promote the hydrolysis of eIF2-bound GTP, the displacement of eIFs and the joining of a 60S subunit with the formation of the elongation-competent 80S ribosome, which leads to translation elongation[25].

Because most regulation occurs at the initiation stage of translation, the molecular basis of this process is being studied intensively to elucidate every potential control points[23] and their deregulation in cancer. Translation initiation can be regulated through the eIF2α phoshorylation and the expression of various initiation factors, and through the mTOR signaling pathway and p70S6K expression[26].

It is well known that rapid responses through the expression of specific proteins may allow tumour cells to grow and survive. Certainly, translation regulation is a rapid and elegant way of tuning gene expression by intensifying protein synthesis from existing mRNAs while silencing others, and also saves transcription-related energy. A further important mechanism that triggers selective translation during the response of cancer cells to stress, including hypoxia or chemotherapy, is part of the unfolded protein response (UPR) during endoplasmic reticulum (ER) stress[21]. The main mechanism to block cap-dependent protein synthesis is the phosphorylation of eIF2α. Upon phosphorylation, the initiation factor is fully capable of forming an initiation-competent eIF2-TC, but following its release, phosphorylated eIF2–GDP tightly binds to and sequesters the guanine nucleotide exchange factor eIF2B, abrogating its activity[25]. There are four mammalian protein kinases that phosphorylate eIF2α on Ser51: haeme-regulated kinase, which is probably significant only in erythroid cells; PKR, which is activated by double-stranded RNAs of more than ~ 40 bp and is important in the antiviral response; PKR-like endoplasmic reticulum kinase (PERK), which is a transmembrane endoplasmic reticulum enzyme with its kinase domain in the cytoplasm, that is activated by ER stress (due to misfolded proteins in the ER lumen); GCN2, a homologue of the only eIF2 kinase in yeast, which is activated by starvation of certain amino acids[25]. In human cancers, induced eIF2α phosphorylation leads to the synthesis of basic leucine-zipper transcription factors such as ATF4 and ATF5, which further support cancer cell survival[21]. Preferential activation of eIF2α upregulates ATF4 target genes involved in amino acid synthesis and transport as well as in response to oxidative or ER stress, and, among others, xCT, the specific subunit of cystine/glutamate antiporter system, and BiP/Grp78, a major ER chaperone essential for protein quality control in the ER[27].

Overexpression of several components of translation initiation machinery was shown to cause or to strongly correlate with malignant transformation. eIF2α, eIF3a,b,c,h, eIF4A, eIF4G1 and eIF5A have been demonstrated to be upregulated in different cancer types[13], such as melanoma, cervix, breast, testis, prostate, hepatocellular, squamous cell lung and ovary cancer, respectively. The cap-binding protein eIF4E, the least abundant eIF and hence considered to be a rate

limiting factor for cap-dependent translation, is found upregulated in bladder, breast, colon, liver, head and neck cancer and non-Hodgkin's lymphoma, and its high expression levels correlate with poor prognosis. Interestingly, the upregulation of eIF4E allows to translate mRNAs with 5'-UTR normally translated with less efficiency, such as many transcription factors, growth factors, receptors and tyrosine kinases[13].

As discussed above, the mitogen and nutrient signalling via mTOR, which regulates cap-dependent translation through controlling both eIF4E and eIF4B via 4E-BP1 and p70S6K respectively[28], results deregulated in cancer cells causing an upregulation of protein synthesis. p70S6K, constitutive activated or amplified and highly expressed in cancer cells, positively regulates initiation and elongation of translation[26]. Indeed, p70S6K phosphorylates a negative regulator of eIF4A, PDCD4 and targets it for degradation by the ubiquitin ligase, βTRCP[28]. Moreover, this kinase phosphorylates eIF4B on Ser422, to enhance the interaction with eIF3. The same authors proposed a model in which a pool of inactive p70S6K is bound to eIF3. Upon phosphorylation by mTOR, it becomes activated, dissociates from eIF3 and phosphorylates its substrates in the pre-initiation complex, including eIF2B and potentially also PDCD4 and the ribosomal protein S6[28].

1.3.2 Elongation and termination steps

The second codon of the open reading frame is present in the A-site of the ribosome waiting for the cognate aminoacyl-tRNA binding. The eukaryotic elongation factor eEF1A, the orthologue of bacterial EF-Tu, binds aminoacyl-tRNA in a GTP-dependent manner and then leads the tRNA to the A-site of the ribosome. After codon recognition by the tRNA, GTP hydrolysis by eEF1A allows the release of the factor and enable the aminoacyl-tRNA to be accommodated into the A-site[29]. The accommodation of the aminoacyl-tRNA into the A-site is rapidly followed by the peptide bond formation with the P-site peptidyl-tRNA. The peptidyl transferase center consisting primarily of conserved ribosomal RNA (rRNA) elements on the large ribosomal subunit, positions the substrates for catalysis. Following peptide bond formation, ratcheting of the ribosomal subunits triggers movement of the tRNAs into so-called hybrid P/E and A/P states with the acceptor ends of the tRNAs

in the E and P-sites and the anticodon loops remaining in the P and A-sites, respectively. The elongation factor eEF2, orthologue of bacterial EF-G, is then required for the translocation of the tRNAs to the canonical E and P sites. Binding of the GTPase eEF2 in complex with GTP seems to stabilize the hybrid state and promote rapid hydrolysis of GTP. Through conformational changes, eEF2 hydrolyses GTP with Pi release and itself is released from ribosome. Moreover, eEF2 seems to alternatively unlock the ribosome allowing tRNA and mRNA movement and then lock the subunits in the post-translocation state. In this state of the ribosome, a deacylated tRNA occupies the E-site and the peptidyl-tRNA is in the P-site, whereas the A-site is vacant and available for binding of the next aminoacyl-tRNA in complex with eEF1A. Recent single molecule and ensemble kinetic analyses indicate that release of the E-site tRNA is not strictly coupled to binding of aminoacyl-tRNA in the A-site[29].

Although initiation has always been considered the main regulatory step of protein synthesis, regulation at elongation is emerging as a key checkpoint in cancer, and overexpression of elongation factor has been associated to cancer cells. For example, the overexpression of EF1A1 and EF1A2 has been found in ovarian and breast cancer[30] respectively. Furthermore, Scaggiante et al[31] suggested that eEF1A2 could be consider a marker for prostate cell transformation and/or possibly as a hallmark of cancer progression. Moreover, a role for p70S6K in controlling the elongation step of translation was demonstrated by the finding that it phosphorylates and inactivates eEF2k, which is a Ca^{2+}/calmodulin-dependent protein kinase that inhibits the translocation step of elongation phase by phosphorylating eEF2 at Thr-56[32-33]. Interestingly, it has been demonstrated that eEF2k has a pivotal role in the adaptation of transformed cells to nutrient withdrawal, a capability severely compromised in cells lacking eEF2K. Its activity is tightly controlled by nutrient availability through direct positive regulation by AMPK and inhibition by mTORC1. Therefore, in presence of nutrients, eEF2k is inactive, whereas under acute nutrient depletion, tumor cells deal with this stress by reactivating the AMPK-eEF2k axis, which confers cell survival by blocking translation elongation[34].

Translation termination takes place when the end of the coding sequence is reached by the ribosome and a stop codon (UAA, UGA, or UAG) enters the A-site. Termination in eukaryotes is catalyzed by

two protein factors, eRF1 and eRF3, that appear to collaborate in the process[29]. Whereas eRF1 is responsible for high-fidelity stop codon recognition and peptidyl-tRNA hydrolysis and promotes peptide release thanks to its middle domain which is functionally analogous to the tRNA acceptor stem, the translational GTPase eRF3 is more closely related to EF-Tu than to EF-G[29].

1.4 The role of ubiquitination and ribosome-bound chaperones in protein quality control

Every stage in proteins production is under a tight regulatory control and it is monitored for errors. All the component of the translation machinery undergo a quality control: cells have evolved pathways to degrade aberrant mRNAs, to detect mutant or damaged rRNAs and ribosomes, and to ensure appropriate tRNA aminoacylation[35]. In addition to the components control, various steps during translation are also monitored, through a kinetic proofreading during codon-anticodon recognition, and protein quality-control pathways that check the folding of nascent polypeptides during and after synthesis. Thanks to all these control steps, cells are able to detect and remove errors at the earliest chance, instead of giving to the polypeptide the opportunity to fold. This opportunity could be useless when, for example, a nascent polypeptide on the ribosome can be deduced to have a low probability of acquiring a fully functional state[35]. There are several sources that could generate defective translation products, such as amino acid misincorporation by noncognate aminoacylated tRNAs, defective co-translational protein folding, stop codon read-through, and ribosome elongation stalling. Sometime, the folding timing itself is a source of errors. In fact, it has been demonstrated that often polypeptides emerging from the ribosome cannot be completely folding until fully synthesized, increasing possibilities of misfolding[36]. However, it has been demonstrated the existence of relatively efficient *de novo* folding in eukaryotic cells, that is performed by an elaborate machinery of ribosome-bound chaperones that interacts with and facilitates folding of nascent polypeptides[36]. Unlike bacteria, where the folding of both newly synthesized and stress-denatured proteins have been proposed to be mechanistically equivalent processes, Albanese et al[37] highlighted the differences between chaperone-mediated *de-novo* and

stress-denatured protein folding in *S. cerevisiae*. Actually, they refer to stress-repressed chaperones associated to translation machinery as Chaperones Linked to Protein Synthesis, or CLIPS, and refer to chaperones induced by stress as Heat Shock Proteins or HSPs. Thus, these two subsets of eukaryotic chaperones have an opposite transcriptional regulation in response to stress and act in two different environments[37]. Moreover, the unusual heterodimeric chaperone complex termed mammalian ribosome-associated complex[38] has been identified in higher eukaryotes. This is a conserved eukaryotic ribosome-bound protein biogenesis factors, which are dynamically interacting factors serving multiple functions, i.e. co-translational sorting, folding, and covalent modification of newly synthesized polypeptides[38].

When nascent proteins fail to fold, they are targeted to degradation. Notably, between 6% and 30% of all eukaryotic newly synthesized proteins are very rapidly degraded by the ubiquitin-proteasome system (UPS)[32]. In eukaryotic cells, UPS is the main pathway for elimination of misfolded proteins. Polypeptides degradation starts with the E1-E2-E3 enzyme cascades that marks UPS substrates with ubiquitin; subsequently, ubiquitinated polypeptides are delivered to the 26S proteasome for degradation[32]. Indeed, it has been established that co-translational ubiquitination is a robust component of quality control system that marks proteins for destruction while they are being synthesized. Moreover, there is an overlap between the machineries that carry out quality control on and off the ribosome, since several ubiquitin-conjugating enzymes have been demonstrated to participate in co-translational process and in a quality control of short lived, misfolded proteins[36].

Thus, both chaperone network and ubiquitination system are active components of the protein quality control exerted on nascent chains to assure a correct folding and an efficient clearance of translation defective products. Furthermore, this two control points are linked to each other, since co-translational folding provides protection from co-translational ubiquitination. Indeed, not every polypeptide emerging from the ribosome is ubiquitinated co-translationally, but there is a subset of nascent chains that is more susceptible to co-translational ubiquitination. It has been assessed that rapid translation of proteins, that already possess challenging folding properties, i.e. aggregation-prone sequences, long sequences, are susceptible to co-translational

ubiquitination[36]. Then, the analysis of the nascent polypeptide-associated complex NAC, a ubiquitously conserved ATP-independent heterodimer with a well-defined nascent interactome, suggests that this complex protects those nascent chains most susceptible to co-translational quality control, giving them a chance to prioritize folding over degradation as they emerge from the ribosome[36].

1.5 TRAP1, Tumor Necrosis Factor receptor-associated protein 1

The Tumor Necrosis Factor receptor-associated protein 1 (TRAP1) is a molecular chaperone that, among the others functions, is involved in protein quality control in mammalian cancer cells. Its cloning as a type I tumor necrosis factor receptor-associated protein[39], and its identification as a retinoblastoma-binding protein, were independently performed by two different groups[40] almost at the same moment. TRAP1, also called HSP75, belongs to the HSP90 chaperone family[41] and shares a 26% identity and 45% similarity with cytosolic HSP90. Even if TRAP1 is sufficiently conserved with HSP90, such that it is sensitive to the HSP90 inhibitors geldanamycin and radicicol, it does not share all the same functions[42], suggesting distinct features for this protein. TRAP1 has different subcellular localizations with distinct functional properties. The attributed cytoplasmic/nuclear localizations to TRAP1 by initial works had not been taken in consideration for farther studies. On the contrary, the mitochondrial localization and the linked functions have been studied for a long period, allowing to discover cytoprotective pathways in which TRAP1 is involved[43]. Taking advantage from a Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, in which a lot of citoplasmatic proteins were reported as putative TRAP1 partners, in 2012 our group identified TRAP1 on the outer side of ER and started to characterize its functions linked to this new subcellular localization[44].

