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ROLE OF TRAP1 IN CHEMORESISTANCE, APOPTOSIS AND PROTEIN QUALITY CONTROL

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In un viaggio non è importante la destinazione, ma il percorso; ancora più importanti sono le persone che ci accompagnano lungo la strada, consentendoci di ritrovare la "giusta direzione" quando ci sentiamo smarriti…
per questo motivo, desidero ringraziare alcune delle persone che mi sono state accanto in questi tre lunghi anni:

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RIASSUNTO

TRAP1 è uno chaperone molecolare con funzione antiapoptotica, identificato in seguito ad analisi di mRNA-differential display effettuate in cellule adattate allo stress ossidativo. L'obiettivo principale della mia tesi di dottorato è stato quello di caratterizzare l'interazione fra TRAP1 e TBP7/Rpt3, una ATPasi appartenente alla subunità regolatoria 19S del proteasoma, identificata come putativo ligando di TRAP1 in seguito ad analisi di spettrometria di massa. Esperimenti di coimmunoprecipitazione ed analisi di FRET (trasferimento di energia fluorescente in risonanza) dimostrano che TRAP1 e TBP7 sono localizzati sul versante citoplasmatico del reticolo endoplasmatico (ER), e che proprio in tale compartimento interagiscono; questa è la prima dimostrazione della localizzazione reticolare di TRAP1. La presenza di queste due proteine nell'ER lascia presupporre un loro ruolo nell'omeostasi di tale organello; il silenziamento di TRAP1 e di TBP7, infatti, sensibilizza le cellule HCT116 allo stress del reticolo indotto da tapsigargina, causando un incremento dell'espressione dello chaperone stress- indotto Bip/Grp78, e causa un notevole aumento dell'ubiquitinazione cellulare, specialmente nei compartimenti extra-mitocondriali. L'azione sul controllo di qualità esercitato da TRAP1 e TBP7 è particolarmente evidente su specifiche proteine destinate al mitocondrio, Sorcina 18 e la subunità ß del complesso F1ATPasi, i cui livelli sono diminuiti in assenza di TRAP1 a causa del loro aumentato livello di ubiquitinazione. Questo meccanismo assume un impatto ancora più rilevante dal momento che la co-regolazione TRAP1-dipendente di substrati specifici è ritrovata anche in campioni di tessuti tumorali prelevati da carcinomi colon-rettali, in cui TRAP1 risulta iperespresso rispetto alle relative mucose sane.
L'interazione TRAP1/TBP7 sull'ER apre nuovi scenari relativi al controllo di qualità delle proteine destinate al mitocondrio, e suggerisce che tale controllo possa avvenire all'interfaccia tra i due organelli; se le proteine neo-sintetizzate sono non correttamente ripiegate, danneggiate o mis-localizzate, vengono riconosciute dal complesso TRAP1/TBP7, ubiquitinate ed indirizzate al proteasoma per la degradazione.

SUMMARY

TRAP1 is an antiapoptotic heat shock protein, identified through an mRNA-differential display analysis in oxidants- adapted osteosarcoma cells. The main objective of my PhD thesis has been the characterization of the interaction between TRAP1 and TBP7 (S6-ATPase 4/Rpt3), a component of the 19S proteasome regulatory subunit, one of the putative TRAP1 "partners" identified by our LC-MS/MS analysis. I demonstrated that TRAP1 and TBP7 are located in the endoplasmic reticulum (ER), on the outer side of this compartment; they directly interact in the ER, as demonstrated by coimmunoprecipitation experiments and by FRET analysis. The information available on the TRAP1 pathway describes just a few well-characterized functions of this protein in mitochondria. This is the first demonstration of TRAP1's presence in this cellular compartment. Given the ER localization of TRAP1 and TBP7, the involvement of these two proteins in ER homeostasis has been investigated. TRAP1silencing by short-hairpin RNAs, in cells exposed to thapsigargin-induced ER stress, correlates with upregulation of BiP/Grp78, thus suggesting a role of TRAP1 in the refolding of damaged proteins and in ER stress protection. Consistently, TRAP1 and/or TBP7 interference enhanced stress-induced cell death and increased intracellular protein ubiquitination. These experiments led us to hypothesize an involvement of TRAP1 in protein quality control for mistargeted/misfolded mitochondria-destined proteins, through interaction with the regulatory proteasome protein TBP7. Remarkably, expression of specific MITO proteins decreased upon TRAP1 interference as a consequence of increased ubiquitination. The proposed TRAP1 network has an impact in vivo, as it is conserved in human colorectal cancers, is controlled by ER-localized TRAP1 interacting with TBP7 and provides a novel model of the ER–mitochondria crosstalk.

The data described in my thesis on the functional characterization of TRAP1/TBP7 interaction allowed us to draw the following hypopthesis: when neo-synthesized proteins are damaged, they are not imported into mitochondria, but sequestered by TRAP1 to be refolded/ repaired; if this attempt fails, these substrates are ubiquitinated, recognised by the regulatory subunits of proteasome to which TBP7 belongs, and delivered to the proteolytic core for degradation.

1 INTRODUCTION

1.1 TRAP1

The cellular chaperone machinery consists of several protein families that facilitate polypeptide folding *in vivo* and prevent misfolding and aggregation. Members of these protein families are often known as stress proteins or heat-shock proteins (HSPs), as they are upregulated under conditions of stress in which the concentrations of aggregation-prone folding intermediates increase. Chaperones are usually classified according to their molecular weight (HSP40, HSP60, HSP70, HSP90, HSP100 and the small HSPs). They are involved in a multitude of proteome-maintenance functions, not only *de novo* folding, or refolding of stress-denatured proteins, but even oligomeric assembly, protein trafficking and assistance in proteolytic degradation.

Among these, TRAP1 was first identified as an Hsp90-like chaperone by screening for proteins associated with the cytoplasmic domain of the type 1 Tumor Necrosis Factor Receptor-1 (TNFR1) using the yeast two-hybrid system (1). In an independent yeast two hybrid screen, an Hsp90-like protein of ~75 kD, designated as Hsp75, was also identified that bound the Retinoblastoma (Rb) protein (2). Sequence analysis later determined that TRAP1 and Hsp75 were identical molecules. Through a mRNA-differential display analysis between oxidant-adapted and control osteosarcoma cells, our group identified, among other proteins, TRAP1 whose expression was highly induced upon oxidant adaptation (3).

Interestingly, our group recently demonstrated that stable and transient transfectants containing high TRAP1 levels: (i) are more resistant to both H_2O_2-induced DNA damage and to the apoptosis induced by cisplatin

(CDDP) and other apoptotic stressors; (ii) don't release apoptosis-inducing factors from mitochondria upon CDDP treatment; (iii) show decreased caspase 3 activation; (iv) contain higher reduced glutathione levels than control cells, which probably contributed to their oxidant-resistant phenotype (4).

Furthermore, TRAP1 exhibited antiapoptotic functions (4), while an involvement of this chaperone in the multi-drug resistance of human colorectal carcinoma (CRC) cells was also established (5), according to the observation that adaptation to oxidative stress is a phenomenon deeply linked to drug resistance to antitumor agents. Moreover, recent data identify TRAP1 as a novel mitochondrial survival factor differentially expressed in localized and metastatic prostate cancer compared with normal prostate, suggesting that TRAP1 could be a novel therapeutic target to enhance tumor cell apoptosis (6). Indeed, TRAP1 and HSP90 were recently described as components of a mitochondrial pathway selectively up-regulated in tumor cells which antagonizes the proapoptotic activity of cyclophilin D (CypD), a regulator of the mitochondrial permeability transition pore (PTP) and responsible for the maintenance of mitochondrial integrity, thus favoring cell survival (7).

A major objective of our research group is the investigation of the molecular mechanisms responsible for TRAP1 protection from apoptosis and its role in drug resistance, with the attempt to characterize TRAP1 pathway as a novel molecular target for cancer therapy. In such a perspective, among several TRAP1 "ligands", recently identified by mass spectrometry analysis, we selected two proteins for further studies, whose interaction with TRAP1 is involved in chemioresistance, endoplasmic stress (ER) stress protection

and regulation of protein quality control, functions all proposed as novel approaches for cancer therapy.

A "fishing for partners" strategy combined with liquid chromatography-mass spectrometry analysis (LC-MS/MS) was carried out to identify TRAP1 protein partners specifically interacting with the bait. Among all the putative ligands, our group selected two proteins for further investigation: the first one is a protein of ~18 kDa identified as Sorcin by LC-MS/MS, a Calcium (Ca^{2+}) binding protein involved in the regulation of intracellular Ca^{2+} homeostasis (8). It was identified as overexpressed in a proteomic screening in gastric cancer cell lines and in chemioresistant tumor cells (9, 10). The involvement of Sorcin in chemioresistance and cell survival and the importance played by calcium in the regulation of PTP opening in mitochondria as well as in global cellular homeostasis prompted us to investigate the functional role of TRAP1-Sorcin interaction in colon cancer cells.

As previously anticipated research activity during my PhD course also focused on the study of a second TRAP1 ligand identified by mass spectrometry, the Tat- binding protein 7 TBP7, a component of 19S regulatory proteasomal subunit (11). The identification of novel pathways for protein quality control, likely altered in tumor cells, in which TRAP1/TBP7 could be involved moved our interest in investigating this interaction.

1.2 CHAPERONES AND PROTEIN QUALITY CONTROL

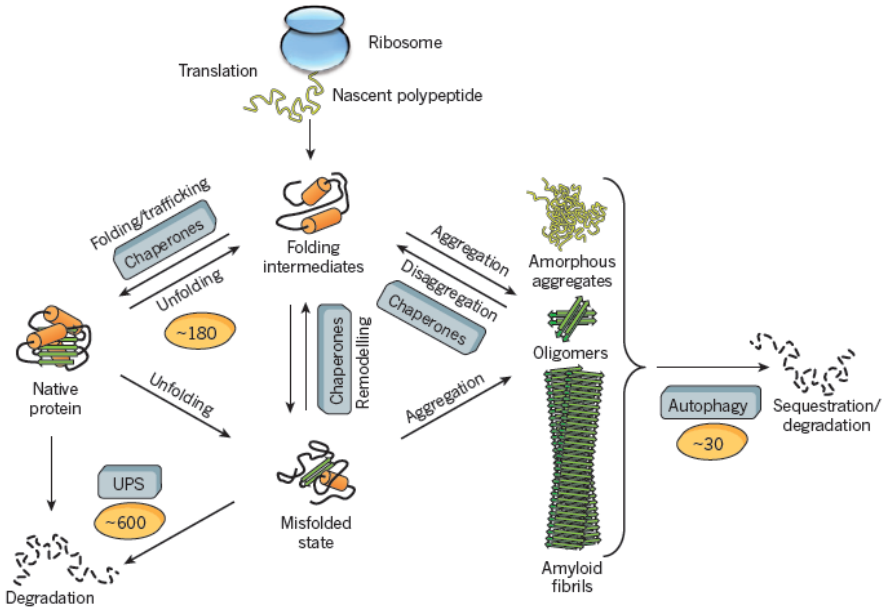
Eukaryotic cells must face with a continuous stream of misfolded proteins that compromise the balance of cellular protein homeostasis and

cell viability. An elaborate network of molecular chaperones and protein degradation factors continually monitors and maintains the integrity of the proteome. Cellular protein quality control relies on distinct yet interconnected strategies whereby misfolded proteins can either be refolded, degraded, or delivered to distinct quality control compartments that sequester potentially harmful misfolded species. Molecular chaperones play a critical role in determining the fate of misfolded proteins in the cell (12).

Mammalian cells typically express in excess of 10,000 different protein species, which are synthesized on ribosomes as linear chains of up to several thousands amino acids. To function, these chains must generally fold into their "native state", an ensemble of a few closely related three-dimensional structures. How this is accomplished and how cells ensure the conformational integrity of their proteome in the face of acute and chronic challenges constitute one of the most fundamental and medically relevant problems in biology. Central to this problem is that proteins must retain conformational flexibility to function, and thus are only marginally thermodynamically stable in their physiological environment. A substantial fraction of all proteins in eukaryotic cells (20–30% of the total in mammalian cells) even seems to be inherently devoid of any ordered three-dimensional structure and adopt folded conformations only after interaction with binding partners. Aberrant behaviour of some of these metastable proteins, such as τ and α-synuclein, can give rise to the formation of fibrillar aggregates that are associated with dementia and Parkinson's disease (12). Since the number of possible conformations that a protein chain can adopt is very large, folding reactions are highly complex and heterogeneous (Fig.1); partially folded or misfolded states are problematic because they tend to

aggregate in a concentration-dependent manner. The formation of these aggregates *in vivo* is strongly restricted by the chaperone machinery, suggesting that they may become more widespread under stress or when protein quality control fails; molecular chaperones typically recognize hydrophobic amino-acid side chains exposed by non-native proteins and may functionally cooperate with other co-chaperones, such as the small HSPs, which function as 'holdases', buffering aggregation (12). The translation process is the first step in which proteins are exposed to the risk of misfolding or aggregation, because an incomplete nascent polypeptide is unable to fold into a stable native conformation and the local concentration of nascent chains in the context of polyribosomes is very high; since partially synthesized nascent chains are particularly prone to aggregation, the presence of molecular chaperones that interact directly with the ribosome near the exit tunnel provides an important link between translation and protein folding. Mistranslated and misfolded proteins may be detrimental for the cell and need to be removed at an early stage, but, to date, the mechanisms and coordination between translation and quality control remain poorly understood, except for the observation that members of HSP70 family seem to be involved even in higher eukaryotes (13).

Figure 1: Role of molecular chaperones in the balance of folding, degradation and aggregation. Molecular chaperones facilitate folding of newly synthesized polypeptides to the native state. Chaperones also bind to non-native intermediates that are generated when native proteins are denatured, for example by stress. Cellular surveillance results in either refolding or degradation. Under some circumstances, such as stress or aging, quality control efforts may fail; in these cases, misfolded proteins may form small soluble aggregates, which, if unresolved by refolding or degradation, go on to form heat- and detergent-resistant amyloidogenic aggregates.



1.3 UBIQUITIN-PROTEASOME SYSTEM (UPS) IN PROTEIN QUALIY CONTROL

The elimination of misfolded proteins represents an important mechanism to maintain cell viability. This protein quality control involves the binding of a chaperone to the misfolded protein and its presentation to the ubiquitin-proteasome degradation pathway (14). Linking of ubiquitin to a protein is a highly controlled process that involves the sequential action of a ubiquitin- activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). This enzymatic cascade results in the attachment of polyubiquitin chains onto specific lysine residues on the substrate; although several types of polyubiquitin chains have been reported, chains that are built on lysine 48 of ubiquitin serve as signal for recognition and degradation by 26S proteasome (Fig. 2). The 26S proteasome comprises two

main particles: the 20S core proteasome, in which proteins are cleaved and digested into short peptides, and the 19S regulatory complex, that is responsible of recognition of ubiquitinated substrates. The 20S proteolytic core particle (CP) has a cylinder-like structure composed of a stack of two α and two β rings, whereas the 19S regulatory particle (RP) comprises a "lid" of eight non-ATPases and a "base" of six ATPases (Rpt1-Rpt6) and three non-ATPases. 19S ATPases provide the molecular chaperonin activity to the 19S RP; furthermore, 19S ATPase activity is essential for the assembly of the 26S proteasome complex and participates in the degradation of the proteins marked by a chain of more than four Lys-48-linked ubiquitin molecules. The lid of the 26S proteasome associates with the polyubiquitin chain, and subsequently the base unfolds the substrate protein in an ATP-dependent manner, finally translocating it to the central chamber of the 20S CP for proteolysis (15).