TRAP1 is strictly linked to tumour biology. Actually, it was found strongly expressed in tumor cells of adenocarcinomas of pancreas, breast, colon, and lung, whereas normal matched epithelia contain very low levels of this chaperone[45]. Moreover, we revealed an overexpression of TRAP1 in human colorectal carcinomas, since we observed an increased expression in 17/26 tumors[46]. It was found also abundantly and ubiquitously expressed in human high-grade prostatic

intraepithelial neoplasia, Gleason grades 3 prostatic adenocarcinomas, and metastatic prostate cancer, but largely undetectable in normal prostate or benign prostatic hyperplasia in vivo[47]. Conversely recent data present a more complex scenario that requires further insights with a lower expression of TRAP1 in lung cancer than in normal lung tissue. Similarly, cisplatin-resistant ovarian cancer cells show opposite TRAP1 regulation[48,49,50]. According to its prevalent mitochondrial distribution and the great research interest in the characterization of TRAP1 mitochondrial functions, the first role assigned to this chaperone was the protection against mitochondrial apoptosis[51]. It has been demonstrated that only tumor cells organize a mitochondrial chaperone network, which involves HSP90, TRAP1 and the immunophilin cyclophilin D in a physical complex that regulates permeability transition pore opening, maintaining mitochondrial homeostasis and antagonizing the pro-apoptotic function of cyclophilin D in permeability transition. Accordingly, inhibition of mitochondrial HSP90 chaperones in cancer cells causes sudden loss of mitochondrial membrane potential, release of cytochrome c, and massive death. TRAP1 involvement in stress-adaptive response of cancer cells has been the main interest of our group: high levels of both TRAP1 mRNA and protein were found in osteosarcoma cells chronically adapted to mild oxidative conditions. Moreover, TRAP1 has been proposed as the link between resistance to antitumor agents and adaptation to oxidative stress, since very high levels of this protein were analogously found in tumor cells resistant to 5-fluorouracil and to platin derivatives. Stable clones expressing constitutively high TRAP1 levels are more resistant to H2O2-induced DNA damage and to apoptosis by cisplatin, contain higher reduced glutathione levels than control cells and do not release the apoptosis-inducing factor into the nucleus upon cisplatin treatment[52]. Furthermore, TRAP1 hyperexpression causes a decrease of cleaved Caspase 3 and PARP, commonly considered as apoptotic markers. TRAP1 interference, as well as the use of dominant negative mutants of TRAP1, sensitized oxidative stress/chemoresistant cells to cell death inducers, thus providing the evidence that TRAP1 is an important player in the development and the maintenance of these phenotypes[53].

It has been suggested that, through its involvement in protection of mitochondria against damaging stimuli via a decrease of Reactve

Oxygen Species (ROS) generation, TRAP1 is also involved in cell migration and invasion, since ROS are reported to stimulate cell invasion[54]. Moreover, through its capability of downregulating mitochondrial respiration and ATP production, TRAP1 knockout or transient suppression dramatically enhances cell invasiveness, both in mouse fibroblasts and in a variety of human cell lines[54]. At the same time, TRAP1 is a pivotal mediator of tumour cell motility and invasion in conditions of nutrient withdrawal: in this view, the mitochondrial HSP90, included TRAP1, could allow to overcome the global tumour-suppressive network under nutrient deprivation allowing cell invasion when it is normally impaired. Accordingly, recent studies reported that transient TRAP1 silencing in cancer cells was associated with upregulation of a number of cell motility and metastasis-associated genes, whereas TRAP1 overexpression was correlated with increased expression of genes associated with cell proliferation. Beyond metabolic regulations, recent studies proposed a link between high TRAP1 expression and increased risk of lymph node metastasis in esophageal squamous cell cancer and enhanced cell migration and invasion in the same cellular model through the STAT3/MMP2 signalling pathway[54].

1.5.1 TRAP1 role in protein quality control

Among the cytoplasmic putative TRAP1-binding partners suggested by LC-MS/MS analysis, we found S6/TBP7/ATPase-4/Rpt3, an ATPase protein of the proteasome regulatory subunit[44]. Thus, we validated this evidence and revealed that TRAP1 and TBP7 interact on the outer side of ER. Then, our study identified a new TRAP1 function linked to this extramitochondrial localization, since we demonstrated that its fundamental role in co-translational protein quality control and in ER homeostasis. Indeed, we proved that TRAP1 is involved in quality control of proteins destined to mitochondria. In particular, the calcium binding protein Sorcin isoform B and F1ATPase β subunit, two nuclear encoded proteins localized in the mitochondria, result less expressed and more ubiquitinated in absence of TRAP1 in HCT116 cells. This phenotype can be attributed only to the extramitochondrial fraction of TRAP1, consistently with the absence of proteasomal machinery in the organelle. Moreover, the analysis of cellular lysates upon TRAP1 and /or TBP7 interference revealed a higher amounts of ubiquitinated proteins than control cells,

a phenotype reverted by re-addition of TRAP1 expression vectors. The finding that TRAP1 is found associated to ribosomes and to the translation factors eIF4A, eEF1A and eEF1G suggested us that this quality control on protein expression performed by TRAP1 is co-translational, thanks to the chaperone simultaneous binding to the proteasome and to the translation machinery[27]. Furthermore, an increased expression of Grp78/BiP, the major ER chaperone and marker of ER stress conditions, was found upon stress induction, in HCT116 cells, in which the expression of TRAP1 was stably knocked down by short hairpin RNAs (sh-RNAs). TRAP1 confers to cancer cells the capability to cope with stress stimuli through an attenuation of global protein synthesis and favoring synthesis of stress-related genes. Indeed, we found that the chaperone modulates the eIF2α pathway either under basal conditions or under stress, favoring the activation of GCN2 and PERK kinases, with consequent phosphorylation of eIF2α and attenuation of cap-dependent translation. This enhances the synthesis of selective stress-responsive proteins, such as the transcription factor ATF4 and its downstream effectors BiP/Grp78, and the cystine antiporter system xCT, thereby providing protection against ER stress, oxidative damage and nutrient deprivation[27].

1.6 Scientific hypothesis and aim of the work

Starting from the previous observation of a translational attenuation in cancer cells by TRAP1, in the present work we aim to shed further light on TRAP1 regulation of protein synthesis and on the signaling pathways involved in this process.

Firstly, the causal role of TRAP1 in translation regulation has been analyzed through different approaches. Starting from the hypothesis that TRAP1 allows cancer cells to face with limiting/stress conditions by favoring translation of stress-related genes, we aim at investigating the ability of TRAP1 to cause a switch from cap-dependent to IRES-mediated translational mechanism. Furthermore, the influence of TRAP1 on PI3K pathway will be addressed to understand whether this signaling pathway is involved in the attenuation of cap-mediated translation by TRAP1 in cancer cells.

Moreover, some reports have underlined an involvement of TRAP1 in cell motility regulation[54]. Starting from this evidence and considering

that a role of PI3K pathway members have been established in cell motility, we also wonder if translational regulation by TRAP1 can affects cell migration behavior in our cellular system, hypothesizing a link between translation regulation and migration.
Finally, we hypothesize that TRAP1 is able to perform the same translational control in mitochondria, since the mitochondrial elongation factor EF-Tu, the orthologue of EF1A, was found among other putative TRAP1 partners. Therefore, to this aim, we evaluated whether and how TRAP1 affects the mitochondrial elongation translational step.

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 McCoy's 5A medium and DMEM, respectively. Both culturing mediums contain 10% fetal bovine serum, 1.5 mmol/L glutamine, penicillin and streptomycin. The authenticity of the cell lines was verified 2 year ago by STR profiling, in accordance with ATCC product description. TRAP1-stable interfered cells were obtained by sh-RNAs as described previously[54].

2.2 Plasmid generation and transfection procedures

Full-length TRAP1-myc and mutant Δ1-59-myc (both in pcDNA 3.1 myc-his vector) were obtained as described in[54]. 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. TRAP1 transient silencing was performed with siRNAs purchased from Qiagen (TRAP1: 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 the manufacturer's protocol.

2.3 WB/immunoprecipitation analysis

Equal amounts of protein from cell lysates and tumour specimens were subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). Protein immunoprecipitations were carried out as described in[54]. Where indicated, protein levels were quantified by densitometric analysis using the software ImageJ (ref 18 BBA). The following antibodies were used for WB, immunofluorescence and immunoprecipitation: anti TRAP1 (sc-13557), anti-β-ACTIN (sc-69879), anti-GAPDH (sc-69778), anti-PI3K (sc-423), anti RSK1 (sc-231), anti-p70S6K (sc-230), anti-TBP7 (PSMC4 sc-166003), anti-ERK1 (sc-94) from Santa Cruz Biotechnology; anti-phospho AKT (Thr308) (#9275S), anti-AKT (#9272), anti-phospho p70S6K (#9205), anti-eIF4G (#2469), anti-eIF4B (#3592), anti-eIF4E (#2067), anti-

phospho eIF4G (#2441), anti-phospho eIF4B (#3591), anti-phospho eIF4E (#9741), from Cell Signaling; anti-E-cadherin (610404) and anti-paxillin (6100052) from BD biosciences; phalloidin–tetramethylrhodamine B isothiocyanate (P1951) from Sigma; and anti-SNAI1 (GTX100754), anti-TUFM (EF-Tu) (GTX101763) from Genetex; anti-phosphoSerine (37430) from Qiagen; anti-rpL11 and anti-rpS19 antibodies have been prepared as described in Sulic et al. (2005) and in Chiocchetti et al. (2005), respectively.

2.4 RNA extraction and qPCR analysis

RNA extraction procedures were performed as described in[54]. The following primers were used for PCR analysis: 18S rRNA forward: 5'-GGCGCCCCCTCGATGCTCTTA-3', reverse: 5'-GCTCGGGCCTGCTTTGAACAC-3'. The sequences of TRAP1, GAPDH, AKT1, AKT2, AKT3, SOX4, PRSS3, F3, E-cadherin and Snail primers are reported in[54], while sequences of p70S6K and RSK1 primers are reported in[55]. When possible, 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.

2.5 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.

2.6 Ribosome analysis

HCT116 or HEK293 cells were collected by scraping and then resuspended in lysis buffer. After incubation on ice for 10 min, the extract was centrifuged at a maximum speed of 4°C with the supernatant (cytoplasmic extract) loaded onto 15 e 50% linear sucrose gradient containing 30 mM TriseHCl (pH 7.5), 100 mM NaCl and 10 mM MgCl2. Gradients were centrifuged for 110 min at 37000 rpm, then collected while monitoring the absorbance at 260 nm. In the case of protein analysis 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 TCA, resuspended in loading buffer and analyzed by WB. The percentage of polysomes has been calculated by quantifying the amount of 18S rRNA by qPCR or on the basis of the intensities of RPS19 signals. 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.

2.7 Wound healing assays

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. Wound healing was followed for 24 h by acquiring digital frames at 10 min intervals. Ribavirin (100 mg/mL), 4EGI-1 (25 μM), PF4708671 (20 μM), LY294002 (10 μM) were used to pre-treat cells for 1 h; Gln deprivation was performed for 16 h before wound. Quantitative analysis of wound invasion by cell populations located at the border was performed by measuring the gap area at 2 hour intervals for 24 h (T0–T24h) using ImageJ. The occupation rate of empty space was evaluated as the ratio between average distance between the two edges at each time point (Lti) and the same distance immediately after the scratch (Lt0). Ribavirin (sc-203238), 4EGI-1 (sc202597) and PF4708671 (sc-361288) were purchased by Santa Cruz Biotechnology; LY294002 (Catalog No 440202), was purchased by Calbiochem.

2.8 Confocal microscopy and apoptosis assay

sh-TRAP1 and scramble HEK293 or HCT116 cells, plated on coverslips, were prepared for immunofluorescence analysis as described in[54]. HCT116 cells were treated with Ribavirin and 4EGI-1 for 48h. Apoptosis was evaluated as described in[27].

2.9 In vitro protein synthesis of eGFP and EmGFP

The RTS 100 *E. coli* HY kit and Wheat Germ extract kit were used to synthesize EmGFP and eGFP respectively according to the manufacturer's manual.

2.10 Duolink in situ proximity ligation assay

Duolink in situ proximity ligation assay (Sigma-Aldrich) was performed according to the manufacturer's instructions.

2.11 Stopped-flow FRET assay

The Cy3-L11 and QSY-EF-Tu were made as described in Liu et al, (2014). Initiation complex was made by mixing in WB buffer 70S (Cy3-L11), mRNA, prf-fMet, IF1, IF2, IF3 and GTP for 25' at 37°C and purified on sucrose cushion. Ternary complex was obtained incubating in WB buffer QSY-EF-Tu, yeast Phe, GTP, PEP, PK for 5' at 37°C. TRAP1 was pre-incubated with Cy3-70SIC (blue trace) and with QSY-TC (purple trace) in two different experiments. The two reaction mixtures, with or without TRAP1 were rapidly mixed and changes in Cy3-L11 fluorescence were monitored at stopped-flow machine. Negative control was obtained using a wild type EF-Tu that is unable to quench Cy3 fluorescence.

2.12 Gene expression analysis

Gene expression analysis was obtained as described in[54].

2.13 Patients

Tumour and normal, non-infiltrated peritumoural mucosa were obtained from patients with CRC during surgical removal of the neoplasm. Samples were prepared for immunoblot analysis. In order to compare expression levels of TRAP1 and other proteins (see tables) in different tumour specimens, protein levels were quantified by densitometric analysis and expressed as time increase/decrease in tumours compared to the levels in the respective peritumoural non-infiltrated mucosa. TRAP1 expression levels were regarded as being upregulated if they had increased at least threefold in comparison to the corresponding non-infiltrated peritumoural mucosa.

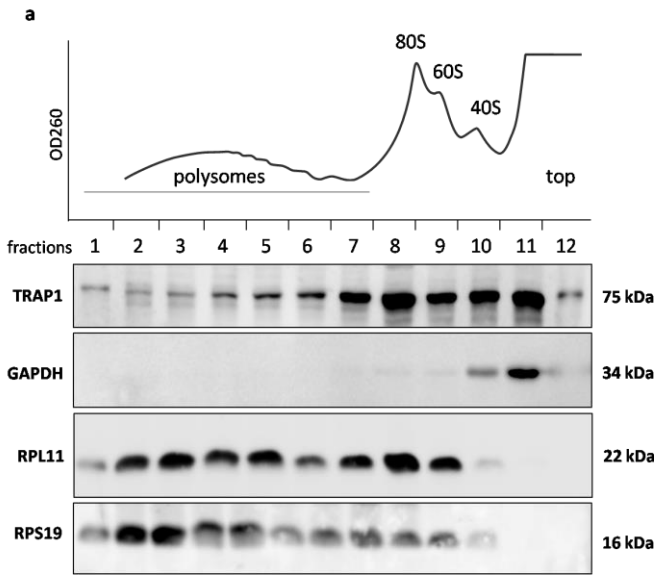
2.14 Statistical analysis

The $\chi 2$ test was used to establish statistical correlation between the expression levels of TRAP1 and those of other proteins (see tables) in human CRCs. The paired Student t-test was used to establish the statistical significance between different levels of gene expression in TRAP1 cells compared with related scramble controls. Student t-test and ANOVA test were used to establish the statistical significance in *in vitro* translational assays.

3. Results

3.1 TRAP1 associates with and influence the amount of active polysomes in cancer cells

A physical interaction between co-translationally acting chaperones and ribosomes has been widely reported as the primary environment for the correct assembling of nascent polypeptides[27]. Consistently, we have already demonstrated that TRAP1 is present in the ribosomal fractions purified from HCT116 cells and that it interacts with initiation and elongation translational factors[27]. To have further indication on the association of TRAP1 with ribosomes, we performed a separation of 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.



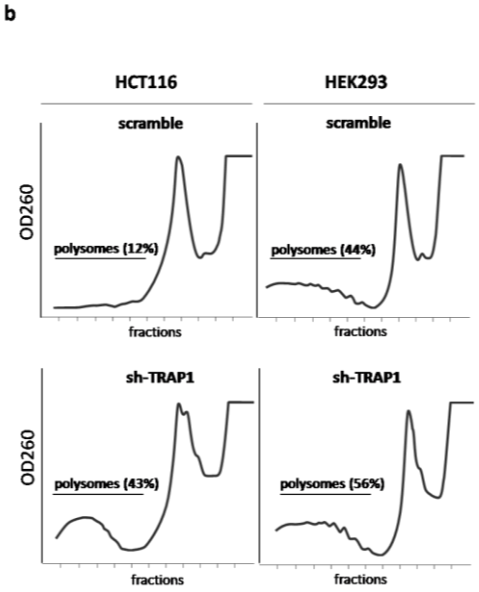


Figure 1 TRAP1 co-sediments with polysomes and regulates protein synthesis. **a)** Separation of cytoplasmic extracts from HCT116 cells was performed by ultracentrifugation on sucrose gradients as described in Materials andmethods. Proteins from the fractions were analyzed by western blot with the indicated 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 HCT116 and HEK293 sh-TRAP1cells. The percentage of polysomes (indicated in the absorbance profiles) is calculated by quantifying the amount of 18S rRNA by qPCR.

3.2 TRAP1involvement in protein synthesis control is confirmed in in vitro translation assays

To have a clear proof that the direct interaction of TRAP1 with translational machinery causes the overall rate change of protein synthesis, as already confirmed in this work (Figure 1b), we performed *in vitro* translational assays using Wheat Germ extract. Indeed, we analyzed the level of enhanced Green Fluorescence Protein (eGFP) mRNA, adding TRAP1 recombinant protein to the reaction. As shown in Figure 2, the eGFP protein amount is slightly higher in the reaction with TRAP1 compared to the control.

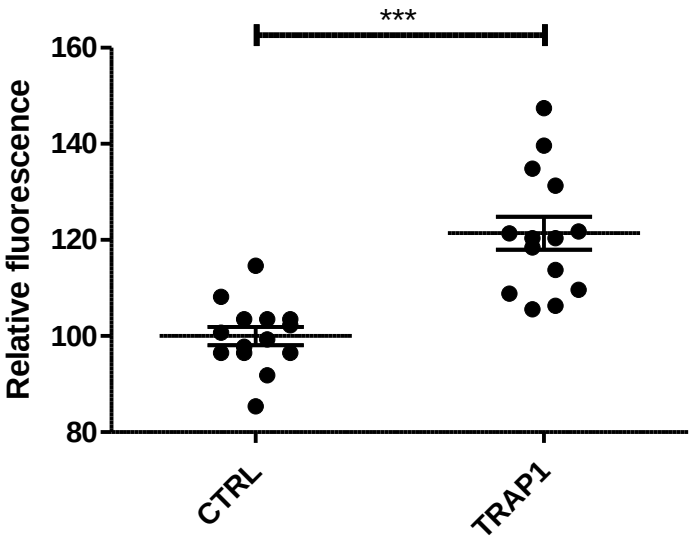


Figure 2 TRAP1 is involved in protein synthesis control. eGFP *in vitro* translation using the Wheat Germ extract kit. eGFP mRNA was added to reactions at a final concentration of 21.95 ng/µL. Where indicated, 0,3 µg/µL TRAP1 recombinant protein was added to the reaction. Data are expressed as mean ± SD from 14 independent experiments; *** P≤ 0.0001.

It has been suggested that the role of TRAP1 in protein synthesis regulation become essential during cellular stress[27]. In agreement with our previous results, we found that TRAP1 capability of allowing translation to adapt and overcome suboptimal conditions is highlighted also in *in vitro* translation experiments. Indeed, we observed that, upon reduction of amino acids amount in reactions (20 fold less than canonical amount), the eGFP protein translation in presence of TRAP1 is comparable with the amount obtained in the control reaction (containing the canonical amount of amino acids), with or without TRAP1. Conversely, amino acids reduction impaired eGFP translation in the sample without TRAP1, where a premature plateau is achieved (Figure 3). Then, the *in vitro* translation results represent an unequivocal proof of TRAP1 involvement in protein synthesis and highlights the capability of TRAP1 of optimizing translation and assure it also in limiting conditions.

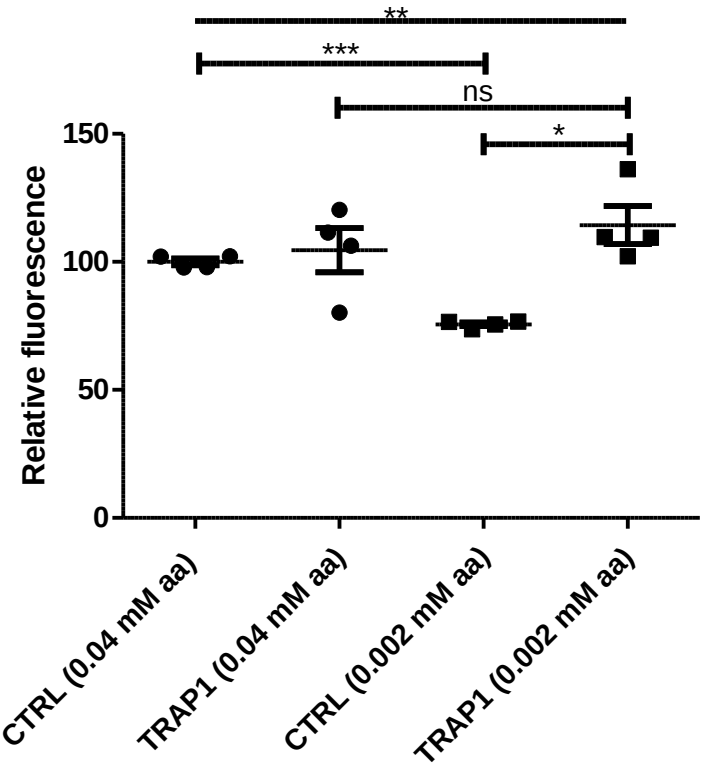


Figure 3 eGFP translation is unaffected by amino acids reduction in presence of TRAP1. eGFP *in vitro* translation using the Wheat Germ extract kit. eGFP mRNA was added to reactions at a final concentration of 21.95 ng/µL. Where indicated, amino acids were reduced (20 fold) and 0,3 µg/µL TRAP1 recombinant protein was added to the reaction. Data are expressed as mean ± SD from 4 independent experiments; * P≤ 0.01; ** P≤ 0.001; *** P≤ 0.0001.

3.3 TRAP1 regulates cap-dependent and IRES-mediated translation mechanisms

It is well known that eIF2α phosphorylation attenuates cap-dependent translation and allows expression of stress responsive genes favoring the IRES-mediated translation mechanism, since most of these genes have an IRES in their 5'-UTR[27]. In order to analyze this translational switch in our cellular model, we measured the ratio between IRES and cap-mediated translation in different experimental conditions by transfecting a dual reporter Cap-Renilla-IRES Luciferase vector: two translation mechanisms from the same transcript were evaluated by assaying the luciferase activity (Figure

4). Ratio between IRES- and cap-mediated translation in each experimental condition was calculated assuming mean level of respective scramble cells equal 1. Results show that the ratio between IRES and cap-mediated translation is lower in sh-TRAP1 cells, both under basal condition or upon translational stress induced by Ribavirin, a translational drug, or Tapsigargin, an ER stress inducer. As a control, cells were treated with Cychloheximide, a protein synthesis inhibitor. These results clearly point to TRAP1 involvement in the attenuation of cap-dependent translation, while favoring the IRES dependent one. Although more experiments are needed to affirm that this phenotype was predicted by *in vitro* experiments, we want to underline that this result is in agreement with wheat germ extract assay, where an mRNA translated by an IRES-mediated mechanism was used.

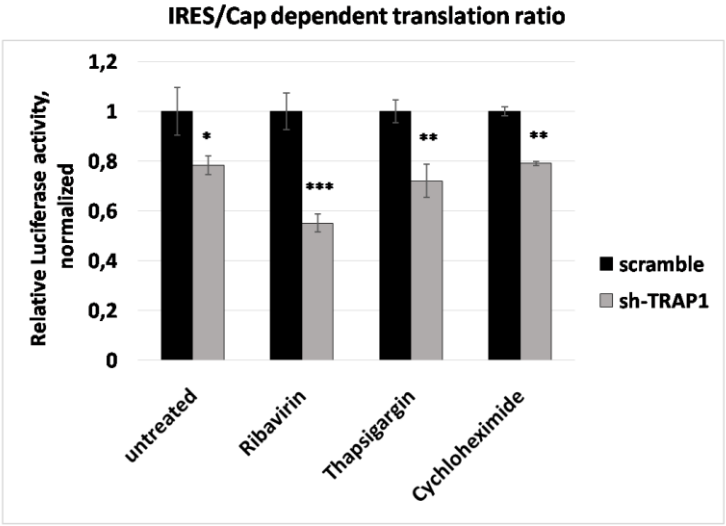


Figure 4 TRAP1 silencing decreases ratio between IRES- and cap-dependent translation. HCT116 sh-TRAP1 and scramble cellswere transfected with pLPL Cap-Renilla-IRES-Luciferase bicistronic dual reporter vector. As indicated, cells were treated with Ribavirin (100 mg/mL) for 16 h, or with Thapsigargin (1 mM) or Cychloheximide (200 mg/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 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 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

3.4 TRAP1 attenuates cap-dependent translation by regulating PI3K pathway

Once demonstrated that TRAP1 down-regulates cap-dependent translation in cancer cells, we decided to study the molecular pathways modulated by TRAP1 and responsible for this regulation. We started to dissect the PI3K pathway, a survival pathway that is constitutively activated in many types of cancer and involved in regulation of protein translation. Notably, we observed an increased expression of AKT upon TRAP1 knock down (TRAP1 KD) in HCT116 and HEK293 cells; consequently, phosphorylated AKT protein also increased in low TRAP1 background (Figure 5a). Conversely, PI3K protein expression levels are not affected by TRAP1modulation. Interestingly, immunoblots performed with antibody specifically directed against single AKT isoforms show that the expressions of AKT2 and AKT3 are the most upregulated upon TRAP1 KD, thus contributing to the regulation of total AKT (data not shown). To analyze the nature of this regulation, i.e., if TRAP1 control is at transcriptional/post-transcriptional level, qPCR experiments were performed in HCT116 and HEK293 sh-TRAP1 cells (Figure 5b). Results show that TRAP1 expression does not affect single AKT isoform mRNA levels thus suggesting that TRAP1-dependent control of AKT occurs at post-transcriptional level. Of note, the transfection of a myc-tagged TRAP1 construct in both sh-TRAP1 cells and scramble controls is able to reduce AKT protein levels, partially rescuing the original phenotype (Figure 5c); this finding further confirms the specificity of TRAP1 role in the regulation of AKT expression. Interestingly, the observed effect is even higher after transfection of the Δ1-59-TRAP1 deletion mutant, which lacks the mitochondrial targeting sequence and is therefore unable to enter mitochondria, supporting the evidence that this regulation is due to the extramitochondrial-localized TRAP1.