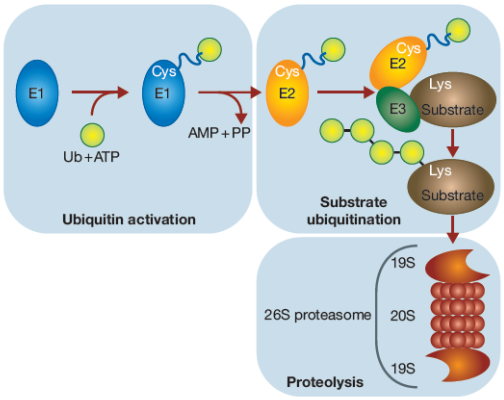


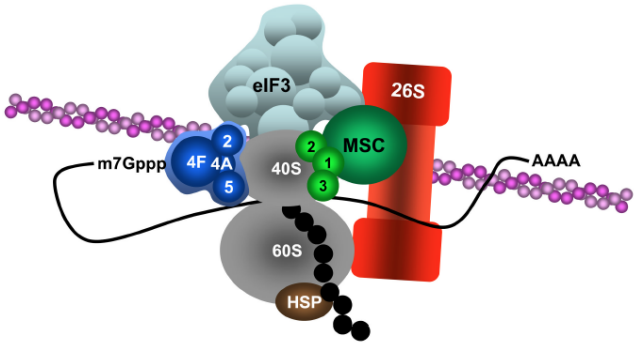
Figure 2: The Ubiquitin- Proteasome System (UPS). After an ATP-dependent activation of an ubiquitin-molecule by E1 and binding of Ub to a cysteine residue of E1, Ub is transferred to E2, binding to a cysteine residue again, while E1 is released. After binding of E2-Ub to the E3-substrate complex, the Ub is transferred to a lysine-residue on the substrate protein. After many cicles, the substrate becomes polyubiquitinated, while both E2 and E3 are released.

The UPS system is involved not only in degradation of yet translated damaged proteins, but even in quality control of nascent polypeptides emerging from ribosomes and not yet folded that can present signals similar

to those recognized by the ubiquitin system in misfolded proteins or ubiquitin moieties; in fact, it has been estimated that up to 50% of newly synthesized proteins are cotranslationally degraded by the ubiquitin-proteasome system (16, 17), implying that efficient quality control mechanisms must exist that couple protein translation, protein folding and proteasomal degradation. Moreover, it has been demonstrated that these nascent damaged proteins can be ubiquitinated while bound to ribosome, this indicating that exists a close coupling between protein synthesis and protein degradation (18).

Furthermore, very recent findings suggest an association between the ribosomal apparatus, the eukaryotic elongation factor EF1A and the proteasome subunits Rpn10 and Rpt1, supporting the idea that proteasome plays an important role in cotranslational protein quality control (19); moreover, recent data obtained in yeast demonstrate the existence of a mechanism that coordinates translation initiation, elongation, and quality control through the formation of an RNA independent supercomplex with eIFs, MSC, eEFs, ribosomes, and the proteasome, called "translasome" (20) (Fig. 3).

Figure 3: The "translasome". Eukaryotic initation factors, elongation factors, ribosomes, chaperones and proteasome associate with the nascent polypeptide to control its stability during translation, before the translated protein is released to reach the final destination.



1.4 QUALITY CONTROL AT ENDOPLASMIC RETICULUM (ERAD)

The ER is an extensive network of cisternae and microtubules, which stretches from the nuclear envelope to the cell surface in all eukaryotic cells; it's the largest organelle, with endomembrane accounting for more than 50% of all the cellular membranes, and occupies a substantial part (> 10%) of the cell volume. The ER plays several vital functions. Firstly, the ER is the site of protein synthesis in the rough ER and correct post-translational "folding" of these proteins. Secondly, the ER serves as a common transport route by which numerous proteins are delivered to their destination. Thirdly, the ER acts as an indispensable source for fast physiological signaling being a dynamic calcium ions reservoir, which can be activated by both electrical and chemical cell stimulation (21).

The ER provides an environment that is optimized for protein folding and maturation. It harbours three groups of molecular chaperones and folding enzymes: chaperones of the heat shock protein family, including Grp78/ BiP and its co-chaperone partners, chaperone lectins such as calnexin and calreticulin, and thiol oxidoreductases of the protein disulfide isomerase (PDI) family. Many of these chaperones interact with translocating nascent

polypeptide chains soon after their entry into the ER lumen and facilitate protein folding, oligomerization, maturation, and post-translational modifications, which may include glycosylation and disulfide bond formation (22).

Substrate proteins are released from ER chaperones once their native structures are attained, and correctly folded and assembled proteins are transported from the ER. However, prolonged interactions of non-native proteins with ER chaperones and folding enzymes during chaperone-mediated folding may well contribute to the retention of non-native proteins in the ER. When mis-folded proteins accumulate in the ER, ER chaperone binding reduces the concentration of free chaperones. To cope with the resulting ER stress, cells elicit an unfolded protein response (UPR). The UPR induces the transcription of gene products that facilitate the processing of aberrant proteins and that attenuate protein translation, which reduces the amount of newly imported proteins into the ER (23, 24). One of the main responses elicited by ER stress is the overexpression of ER-resident chaperones, the Grp proteins (glucose- responsive proteins), like Grp78/ BiP and Grp94, that can be induced by stress conditions like perturbation of ER-calcium stores or inhibition of glycosylation. As these stress conditions generally lead to the accumulation of misfolded proteins in the ER, induction of *Grp* gene expression has been extensively used as a marker for the unfolded protein response (UPR) (25). Pathological conditions such as tumor growth correlate with Grp78 and Grp94 overexpression (26, 27). This could be partly caused by the activation of *Grp* gene expression through glucose starvation, acidosis and hypoxia, which are hallmarks of the

microenvironment of poorly vascularized solid tumors and seem to represent the physiological stresses for Grp activation *in vivo* (28).

In eukaryotes, endoplasmic reticulum-associated degradation (ERAD) functions in cellular quality control and regulation of normal ER-resident proteins (29). Protein folding in endoplasmic reticulum is monitored by UPS: in fact, when misfolded proteins are recognized as aberrant by luminal chaperone BiP and the repair fails, they are retrotranslocated, ubiquitinated and recognized by proteasome to be degraded (Fig. 4); this hypotesis is supported by the fact that polyubiquitination is necessary for the retrotranslocation process, and by the association of proteasome to the endoplasmic reticulum and by accumulation of ubiquitinated proteins on the cytoplasmic side of ER when the proteasome is blocked (30, 31).

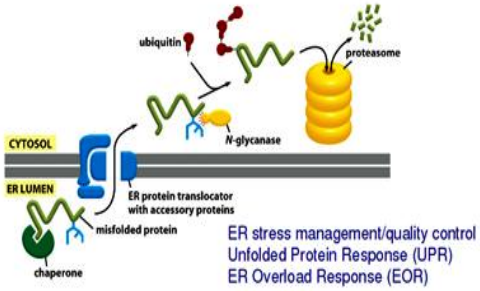


Figure 4: Ubiquitin Proteasome System at ER. Damaged proteins in the ER are retrotranslocated, ubiquitinated and degraded via UPS. Failure in this quality control leads to ER stress, activating Unfolded Protein Response (UPR).

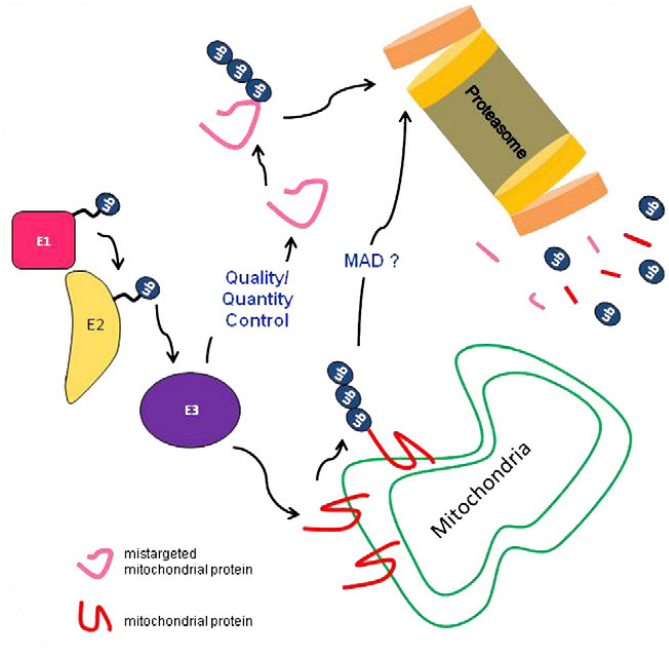
1.5 QUALITY CONTROL OF MITOCHONDRIAL PROTEINS

Protein quality control in mitochondria is a still unclear issue because of the complexity of the organelle. The mitochondrion contains two membranes, the outer membrane (OM) and the inner membrane (IM); the later forms invaginations called *cristae* and contains the matrix where mitochondrial DNA resides. The space between the inner and outer

membranes is referred to as the intermembrane space (IMS) and contains several proteins, most of them are best known for their roles in the initiation of apoptosis upon their release from the mitochondria like cytochrome *c*, smac, HtrA2/Omi, and endonuclease-G. Protein quality control (PQC) inside mitochondria seems to exclude UPS, because it is mainly exherted by AAA-ATPases, the mitochondrial proteases that are responsible of degradation of misfolded-damaged proteins; furthermore, no evidences of the presence of proteasome have been reported in mitochondria (32). Recent findings suggest that ubiquitination may be involved in degradation of many OMM proteins (33) and of the IMM protein UCP3 (34) with a mechanism of ubiquitination and retrotranslocation that reminds ERAD pathways; in fact, upon inhibition of the proteasome, proteins accumulate in the mitochondria. Furthermore, these results raised the possibility that a proteasome-dependent PQC, exerted in concert with chaperones, normally acts to limit the import of excess or misfolded mitochondrial proteins. In agreement with this possibility, the accumulation of mitochondrial proteins and the appearance of electron-dense mitochondria was also reported following treatment with an inhibitor of the chaperone HSP90 (35). Interestingly, a quality control for one of the nuclear-encoded mitochondrial proteins, that have to be imported to the final destination, requires the ubiquitin-proteasome system outside the mitochondria. Data presented by Radtke et al (36) support the hypotesis that misfolded IMS proteins, like the IMS protein endoG, are ubiquitinated and eliminated in the cytoplasm prior to their import into the mitochondria, indicating presence of a PQC that monitors the folding state of proteins targeted to the IMS of the mitochondria. This PQC appears to act prior to the import into the IMS

based on the observation that deletion of the mitochondrial targeting signal did not abolish the ubiquitination of mutant endoG (Fig. 5). Is still unclear if this pre-import quality control happens at MAMs (mitochondrial associated membranes), points of contact between ER and mitochondria that could couple synthesis, import and quality control of mitochondria destined proteins.

Figure 5 :The emerging role of UPS in quality control of mitochondrial proteins. Proteins of OMM are retrotranslocated, than ubiquitinated and degraded by UPS in an ERAD- like mechanism. For other mitochondrial proteins, a pre- import quality control via ubiquitin has been recently suggested.



1.6 AIM OF THE PROJECT

Disorders of protein folding and degradation are emerging as fundamental mechanisms in the pathogenesis of many diseases. Many studies raise the intriguing possibility that quality-control pathways may

unexpectedly have a role in tumour progression (37). In this context the identification of TBP7, a regulatory proteasome subunit particle, as TRAP1-interacting protein, appears a very suitable tool to further analyze a novel pathway contributing to cancer biology.

The aim of my PhD research is a structural and functional characterization of the interaction between TRAP1 and its putative ligand TBP7. Given the well known role exerted by TRAP1 in the protection against stress and apoptosis, and the involvement of TBP7 and the proteasome in the cleareance of misfolded proteins, the characterization of this interaction in the endoplasmic reticulum could elucidate the molecular pathways that link PQC, UPR and cell survival (Fig. 6). This novel TRAP1 regulatory function may be relevant for its antiapoptotic activity and involvement in drug resistance in human malignancies. Indeed, the proteotoxic stress generated by accumulation of misfolded proteins and the consequent heat shock response is currently under evaluation as a potential anticancer treatment target (38), since many tumor cells display constitutive proteotoxic stress and dependence on heat shock response due to their rapid rates of proliferation and translation.

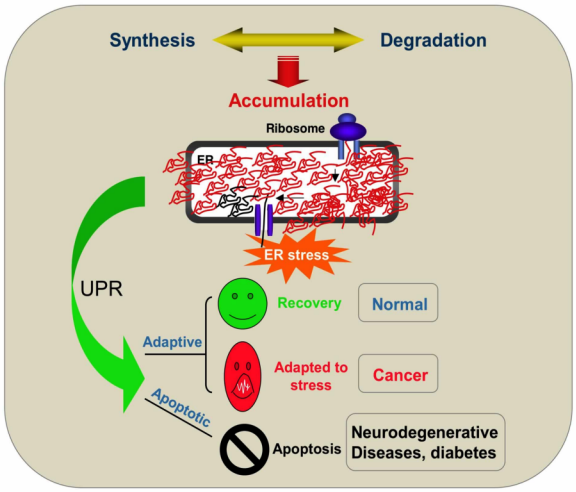


Figure 6: Protein quality control, UPR and cell survival An unbalance between synthesis and degradation could lead to accumulation of misfolded proteins: a cell can responde and return to a physiological situation, or can activate apoptotic pathways , or can overwhelm misfolding thus becoming tumorigenic.

2 MATERIALS AND METHODS

2.1 Cell culture, plasmids generation and transfections procedures

HCT116 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in standard conditions. Full-length TRAP1 and Sorcin expression vectors were obtained as described elsewhere (39). Mutant **Δ1-59-Myc** was generated with the following primers:

Δ59-myc Fw:

5' - ATTAGAATTCATGAGCACGCAGACCGCCGAGG - 3';

Δ59-myc Rv:

3' - ATTACTCGAGGTGTCGCTCCAGGGCCTTGA - 5'.

PCR-amplified fragments were gel-purified and cloned in frame into pCDNA 3.1 plasmid (INVITROGEN) at the Eco-RI and XhoI restriction sites.

Mutant **Δ101-221- HA** was generated using the following primers:

TRAP1-HA fw:

5'-atta GCGGCCGCGCAGCCAACATGGCGCGCGAGCCTGCGGG-3';

TRAP1-HA rev:

5'attaTCTAGATTAAGCGTAATCTGGAACATCATATGGGTATCAGT GTCGCTCCAGGGCCTTGA-3';

ΔATPase-HA fw 221: 5'-atta CCGCGGTCGGCAGCCCCGGGGAGCCT-3';

ΔATPase-HA 110 rev: 5'-atta CCGCGGAAACACCTCTTTTTCTGAGT-3'.

The PCR products obtained with the primers TRAP1-HA fw and ΔATPase-HA rev were cloned in pRc-CMV vector (INVITROGEN); the PCR product obtained with the primers ΔATPase-HA fw and TRAP1-HA rev was subcloned in the same plasmid. All clones were sequenced to confirm identity and PCR fidelity.

The plasmid pCMV5L/S6 (TBP7-HA) was a kind gift of Dr. Simon Dawson, University of Nottingham. Transient transfection of DNA plasmids was performed with Polyfect Transfection reagent (Qiagen, Milan, Italy). siRNAs of TRAP1 and TBP7 were purchased from Qiagen, Milan, Italy (Cat. No. S100301469 for TBP7, SI00115150 for TRAP1). For knockdown experiments, siRNAs were diluted to a final concentration of 20 nmol/L and transfected according to the manufacturer's protocol. For control experiments, cells were transfected with a similar amount of scrambled RNA (Qiagen, Milan, Italy, Cat. No. SI03650318). Transient transfections of siRNAs were performed by using HiPerFect Transfection Reagent (Qiagen, Milan, Italy). TRAP1-stable interference was achieved by transfecting HCT116 cells with TRAP1 (TGCTGTTGACAGTGAGCGACCCGGTCCCTGTACTCAGAAATAGT GAAGCCACAGATGTATTTCTGAGTACAGGGACCGGGCTGCCTAC TGCCTCGGA) or scrambled (sequence containing no homology to known mammalian genes) short hairpin RNAs (shRNA) (Open Biosystems, Huntsville, AL, USA).

2.2 Cell extracts and treatments

Total cell lysates were obtained by homogenization of cell pellets and tumor specimens in cold lysis buffer (20mM Tris, pH 7.5 containing

300mM sucrose, 60mM KC1, 15mM NaC1, 5% (v/v) glycerol, 2mM EDTA, 1% (v/v) Triton X-100, 1mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2% (w/v) deoxycholate) for 1 min at 4°C and further sonication for 30 sec at 4°C. Cytosolic, microsomal and mitochondrial fractions were prepared with the Qproteome Mitochondria Isolation Kit (Qiagen, Milan, Italy). For ER stress induction, cells were treated overnight with 1μM thapsigargin (Sigma-Aldrich, Milan, Italy) before harvesting.