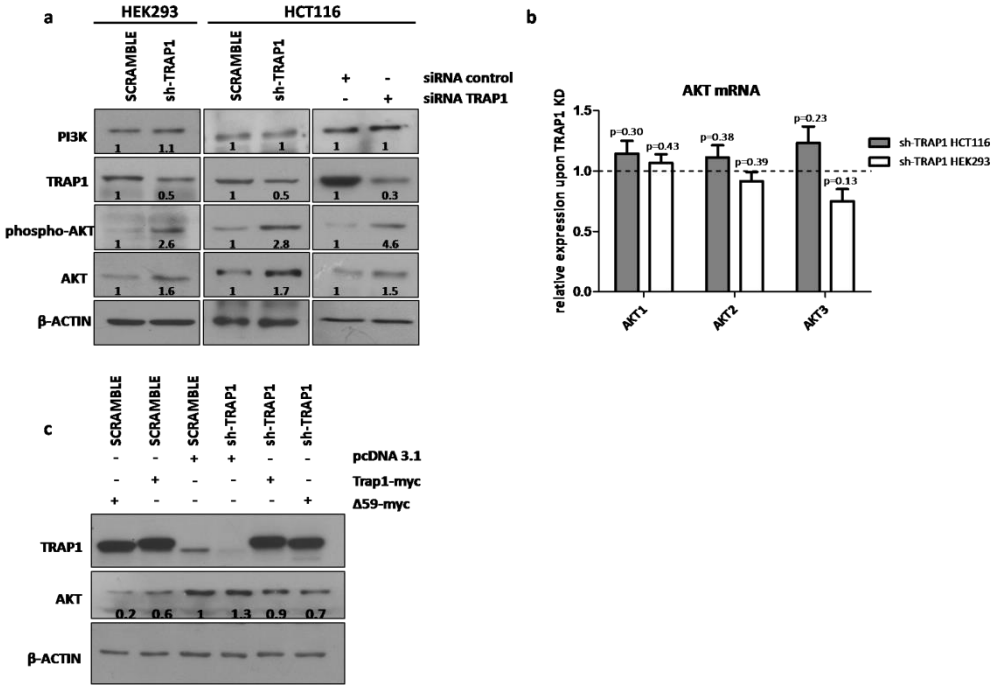


Figure 5 TRAP1 regulates AKT expression. a) Total lysates obtained from HCT116 cells transfected for 72 h with non targeted control siRNA or TRAP1-directed siRNA and from HCT116 and HEK293 sh stable clones were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, which have been calculated by assuming protein levels of the control equal to 1. Images are representative of three independent experiments. **b)** qPCR analysis of single AKT isoform mRNA expression in HCT116 and HEK293 sh-TRAP1 cells. All data are expressed as mean with SEM from three independent experiments with technical triplicates each. The p-values indicate the statistical significance between relative expression levels. **c)** HEK293 sh-TRAP1 and scramble 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 48 h from transfection, separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, which have been calculated by assuming protein levels of the control (scramble) equal to 1. Images are representative of 3 independent experiments.

p70S6K, a translation regulatory kinase that is downstream AKT and is activated by mTORC1, is involved in a positive regulation of cap-dependent translation through the phosphorylation of rpS6 and translation initiation factors. The expression levels of this enzyme was analyzed in TRAP1 KD cells vs controls in HCT116 and HEK293 cell lines. As shown in Figure 6a this enzyme is hyper-expressed in sh-TRAP1 cells compared to their scramble controls. Remarkably, also

RSK1, another kinase involved in positive translation regulation and activated by RAS pathway shows an increased expression upon TRAP1 KD (Figure 6a). 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 6a-b).

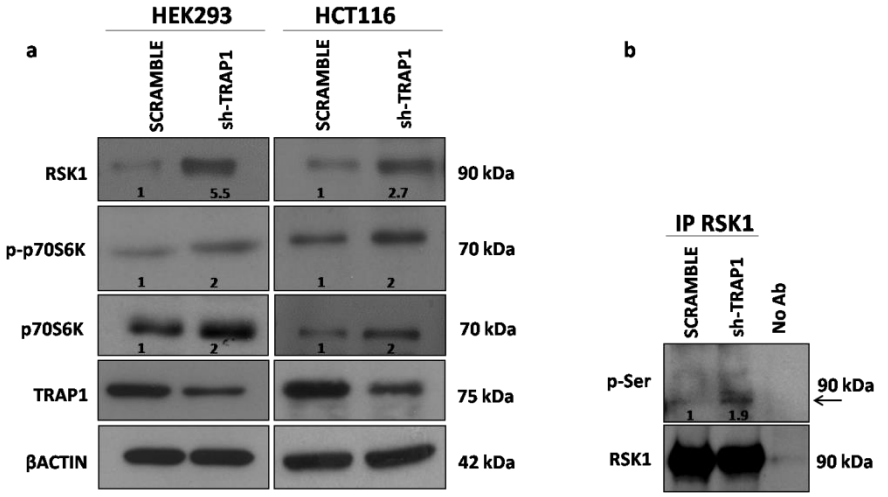


Figure 6 TRAP1 silencing upregulates p70S6K and RSK1 expression/phosphorylation. a) HCT116 and HEK293 stable clones total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric bandintensities, eachnormalized to the respective ACTIN band, which have been calculated by assuming protein levels of the control (scramble) equal 1 **b)** HCT116 scramble and sh-TRAP1 cells were immunoprecipitated with anti-RSK1 and immunoblotted with anti-phospho-Serine antibody. Numbers indicate densitometric bandintensities, eachnormalized 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 antibody.

Remarkably, TRAP1 expression/function is important for these two S6 kinases (S6Ks) regulation: in fact, transient downregulation of TRAP1 expression upon siRNA transfection yielded an increase of p70S6K and RSK1 protein levels (Figure 7a), findings that demonstrate a causal role of TRAP1 for 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 7b), thus

allowing us to conclude that regulation of p70S6K and RSK1 expression occurs at post-transcriptional levels.

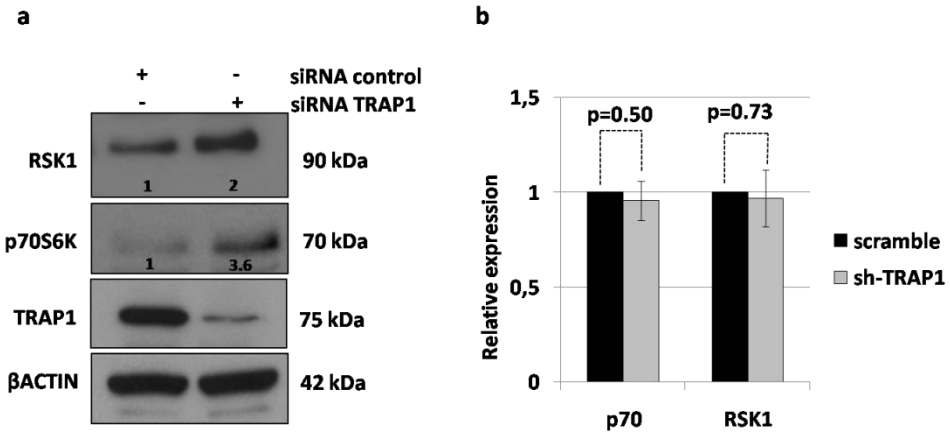


Figure 7 TRAP1 regulates p70S6K and RSK1 expression at post-transcriptional level. a) HCT116 cells were transfected with non-targeted control siRNA or TRAP1-directed siRNA. 48 h after transfection, total lysates were harvested, separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, each normalized to the respective ACTIN band, which have been calculated by assuming protein levels of the control equal 1. **b)** qPCR analysis of p70S6K and RSK1 mRNAs expressionin HCT116 sh-TRAP1 and scramble cells. All data are expressed as mean ± S.D. from 3 independent experiments. The p-values indicate the statistical significance between relative expression levels.

Furthermore, the transfection in HEK293 sh-TRAP-1 cells of constructs expressing either a full-length TRAP1 or TRAP1 mitochondrial-import deletion mutant Δ1-59, is sufficient to recapitulate p70S6K protein levels (Figure 8a). 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. Moreover, co-immunoprecipitation (co-IP) experiments were performed to evaluate whether this regulation is due to a direct interaction between TRAP1 and these kinases. Data in Figure 8b 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[44] and between RSK1 and ERK1/2[41], used as positive controls of these experiments, could easily be detected. All these data suggest that TRAP1 influences this

pathway downstream PI3K, through an indirect modulation of AKT and p70S6K protein translation.

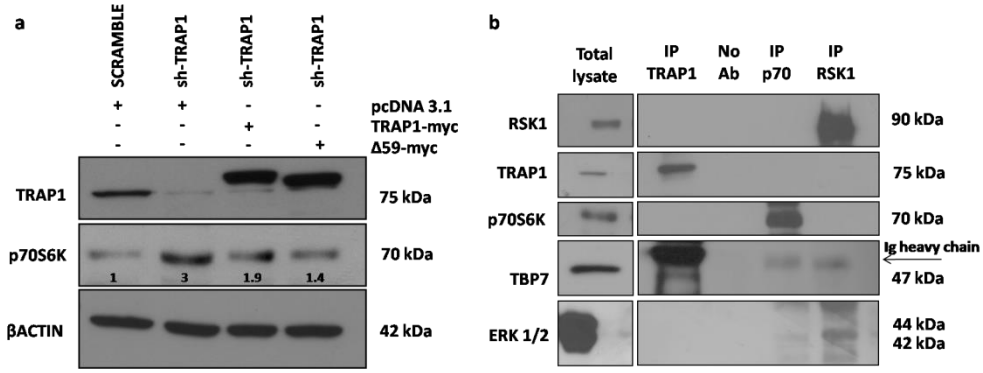


Figure 8 TRAP1 modulates p70S6K and RSK1 through an indirect regulation of their translation. a) HEK293 sh-TRAP1 and scramble cells were transfected with TRAP1-myc and Δ1-59-myc expression vectors (pcDNA 3.1 vector was used as control). Total cell lysates were harvested after 24 h from transfection, 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. **b)** 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-IP. 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.

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[55]. Accordingly, we analyzed phosphorylation levels of the main translation initiation factors. As represented in Figure 9, initiation factors eIF4G, eIF4B and eIF4E show higher phosphorylation levels in HCT116 cells with a stable or transient TRAP1 KD, whereas their expression levels are unchanged.

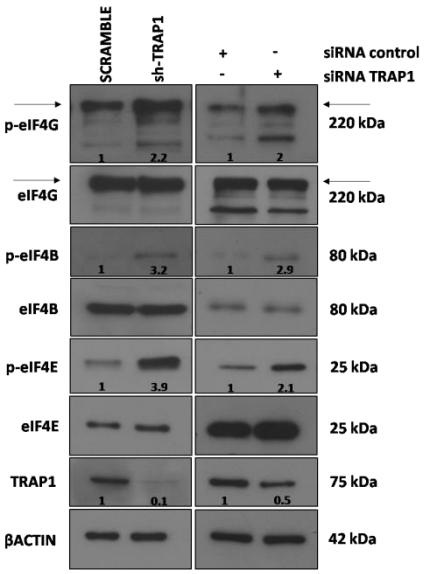


Figure 9 TRAP1 silencing enhances translation initiation. Total extracts were obtained from HCT116 stable clones and from HCT116 cells transfected with non-targeted control siRNA or TRAP1-directed siRNA for 48 h. Total lysates were separated by SDSPAGE and immunoblotted with the indicated antibodies. 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.

3.5 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[41]. 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 formation in scramble vs sh-TRAP1 cells. As shown in Figure 10, Thapsigargin treatment induces stress granules in sh-TRAP1 cells.

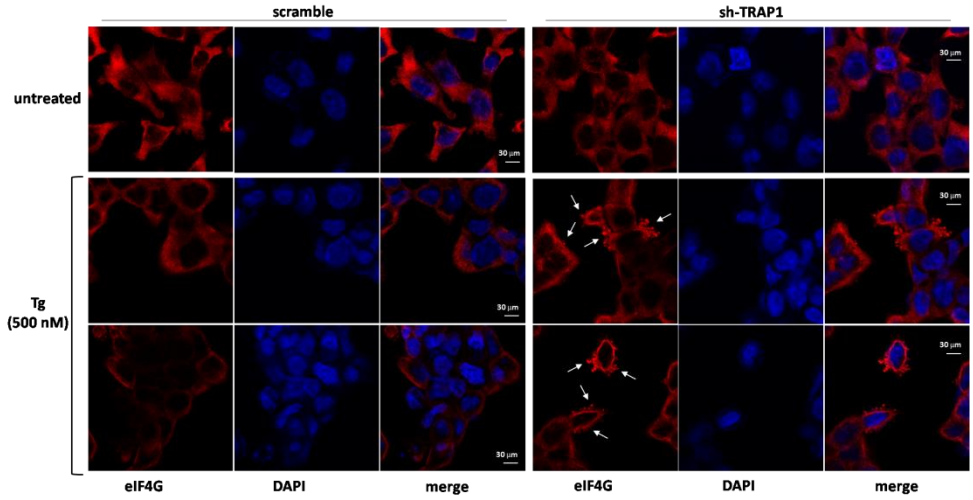


Figure 10 TRAP1 silencing sensitizes cells to translational stress. HEK293 scrambled and sh-TRAP1 cells were treated with Thapsigargin (Tg) (500 nM) for 50 min. Stress granules were analyzed using rabbit monoclonal anti-eIF4G antibody and goat anti-rabbit Alexa Fluor 568. DAPI staining is also shown to detect nuclei.