2.3 Western blot analysis and antibodies

Equal amounts of protein from cell lysates and tumor specimens were subjected to 10% (v/v) SDS-PAGE and transferred to a PVDF membrane (Millipore, Temecula, CA, USA). The membrane was blocked with 5% (w/v) skim milk and incubated with primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (GE Healthcare, Waukesha, Wisconsin, USA). The following antibodies from Santa Cruz Biotechnology, Segrate, Italy were used for WB analysis and immunoprecipitation: anti-TRAP1 (sc-13557), anti-Sorcin (sc-100859), anti-TBP7 (sc-166003), anti-cMyc (sc-40),CypD, VDAC, HSP60, anti-ubiquitin (sc-8017), anti-COX4 (sc-58348), anti-F1ATPase (ATP5B subunit, sc-58619), anti-tubulin (sc-8035), anti-HA (sc-805), and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-69778). Rabbit polyclonal anti-calnexin antibody (BD Biosciences, Milan, Italy), mouse monoclonal anti- FLAG antibody (SIGMA ALDRICH, Milan, Italy) and mouse monoclonal anti-BiP antibody (StressGen, Milan, Italy) were also used.

2.4 Membrane fractionation , proteinase K digestion and alkaline treatment

Mitochondria and ER were purified using the Q- proteome Mitochondria Isolation Kit (Qiagen, Milan, Italy) according to the manufacturer's protocol and as elsewhere described . Briefly, HCT116 cells were washed and suspended in lysis buffer, which selectively disrupts the plasma membrane without solubilizing it, resulting in the isolation of cytosolic proteins. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and ER, remained intact and were pelleted by centrifugation. The resulting pellet was resuspended in disruption buffer, repeatedly passed through a narrow-gauge needle (to ensure complete cell disruption), and centrifuged to pellet nuclei, cell debris, and unbroken cells. The supernatant (containing mitochondria and the microsomal fraction) was recentrifuged to pellet mitochondria. The resulting supernatant (microsomal fraction) was treated with proteinase K for 20 min on ice ± NP40 (Igepal, Sigma-Aldrich, Milan, Italy) according to Hassink et al. (40) or with 0.1 M Na2CO3 pH 11.3 for 30 min to remove peripheral ER membrane proteins as described in (41). Ribosomes were purified on a sucrose cushion as described in (42).

2.5 RNA extraction and Real Time RT-PCR analysis.

Total RNA from cell pellets and tumor specimens was extracted using the TRIzol Reagent (Invitrogen, Italy). For the first strand synthesis of cDNA, 3μg of RNA were used in a 20 μl reaction mixture utilizing a cDNA Superscript II (Invitrogen, San Giuliano Milanese, Italy). For Real Time PCR analysis, 1 μl of cDNA sample was amplified using the Platinum

SYBR Green qPCR Supermix UDG (Invitrogen, San Giuliano Milanese, Italy) in an iCycler iQ Real Time Detection System (BioRad Laboratories GmbH, Segrate, Italy). The following primers were utilized: GRP78/BiP forward 5'-*CGTGGATGACCCGTCTGTG*-3', reverse 5'-*CTGCCGTAGGCTCGTTGATG*-3' (PCR product 308 bp); GAPDH, forward 5'-*CAAGGCTGAGAACGGGAA*-3', reverse 5'-*GCATCGCCCCACTTGATTTT*-3' (PCR product 90 bp). Primers were designed to be intron spanning. Reaction conditions were 50° C for 2 min, 95°C for 2 min, followed by 45 cycles of 15 s at 95°C, 30 s at 60°C, 30 s at 72°C. GAPDH was chosen as an internal control.

2.6 Cytotoxicity assay

HCT116 cells were subjected to downregulation of TRAP1 and TBP7 expression by siRNA transfection Apoptosis was evaluated by cytofluorimetric analysis of annexin V and 7-amino-actinomycin D (7-AAD) positive cells using the FITC-Annexin V/7-AAD Kit (Beckman Coulter, Cassina De' Pecchi – Milan, Italy). Stained cells were analyzed by the "EPICS XL" Flow Cytometer (Beckman Coulter, Cassina De' Pecchi – Milan, Italy). Ten thousand events were collected per sample. Positive staining for annexin V, as well as double staining for annexin V and 7-AAD were interpreted as signs of, respectively, early and late phases of apoptosis. Experiments were performed three times using three replicates for each experimental condition.

2.7 Immunofluorescence, confocal-microcopy and EM analysis

HCT116 cells were fixed with 0.1M phosphate buffer containing 4% (w/v) paraformaldehyde for 15 minutes, then blocked and permeabilized with 5% (w/v) BSA, 0.1% (v/v) Triton X-100, 10% (v/v) FBS in PBS for 20 min at RT before staining with primary antibodies (for TRAP1, CALNEXIN and TBP7) and corresponding secondary TEXAS RED/FITC-conjugated antibodies. The analysis of immunofluorescence was performed with a confocal laser scanner microscopy Zeiss 510 LSM (Carl Zeiss Microimaging, Göttingen, Germany), equipped with Argon ionic laser (Carl Zeiss Microimaging, Göttingen, Germany) whose λ was set up to 488 nm, a HeNe laser whose λ was set up to 546 nm, and an immersion oil objective, 63 ×/1.4 f. For the immuno-EM analysis, cells were fixed with a mixture of 4% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde, labeled with a monoclonal antibody against HA using the gold-enhance protocol, embedded in Epon-812, and cut as described previously (43). EM images were acquired from thin sections using an FEI Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (FEI, Eindhoven, The Netherlands). Thin sections were also used for quantification of gold particles residing within mitochondria using the AnalySIS software (Soft Imaging Systems GmbH, Munster, Germany).

2.8 FRET experiments

FRET was measured by using the acceptor photo-bleaching technique (44), where, upon irreversible photo-bleaching, the donor fluorescence increase was recorded. Cells on coverslips were fixed; immunostained with specific anti-TBP7 and anti-TRAP1 antibodies, and secondary antibodies

conjugated, respectively, to Cy3 and Cy5; and mounted in PBS/glycerol (1□:□1). Images were collected using a laser-scanning confocal microscope (Zeiss LSM 510 Meta) equipped with a planapo × 63 oil-immersion (NA 1.4) objective lens. Laser lines at 543 and 633□nm were used to excite, respectively, the fluorophores Cy3 and Cy5. For Cy5 bleaching, the 633-nm He–Ne laser light with 100% output power was used and pinhole diameters were set to have 1.0-μm optical slices.

FRET measurements were performed by using the LSM software (LSM Zeiss, Göttingen, Germany) after photo-bleaching of a selected squared ROI of 6□μm^2. We calculated the FRET efficiency on the basis of the following equation: E=(Fluorescence intensity of Cy3 after bleaching−Fluorescence intensity of Cy3 before bleaching)/Fluorescence intensity of Cy3 after bleaching.

As control we measured FRET on cells expressing TBP7 alone labeled with Cy3 in order to ensure that photo-bleaching per se does not affect the fluorescence of the donor and that photo-conversion does not occur during the photo-bleaching analysis. We calculated the background raised by the photo-bleaching per se by bleaching Cy5 in cells negative for this fluorophore. The background value was subtracted from all samples.

2.9 Pulse-chase assay

Pulse-chase analysis was performed as described elsewhere (45). In brief, HCT116 cells were incubated in cysteine/methionine-free media (Sigma-Aldrich, Milan, Italy) for 1 h followed by incubation in cysteine/methionine-free media containing 50 mCi/mL ^{35}S-labeled cysteine/methionine (GE-Healthcare, Waukesha, Wisconsin, USA) for 1 h.

After labelling, cells were washed once with culture media containing 10-fold excess of unlabeled methionine and cysteine (5mM each) and incubated further in the same medium for the indicated times. Cells were collected at the indicated time points, and separated on 10% SDS–PAGE. Proteins were transferred onto a PVDF membrane (Millipore, Temecula, CA, USA) and probed by WB analysis.

2.10 Patients

Between May 2008 and November 2010, specimens from both tumor and normal, non-infiltrated peritumoral mucosa were obtained from 51 patients with CRC during surgical removal of the neoplasm. Samples were divided into 125 mm^3 pieces, one specimen was fixed in formalin and used for the histopathological diagnosis, while the others were immediately frozen in liquid nitrogen and stored at –80°C for immunoblot analysis. Samples were analyzed within 30 days after collection and were thawed only once. Express written informed consent to use biological specimens for investigational procedures was obtained from all patients.

2.11 Statistical Analysis.

Chi-square test was used to establish the statistical correlation between the expression levels of TRAP1 and those of Sorcin, F1ATPase and TBP7 in human colorectal carcinomas. Statistically significant values ($p<0.05$) are reported in the Results.

RESULTS

3.1 TRAP1/SORCIN INTERACTION: PROTECTION AGAINST ANTIBLASTIC AGENTS

A proteomic analysis of TRAP1 co-immunoprecipitation complexes was performed in our laboratory, in order to further characterize the TRAP1 network and evaluate protein interactors relevant for its roles. The first putative ligand analyzed by our group is Sorcin. Sorcin is a 21.6-kDa Ca^{2+}-binding protein that is a member of the penta EF-hand protein family and is widely distributed among mammalian tissues such as skeletal muscle, kidney, and brain, but most abundantly in cardiac muscle (8, 9, 10). Our group is involved in the study of molecular mechanisms responsible of chemoresistance in colorectal cancers, and the role played by Sorcin in the development of these mechanisms led us to investigate the functional role of the interaction between TRAP1 and Sorcin.

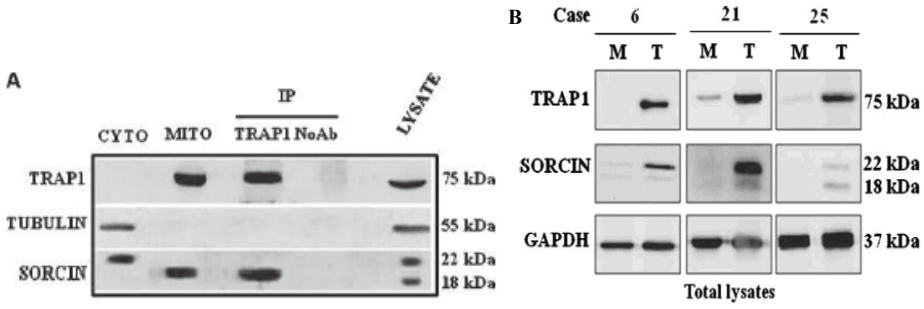
Mass spectrometry analysis revealed interaction of TRAP1 with a smaller isoform of Sorcin, of about 18 kDa, generated by alternative splicing, whose function has never been identified. Coimmunoprecipitation experiments showed that TRAP1 and Sorcin interact in HCT116 and HT29 colon carcinoma cells and, in particular, TRAP1 seems to interact with the Sorcin band with a lower electrophoretic mobility. Considering the abundance of TRAP1 in mitochondria and given the cytosolic localization previously attributed to this protein, to further define the subcellular localization of sorcin isoforms detected by antibodies, cellular subfractionations were performed, and the result showed that the 18 kDa isoform localizes in mitochondria (Fig.7, panel A); the mitochondrial localization of the lower

band of Sorcin was confirmed by immunofluorescence experiments, that showed colocalization between 18-Sorcin and a red fluorescent protein targeted to mitochondria (MITO-RFP). Coimmunoprecipitates from mitochocondrial lysates allowed us to detect the interaction between the two proteins specifically in this compartment; this interaction is highly sensitive to calcium concentration, in fact it was abolished after exposure to calcium chelators (data not shown).

Once demostrated the interaction, our aim was to functionally characterize the binding between TRAP1 and Sorcin; first, we tested if a regulation between the levels of the two proteins occurs and indeed demonstrated that in TRAP1 stable interfered clones the levels of Sorcin 18 were dramatically decreased, while upon readdition of TRAP1 this phenotype was rescued. Finally, to study the functional effects of TRAP1/Sorcin interaction on the protection from apoptosis, we evaluated the rates of apoptotic cell death in colorectal carcinoma HCT-116 cells treated with anticancer compounds upon transient (siRNA) or stable (shRNA) downregulation of TRAP1 and/or Sorcin gene expression, and we found that silencing of either TRAP1 or Sorcin by siRNA enhanced drug-induced apoptosis. However, TRAP1/Sorcin double knockdown did not induce any additional effect on cell death, thus suggesting that the two proteins concomitantly contribute to cytoprotection and to development of a multi-drug resistant phenotype in colon cancer cells by interacting and working together in a common pathway (Table 1). This functional link is demonstrated even in human colorectal cancer tissues, where TRAP1 and Sorcin are co-upregulated (Fig. 7, panel B); these findings suggest that TRAP1/Sorcin network in

mitochondria may represent a novel therapeutic strategy for colorectal tumors (39).

Figure 7: TRAP1 and Sorcin interact in mitochondria and are co-upregulated in colorectal carcinomas specimens. **A)** HCT116 cells were sufractionated in the cytosolic (CYTO) and mitochondrial (MITO) fractions, separated by SDS-PAGE and immunoblotted with the indicated antibodies. No Ab, total cellular extracts incubated with protein A/G-Sepharose without antibodies; IP, immunoprecipitation with the corresponding antibodies. **B**) TRAP1 and Sorcin levels in colorectal carcinomas specimens (T) respect to the corresponding non infiltrated peritumoral mucosas (M)



	Apoptosis (% ± SD)	Ratio (± SD)	*P*
siRNA-negative control			
Control	2.6 ± 0.2		
10 μmol/L I-OHP	5.9 ± 0.4	2.3 ± 0.3	
30 μmol/L I-OHP	23.6 ± 1.2	9.1 ± 1.2	
siRNA Sorcin			
Control	1.1 ± 0.1		
10 μmol/L I-OHP	7.5 ± 0.5	6.8 ± 1.2	0.003
30 μmol/L I-OHP	19.0 ± 1.4	17.3 ± 3.1	0.013
siRNA TRAP1			
Control	1.6 ± 0.2		
10 μmol/L I-OHP	21.3 ± 1.4	13.3 ± 2.9	0.003
30 μmol/L I-OHP	28.0 ± 1.2	17.5 ± 3.3	0.014
siRNA TRAP1/Sorcin			
Control	1.7 ± 0.3		
10 μmol/L I-OHP	15.9 ± 1.1	9.3 ± 2.8	n.s.*
30 μmol/L I-OHP	28.6 ± 1.3	16.8 ± 4.5	n.s.*

Table 1: **Rates of apoptotic cell death in colorectal carcinomas HCT116 cells.** Cells treated with I- OHP upon transient silencing of TRAP1 (siTRAP1), or Sorcin (si Sorcin), or both. The statistical analysis were performed as described in Materials and Methods.

Our findings are in agreement with data obtained by Altieri's group. They found that differential expression of TRAP1 in cancer, as opposed to normal tissues, has been implicated in inhibition of mitochondrial apoptosis, suppression of ROS production, and acquisition of resistance to standard chemotherapeutics. Effective cytoprotection under these conditions may require TRAP1 phosphorylation by the mitochondrial-localized kinase PINK1, which associates with TRAP1. The Ca^{2+} binding protein, Sorcin is also a TRAP1-associated molecule, which collaborates with TRAP1 in maintaining the cytoprotective network in mitochondria through the regulation of the activity of the PTP (46).