We then selected two drugs inhibitors of mRNA translation, with the aim of analyzing the response of control and TRAP1 KD cells: Ribavirin, which inhibits cap-mediated translation and 4EGI-1, a synthetic peptide that binds the translational initiation factor eIF4E and prevents its interaction with eIF4G[27]. As shown in Figure 11a-b, a significant increase in the rate of apoptotic cell death can be observed in sh-TRAP1 stable transfectants. Conversely, cells containing higher TRAP1 levels seem to be less sensitive, especially for the apoptotic response to Ribavirin. The low sensitivity of TRAP1-containing cells to blockers of cap-dependent mRNA translation is not surprising, considering that an attenuation of protein synthesis is already present in these cells.

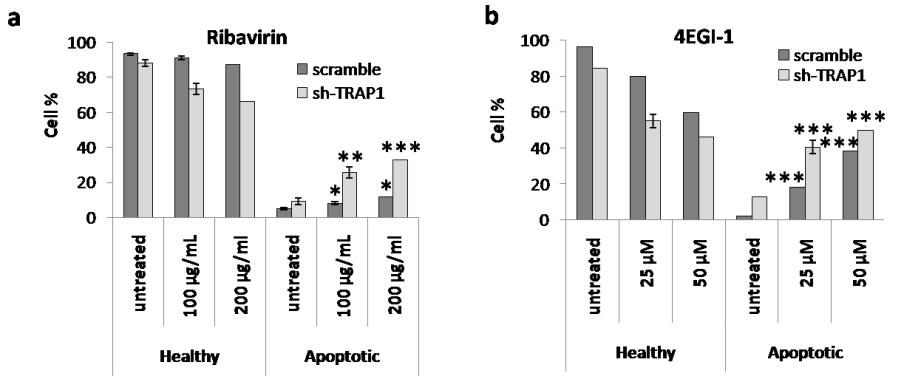


Figure 11 Downregulation of TRAP1 sensitizes cancer cells to drugs targeting cap-dependent translation. (a, b) Rates of apoptotic cell death in HCT116 cells treated with Ribavirin **(a** (100 or 200 mg/ml) or 4EGI-1 **(b** (25 or 50 mM) for 48 h upon stable downregulation of TRAP1. All data are expressed as mean±S.D. from 3 independent experiments; *P<0.01, **P<0.001, ***P<0.0001.

It has been proposed that agents interfering with the regulatory mechanism of gene translation, could be regarding as leading compounds in the antimetastatic drug development process[27]. Moreover, few studies suggest an involvement of TRAP1 in the regulation of the motile behavior of cancer cells[55]. Thus, in collaboration with the Professor Paolella's group (University of Naples), we analyzed the migratory potential of HEK293 scramble and sh-TRAP1 cells in the presence/absence of Ribavirin and 4EGI-1, in a wound healing assay. In Figure 12, a quantitative analysis as linear progression (left) and rate of advancement (right) of the wound edge during time is shown. In the reported experiments carried out on untreated cells (Figure 12a), TRAP1 interfered cells are faster than control cells and completely fill the gap within 16 h; edge advancement becomes higher than control cells after the scratch and stays higher for several hours, until it is reduced when the wound starts to close. Upon Ribavirin treatment (Figure 12b), 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 HEK293 sh-TRAP1 cells is consistently lower than scramble cells for most of the observation times, 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 (Figure 12c). Taken together, the data reveal a role of TRAP1 in counteracting the anti-migratory effect of translation inhibitory drugs.

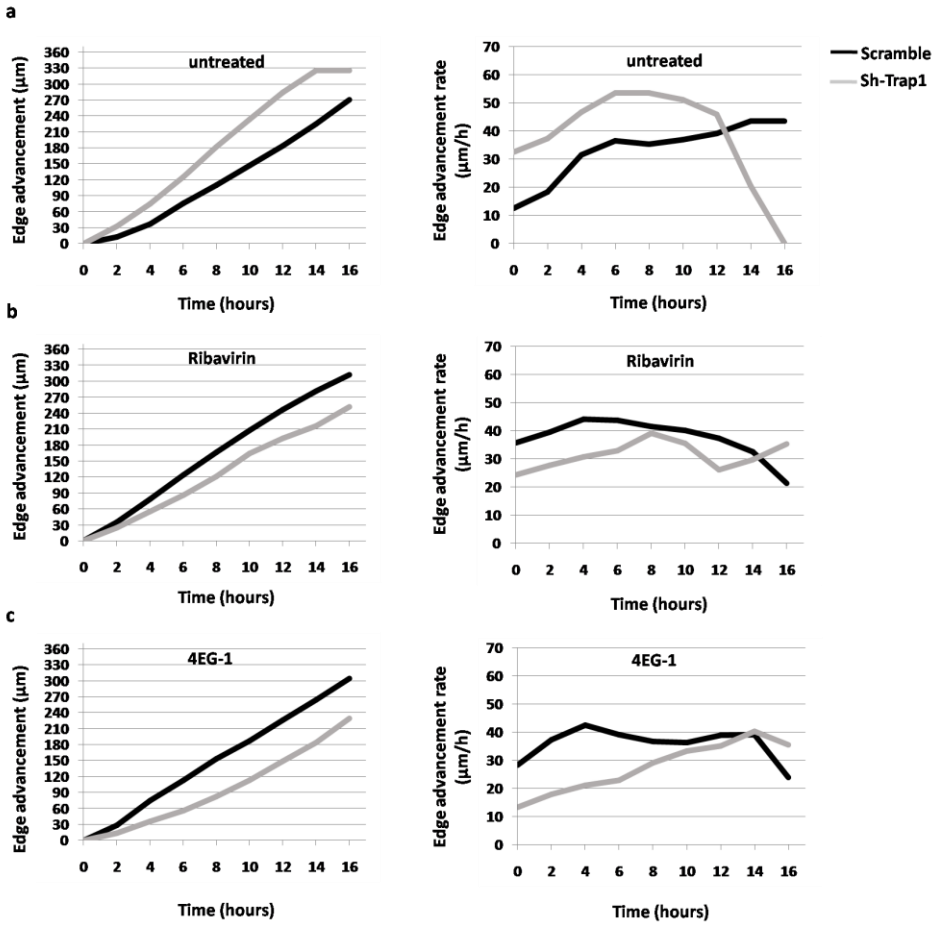


Figure12 TRAP1 affects cell migration. Wound healing assay with scrambled and sh-TRAP1 HEK293 cells. Wound closure, expressed as linear progression (left) and rate of advancement (right) during time (see Materials and methods), of scramble (black) and sh-TRAP1 (gray) HEK293 cells **a)** under control conditions andupon treatment with **b)**100 mg/mL Ribavirin , **b)** 25 µM 4EGI-1.

To further characterize the molecular environment and players of TRAP1 regulation of cell migration, we focused on p70S6K pathway and analyzed its involvement in the motility of HEK293 TRAP1 KD cells compared to controls. Cell migration has been studied during wound healing experiments and quantitatively evaluated in terms of occupation rate of empty space. According to our previous results, we show that, under basal conditions, sh-TRAP1 cells move faster than scramble control; however, treatment with the p70S6K inhibitor PF4708671 selectively reduces the rate of edge advancement of

HEK293 sh-TRAP1 cells, whereas scramble cells are unaffected. As shown in the Figure 13a, the curve for sh-TRAP1 PF4708671-treated cells (closed gray symbols) consistently runs above the curve for untreated cells (gray curve), whereas the curves for treated and untreated scramble cells (black curves) run together. Unlike p70S6K inhibition, treatment with LY294002, a PI3K inhibitor, reduces wound healing progression of sh-TRAP1 cells and also of scramble cells, even if at a lower extent; treatment with LY294002 makes the scramble and sh-TRAP1 curves similar, as reported in the Figure 13b (see black and gray curves with closed symbols). These findings suggest that TRAP1 silencing enhances cell migration by acting downstream PI3K through the AKT/p70S6K axis, thus making these cells addicted to such pathway. Consistently, sh-TRAP1 cells show higher sensitivity to PF4708671 treatment than their scramble counterpart, as suggested by reduction of phosphorylation levels of the specific p70S6K downstream target eIF4B (Figure 13c), whereas PI3K inhibition has the same effect on p70S6K activity in sh-TRAP1 cells and in control cells (Figure 13d).

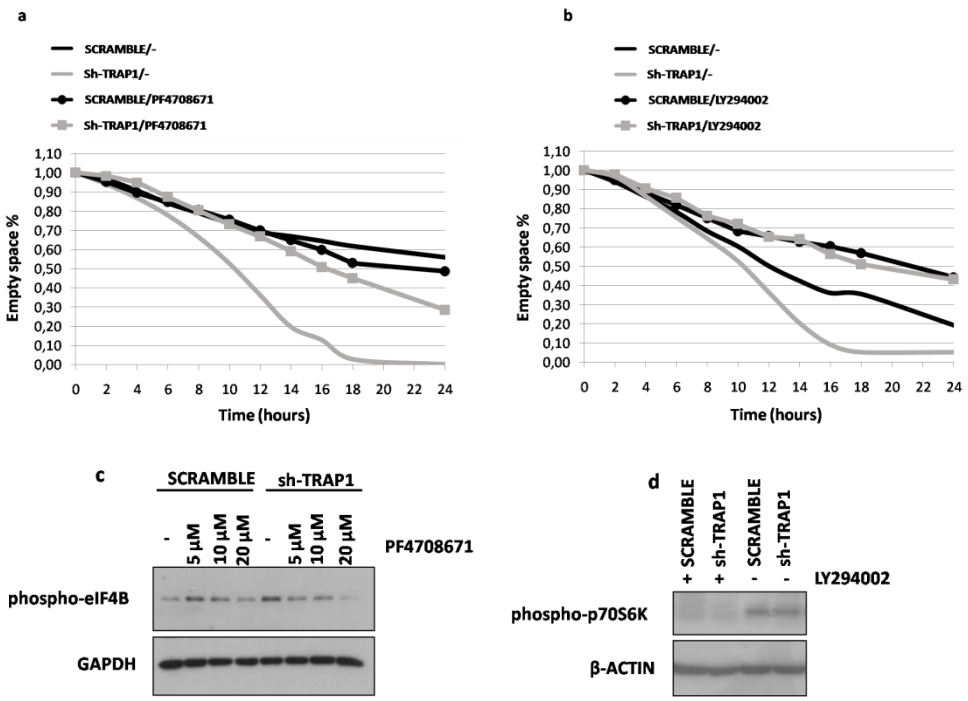


Figure 13 Effect of PF4708671 and LY294002 on HEK293 scramble and sh-TRAP1 cells during wound healing. Wound healing assays of HEK293 cells upon **a)** PF4708671 and **b)** LY294002 treatments. The occupation rate of empty space, evaluated as the ratio between average distance between the two edges at each time point (Lti) and the same distance immediately after the scratch (Lt0), is reported as a function of time for HEK293 scramble (black) and sh- TRAP1 (grey) cells, by using time points corresponding to snapshots taken at 2 hour intervals up to 24 h after the wound. Closed symbols are used for cultures in the presence of 20 μM PF4708671 and 10 μM LY294002; simple traces are used for untreated cultures. **c)** and **d)** HEK293 sh-stable clones were treated with indicated concentrations of PF4708671 and LY294002 for 1 h. Total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Images are representative of 3 independent experiments.

3.6 Downstream effects of TRAP1-mediated regulation of AKT/p70S6K axis

The AKT/p70S6K pathway is considered an important player in tumor cell biology, since promote cell cycle progression, cell survival, and tumor cell invasion. The latter can be either due to cytoskeleton remodelling, induction of epithelial–mesenchymal transition, or metabolic reprogramming[54]. To evaluate whether AKT/p70S6K pathway regulation by TRAP1 influences the observed cell migratory behaviour through cytoskeleton organization and/or focal adhesion expression, we analyzed actin and paxillin distribution by immunofluorescence. Results show no differences between scramble and sh-TRAP1 cells (Figure 14a-b). The expression levels of the two proteins were also observed by immunoblot analysis performed in HEK293 stable clones, with comparable results (Figure 14c). Our data suggest that TRAP1 does not affect actin cytoskeleton neither cell–matrix adhesion in our cellular system.

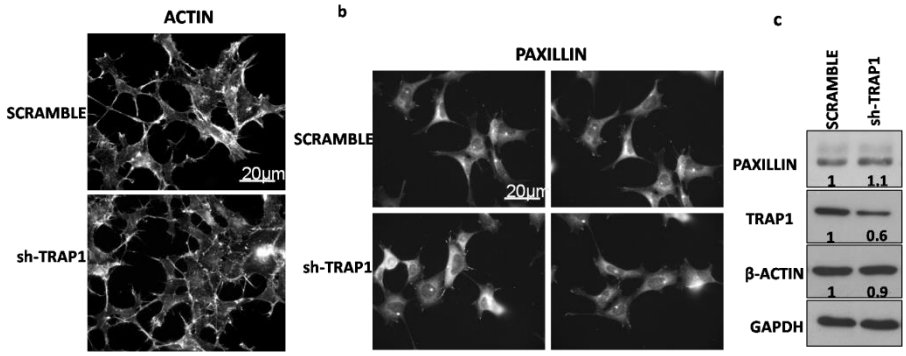


Figure 14 TRAP1 expression does not influence cytoskeleton organization and paxillin distribution. a, b) F-actin and paxillin spikes were visualized in HEK293 scramble and sh-TRAP1 cells by immunofluorescence staining. **c)** HEK293 sh stable clone total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, which

have been calculated by assuming protein levels of the control (scramble) equal to 1. Images are representative of 3 independent experiments.

Interestingly, it has been shown in ovarian cancer cells that, besides AKT, the downstream target p70S6K is directly involved in repression of E-cadherin, the transmembrane protein involved in cell adhesion, whose downregulation is considered as a hallmark of EMT and is typically associated with cancer progression and metastasis[54]. As already explained, p70S6K induction can induce the expression of the transcription factor Snail with consequent downregulation of E-cadherin. Therefore, we questioned whether this pathway is conserved in our cellular system and whether the observed differences in cell migration are due to E-cadherin expression regulation. qPCR and immunoblot analyses (Figure 15a-b) show no changes of Snail mRNA and protein levels in HEK293 sh-TRAP1 cells, whereas HCT116 sh-TRAP1 cells show a slight Snail protein upregulation. Consistently, there is no significant change of E-cadherin expression in HCT116 cells upon TRAP1 KD, as shown by qPCR (Figure 15c), immunoblot (Figure 15d) and immunofluorescence (Figure 15e), while a decrease of E-cadherin mRNA is observed in HEK293 cells upon both siRNA and shRNA-mediated TRAP1 silencing (Figure 15c); however, as shown in Figure 15c, HEK293 sh- TRAP1 contain very low level of E-cadherin when compared to HCT116 cells, to such an extent to be undetectable by immunoblot and immunofluorescence analyses (data not shown). These results suggest that the observed effects of TRAP1 on cell motility are independent from the regulation that the AKT/p70S6K axis exerts on actin cytoskeleton dynamics and EMT; however, although a possible role of AKT in EMT program upon TRAP1 KD is supported by E-cadherin modulation in HEK293 cells, these findings require further study in cell models in which the role of the AKT/p70S6K pathway in the EMT program has already been assessed.

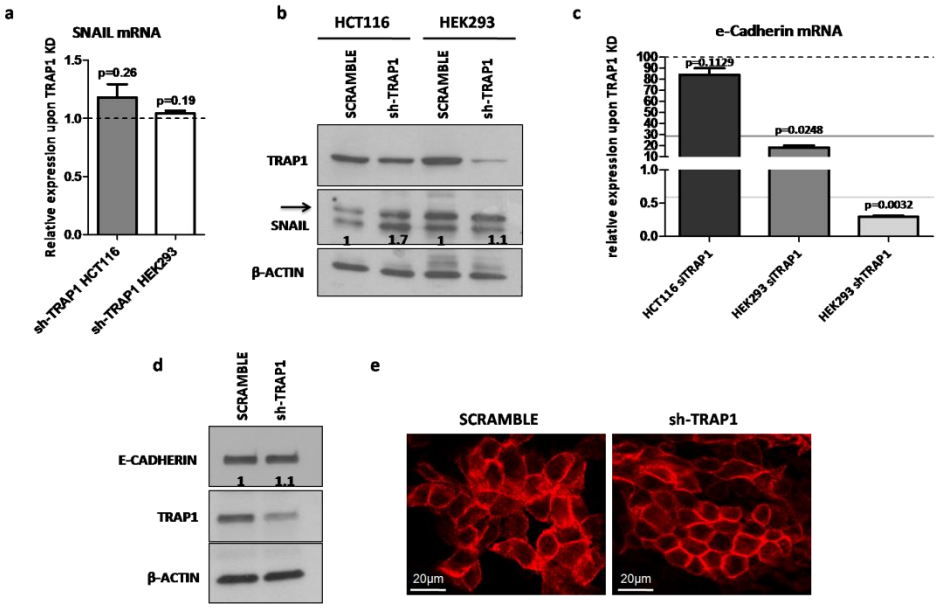


Figure 15 Correlation between TRAP1 and Snail gene expression and effects of AKT/p70S6K pathway on E-cadherin expression. a) qPCR analysis of Snail mRNA expression in HCT116 and HEK293 sh stable clones. All data are expressed as mean with SEM from 3 independent experiments with technical triplicates each. The p-values indicate the statistical significance between relative expression levels. Dashed line indicates expression level of scramble controls. **b)** HCT116 and HEK293 sh stable clone total lysates were separated by SDSPAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, which have been calculated by assuming protein levels of the control (scramble) equal to 1. **c)** qPCR analysis of E-cadherin mRNA expression in HEK293 sh stable clones and siRNA-mediated TRAP1 interfered HEK293 and HCT116 cells, harvested 96 h after transfection. All data are expressed as mean with SEM from 3 independent experiments with technical triplicates each. The p-values indicate the statistical significance between relative expression levels. Dashed line, dark grey line and light grey line indicate reference expression levels of the scramble controls for HCT116 siTRAP1, HEK293 siTRAP1 and HEK293 sh-TRAP1 respectively. **d)** HCT116 sh stable clone total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate densitometric band intensities, which have been calculated by assuming protein levels of the control (scramble) equal to 1. **e)** Confocal microscopy analysis of E-cadherin in scramble and sh-TRAP1 HCT116. All images are representative of 3 independent experiments.

To complete our analysis of possible mechanisms involved in TRAP1 control of cell migration, we focused on the regulation of metabolic processes by TRAP1 and analyzed their correlation with cell migration. To this aim, we withdraw from culture medium glutamine (Gln), which is an important biosynthetic amino acid source, especially in cells with high energy demands for the synthesis of large amounts of proteins and nucleic acids. The results of these experiments show that Gln removal causes a reduction of cell motility, with effects being particularly significant in TRAP1 KD cells (Figure

16a). This abolishes the increased cell migration observed upon TRAP1 KD under basal conditions. Consistently, Gln deprivation reduces phosphorylation of p70S6K (Figure 16b), compromising cell motility in TRAP1 KD, addicted to the p70S6K pathway. Taken together, these results suggest that TRAP1 is an important regulator of AKT/p70S6K activity, through the regulation of their expression; this, in turn, confers resistance to nutrient deprivation and to p70S6K inhibitory drugs, thus enabling cell motility under condition in which it would be normally impaired.

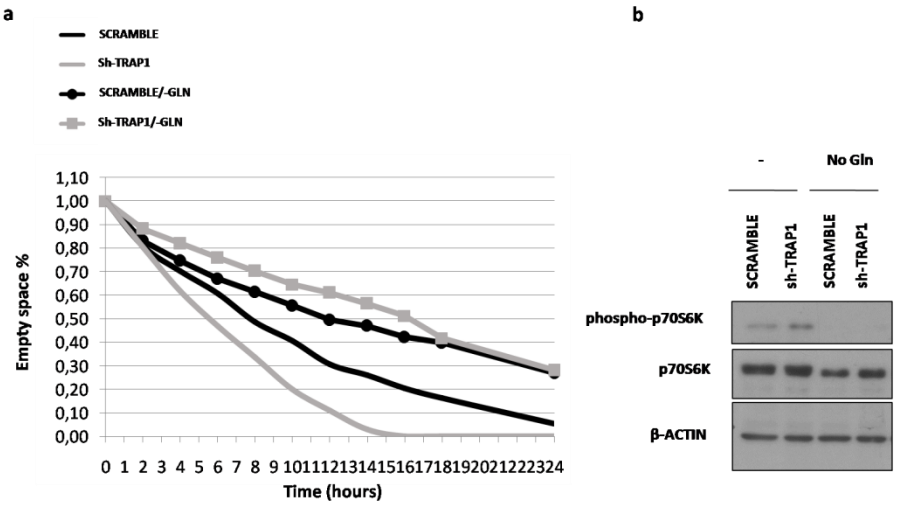


Figure16 Effect of glutamine deprivation on scramble and sh-TRAP1 cells in wound healing assay. a) Wound healing assays of HEK293 cells upon Gln deprivation: the occupation rate of empty space, evaluated as the ratio between average distance between the two edges at each time point (Lti) and the same distance immediately after the scratch (Lt0), is reported as a function of time for HEK293 scramble (black) and sh-TRAP1 (gray) cells by using time points corresponding to snapshots taken at 2 hour intervals up to 24 h after the wound. Closed symbols are used for glutamine-deprived cultures; simple traces are used for untreated cultures. **b)** HEK293 sh stable clones were cultured in a Gln. Total lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Images are representative of 2 independent experiments.

3.7 TRAP1 regulates genes involved in cell movement and metastases

To further evaluate the influence of TRAP1 on the migratory phenotype, we took advantage of a whole genome gene expression profiling recently performed in HCT116 sh-TRAP1 cells (Array Express, accession number E-MTAB-2500). This allowed the identification of 504 genes significantly modulated in TRAP1-

silenced cells ($p<0.001$), with 246 up- and 258 downregulated. The analysis of the dataset with the Ingenuity Pathway Analysis identified cell movement among the top 5 predicted biofunctions. Among all the genes associated with cell movement, 3 of them, SOX4, F3 and PRSS3, have a cutoff value of fold change >3. The expression of the three genes was validated by qPCR, and SOX4 and F3 confirmed their transcriptional regulation in HCT116 upon TRAP1 interference (Figure 17). Among those, the regulation of F3/Tissue Factor, a gene playing an important role in tissue repair, inflammation, angiogenesis, and tumour metastasis[54], was also confirmed in HEK293 cells, in which TRAP1 downregulation yields a significant decrease in F3 mRNA levels.

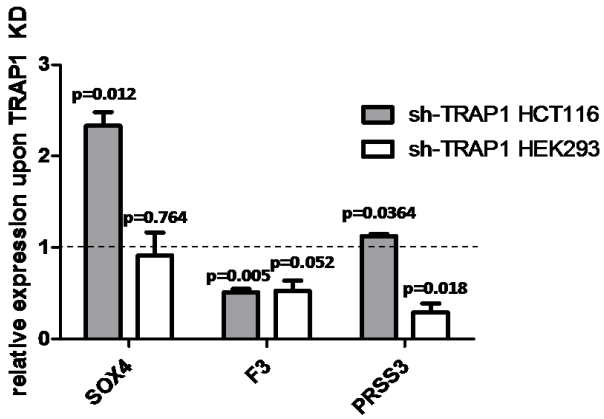


Figure 17 TRAP1 expression modulates genes involved in cell movement. qPCR analysis of SOX4, F3 and PRSS3 mRNA expression in HCT116 and HEK293 TRAP1 stably interfered clones. All data are expressed as mean with SEM from 3 independent experiments with technical triplicates each. The p-values indicate the statistical significance between relative expression levels. Dashed line indicates expression level of scramble controls.

3.8 The role of TRAP1 in protein synthesis is relevant in cancer

Finally, we evaluated whether TRAP1-dependent regulation of protein synthesis rate and of AKT/p70S6K axis may be relevant in human colorectal cancer (CRC).

We used a tissue collection of CRCs and analyzed 10 TRAP1-positive and 10 TRAP1-negative human CRCs for eEF1G, eEF1A, eIF4A and eIF4E expression. Remarkably, the majority of the TRAP1-positive tumors exhibited upregulation of eEF1G (7/10 cases), eEF1A (8/10),

eIF4A (5/10 cases) and eIF4E (8/10 cases). In contrast, among 10 tumors with low expression of TRAP1, all exhibited low levels of eIF4A and eIF4E, 9/10 exhibited low expression of eEF1G and 7/10 exhibited low expression of eEF1A. A $\chi 2$ test demonstrated a positive statistical correlation between the expression levels of TRAP1 and those of eEF1G (P=0.02), eIF4A (P=0.039) and eIF4E (P=0.001) and a trend toward a positive correlation between TRAP1 and eEF1A levels (P=0.07) (Table 1).

Moreover, a correlation between TRAP1 and AKT/phospho-AKT and a correlation between TRAP1 and p70S6K/phospho-p70S6K levels were confirmed in our CRC collection. Table 2 reports the immunoblot densitometric analysis of AKT and phospho-AKT in 14 tumour samples, where the majority of TRAP1-upregulated tumours exhibited the downregulation of AKT (10/14 cases). Phosphorylation levels were also assessed, with 12/14 TRAP1-upregulated tumours exhibiting the downregulation of phospho-AKT, as confirmed by the $\chi 2$ test ($p<0.01$).

The majority of TRAP1-upregulated tumors exhibited the downregulation of p70S6K (12/17 cases), as confirmed by the $\chi 2$ test ($p=0.04$); phosphorylation levels were also assessed, with similar results, as reported in Table 3. By contrast, tumors with non upregulated TRAP1 levels showed stable or upregulated levels of p70S6K and phospho-p70S6K.

Taken together, these observations suggest that TRAP1-dependent regulation of AKT/p70S6K axis and, likely, its downstream pathway, is conserved in human colorectal tumors with high TRAP1 expression.

Table 1 TRAP1 regulation of protein synthesis in CRCs.