3.2 TRAP1/TBP7 INTERACTION: IMPLICATIONS IN PROTEIN QUALITY CONTROL AND CELL SURVIVAL

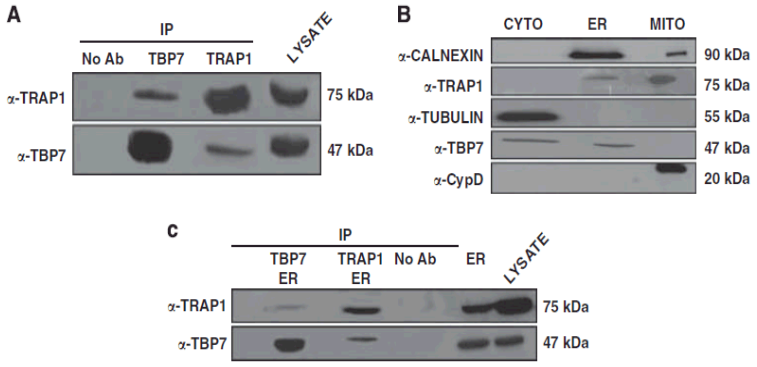
3.2.1. TRAP1 and TBP7 colocalize and directly interact in the endoplasmic reticulum (ER)

The main aim of my PhD project has been the characterization of the interaction between TRAP1 and TBP7 (S6-ATPase 4/Rpt3), a component of the 19S proteasome regulatory subunit (11, 47, 48), another putative partner identified by our LC-MS/MS analysis. I've performed western blot (WB) and co-immunoprecipitation (co-IP) analyses from total extracts of HCT116 colon carcinoma cells to verify the effective interaction between TRAP1 and TBP7 in my experimental model (Fig. 8, panel A). To understand the subcellular compartment in which this interaction could occur, I performed cellular subfractionation of HCT116 cells; in fact, a prevalent mitochondrial localization has been previously reported for TRAP1 (5). WB analysis of

the fractions showed that: i) TBP7 is absent from mitochondria, but expressed both in the cytosol and microsomal (ER) fractions; ii) TRAP1 is present in mitochondria and, surprisingly, also in ER (Fig. 8, panel B). This observation lead us to speculate that this interaction could happen specifically in endoplasmic reticulum; the hypothesis was confirmed by Co-IP analyses from microsomal fraction, that confirmed TRAP1/TBP7 interaction in the ER (Fig. 8, panel C). The TRAP1/TBP7 molecular interaction was further investigated by using a fluorescence resonance energy transfer (FRET) approach in fixed cells (Fig. 9, panel A). In cells coexpressing wild-type TRAP1 and TBP7 about 12% of FRET efficiency was found, indicating that TRAP1 and TBP7 are close enough to allow energy transfer. Interestingly, FRET was found exclusively after bleaching of ER regions, selected on the basis of their morphological features (one example in Fig. 9D, a–f). Thus, these data indicate that TRAP1 and TBP7 directly interact with each other and this interaction occurs specifically in the ER compartments.

Figure 8 : TRAP1 and TBP7 interact and colocalize in the ER. **A**) Total HCT116 lysates were harvested and immunoprecipitated using anti-TRAP1 and anti-TBP7 antibodies as described under Materials and Methods, separated by SDS-PAGE and immunoblotted using the indicated antibodies. **B**) Total HCT116 lysates were fractionated into mitochondrial (MITO), cytosolic (CYTO) and microsomal (ER) fractions as described under Materials and Methods, separated by SDS-PAGE and immunoblotted using the indicated antibodies to verify TRAP1 and TBP7 localization and to assess the purity of the fractions. **C**) TRAP1 and TBP7 co-IP analysis on the microsomal fraction (ER), obtained as described under Materials and Methods. WB of immunoprecipitates was performed by using the indicated antibodies.

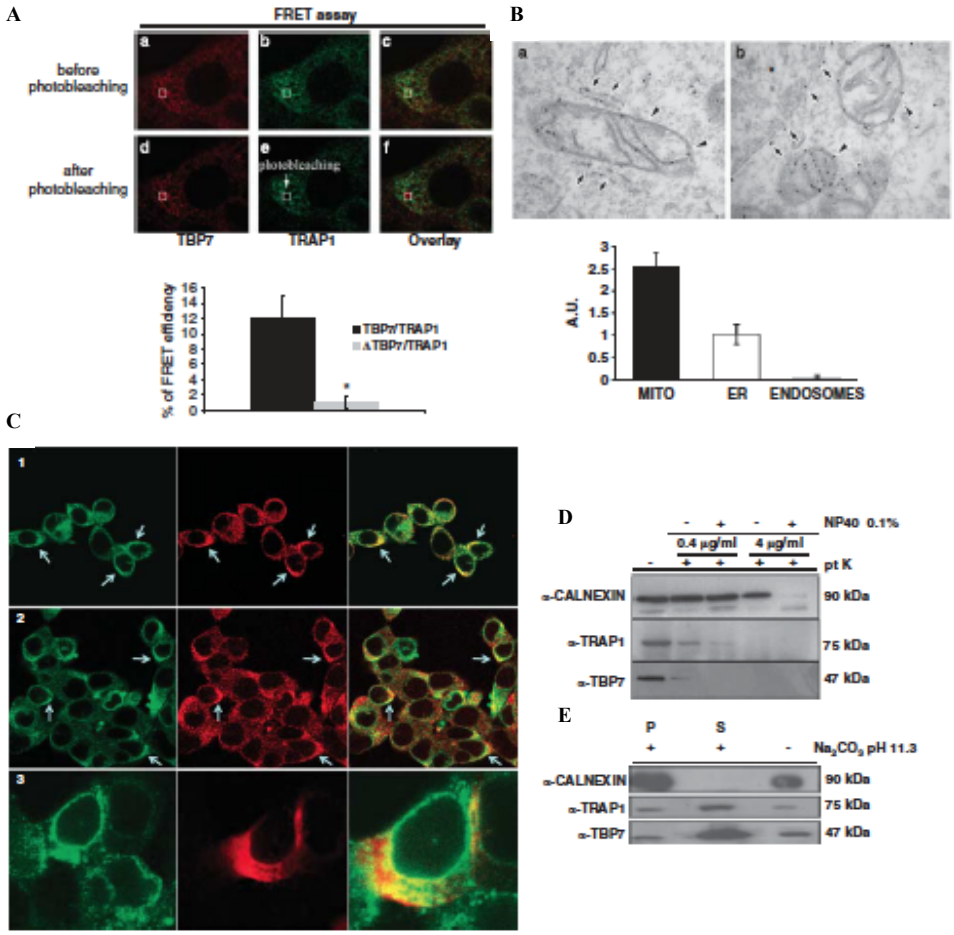
RESULTS



To demonstrate the new finding of the ER localization of TRAP1, confocal and electron microscopy were used. First, HCT116 cells stably-iperexpressing a plasmid for TRAP1-HA were fixed and analyzed for immuno- electron microscopy (EM). Labeling with gold particles revealed a main staining in mitochondria, as expected, but also a signal associated to elongated membranes that on the basis of their ultrastructural features (such as attached ribosomes) can be attributed to the rough ER compartment; moreover, signals for TRAP1 were found even in corrispondence to nuclear envelope, that can be considered a part of ER network (Fig.9, panel B). Densitometric analysis (in arbitrary units, A.U.) of the labeling associated to TRAP1 confirmed the enrichment of this protein in mitochondria (2.53±0.34), but revealed the association of a consistent fraction of TRAP1 to endoplasmic reticulum (1.02±0.22). Confocal microscopy confirmed the reticular localization of TRAP1 and TBP7; in fact, the two proteins colocalize with calnexin, an ER resident protein, and the signals given by anti-TRAP1 and anti-TBP7 antibody show a significant merge (Fig. 9, panel C: 1, 2, 3).

Even if these findings demonstrate that the interaction between TRAP1 and TBP7 occurs in endoplasmic reticulum, there is no indication about the topology of these two proteins; to reveal a more detailed localization for TRAP1 and TBP7, biochemical assays were performed. First, protease digestion of microsomal fractions shows that, in contrast to calnexin, that is tightly inserted into ER membranes, both TRAP1 and TBP7 are sensitive to proteinase K treatments (Fig. 9, panel D), thus indicating that they are located on the outer side of ER, facing the cytoplasm. Moreover, alkaline treatment of ER fractions to remove peripheral membrane proteins demonstrates that both TRAP1 and TBP7 are loosely associated to ER membranes (Fig. 9, panel E).

Figure 9 : **TRAP1/TBP7 direct interaction and topology in ER**. **A**) FRET was measured by using the acceptor photo-bleaching technique as described under Materials and Methods. The images show the signal of TBP7 (red) and TRAP1 (green) before (a–c) and after photo-bleaching (d–f). Error bars:±S.D. **B**) ER Distribution of TRAP1 in HCT116 cells (EM). Cells expressing TRAP-HA vector were fixed and prepared for immuno-EM and labeled with the anti-HA antibody as described in Materials and Methods. The density of immuno-gold labeling (in arbitrary units; average±S.D.) in mitochondria (MITO), ER and endosomes (as a negative control) is reported in the lower histogram. **C**) ER TRAP1/TBP7 colocalization (confocal microscopy). Immunofluorescence shows colocalization of TBP7 with TRAP1 and with the ER protein calnexin. Panel-1: double immunofluorescent staining for TRAP1 (green) and TBP7 (red). Panel-2:double immunofluorescent for calnexin (green) and TBP7 (red). Panel-3: in cells expressing the Myc-tagged TRAP1 construct (red) the protein co-distributes to a great extent with endogenous calnexin (green). **D** and **E**) TRAP1/TBP7 'topology' in the ER. WB of HCT116 microsomal fractions treated with 0.4 μg/ml or 4 μg/ml proteinase-K (pt K) ±1% NP-40 for 20 min on ice (**D**) or with 100mM Na_2CO_3 (pH 11.3) for 30 min (**E**) as described under Materials and Methods. Specific proteins were revealed using the indicated antibodies. **E**): S, supernatant; P, pellet.



3.2.2 TRAP1 and TBP7 interference sensitizes HCT116 cells to thapsigargin- induced ER stress

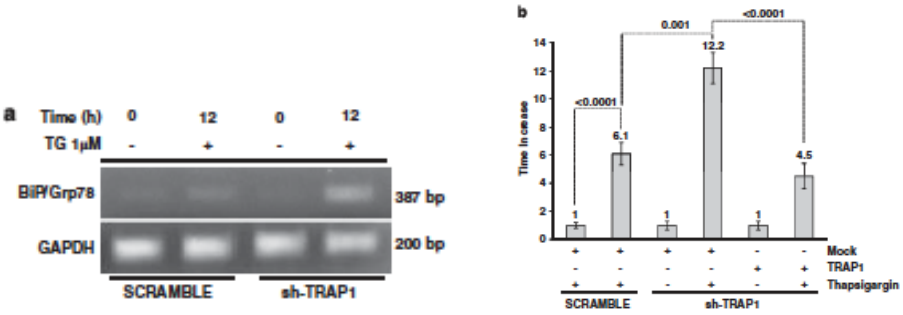
Given the ER localization of TRAP1 and TBP7, I decided to investigate the involvement of these two proteins in ER homeostasis. Although oxidative stress can disrupt protein folding, how protein misfolding and oxidative stress impact each other has not been explored, but it is well known that antioxidant treatment reduces UPR activation,

oxidative stress, and apoptosis, and that ROS concentration in ER is controlled by networks of molecular chaperones (49). Given the well characterized antioxidant role of TRAP1, we hypothezised that TRAP1/TBP7 interaction could constitute an additional check point to monitor the state/folding of proteins damaged in ER. To induce protein misfolding, HCT116 colon carcinoma cells were treated with thapsigargin (TG), an agent that induces ER stress through mobilization of calcium stores from the ER lumen (50); as a marker of ER stress induction we analyzed for REAL-TIME PCR (RT-PCR) the expression of the luminal chaperone BiP/Grp78, a central regulator of the UPR that is highly induced by thapsigargin (25). Upon TG treatment, shTRAP1 stable clones show an increased BiP expression compared to control cells (Fig.10, panels a, b); a rescue of the phenotype (decreased BiP iperexpression upon TG) is observed upon transfection of TRAP1 expression vector. These results show that TRAP1 sensitizes cells to ER and is involved in stress responses upon TG treatment, as recently indicated by Takemoto et al (51).

It has been previously demonstrated that ER stressors induce apoptosis after prolonged treatments (52); TRAP1 is well characterized as an anti-apoptotic protein, so we asked if TRAP1, in concert with TBP7, could sensitize HCT116 cells to apoptosis induced by reticular stress. As expected, 48 hours TG treatments make TRAP1 and TBP7 interfered cells more sensitive to apoptosis induced by ER stress, and the same results were obtained after treaments with genotoxic agents (e.g. oxaliplatin, data not shown). Taken togheter, these results confirm the antiapoptotic function of TRAP1, demonstrating, in particular, that TRAP1 sensitizes cells to apoptosis induced by ER stressors and that TBP7 could be involved in this

cytoprotective mechanisms, highlighting a new role for proteasomal regulatory proteins in apoptotic control.

Figure 10: **BiP mRNA levels in sh-TRAP1 stable clones. a**) Semi-quantitative RT-PCR analysis of BiP/Grp78 mRNA expression in sh-TRAP1 stable clones with respect to scrambled transfectants after 12 hours (h) treatment with 1 μM TG. As control, the levels of GAPDH transcript were analyzed. **b**) RT-PCR analysis of BiP/Grp78 mRNA expression in scrambled and sh-TRAP1 HCT116 cells exposed to 1 μM TG for 12 h and in sh-TRAP1 HCT116 cells transfected with TRAP1 cDNA before treatment with TG. The P-values indicate the statistical significance between different BiP/Grp78 levels under the indicated conditions.



3.2.3 TRAP1 and TBP7 are involved in control of cellular ubiquitination acting on ER .

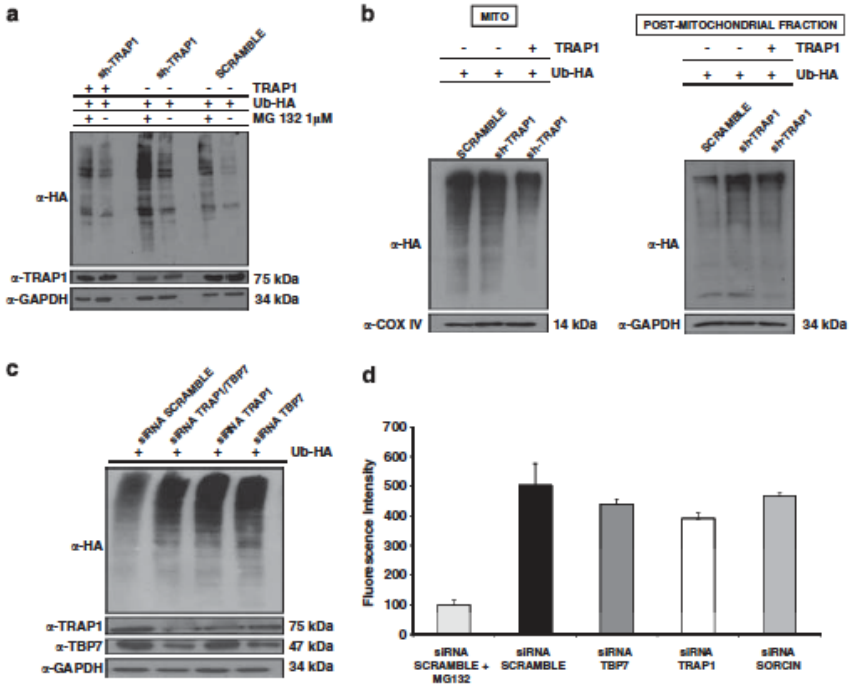
Ubiquitin modification irreversibly or reversibly changes the fate of a target protein. Not much is known about the regulation of ubiquitination in cellular processes like apoptosis and cell proliferation; the involvement of the UPS system in cellular processes like proliferation and apoptosis is well estabilished, but less is known about the cause-effect relashionships in cellular homeostasis or in stress responses. Several molecular chaperones have been reported to regulate cellular ubiquitination, in physiological conditions or in stress responses (53); given the interaction of TRAP1 with the proteasomal subunit TBP7, I tested if these two proteins could collaborate in regulation of cellular ubiquitination. Analysis of cellular lysates of TRAP1 stably interfered clones (sh-RNAs) shows an increased

amount of ubiquitinated proteins respect to control cells; this phenotype could be reverted by readdition of TRAP1 expression vector (Fig. 11, panel a). Cellular subfractions revealed additional details about TRAP1 control: in fact, proteins contained in post-mitochondrial (PM) fraction (microsomes+ cytosol) were found more ubiquitinated in absence of TRAP1, accordingly with the fact that protein quality control via ubiquitin is more requested in compartments assigned to protein synthesis and folding, where the proteasome is present and active. Mitochondrial lysates, on the other hand, don't show a TRAP1-dependent regulation of protein ubiquitination, according to the fact that TBP7 and the proteasome are absent in mitochondria (Fig.11, panel b). The interference of TBP7 with specific siRNAs mimics the effect of TRAP1 interference, while in cells interfered for both TRAP1 and TBP7 the phenotype does not seem to be additional, confirming the idea that TRAP1 and TBP7 could work in concert in regulation of protein ubiquitination (Fig.11, panel c). These effects seem to be independent from proteasome inhibition (MG132 treatments), and, according to this observation, TRAP1 and TBP7 interference does not affect proteasome activity, as demonstrated by *in vitro* proteasomal activity assays (Fig.11, panel d). These data support the hypothesis that TRAP1 and TBP7 act upstream the proteasome function and that these two proteins don't compromise the proteasomal catalytic activity, but may decide if a substrate can be ubiquitinated and then destined to proteasome.

Since the endoplasmic reticulum, the extramitochondrial compartment in which TRAP1 is enriched, is the main site of protein quality control (after or during translation), I wanted to test the hypothesis that TRAP1 could exhert

the control of cellular ubiquitination outside from mitochondria, through its ER location, and that this control requires TRAP1/ TBP7 interaction.

Figure 11 : **Ub levels and proteasomal activity in HCT116 cells**. **a**) Total cell lysates from sh-TRAP1 and scrambled HCT116 stable clones obtained as described in Materials and Methods were analyzed with the indicated antibodies to detect ubiquitin levels. **b**) Sub-cellular fractions: PM fraction (microsomes +CYTO fraction) and mitochondria (MITO, see Materials and Methods) were obtained from sh-TRAP1 and scrambled HCT116 stable transfectants and analyzed using the indicated antibodies. **c**) HCT116 cells were co-transfected with a Ub-HA vector and an siRNA negative control (scramble), or with siRNAs specific for TRAP1, TBP7, or both (as indicated) as described in Materials and Metohds, and the lysates were analyzed with the indicated antibodies. **d**) Proteasome activity is not affected by TRAP1 and TBP7 silencing. Total cellular extracts were prepared after 48 h of transfection with specific siRNA for TRAP1, TBP7 or Sorcin, as control, or with an siRNA negative control (scramble), and incubated in the presence of assay buffer and the fluorogenic substrate Suc-LLVY-AMC, as described under Materials and Methods. Samples were analyzed in triplicate using an excitation wavelength of 360 nm and an emission wavelength of 450 nm to detect chymotryptic proteasome activity. The data represent the mean of three independent experiments.



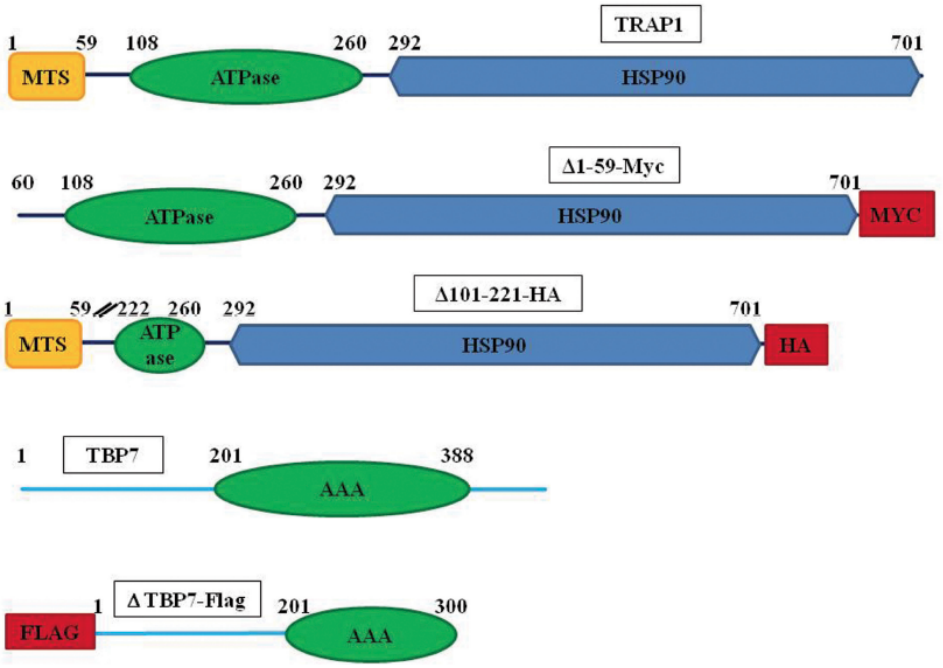
To demonstrate this, I generated the Δ1-59 mutant of TRAP1 (in which the first 59 aminoacids containing the mitochondrial targeting sequence (54) were removed from the N-terminus, yielding TRAP1 mutant defective for mitochondrial import but still able to bind TBP7), and the TRAP1 mutant Δ101-221 able to localize into mitochondria but unable to bind TBP7 which, as previously shown, is absent in the mitochondrial fraction (Fig. 12, Panels A-B-C-E-F).

Transfection of Δ1-59 mutant in sh-TRAP stable clones rescues the high amount of ubiquitinated proteins accumulated in absence of TRAP1, while the Δ101-221 mutant does not (Fig.12, panels D-G).

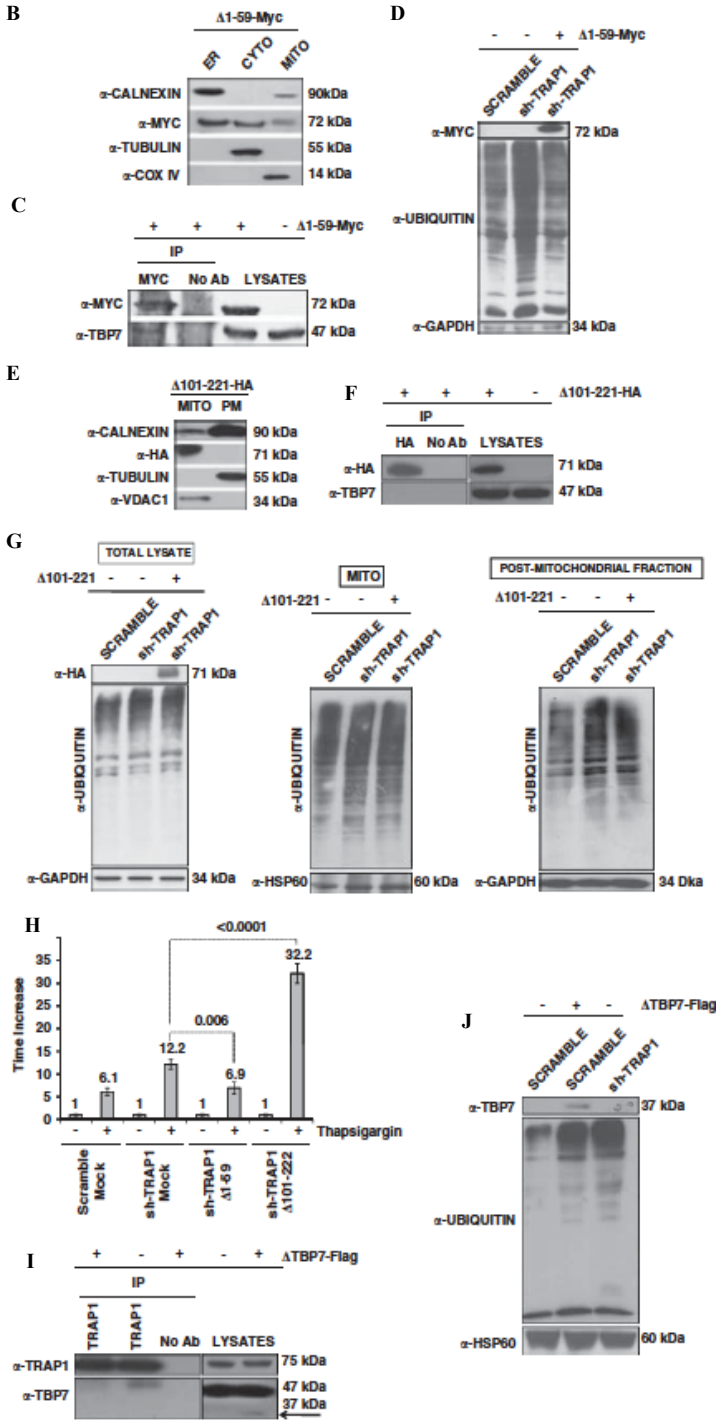
A TBP7 deletion mutant, lacking a portion of one ATPase domain (ΔTBP7-Flag, Fig.12, panel A), was generated to confirm that TRAP1/TBP7 interaction was necessary in ubiquitination control; this mutant disrupts TRAP1/TBP7 interaction, probably acting like a dominant-negative mutant, and, as a consequence of this, increases the cellular ubiquitin levels in scramble control cells , inducing a phenotype very similar to sh-TRAP cells (Fig. 12, panels I- J). Moreover, the transfection of TRAP1 Δ1-59 mutant decreased Grp78/BiP mRNA levels present in sh-TRAP1 clones upon thapsigargin-induced ER stress (Figure 12, Panel H), while the mitochondrial TRAP1 mutant Δ101-221 is not only unable to counteract ER stress, but even further increased BiP levels. Taken together, these observations demonstrate that the mitochondrial localization of TRAP1 is not necessary for cellular ubiquitination control, and that ER TRAP1 localization is required for this check point; furthermore, these results show

that TRAP1/ TBP7 interaction in ER is crucial and essential for protein quality control via ubiquitin-proteasome system.

Figure 12: TRAP1/ TBP7 interaction is required in ER to control cellular ubiquitination. A) Scheme of TRAP1 mutants Δ1-59 and Δ101-221, and of TBP7 mutant ΔTBP7-Flag. For details about the generation of mutants, see Materials and Methods. **B**) and **E**) Subcellular localization of TRAP1 deletion mutants; the cellular subfractions were probed with the indicated antibody to detect the expression of mutants and assess the purity of fractions. **C**) and **F**) The indicated TRAP1 mutants were transfected, immunoprecipitated and immunoblotted with the indicated antibodies to verify the interaction with TBP7. **D**) and **G**) Ubiquitination levels upon transfection of TRAP1 mutants were analyzed as described in Materials and Methods. **H**) Real-time (RT-PCR) analysis of BiP/Grp78 mRNA expression was peformed as described in Materials and Methods. **I**) TRAP1/ TBP7 interaction in presence of Δ TBP7 deletion mutant. Immunoprecipitation analysis were performed in presence of the Δ TBP7 mutant respect to mock- transfected cells; the indicated antibodies were used to verify the interaction. The arrow indicates TBP7-Flag mutant. **J**) Ubiquitination levels upon transfection of the Δ TBP7-Flag deletion mutant. All the indicated data are representative of three independent experiments with similar results.



RESULTS


B
Δ1-59-Myc
ER
CYTO
MITO
α-CALNEXIN
α-MYC
α-TUBULIN
α-COX IV
90kDa
72 kDa
55 kDa
14 kDa
C
+ + + - Δ1-59-Myc
IP
MYC No Ab LYSATES
α-MYC
α-TBP7
72 kDa
47 kDa
D
- - + Δ1-59-Myc
SCRAMBLE
sh-TRAP1
sh-TRAP1
α-MYC
72 kDa
α-UBIQUITIN
α-GAPDH
34 kDa
E
Δ101-221-HA
MITO PM
α-CALNEXIN
α-HA
α-TUBULIN
α-VDAC1
90 kDa
71 kDa
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sh-TRAP1
sh-TRAP1
α-HA
71 kDa
α-UBIQUITIN
α-GAPDH
34 kDa
MITO
Δ101-221 - - +
SCRAMBLE
sh-TRAP1
sh-TRAP1
α-UBIQUITIN
α-HSP60
60 kDa
POST-MITOCHONDRIAL FRACTION
Δ101-221 - - +
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sh-TRAP1
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34 Dka
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35
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Scramble Mock
sh-TRAP1 Mock
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I
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α-TRAP1
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SCRAMBLE
SCRAMBLE
sh-TRAP1
α-TBP7
37 kDa
α-UBIQUITIN
α-HSP60
60 kDa

3.2.4 TRAP1 and TBP7 are responsible of quality control for specific mitochondrial-destined proteins

The control of ubiquitination performed by TRAP1 and TBP7 could be exherted at different levels; the ubiquitin is the first signal that leads the proteins to degradation, recognizing their misfolding, therefore affecting their stability. I tested the hypothesis that TRAP1 could influence the overall protein half-life performing pulse–chase experiments on total lysates of sh-TRAP1 stable clones and the respective scrambled controls. Surprisingly, the total rate of degradation seems unchanged between scrambled and sh-TRAP1 cells (data not shown), indicating that TRAP1 doesn't affect total protein stability, although influencing total ubiquitination; this observation led us to investigate the expression of specific proteins, in particular TRAP1 "substrates". We analyzed the expression levels of F1ATPase βsubunit, a nuclear-encoded mitochondrial protein and a potential TRAP1 interactor as suggested by MS analysis, and the amount of 18 Sorcin, the mitochondrial TRAP1 interactor previously described (39). In sh-TRAP1 stable clones the levels of both 18 sorcin and F1ATPase are significantly reduced respect to control cells (Fig.13, panel A) and, interestingly, the basal levels of these proteins were rescued upon transfection of Δ1-59 mutant of TRAP1, and not by expression of the "mitochondrial" mutant Δ101-221 (data not shown); these data demonstrate that TRAP1 is responsible for the control of the levels of the two analyzed substrates and, more importantly, the mitochondrial localization of TRAP1 is not required for this specific function. In agreement with these data, quantification of relative levels of TRAP1 and the above mentioned

substrates in tissue specimens of colorectal carcinomas confirmed a significant correlation between TRAP1 overexpression and upregulation of levels of 18 Sorcin and F1ATPase ß subunit (Fig.13, panel E). Again, pulse-chase analysis performed by immunoprecipitation of 18 Sorcin and F1ATPase in scrambled and shTRAP1 cells demonstrated that the stability/half-life of these substrates is not affected by TRAP1 interference (data not shown). Therefore, the more probable case was that the decreased expression of F1ATPase and p18 Sorcin in mitochondria of TRAP1-interfered cells was dependent on increased ubiquitination. To this aim, the respective ubiquitination levels in scramble and TRAP1-inteferred cells were analyzed. In figure 13 (panels B and C) is shown that both proteins are more ubiquitinated in sh-TRAP1 transfectants; accordingly, increased levels of ubiquitinated F1ATPase accumulated upon TBP7 interference (Figure 13, panel D).

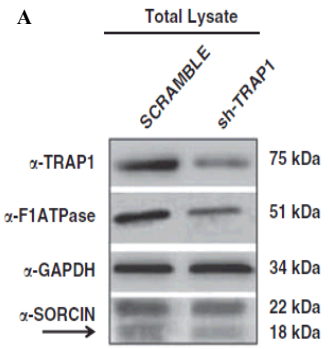
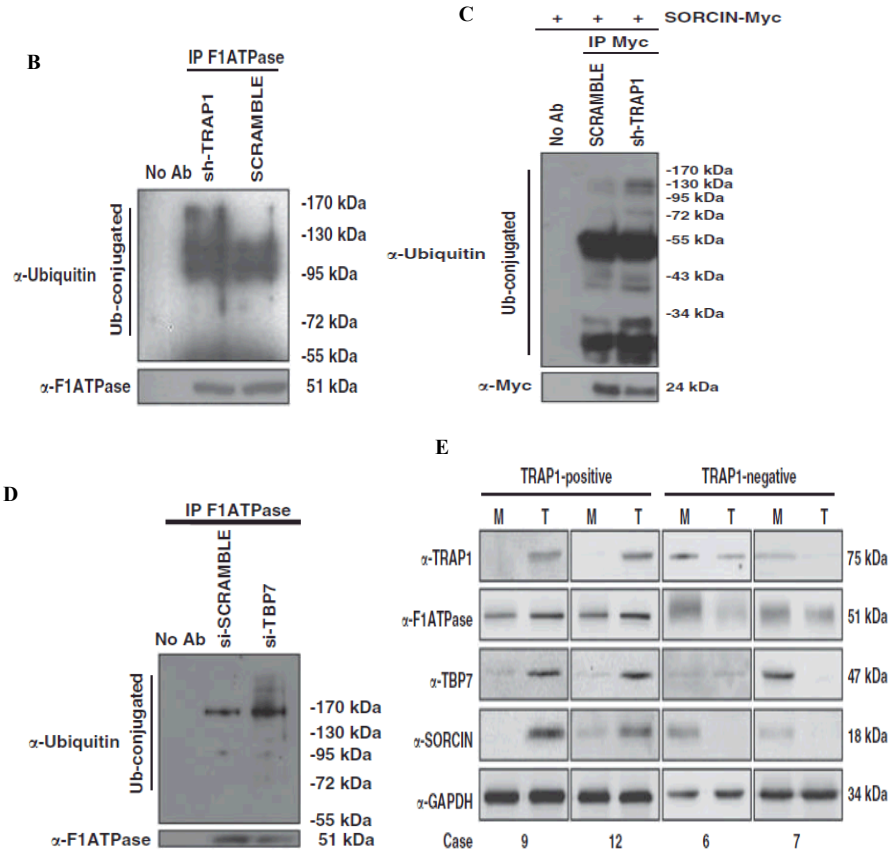


Figure 13: TRAP1 and TBP7 regulate the levels of specific mitochondrial substrates. A) The levels of 18 Sorcin and F1ATPase in shTRAP1 and scrambled stable clones were analyzed by Western Blot analysis with the indicated antibodies. **B**) and **D**) Scrambled and sh-TRAP1 HCT116 clones (or HCT116 cells upon 48 h transfection with siRNA specific for TBP7) were treated with 1 μM MG132 for 24 h before harvesting, immunoprecipitated with the indicated antibodies, subjected to SDS-PAGE and immunoblotted using mouse monoclonal anti-Ub antibody. Three independent experiments were performed, with similar results. **C**) Scrambled and sh-TRAP1 HCT116 clones were transfected with an expression vector containing the 18-Sorcin-Myc expression vector and analyzed as described in Materials and Methods. **E**) TRAP1, TBP7, F1ATPase and Sorcin expression in human CRCs. Total cell lysates from four human CRCs (T) and the respective non-infiltrated peritumoral mucosas (M) were analyzed as described in Materials and Methods.