Sample #	Trap1	eEF1G	eIF4A	eIF4E	eIF1A
1	5.5	2.0	1.01	7.3	2.3
2	5.9	1.4	2.6	2.0	5.0
3	4.0	6.7	0.6	1.8	3.23
4	19.7	3.8	14.0	4.6	3.6
5	12.5	1.8	2.8	3.0	2.23
6	6.31	2.4	1.7	3.15	3.64
7	4.53	1.92	3.4	1.3	8.24
8	4.0	3.66	1.7	2.0	1.65
9	5.7	8.9	1.0	3.8	10.4
10	9.4	0.98	1.3	2.3	2.9
1	1.13	0.98	0.51	1.04	0.4
2	1.5	1.2	0.2	0.98	1.5
3	0.66	0.6	0.23	0.98	1.5
4	0.04	1.3	1.0	1.24	1.7
5	0.4	0.62	0.2	0.9	0.35
6	0.8	0.99	1.09	0.6	1.1
7	0.96	1.74	0.7	0.9	2.3
8	0.2	2.5	0.3	0.4	0.9
9	0.75	1.54	0.81	0.98	3.0
10	0.92	1.62	0.63	1.09	1.9

Table 2 Inverse correlation between TRAP1 and AKT expression and phosphorylation

Sample #	Trap1	p-AKT	AKT
1	15.47	1.0	1.5
2	4.3	0.2	0.6
3	3.9	0.1	0.6
4	3.31	0.3	0.6
5	4.3	0.2	0.4
6	4.0	0.4	0.4
7	5.16	0.3	0.6
8	4.53	0.4	0.6
9	6.31	0.3	0.6
10	12.5	1.1	1.1
11	19.7	0.4	1.0
12	4.0	0.4	0.5
13	5.9	0.3	0.5
14	5.5	0.5	1.6

Table 3 Inverse correlation between TRAP1 and p70S6K expression and phosphorylation.

Sample #	Trap1	p-p70S6K	p70S6K
1	5.70	0.21	0.24
2	9.40	1.26	1.05
3	0.75	0.68	0.25
4	0.92	0.73	3.50
5	1.13	1.07	2.30
6	1.50	0.88	1.60
7	0.33	0.83	0.97
8	0.04	0.66	0.60
9	0.44	0.37	0.22
10	0.80	1.11	1.40
11	0.96	1.02	1.56
12	0.90	1.16	1.60
13	1.40	0.63	0.89
14	1.75	1.12	0.12
15	0.79	0.67	0.21
16	1.95	1.79	0.10
17	0.75	2.00	1.07
18	5.50	0.50	0.50
19	5.90	0.50	0.21
20	4.00	0.08	0.25
21	19.7	0.37	0.15
22	12.50	0.99	4.30
23	6.31	0.66	0.40
24	4.53	0.53	0.25
25	4.00	0.48	0.31
26	4.30	0.54	0.19
27	3.31	1.03	1.17
28	3.90	0.40	0.01
29	4.30	0.49	0.48
30	15.47	0.77	0.89
31	5.16	0.46	0.05
32	7.90	0.32	0.62
33	0.06	0.75	0.94
34	0.08	2.80	1.70

3.9 TRAP1 exerts control on protein synthesis also in mitochondria

The mitochondrial EF-Tu (mEF-Tu), the orthologue of EF1A, is a putative partner of TRAP1 in our LC-MS/MS analysis. Starting from this evidence, we wondered whether TRAP1 controls protein synthesis also in mitochondria, the most TRAP1-enriched organelle. Interestingly, a duolink in situ proximity ligation assay and a microscopy analysis showed that TRAP1 binds mEF-Tu and both colocalize in mitochondria (Figure 18a-b). Since the mitochondrial and prokaryotic EF-Tu shares a 55-60% of identity, we took advantage from prokaryotic tools to further investigate this interaction. Preliminary data obtained using an *E.coli* cell-free system for *in vitro* transcription/translation of GFP variant Emerald (EmGFP) (Figure 18c) show that TRAP1 is able to influence prokaryotic protein synthesis, as already observed in wheat germ extract assays.

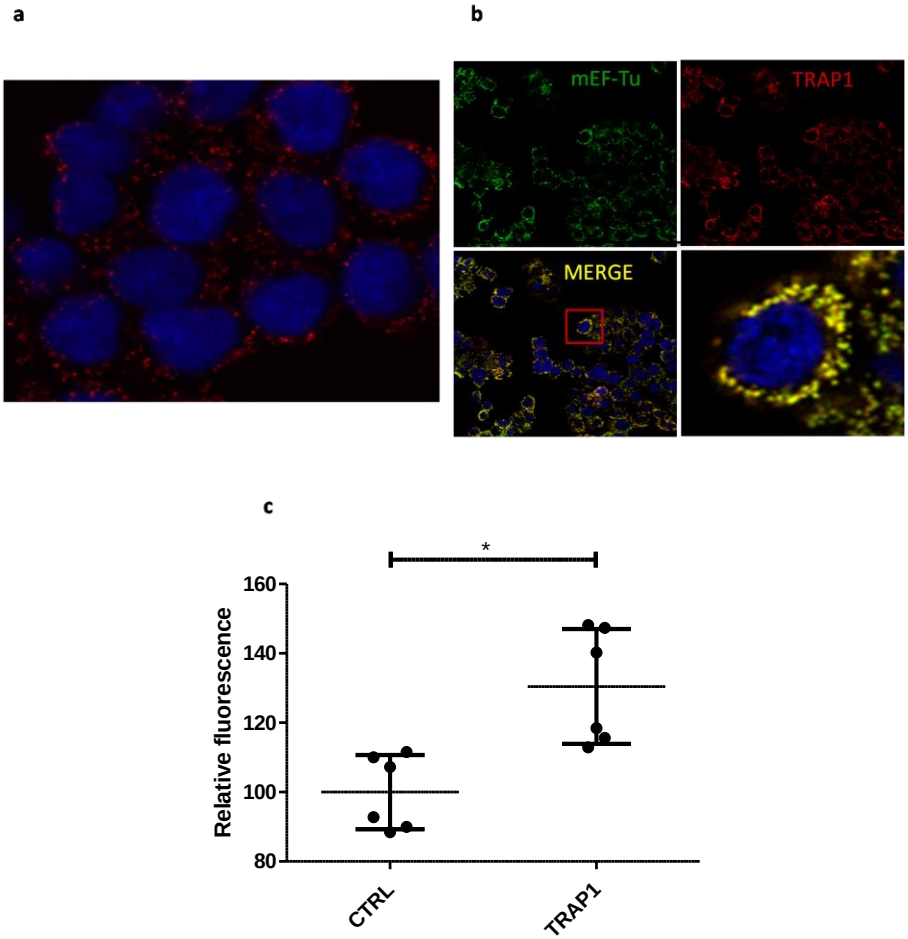


Figure 18 TRAP1 is involved in mitochondrial protein synthesis through its interaction with mEF-Tu. a) Duolink in situ proximity ligation assay imaging were obtained by incubating cells with primary antibodies, with secondary antibodies conjugated with MINUS and PLUS oligonucleotides, followed by a ligation and amplification reaction. Proximity ligation assay dots (red) are generated if two proteins are in close proximity (<40 nm). **b)** TRAP1/mEF-Tu colocalization: HCT116 cells were fixed and treated as described in Materials and Methods. **c)** EmGFP *in vitro* transcription/translation using *E. coli* lysates. EmGFP expressing vector was added to reactions at a final concentration of 10 ng/μL. Where indicated 0,2 μg/μL TRAP1 recombinant protein was added to the reaction. Data are expressed as mean ± SD from 6 independent experiments; * P≤ 0.01.

To further investigate whether TRAP1/EF-Tu interaction could affect the activity of the elongation factor, we analyzed the association and dissociation of prokaryotic EF-Tu from the ribosome by stopped-flow experiments. A Ternary Complex in which EF-Tu is labeled with the QSY9 fluorescence quencher (QSY-TC), and a bacterial 70S Initiation Complex labeled with a Cy3 fluorophore on protein L11 (Cy3-

70SIC), have been used in these assays. Upon entering in the A-site, the quencher-labeled EF-Tu decreases the Cy3–labeled ribosome fluorescence, whereas its dissociation from the ribosome allows Cy3 fluorescence recovery. As shown in Figure 19, TRAP1 is able to inhibit dissociation of prokaryotic EF-Tu from 70SIC, and this is more evident upon TRAP1 and QSY-TC preincubation (purple trace).

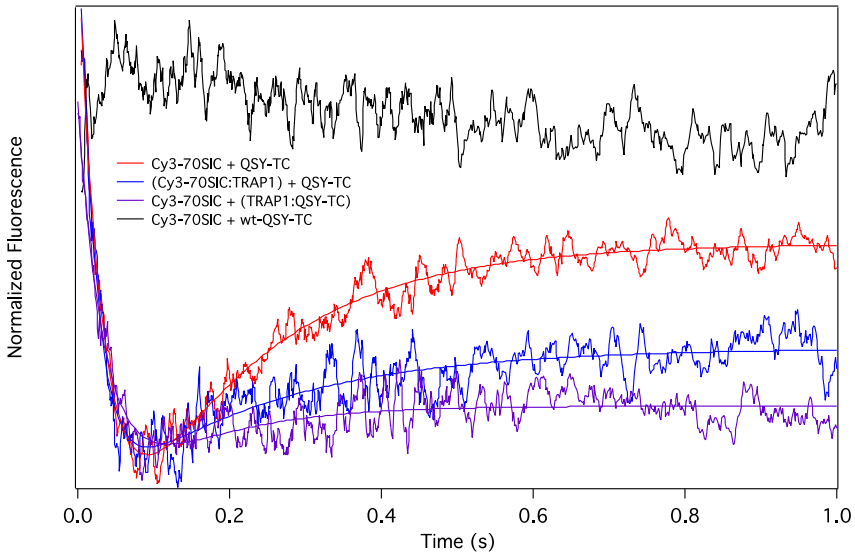


Figure 19 TRAP1 inhibits EF-Tu release from 70SIC. Stopped-flow assays: 0,3 µg/µlTRAP1 recombinant protein was pre-incubated with QSY-TC (purple trace) or with Cy3-70SIC (blu trace); upon a rapid mixing of the two mixtures, change in Cy3 fluorescence was monitored using a stopped-flow instrument (see Material and Methods). Black trace, negative control; red trace, positive control

All together these preliminary results suggest that TRAP1 could attenuate mitochondrial protein translation through a direct inhibition of elongation step.

4. Discussion/Conclusions

TRAP1 is upregulated in most tumor types and it is involved in the protection from oxidative stress and mitochondrial cell death, in signaling circuitries of mitochondrial integrity and cellular homeostasis[27]. Recently, we have shown that TRAP1 is involved in the crosstalk between mitochondria and ER and in ER stress protection of tumor cells: indeed, we demonstrated a role of TRAP1 in protein quality control due to its interaction with proteasomal and translational machinery components in the ER[27,44]. The association of the chaperone apparatus to protein synthesis machinery has been already demonstrated in eukaryotic system as well as the contribute of ribosome-bound molecular chaperones in the protection of nascent chains from premature co-translational ubiquitination[27]. We found TRAP1 associates with polysomes in cancer cells; moreover, we observed a change in the total amount of active polysomes upon TRAP1 inhibition, highlighting that this interaction to translational components has more implications than the classical *de novo* protein folding already described for chaperones. Consistently, the *in vitro* translation assays clearly confirm TRAP1 causal role in protein synthesis attenuation. The optimization of protein synthesis in *in vitro* translational assays using both wheat germ and *E.coli* extracts could be considered as an unequivocal proof of TRAP1 influence on translational components. Further *in vitro* experiments by using eukaryotic translational tools and an mRNA translated by a cap-mediated mechanism could allow us to determine if this *in vitro* assay may provide more information, such as a prediction of cellular phenotype.

A very interesting finding in the present work is the identification of a new cytoprotective role carried out by TRAP1: through regulation of protein synthesis, TRAP1 confers cells the capability to cope with stresses, therapy-induced or normally found in tumour microenvironment. Indeed, it is well known that the post-transcriptional regulation represents a fast way to handle stress stimuli: it is clear that in such situations the usual order of events, with transcription and subsequent translation, may be too slow for an appropriate physiological reaction[56]. Thus, the concept of "translation on demand" has been proposed as the mechanism to characterize the responses of tumor cells in different biological phenotypes[54]. 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. We demonstrated that TRAP1 is involved in the attenuation of cap-dependent synthesis, suggesting that this translational control mechanism would provide a survival advantage to cancer cells, expanding indefinitely their growth even under unfavorable conditions. Moreover, this is in agreement with demonstration that high rates of translation elongation negatively affect both the fidelity of translation and the co-translational folding of nascent polypeptides. As a consequence, by slowing down translation, cancer cells can efficiently improve the correct folding of proteins relevant for tumorigenesis. Furthermore, 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, a large number are found in cancer related genes[55].