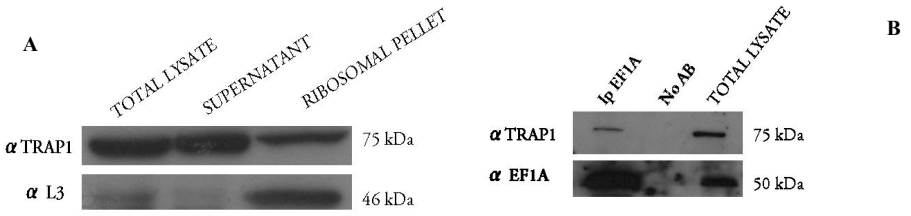


3.2.5 TRAP1 interacts with the translational apparatus on ribosomes

As discussed above, it's well known that de novo folding in eukaryotes starts cotranslationally (55), with the help of a specialized chaperone machinery that is physically and functionally linked to ribosomes (and to ribosome bound nascent polypeptides) (56, 57), coupling folding to translation and to degradation of misfolded- newborn polypeptides via UPS (16, 17). Considering that my previous results show that TRAP1 doesn't affect protein stability and post- translational degradation, even if involved

in ubiquitin dependent quality control, I decided to test the hypothesis that TRAP1 could be linked to the translational apparatus, being involved in cotranslational quality control via ubiquitin, given its interaction with the regulatory proteasomal subunit TBP7. I obtained very preliminary data that give an indication about TRAP1 implication in this physiological process. The first hypothesis that I wanted to test was to verify the physical association between TRAP1 and the ribosomes; Western blot analysis of purified ribosomes shows that a fraction of TRAP1 is present in ribosomal pellet, with the presence of the specific ribosomal marker L3 (Fig.14, panel A). To confirm the association of TRAP1 with the translational apparatus, I performed coimmunoprecipitation experiments to verify the physical interaction of TRAP1 with the elongation factor eEF1A, identified as a putative TRAP1 interactor by mass spectrometry analysis; coIp experiments show that TRAP1 and eEF1A are physically interacting in HCT116 cells (Fig 14, panel B). These preliminary observations suggest that TRAP1 could be one of the chaperones associated to the translational machinery involved in cotranslational protein quality control; it remains to evaluate if TRAP1 could play this function in concert with TBP7, thus linking cotranslational protein quality control and ubiquitination.

Figure 14: TRAP1 and the translational apparatus. A) Ribosomes from HCT-116 cells were isolated by centrifugation through a sucrose cushion as described in Materials and Methods. Equivalent amounts of supernatant and ribosomal-containing pellet were subjected to immunoblot analysis with the indicated antibodies. B) Interaction between TRAP1 and EF1A. Total HCT 116 lysates were immunoprecipitated with anti EF1A antibody and subjected to SDS PAGE, then immunoblotted with the indicated antibodies.



4 DISCUSSION

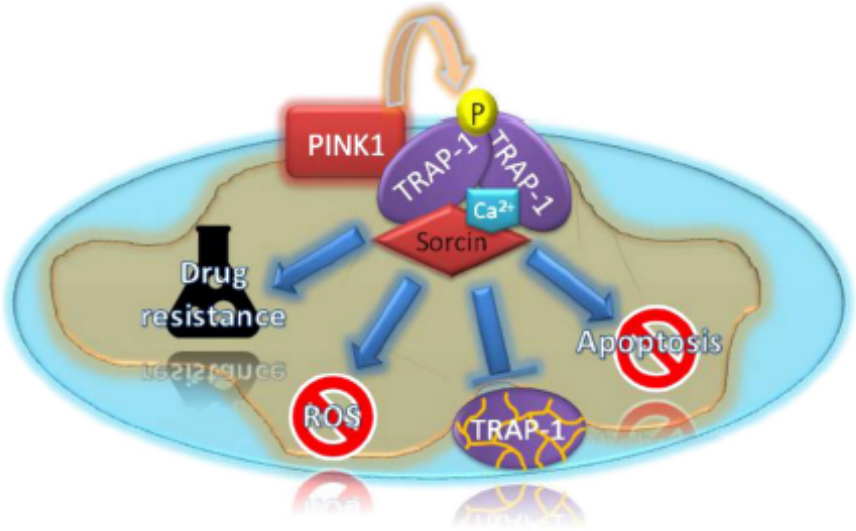
TRAP1 was identified by our group as a molecular chaperone iperexpressed in osteosarcoma cells adapted to mild oxidative stress (4). For its well demonstrated mitochondrial localization and cytoprotective effect against antiblastic agents, we and others demonstrated for this molecular chaperone a role in protection against apoptosis acting in concert with the other components of mitochondrial transition pore (6, 7).

Despite its well defined and accepted role in protection from apoptosis, less is known about the molecular pathways in which TRAP1 is involved neither about its interactors. For this reason, a mass spectrometry (LS/ MS) analysis was performed to identify putative "ligands" of TRAP1, and among all we further characterized two proteins identified as Sorcin and TBP7.

Sorcin is a Calcium binding protein identified as overexpressed in many tumor cell lines and involved in calcium homeostasis and chemoresistance development (8, 9, 10). Our group identified for the first time a novel low molecular weight Sorcin isoform that interacts with TRAP1 and is localized in the mitochondrial matrix; moreover, Sorcin is involved in protection from apoptosis acting in concert with TRAP1 (39). The role of this Calcium binding protein in the protection from apoptosis and its interaction with TRAP1 allowed us to include these proteins in a well charachterized network of mitochondria -localized molecular chaperones: as described and summarized by Altieri et al (45), this pathway antagonizes CypD-dependent pore-forming properties, and oxidative cell death in tumors, potentially via ATPase-directed protein (re)folding (Fig.15) . For its ubiquitous differential expression in cancer, as opposed to normal tissues, this mitochondrial

chaperone network is considered a potentially attractive target for cancer therapy.

Figure 15: Cytoprotective network in mitochondria involving TRAP1 and Sorcin. The interaction between TRAP1 and Sorcin is crucial in cancer cells in regulating calcium homeostasis, counteracting ROS production and inhibiting PTP opening, thus conferring resistance to apoptosis and to antiblastic agents (46).



My PhD project mainly focused on the study and characterization of TRAP1-TBP7 interaction. TBP7 is an AAA-ATPase subunit of the proteasome regulatory particle, previously identified as a synphylin-interacting protein and probably involved in the pathogenesis of Parkinson's disease (58). In my PhD thesis, I demonstrated for the first time that TRAP1 and TBP7 interact in the endoplasmic reticulum (ER), an organelle crucial for protein quality control. TRAP1 and TBP7 interference sensitizes HCT116 cells to apoptosis induced by thapsigargin, an ER stress inducer; in addition, TRAP1 interference causes a dramatic increase of BiP levels upon thapsigargin treatments. Moreover, TRAP1 and/or TBP7 interference

induces a significant increase of cellular ubiquitination: in particular, this accumulation of ubiquitin levels is not detectable in mitochondrial fraction, where TBP7 is absent. These phenotypes are selectively rescued by a TRAP1 deletion mutant (Δ1-59) that lacks mitochondrial targeting sequence (MTS) and is able to bind TBP7; the rescue doesn't occur after overexpression of a mitochondrial TRAP1 mutant (Δ101-221), which is unable to bind TBP7. Furthermore, the overexpression of a TBP7 mutant (ΔTBP7), which disrupts TRAP1-TBP7 interaction, causes an accumulation of ubiquitinated proteins similar to that caused by TRAP1 or TBP7 interference. These results show that the ER localization of TRAP1 is essential to perform its function of protein quality control in concert with TBP7, and that this interaction is crucial in maintaining protein homeostasis, without affecting the proteasomal function. TRAP1 regulates the levels of two specific mitochondrial nuclear-encoded proteins, 18 Sorcin and the β-subunit of F1ATPase complex, whose expression decreased in shTRAP1 cells, as a consequence of their elevated ubiquitination levels. All these observations open interesting questions about the new discovered role of ER localized TRAP1 in controlling protein ubiquitination through TBP7 interaction. We hypothezise that when a protein is misfolded or damaged, it is controlled by a series of molecular chaperones that try to refold it; if this is not possible, the damaged protein is directed, in most cases, to ubiquitination and proteasomal degradation. These mechanisms are prevalently controlled on endoplasmic reticulum, where a wide number of molecular chaperones, inside the lumen and just outside the ER, controls the folding state of the new translated polypeptides, protecting them, or destining them to degradation (59). The ubiquitin-proteasome system (UPS)

is crucial in the elimination of damaged proteins; in fact the presence of proteasome associated to the outer face of ER, where both TRAP1 and TBP7 are located, is well demonstrated (60). An accumulation of misfolded proteins triggers ER stress that, in turn, elicits an Unfolded Protein Response (UPR), leading to: i) the immediate overexpression of the luminal chaperone BiP, ii) a translational block and iii) the transcription of stress-responsive genes (24). The fact that TRAP1 interference induces an increase in BiP expression suggests that shTRAP1 cells are subjected to an higher stress level. It is reasonable to think that this elevated stress condition is induced by the accumulation of ubiquitinated proteins, escaped from the quality control exherted by TRAP1 and TBP7 on ER. The increase in BiP expression could represent a compensatory response to fight against the high levels of misfolded and potentially toxic proteins accumulated in absence of TRAP1, supporting the emerging idea of a "chaperones compensation". The finding that two specific mitochondrial proteins, Sorcin and F1ATPase, are more ubiquitinated in absence of TRAP1 and TBP7 arises the question of mitochondrial proteins quality control, a still poorly characterized issue. Mitochondrial proteins are controlled, in their compartment, by mitochondrial proteases, that eliminate misfolded proteins; there are no evidences of the presence of proteasome in mitochondria, even if ubiquitin ligases have recently been found in these organelles, thus suggesting that many mitochondrial damaged proteins could be ubiquitinated inside mitochondria, then retrotranslocated and degraded by the proteasome, as recently supposed for the mitochondrial inner membrane protein UCP (34). The identification of TRAP1/TBP7-specific "substrates" strongly contributes to the complex study of mitochondrial protein quality

control, and supports a role of the cytosolic UPS in controlling the levels and/or the quality of proteins destined to mitochondria. In fact, recent findings demonstrate that a pre-import control could by exherted on mitochondria-destined proteins, in association with ubiquitin proteasome system, that targets damaged or mistargeted nuclear encoded mitochondria-destined proteins to prevent their import into the organelle and the accumulation in mitochondria of damaged products (36). We suppose that this pre-import control could be located at ER, that is in close proximity to mitochondria and harbours a rich machinery of chaperones- including TRAP1- that controls the correct folding and targeting of new translated proteins. Supporting this hypothesis there are very recent evidences showing that protein targeting and degradation are coupled for elimination of mislocalized proteins; this mechanism has been specifically demonstrated for membrane proteins translated at ER site that, when are inappropriately released to the cytosol, become substrate of the ubiquitin-proteasome pathway. This process seems to be controlled by an HSP70 client protein, Bag6, a molecule that links targeting and ubiquitination pathways (61).

Finally, the finding that TRAP1-dependent regulation of mitochondrial substrated is conserved in CRCs tumor specimens confirms the presence of a conserved network that regulates tumor growth and maintainance of the malignant phenotype, thus providing a novel molecular target for treatment of human CRCs.

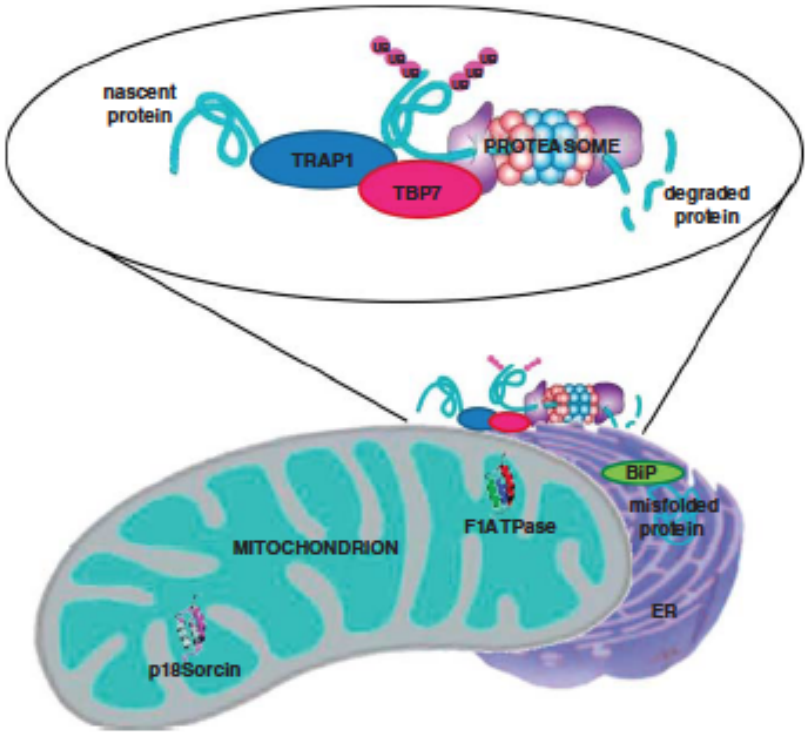
The importance of protein quality control machinery in cellular survival and, in particular, in the development of malignant phenotypes was the main reason to select TBP7 to study TRAP1 network. Protein quality control is a basic cellular phenomenon through which aberrant proteins become

eliminated. The quantity and severity of misfolded proteins can overwhelm the capacity of the PQC system and may lead to protein aggregate accumulation; misfolded proteins that cannot be degraded tend to be more toxic to the cells. Tumorigenesis is regulated by several mechanisms including signalling, transcription and DNA replication; in breast cancer, recent discoveries show that a cytoplasmic protein quality-control pathway is implicated in the suppression of breast cancer cell growth, suggesting a new role for quality-control mechanisms in suppressing cells with malignant potential (62). Furthermore, Kajro and collegues reported the intriguing observation that iperexpression of the cytoplasmic protein quality-control ubiquitin ligase, CHIP (carboxy terminus of Hsp70-interacting protein), suppresses tumorigenesis and metastatic cellular phenotypes in cultured breast cancer cells (63). These studies raise the unexpected possibility that quality-control pathways may have a role in tumour progression. Molecular chaperones are strictly involved in quality control mechanisms, especially in cytoplasm and in endoplasmic reticulum, even if in a recent paper Altieri et al (64) suggest that perturbation of mitochondrial chaperones triggers an UPR response similar to that induced by ER stress; nevertheless, these data confirm the close link between mitochondria and ER, strongly supporting the emerging idea of a structural and functional crosstalk between the two compartments.

Our final model (Fig. 16) supports the idea of a close correlation between mitochondria and endoplasmic reticulum in quality control of mitochondria targeted proteins. We speculate that Sorcin and F1ATPase (and, in a similar way, other mitochondrial proteins subjected to TRAP1 and TBP7 control) are translated in proximity of mitochondria, on ribosomes associated to the

outer side of the ER, where TRAP1 and TBP7 are located. If these neo-synthesized proteins are damaged, they will be not imported into mitochondria, but sequestered by TRAP1 with an attempt to refold or repair them; if this attempt fails, the substrates are ubiquitinated, recognised by the regulatory subunits of proteasome to which TBP7 belongs, and delivered to the proteolitic core for degradation. We still have to analyze whether this process could happen in proximity of MAMs, reticular membranes physically linked to mitochondria. Furthermore, future approaches will elucidate if the quality control of these mitochondria-destined proteins happens in a co-translational way, when they are still attached to ribosomes, or after release of the polypeptides from the translational machinery. Very preliminary data obtained in the last part of my PhD work indicate the association of TRAP1 to ribosomes and to members of the translational machinery (in particular, the elongation factor EF1A), thus suggesting that TRAP1 quality control could happen in a very preliminary phase of a protein's life, soon after synthesis or co-translationally. If these data will be confirmed, TRAP1 could be functionally and physically included into ribosome associated chaperone machinery, whose members are well known in yeast (HSP70 family members), but remain still unknown in higher eukaryotes (65).

Figure 16: Crosstalk between ER and mitochondria in quality control of mitochondrial destined proteins. TRAP1 forms a supra-molecular complex with TBP7 on the outside of the ER, in a cellular compartment of tight ER–mitochondria contact sites, where proteasomes are also present. This TRAP1/TBP7 complex is involved in the control of protein stability and intracellular protein ubiquitination of mitochondria-destined proteins. These two proteins, each with independent but related functions, help to judge whether a protein can be repaired and reach the final mitochondrial destination or, if damaged, needs to be degraded through the ubiquitin- proteasome system.



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Review

Heat shock proteins, cell survival and drug resistance: The mitochondrial chaperone TRAP1, a potential novel target for ovarian cancer therapy

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ABSTRACT

Background. Protein homeostasis is a highly complex network of molecular interactions governing the health and life span of the organism. Molecular chaperones, mainly heat shock proteins (HSP) and other stress-inducible proteins abundantly expressed in multiple compartments of the cell, are major modulators of protein homeostasis. TRAP1 is a mitochondrial HSP involved in protection against oxidant-induced DNA damage and apoptosis. It was recently described as a component of a mitochondrial pathway selectively up-regulated in tumor cells which antagonizes the proapoptotic activity of cyclophilin D, a mitochondrial permeability transition pore regulator, and is responsible for the maintenance of mitochondrial integrity, thus favoring cell survival. Interestingly, novel TRAP1 antagonists cause sudden collapse of mitochondrial function and selective tumor cell death, suggesting that this pathway may represent a novel molecular target to improve anticancer therapy. Preliminary data suggest that TRAP1 may be a valuable biomarker in ovarian cancers: in fact, TRAP1 levels are significantly higher in cisplatin-resistant ovarian tumors and ovarian carcinoma cell lines.

Conclusions. While major advances have been made in understanding the genetics and molecular biology of cancer, given the considerable heterogeneity of ovarian cancer, the introduction of novel targeted therapies and the consequent selection of treatments based on the molecular profile of each tumor may have a major impact on the management of this malignancy and might contribute to building a new era of personalized medicine.

Contents

Heat shock proteins in cell survival

A highly conserved and functionally interactive network of intracellular "chaperones" disaggregate, refold and renature mis-folded proteins following different environmental, physical and chemical stress. Heat shock proteins (HSPs), and other HSP-controlled cellular responses limit the damage caused by stress, thus facilitating cellular recovery. The major HSPs interact with components of the apoptotic pathways and promote cell survival by preventing mitochondrial outer membrane permeabilization and subsequent cytochrome *c* release, caspase activation and apoptosome assembly [1]. As a result of protein misfolding, protein aggregation, or disruption of regulatory complexes, inappropriate

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activation of signaling pathways could occur in several pathophysiological conditions, mainly during acute or chronic stress. Consequently, the levels of HSPs are elevated in many cancers, and HSP overexpression results in a poor prognosis in terms of patient survival and response to therapy in specific cancer types [2,3]. Indeed, elevated HSP expression in malignant cells plays a key role in protecting against the spontaneous apoptosis associated with malignancy as well as the apoptosis generated by therapy, mechanisms which may underlie the role of HSPs in tumor progression and resistance to treatment [3,4].

The 90-kDa heat shock proteins (HSP90), which are integrally involved in cell signaling, proliferation, and survival, are ubiquitously expressed in cells. Many proteins in tumor cells are dependent upon the HSP90 protein folding machinery for their stability, refolding, and maturation [5]. Thus, HSP90 has emerged as a promising target for the treatment of cancer [6]. HSP90 exists as a homodimer, containing three domains. Interestingly, while the N-terminal domain contains an ATP-binding site that binds the natural products geldanamycin and radicicol, a second ATP-binding site in the C-terminus of HSP90 has been identified which not only binds ATP, but also cisplatin (CDDP), novobiocin, epilgallocatechin-3-gallate and taxol [7], all well-known HSP90 inhibitors and powerful antitumor agents.

Several lines of evidence suggest a direct correlation between HSP27 overexpression and resistance to chemotherapy in several human malignancies, such as ovarian cancer, head and neck cancer, esophageal squamous-cell carcinoma, and leukemia [3]. Similarly, HSP70 and HSP27 are emerging as predictors of resistance to chemotherapy and shorter disease-free survival in breast cancer [3,8].

The molecular mechanisms involving HSPs in resistance to cancer therapies can be explained in several ways: (i) as molecular chaperones they can confer cytoprotection by more efficiently repairing the damaged proteins resulting from cytotoxic drug administration; (ii) protecting cancer cells against apoptosis [9], (iii) protecting the microvasculature inside tumors, because HSP27 is found in endothelial cells [10], and (iv) enhancing DNA repair [11].

In such a perspective, neutralizing HSPs is therefore an attractive strategy for anticancer therapy. Up to now, the only inhibitors to have been developed are against HSP90 and they are now under clinical evaluation However, an inhibitor of HSP70 or other HSPs would be very useful in cancer therapy alone and in combination with the above-mentioned inhibitors of HSP90. Indeed, several reports suggest that HSP70 or HSP27 antisense constructs have chemosensitizing properties and may even kill cancer cell lines in the absence of additional stimuli [12,13]. Interestingly, the cytotoxic effect of HSP70 down-modulation is particularly strong in transformed cells yet undetectable in normal, non transformed cell lines or primary cells [14]. This body of evidence suggests that targeting HSPs is one of the most promising approaches to anticancer therapy.

TRAP1 as a candidate biomarker in cancer

TRAP1 function

TRAP1 (TNF receptor-associated protein 1) is a mitochondrial heath shock protein 75 with antioxidant and antiapoptotic functions [15,16]. Several lines of evidence suggest that TRAP1 is part of a complex network involved in protecting cells against oxidative stress and apoptosis. Indeed, TRAP1 and HSP90 were recently described as components of a mitochondrial pathway selectively up-regulated in tumor cells which antagonizes the proapoptotic activity of cyclophilin D (CypD), a regulator of the mitochondrial permeability transition

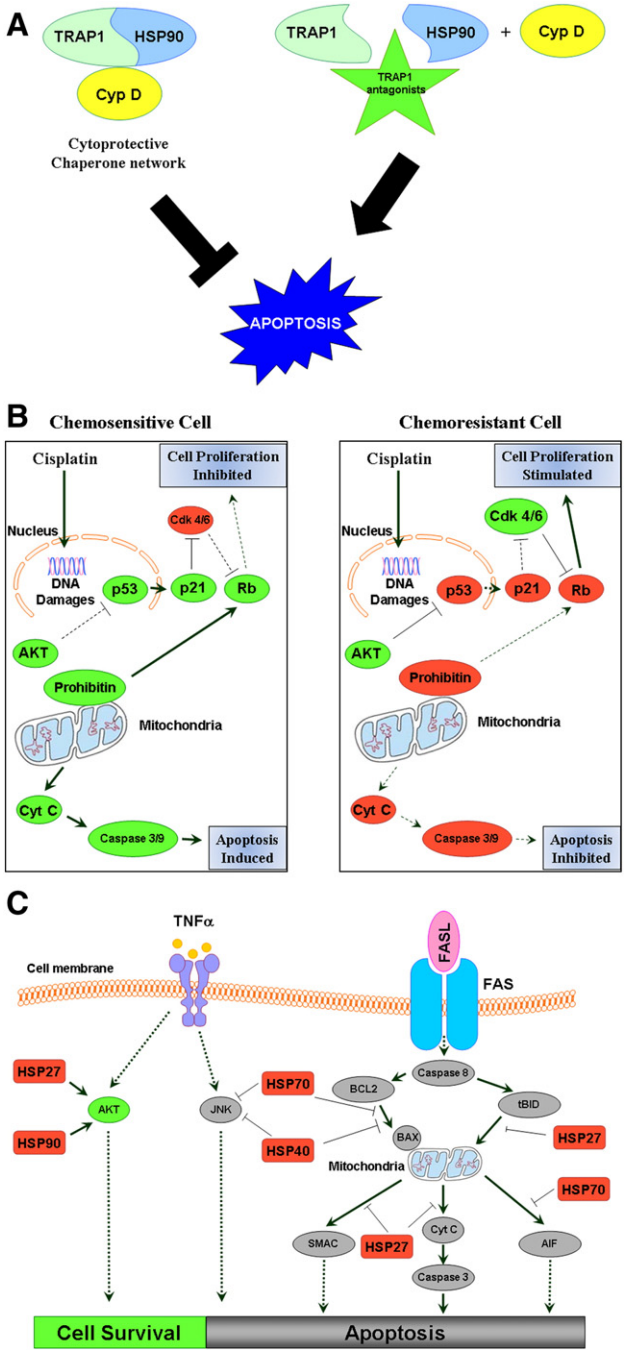

Fig. 1. Examples of signaling pathways and target molecules altered in ovarian cancer. Panel A. Regulation of tumor cell mitochondrial homeostasis by a Hsp90/TRAP1/cyclophilin D network. TRAP1 antagonists include novel agents (known and/or unknown) directed to mitochondria which induce apoptosis by disabling the cytoprotective pathway (for details see the text and Ref. [22]). CypD, cyclophilin D. Panel B. Regulation of cell proliferation and apoptosis in cisplatin chemosensitive/chemoresistant human ovarian cancer cells. Examples of target molecules undergoing quali-quantitative activation (green/grey) or inhibition (red/white). (A) Cisplatin treatment induces cell cycle arrest through a p53-dependent up-regulation of cell cycle-regulatory proteins, such as p21, and of pro-apoptotic proteins (green/grey colors). This activates programmed cell death pathways, (i.e. activation of the caspases). In these cells, cell survival mediators such as Akt, are downregulated or are in their inactive state. Prohibitin may also play a role in inhibiting cell cycle progression through the Rb-E2F pathway by binding to Rb. (B) In the presence of low levels of p53 (upon ubiquitination and/or inactivation) pathways involving Akt and other proteins with pro-survival/cytoprotective roles are in an active state despite the presence of cisplatin. Overall, failure to activate apoptosis in response to chemotherapeutic agents is a major cause of uncontrolled cell proliferation and chemoresistance. Panel C. Key role of heat shock proteins (HSPs) in the regulation of cell survival and apoptotic pathways. Most HSPs function as inhibitors of crucial molecules in the apoptosis pathway such as JNK, Cyt C, caspase-3 and other proteins with cytoprotective roles. Meanwhile, Hsp27 and Hsp90 can also promote the function of AKT in maintaining cell survival.

pore and responsible for the maintenance of mitochondrial integrity, thus favoring cell survival (Fig. 1A) [17]. Interestingly, our group recently demonstrated that stable and transient transfectants containing high TRAP1 levels (i) are more resistant both to H_2O_2-induced DNA damage and to the apoptosis induced by CDDP and other apoptotic stressors; (ii) inhibit the release of apoptosis-inducing factors from mitochondria upon CDDP treatment; (iii) interfere with caspase 3 activation; (iv) contain higher reduced glutathione levels than control cells, which probably contributed to their oxidant-resistant phenotype [16].

TRAP1 and cancer drug-resistance

Based on previous comparisons between various tumoral and normal tissues, our group and other Authors proposed TRAP1 as a candidate cancer biomarker. Our group is one of the first to demonstrate the up-regulation of TRAP1 [18] in human osteosarcoma cells chronically adapted to grow in mild oxidizing conditions [19]. Specifically, TRAP1 mRNA expression is concomitantly increased in oxidant-adapted cells as well in tumor cells resistant to 5-fluorouracil and platin derivatives, thus further supporting the correlation between resistance to oxidative stress and chemoresistance [20]. In addition, we recently demonstrated that TRAP1 is responsible for a multi-drug resistant phenotype in human colorectal carcinoma cells [21] since TRAP1 overexpression induces resistance to 5-fluorouracil, oxaliplatin and irinotecan, three agents widely used in the treatment of human colorectal cancer [22]. Conversely, the inhibition of TRAP1 ATPase activity either by using a mitochondria-directed ATPase peptidomimetic Shepherdin [17] or by stable transfection of a dominant-negative amino-terminal TRAP1 deletion mutant, increases the sensitivity to apoptosis induced by oxaliplatin and irinotecan in wild type colon cancer cells and in cells resistant to the single agents [21]. Finally, we further supported the role of TRAP1 as a cancer biomarker, by demonstrating that TRAP1 expression is increased in 62% of colorectal carcinomas examined [21]. Taken together, these results suggest that TRAP1 may be proposed as a novel molecular target to overcome drug resistance. This hypothesis is further supported by studies showing that TRAP1 and other proteins involved in the activation of cell growth and signal transduction pathways, such as MAPK and other targets involved in VEGF signaling, were upregulated in nasopharyngeal carcinoma [23].

TRAP1 and ovarian cancer

Few data are available suggesting a role of TRAP1 in ovarian cancers. In such a perspective, resistance to tumor necrosis factor-α (TNFα)-mediated apoptosis in ovarian cancer-derived cells has been observed [24]. However, the relevance of these studies to the heterogeneity of ovarian cancer has yet to be established. Furthermore, in previous studies aiming to understand the molecular basis for the failure of CDDP-based chemotherapy, a gene expression comparison between CDDP-sensitive and CDDP-resistant ovarian tumor cell lines, by mRNA differential display, highlighted TRAP1, HSP27 and HSP70 as the most significant up-regulated genes in resistant cells [24]. Furthermore, transient transfection with HSP27 expression vectors made the sensitive cell more resistant, while transfection with the specific antisense oligonucleotides made the ovarian tumor cell line more sensitive [25]. These results are in agreement with the direct correlation between HSP overexpression and resistance to chemotherapy in several human malignancies (see previous chapter in this review). Recent proteomic approaches identified other redox regulatory proteins, such as peroxiredoxins, thioredoxin-2, and several HSPs such as tumor-associated protein in ovarian serous cystadenocarcinoma [26].

Another study was performed to identify the mechanisms underlying progesterone's growth suppressive and immunomodulatory effects in endometrial cancer. Expression analysis demonstrated a significant effect of progesterone on a number of genes, including cell signaling, DNA remodeling, apoptotic, tumor-suppressor, and transcription factors. Specifically, a consistent modulation of cytokines was demonstrated: pro-inflammatory genes such as TNFalpha, IL-1beta, and MCP-1/MCAF-1 were down-regulated and anti-inflammatory genes such as TRAP1 and SMAD4 were induced [27]. Furthermore, a CDDP-resistant ovarian carcinoma cell line exhibited a 20-fold level of drug resistance (PE01CDDP), compared to the parental PE01 line. Microarray analysis revealed 51 genes whose mRNA increased by at least 2 fold in PE01CDDP cells relative to PE01, including TRAP1 [28]. Finally, other studies suggest that expression levels of certain proteins in ovarian cancers are estrogen-regulated and this could help to identify patients who would benefit from endocrine therapy. Interestingly, TRAP1 expression is increased in estrogen receptor-positive ovarian cell lines while TRAP1 up-regulation by estrogen is reversed by the anti-estrogen tamoxifen. These studies also aimed to explore the predictive value of estrogen-regulated gene changes as indicators of sensitivity in ovarian cancer patients treated with the aromatase inhibitor letrozole [29]. Significant differences in expression levels of TRAP1 were observed between tumors from CA125 responsive/stable patients as opposed to tumors from patients whose disease was progressing, using serum levels of CA125 as an indicator of response. Aromatase expression in the ovarian cancers also differed between these 2 groups of patients [29].