The importance of this regulation in tumour biology, led us to further analyze the pathways of protein synthesis in cancer cells regulated by TRAP1. Then, we 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. S6Ks have been shown to accelerate the initiation step of translation by phosphorylating the cap binding complex component eIF4B at serine 422[55]. Consistently, we show that phosphorylation levels of translation initiation factors, namely eIF4G, eIF4B and eIF4E, are higher in colorectal cancer cells upon TRAP1 KD, thus indicating a condition of improved cap-dependent translation. Remarkably, we have unveiled a link between translational stress response and cell migration behavior, both processes in which TRAP1-regulated S6Ks are involved[54]. The wound healing assays in the presence and absence of translational drugs, such as Ribavirin and 4EGI-1, show that TRAP1 influences global mRNA translation and favors the synthesis of pro-motility molecules, as also revealed by the gene expression performed in HCT116 stable clones, thus allowing migration under conditions where cell migration is normally impaired. Moreover,

lower TRAP1 background makes cancer cells more dependent from protein synthesis, as demonstrated by apoptosis increase in sh-TRAP1 cells upon Ribavirin and 4EGI-1 treatments. 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[54]. 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[54]. We have also demonstrated that TRAP1 affects cell migration through a regulation of the AKT/p70S6K axis, which is upregulated in TRAP1 KD cells. As a consequence, upon TRAP1 silencing cancer cells show a higher migratory potential under condition of full nutrient availability and in the absence of cellular stress, whereas in low TRAP1 background, cells seem to be addicted to the activation of this pathway, as demonstrated by stronger inhibition of cell migration in sh-TRAP1 cells upon treatment with PF4708671, a p70S6K inhibitor. Analogously, glutamine deprivation profoundly affects the ability of cells to migrate in low TRAP1 background, whereas motility of TRAP1 expressing cells is marginally impaired. The observed high motile behavior in low TRAP1 background is therefore p70S6K- and glutamine-dependent. Accordingly, Caino et al[57]. showed that mitochondrial HSP90s, including TRAP1, are crucial for tumour cell motility in condition of poor nutrient availability. Moreover, in the absence of metabolic stress, cells most rely on AKT/p70S6K pathway for cell motility. Interestingly, it has been shown that cells expressing constitutively active AKT are highly sensitized to cell death induced by nutrient and growth factor deprivation[54]. In this view, TRAP1 expression could represent a mechanism of resistance adopted by cancer cells when nutrient scarcity requires downregulation of the AKT pathway.

According to the classical multistep model, metastases generated from tumour cells that are able to infiltrate vessels, survive to circulation in the blood stream and colonize new sites. To do this, cells must undergo several morphologic and metabolic changes that go under the definition of epithelial to mesenchymal transition. In this context, it has been reported that p70S6K is involved in the regulation of the

Snail gene[54]. We have therefore analyzed the expression of Snail and E-cadherin in two different cell models, finding that a slight regulation in the expression of both genes is detectable upon TRAP1 KD, although this seems not to be the main mechanism responsible for the changes in the motile behavior of these cells. Conversely, we demonstrated, at least in our experimental systems, a prevalent function of metabolic balance for the TRAP1-dependent regulation of the AKT/p70S6K pathway. Possibly, more suitable experimental systems, in which many data already confirmed the causal correlation between cell migration, EMT and the PI3K/AKT/p70S6K pathway, will provide further insights into TRAP1 role in metastatic dissemination. Of note, it has been demonstrated[54] that in two different cell lines the expression of TRAP1 is inversely related to the expression of genes involved in metastasis, suggesting that, while induction of TRAP1 expression promotes cell proliferation and tumour growth through the TNF pathway, its downregulation may lead to decreased proliferation and increase of metastatic potential. We also analyzed the gene expression pattern of HCT116 cells following TRAP1 downregulation, and found several genes involved in cell motility and EMT to be differentially regulated in TRAP1 KD cells. In particular, we have analyzed the expression of SOX4, F3 and PRSS3. SOX4, which is considered the master regulator of EMT[54], is substantially upregulated in HCT116 sh-TRAP1 cells. SOX4 KD has been related to reduced tumour cell migration, invasion, in vivo tumourigenesis and metastasis in hepatocellular carcinoma[54], and overexpression of nuclear SOX4 was significantly correlated with invasion, metastasis and stage in CRC patients[54]. Conversely, we found that the F3/Tissue Factor gene, which has a role not only in coagulation control, but also in angiogenesis[54], is two-fold downregulated in the highly motile sh-TRAP1 cells. Interestingly, F3 is the only gene that we have found significantly downregulated in both cell lines in which TRAP1 has been silenced. Taken together, these results confirm a dual role of TRAP1 in the regulation of cell motility, enabling cell movement under limiting conditions, while possibly reducing the maximum migratory potential of cells when plenty of energy source are available.

The regulation of protein synthesis and AKT/p70S6K axis is conserved in CRC specimens; moreover, our preliminary observation shows high tendency of TRAP1-positive CRCs to produce distant

metastases, despite low AKT expression. This data suggest that TRAP1 has a pivotal role in CRC development and migration, and underline that this chaperone is an important element in the multistep process of tumor progression and metastasis dissemination. Hence, targeting TRAP1 may enhance the efficacy of antimetastatic treatments selectively for those cancers in which signaling pathway by this chaperone contributes to resistance to tumour-suppressive mechanisms and metabolic stress.

TRAP1 role in protein synthesis control has left some open questions. Whereas the regulation of AKT/p70S6K pathway has been extensively analyzed, it is possible that TRAP1 is able to inhibit a translational step by its direct interaction with initiation and elongation factors. Our preliminary data show that TRAP1 binds the mitochondrial EF-Tu, the counterpart of the cytosolic EF1A. Taking advantage from sequence identity (more than 50%) between the mitochondrial and the prokaryotic EF-Tu, we demonstrated that TRAP1 inhibits the elongation factor release from the prokaryotic ribosome, slowing down the elongation step. A similar regulation of EF1A factor has been reported in several papers. Sivan et al[58] demonstrated that, during mitosis, the elongation step is regulated through the phosphorylation of eEF1B, a factor necessary to catalyze the GDP /GTP exchange on eEF1A, that causes a lower affinity to its substrate. This modification is correlated with reduced availability of eEF1A-tRNA complexes, as well as reduced delivery of tRNA to and association of eEF1A with elongating ribosomes. Moreover, Howe et al[20] identified the eukaryotic EF1A1 as an integral component of a complex that binds to a structural element in the 3′-UTR of two mRNAs inhibiting their translation. A component of this complex blocks progression of the 80S ribosome by preventing the release of eEF1A1 from the ribosomal A site post GTP hydrolysis. We have hypothesized that TRAP1 could directly affect the release of EF-Tu from ribosome, since the stopped-flow reaction composition allows to observe only one round of bound/release of the elongation factor from the ribosome, excluding a recycling of EF-Tu. However, further studies are needed to clarify which step of elongation stage is affected by TRAP1, i.e. GTP hydrolysis, EF-Tu-GDP dissociation from the ribosome, and to understand if EF1A undergos a similar regulation.

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Translational control in the stress adaptive response of cancer cells: a novel role for the heat shock protein TRAP1

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TNF receptor-associated protein 1 (TRAP1), the main mitochondrial member of the heat shock protein (HSP) 90 family, is induced in most tumor types and is involved in the regulation of proteostasis in the mitochondria of tumor cells through the control of folding and stability of selective proteins, such as Cyclophilin D and Sorcin. Notably, we have recently demonstrated that TRAP1 also interacts with the regulatory protein particle TBP7 in the endoplasmic reticulum (ER), where it is involved in a further extra-mitochondrial quality control of nuclear-encoded mitochondrial proteins through the regulation of their ubiquitination/degradation. Here we show that TRAP1 is involved in the translational control of cancer cells through an attenuation of global protein synthesis, as evidenced by an inverse correlation between TRAP1 expression and ubiquitination/degradation of nascent stress-protective client proteins. This study demonstrates for the first time that TRAP1 is associated with ribosomes and with several translation factors in colon carcinoma cells and, remarkably, is found co-upregulated with some components of the translational apparatus (eIF4A, eIF4E, eEF1A and eEF1G) in human colorectal cancers, with potential new opportunities for therapeutic intervention in humans. Moreover, TRAP1 regulates the rate of protein synthesis through the eIF2α pathway either under basal conditions or under stress, favoring the activation of GCN2 and PERK kinases, with consequent phosphorylation of eIF2α and attenuation of cap-dependent translation. This enhances the synthesis of selective stress-responsive proteins, such as the transcription factor ATF4 and its downstream effectors BiP/Grp78, and the cystine antiporter system xCT, thereby providing protection against ER stress, oxidative damage and nutrient deprivation. Accordingly, TRAP1 silencing sensitizes cells to apoptosis induced by novel antitumoral drugs that inhibit cap-dependent translation, such as ribavirin or 4EGI-1, and reduces the ability of cells to migrate through the pores of transwell filters. These new findings target the TRAP1 network in the development of novel anti-cancer strategies.

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TNF receptor-associated protein 1 (TRAP1), the only mitochondrial member of the heat shock protein (HSP)90 protein family, is involved in protection from oxidative stress and apoptosis induced by several antitumor agents and other stressors.[1] Acute silencing of TRAP1 in tumor cells has been consistently associated with CypD-dependent mitochondrial apoptosis.[2] TRAP1-dependent organelle-directed regulation of folding and stability of selective proteins involved in mitochondrial homeostasis, such as Cyclophilin D and Sorcin, is pivotal for the control of tumor cell proteostasis, leading to resistance to apoptosis.[3] Remarkably, aberrant deregulation of TRAP1 function has been observed in colorectal[4] and prostate carcinomas,[5] with potential new opportunities for therapeutic intervention in humans. Evidence suggests that, despite the high homology between all members of the HSP90 chaperone family, TRAP1 has distinct functional properties.[6] TRAP1 is involved in endoplasmic reticulum (ER) stress protection[7,8] and some recent findings have reported other sub-cellular localizations of this chaperone.[6] In fact, we have recently demonstrated that TRAP1 also localizes in the ER, where it directly interacts with the proteasomal particle TBP7 and controls ubiquitination/

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Keywords: TRAP1; stress-adaptive response; protein synthesis; protein quality control; co-translational ubiquitination
Abbreviations: TRAP1, TNF receptor-associated protein 1; HSP, heat shock protein; KD, knockdown; UPR, unfolded protein response; ER, endoplasmic reticulum; CRC, colorectal carcinoma; TG, thapsigargin; Ub, ubiquitin; UPS, ubiquitin–proteasome system; shRNA, short-hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHX, cycloheximide
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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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TRAP1 controls cell migration of cancer cells in metabolic stress conditions: Correlations with AKT/p70S6K pathways

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ABSTRACT

Cell motility is a highly dynamic phenomenon that is essential to physiological processes such as morphogenesis, wound healing and immune response, but also involved in pathological conditions such as metastatic dissemination of cancers. The involvement of the molecular chaperone TRAP1 in the regulation of cell motility, although still controversial, has been recently investigated along with some well-characterized roles in cancer cell survival and drug resistance in several tumour types. Among different functions, TRAP1-dependent regulation of protein synthesis seems to be involved in the migratory behaviour of cancer cells and, interestingly, the expression of p70S6K, a kinase responsible for translation initiation, playing a role in cell motility, is regulated by TRAP1. In this study, we demonstrate that TRAP1 silencing enhances cell motility in vitro but compromises the ability of cells to overcome stress conditions, and that this effect is mediated by the AKT/p70S6K pathway. In fact: i) inhibition of p70S6K activity specifically reduces migration in TRAP1 knock-down cells; ii) nutrient deprivation affects p70S6K activity thereby impairing cell migration only in TRAP1-deficient cells; iii) TRAP1 regulates the expression of both AKT and p70S6K at post-transcriptional level; and iii) TRAP1 silencing modulates the expression of genes involved in cell motility and epithelial–mesenchymal transition. Notably, a correlation between TRAP1 and AKT expression is found in vivo in human colorectal tumours. These results provide new insights into TRAP1 role in the regulation of cell migration in cancer cells, tumour progression and metastatic mechanisms.

1. Introduction

TRAP1 (*T*umour Necrosis Factor *R*eceptor-*A*ssociated *P*rotein 1) is a molecular chaperone, member of the HSP90 family, that contributes to survival of cancer cells and is induced in most tumour types [1]. Recent reports have shown that TRAP1 stays at the crossroad of multiple crucial processes in the onset and progression of the malignant phenotype. Indeed, TRAP1: i) controls protein homeostasis through a direct involvement in the regulation of protein synthesis and protein co-translational degradation [2]; ii) contributes to tumour cell bioenergetic regulation through the control of mitochondrial respiratory complexes [3–5]; iii) is part of a pro-survival signalling pathway aimed at evading the toxic effects of oxidants and anticancer drugs and protects mitochondria against damaging stimuli via a decrease of ROS generation [6]. Since elevated ROS are reported to stimulate cell invasion [7], Yoshida and colleagues [4] evaluated whether TRAP1 expression might affect this phenotype by transwell migration assays. Interestingly, they demonstrated that TRAP1 knockout or transient suppression dramatically enhances cell invasiveness, both in mouse fibroblasts and in a variety of human cell lines. The authors hypothesize that the contribution of TRAP1 to this phenotype may be attributed, at least in part, to its impact on cellular bioenergetics. By contrast, TRAP1-directed tumour cell metabolism has been proposed as a pivotal mediator of tumour cell motility and invasion in conditions of nutrient withdrawal [8]. In this view, mitochondrial HSP90-directed bioenergetics could represent an adaptive mechanism overcoming the global tumour-suppressive

Abbreviations: TRAP1, TNF receptor-associated protein 1; HSP, heat shock protein; KD, knockdown; ER, endoplasmic reticulum; CRC, colorectal carcinoma; EMT, epithelial–mesenchymal transition; shRNA, short-hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S6K, S6 kinases; siRNA, small interfering RNA.

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