Taken together, the identification of TRAP1 as a potential target in ovarian cancer may provide an opportunity to improve the treatment options in this tumor through customized clinical trial designs, and important therapeutic advances for patients with this invalidating malignancy.

Major issues in the clinical management of advanced ovarian cancer

The majority of patients with epithelial ovarian cancer are diagnosed with locally-advanced or metastatic disease (FIGO stages III and IV), making this the gynaecologic cancer associated with the highest morbidity in the western world. While surgery is a fundamental component of initial therapy, most patients cannot be cured by surgery alone, due to residual microscopic and macroscopic peritoneal implants or distant metastases. However, ovarian carcinoma is a chemosensitive tumor and is one of the malignancies for which the strategy of delivering cytotoxic pharmaceutical agents produced more favorable clinical benefits. Numerous chemotherapeutics have been shown to induce objective responses following surgery, and current treatment with combination chemotherapy results in clinical complete remission in the majority of patients with locally-advanced ovarian cancer. Indeed, median progression-free survival (PFS) ranges from 16 to 21 months and median overall survival (OS) from 24 to 60 months, depending upon the volume of disease at the time chemotherapy is started [30]. Consequently, 5-year survival rates have increased from 30% in the 1960s to almost 50% in the current decade. However, there are a considerable number of patients who relapse after initial chemotherapy and all of them will ultimately succumb to their disease. Median survival following relapse is approximately 2 years [31].

Platin derivatives are the backbone of chemotherapy in ovarian cancer. Therefore, from this point of view, the identification of TRAP1 as the target gene involved in platin-derived chemoresistance becomes even more interesting. Initial studies employed CDDP in combination with cyclophosphamide, and therefore, this doublet became the reference regimen for subsequent studies until the GOG 111 trial showed the PFS and OS superiority of CDDP and paclitaxel [32,33]. At present, the combination of carboplatin and paclitaxel is considered the standard of care, since two large non-inferiority trials

demonstrated that carboplatin can be preferred–because of its lower toxicity with at least equivalent efficacy–over its CDDP counterpart [34,35]. While these studies clearly suggest that platin-based combination chemotherapy represents an improvement in the treatment of advanced ovarian cancer, most patients still die from their disease. Thus, an important challenge in the management of ovarian cancer is the development of novel strategies to improve the efficacy of chemotherapeutic regimens, both for induction chemotherapy and for patients at the time of relapse.

Several studies have tried to increase the efficacy of the carboplatin/paclitaxel doublet, with no success. After exposure to platin derivatives, various agents have been tested with disappointing activity or relevant critical toxicities. Topotecan, gemcitabine and pegylated liposomal doxorubicin came into common use due to their reproducible activity and reasonable therapeutic index [36]. However, the addition of the latter three agents into the five-arm GOG 182 trial did not prove advantageous over the carboplatin/paclitaxel doublet as first-line chemotherapy [37]. When used as short–term consolidation or maintenance after initial platinum-based induction therapy, only paclitaxel showed improvement in PFS [38].

A relevant issue in ovarian cancer therapy is the management of patients who recur after platin-based chemotherapy. It is well recognized that tumors which have responded to platinum-based regimens might respond a second or third time to this class of agents [39]. The probability of such a response is closely related to the duration of the platinum-free interval, which is the time from completion of the last regimen and the documentation of disease progression. In platinum-sensitive recurrence (i.e., evidence of disease >6 months since the last platinum treatment), two randomized phase III trials documented the favorable impact of the re-challenge of platinum-based combination therapy (platin/paclitaxel or platin/gemcitabine) compared to single-agent platinum [40,41]. By contrast, the outcome of patients with early recurrence after platin-based chemotherapy (i.e., evidence of disease <6 months since the last platinum treatment) is generally worse since the clinical activity of platinum derivative re-challenge or other chemotherapeutic regimens is poor. Thus, it is critical to characterize novel molecular markers able to predict a condition of resistance to platin derivatives and develop novel strategies to overcome drug resistance and improve the outcome for this subset of patients.

TRAP1: a novel therapeutic molecular target in ovarian carcinoma?

The results reported above support the working hypothesis that TRAP1 could be a novel molecular target in ovarian carcinoma. This is supported by two major observations: (i) platin-based therapies are at present the most successful in the advanced setting of this disease and (ii) the up-regulation of TRAP1 expression in human CDDP-resistant ovarian carcinomas and ovarian cancer cell lines suggest that TRAP1 is involved in CDDP resistance [24,28] and thus may be regarded as a novel biomarker of drug-resistance in this malignancy. Furthermore, the mitochondrial heat shock protein TRAP1 is a critical component of a mitochondrial pathway involved in protecting tumor cells from apoptosis and favoring drug-resistance [17]. This hypothesis would indicate that targeted molecular strategies mainly aimed at inhibiting the pro-survival activity of the TRAP1/HSP90 pathway may improve the efficacy of anticancer treatments by decreasing drug resistance. Thus, it is tempting to speculate that elucidation of the exact molecular mechanisms that govern TRAP1-mediated regulation of cell survival and drug-resistance in ovarian cancer may provide novel diagnostic tools for drug-resistance assessment, and may lead to an improvement in ovarian cancer treatment by providing the rationale to develop new therapeutic strategies.

In such a context, it is significant that several agents which target the TRAP1/HSP90 pathway are under preclinical/clinical evaluation in several human malignancies (Table 1). A derivative of geldanamycin, 17AAG, has been proposed for clinical use in cancer patients for its ability to block the HSP90 pathway, by binding the regulatory pocket in the N-terminal domain of HSP90 [42]. 17AAG has shown significant antitumor activity *in vitro* and in animal models and, thus, has entered clinical testing in cancer patients [43]. Down-regulation of client proteins (i.e., Raf-1, cdk4 and Akt, see also Figs. 1B and C as an example) has been reported in lymphocytes at well-tolerated doses of 17AAG [44] and early evidence of therapeutic activity has been described in human solid tumors by using 17AAG alone or in combination with paclitaxel [45,46]. However, recent reports suggest that 17AAG is unable to accumulate in mitochondria [17,47]: hence, HSP90 inhibition as well as cytochrome *c* release cannot occur, and apoptosis is therefore not activated. Recently, it has been reported that the mitochondria-directed ATP binding peptidomimetic shepherdin induces the disruption of TRAP1/HSP90 interaction and favors CypD release and the consequent opening of the mitochondrial permeability transition pore [17]. Furthermore, we have recently shown that the inhibition of TRAP1 activity by shepherdin rescues the resistance to apoptosis induced by oxaliplatin and irinotecan in colorectal carcinoma cells resistant to the single agents [21]. The advantage of using shepherdin to block ATP binding and ATPase activities, compared to the widely used pharmacological ATPase inhibitors 17AAG, geldanamycin and radicicol [17,47,48], depends on the specificity of its effects. In fact, shepherdin is directed to mitochondria due to the Antennapedia sequence, where TRAP1 and

Table 1
HSP90/TRAP1 antagonists under preclinical/clinical evaluation.

Pharmaceutical agents	Target domain	Human cancer models	References
Geldanamycin	HSP90 ATP-binding N-terminal domain	Pancreas carcinoma, rhabdomyosarcoma,	[42-44, 51,52]
Geldanamycin Analogs	HSP90 ATP-binding N-terminal domain		
17AAG		Prostate, breast, ovarian and hepatocellular carcinoma, melanoma, neuroblastoma, glioblastoma, advanced solid malignancies	[42,45-46, 53-54, 55-61]
Radicicol		n.a.	[42-44]
PU-H71		Hepatocellular and breast carcinoma	[53,62]
BIIB021		Multidrug-resistant tumor cells	[63]
SNX-2112		Multiple myeloma	[64]
17-DMAG		Cervical, gastric, colon hepatocellular and pancreas carcinoma, melanoma, multiple gynecologic cancer cell types	[53,56, 65-69]
IPI-504		Multiple myeloma, pancreas and HER-2 positive breast carcinoma	[70-72]
Shepherdin	Mitochondrial HSP90/TRAP1 complex	n.a.	[17,47-48]
Gamitrinibs	HSP90 ATP-binding N-terminal domain (mitochondria-directed)	n.a.	[49]
Novobiocin	HSP90 ATP-binding C-terminal domain	Drug-resistant human tumors	[7,73]
Epigallocatechin-3-Gallate (EGCG)	HSP90 ATP-binding C-terminal domain	Kidney, prostate, breast and pancreas carcinoma	[7,74-78]

n.a., not available.

HSP90 are active in protecting against apoptosis. Since TRAP1 (i) is present only in mitochondria, while HSP90 is also localized in the cytoplasm, and (ii) shows an ATP binding affinity higher than that of HSP90 (KM 33 μM vs. 100 μM, respectively), the efficiency and selectivity of blocking TRAP1 ATPase activity by shepherdin in mitochondria becomes highly relevant [15]. These data also suggest that strategies aimed at inhibiting TRAP1 function, based on novel TRAP1 ATPase antagonists, may induce sudden collapse of mitochondrial function and apoptosis, thus improving the efficacy of anticancer treatments.

Recently, gamitrinibs, a novel class of small molecules, have been described. These agents are designed to selectively target HSP90 in human tumor mitochondria and have been shown to accumulate in the mitochondria of human tumor cell lines and to inhibit HSP90 activity by acting as ATPase antagonists [49]. Taken together, these results suggest that TRAP1 may be regarded as a novel molecular target to overcome drug resistance in ovarian cancer and TRAP1 antagonists deserve to be evaluated at preclinical and clinical levels as pharmacological tools potentially able to restore/improve the sensitivity of cancer cells to antiblastic agents. Such studies would provide critical information for the understanding of the molecular mechanisms involved in the resistance to anticancer agents in ovarian cancer and would be extremely useful when designing novel therapeutic strategies based on the combination of traditional chemotherapeutic agents and TRAP1-specific antagonists with the aim of rescuing drug-resistance and improving clinical outcome.

Concluding remarks

Nowadays, great clinical interest is focused on novel molecular-targeted tumor-specific agents and on the design of multi-target strategies based on the simultaneous inhibition of several tumor-specific pathways, with the aim of reversing drug-resistance and improving antitumor activity. In such a context, molecular chaperones and stress responsive proteins may be potential molecular targets for improving the cytotoxic effects of drug therapies and overcoming drug resistance. These studies, although still preliminary, will also provide critical information for the understanding of the molecular mechanisms involved in resistance to molecular-targeted agents.

At the cellular and molecular levels, ovarian cancers are remarkably heterogeneous. At least 15 oncogenes have been implicated and at least seven signaling pathways are activated in >50% of ovarian cancers, such as the PI3K–Akt–PTEN pathway, and these are potential targets for anticancer therapeutics [50]. Examples of target molecules and signaling pathways altered in ovarian cancer are shown in Fig. 1. Given the heterogeneity of this disease, increases in long-term survival might be achieved by translating recent insights at the molecular and cellular levels into personalized individual strategies for treatment and by optimizing early detection. Our studies on TRAP1 and other predictive biomarkers will provide proof-of-principle of target modulation, could be used for patient selection, and will be essential in the development of novel molecular-targeted therapies for ovarian cancer.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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Cancer Research

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Therapeutics, Targets, and Chemical Biology

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Mitochondrial Chaperone Trap1 and the Calcium Binding Protein Sorcin Interact and Protect Cells against Apoptosis Induced by Antiblastic Agents

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Abstract

TRAP1, a mitochondrial chaperone (Hsp75) with antioxidant and antiapoptotic functions, is involved in multidrug resistance in human colorectal carcinoma cells. Through a proteomic analysis of TRAP1 coimmunoprecipitation complexes, the Ca^{2+}-binding protein Sorcin was identified as a new TRAP1 interactor. This result prompted us to investigate the presence and role of Sorcin in mitochondria from human colon carcinoma cells. Using fluorescence microscopy and Western blot analysis of purified mitochondria and submitochondrial fractions, we showed the mitochondrial localization of an isoform of Sorcin with an electrophoretic motility lower than 20 kDa that specifically interacts with TRAP1. Furthermore, the effects of overexpressing or downregulating Sorcin and/or TRAP1 allowed us to demonstrate a reciprocal regulation between these two proteins and to show that their interaction is required for Sorcin mitochondrial localization and TRAP1 stability. Indeed, the depletion of TRAP1 by short hairpin RNA in colorectal carcinoma cells lowered Sorcin levels in mitochondria, whereas the depletion of Sorcin by small interfering RNA increased TRAP1 degradation. We also report several lines of evidence suggesting that intramitochondrial Sorcin plays a role in TRAP1 cytoprotection. Finally, preliminary evidence that TRAP1 and Sorcin are both implicated in multidrug resistance and are coupregulated in human colorectal carcinomas is provided. These novel findings highlight a new role for Sorcin, suggesting that some of its previously reported cytoprotective functions may be explained by involvement in mitochondrial metabolism through the TRAP1 pathway. *Cancer Res; 70(16); 6577–86. ©2010 AACR.*

Introduction

The intrinsic or acquired resistance to anticancer drugs remains one of the most serious problems responsible for the failure of cancer chemotherapy (1). The cellular basis underlying multidrug resistance is not fully understood, and several mechanisms have been proposed. Our group recently showed that TRAP1, a mitochondrial chaperone (Hsp75) with antioxidant and antiapoptotic functions (2), is involved in the multidrug resistance of human colorectal carcinoma cells (3). In fact, TRAP1 protein levels are increased in HT-29 colorectal carcinoma cells resistant to 5-fluorouracil (FU), oxaliplatin (l-OHP), and irinotecan (IRI), and in the majority of human colorectal cancers. Furthermore, HT-29 colorectal carcinoma and Saos-2 osteosarcoma cells transfected with TRAP1 exhibited a phenotype resistant to FU-, l-OHP–, and IRI-induced apoptosis, whereas a TRAP1 dominant-negative deletion mutant sensitized tumor cells to apoptotic cell death (3). Recent studies showed that TRAP1 can be proposed as a novel molecular target in localized and metastatic prostate cancer (4), and some other tumors (5), whereas other authors have pointed out that TRAP1 protects against oxidative stress and against focal ischemia in rat brain mitochondria (6).

Little is known about TRAP1 signal transduction. Kang and colleagues (7) identified TRAP1 as a member of a cytoprotective network selectively active in mitochondria of tumor tissues. Other studies suggested an involvement of this chaperone in cell adhesion and synaptic morphology through the modulation of N-cadherin expression (8). Starting from this limited information, a proteomic analysis of TRAP1 coimmunoprecipitation complexes was performed in our laboratory to further characterize the TRAP1 network and evaluate protein interactors relevant for TRAP1 role in multidrug resistance. Among several proteins, we identified Sorcin as a new TRAP1 interactor. Sorcin is a 21.6-kDa Ca^{2+}-binding protein that is a member of the penta EF-hand protein family

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and is widely distributed among mammalian tissues such as skeletal muscle, kidney, and brain, but most abundantly in cardiac muscle (9). After Ca^{2+} binding, the overall hydrophobicity of the protein increases, and this leads to the translocation from the cytoplasm to cell membranes, resulting in Ca^{2+}-dependent interaction with other proteins (e.g., the ryanodine receptor; ref. 10). Although several biochemical studies are available on the direct binding of Sorcin to both cardiac ryanodine receptor RyRs (9) and voltage-dependent L-type calcium channel CaV1.2 (11, 12), little is known about the function of this soluble Ca^{2+}-binding protein in chemoresistance. Indeed, intracellular Ca^{2+} concentration may play a role in the development of chemoresistance, as suggested by clinical trials with verapamil, a Ca^{2+} channel blocker which selectively enhances drug cytotoxicity in MDR cell lines (13). Thus, some studies, aimed at investigating the regulatory role of Ca^{2+} in Taxol resistance, have suggested that chemoresistant cells express higher levels of the Ca^{2+}-binding protein Sorcin and the antiapoptotic Bcl2, compared with the parent cell line (14). Both these proteins have previously been implicated in chemoresistance, in part due to their ability to modulate Ca^{2+} levels. Other reports suggest a correlation between Sorcin and the multidrug-resistant MDR1/P-glycoprotein: it has been shown that the knockdown of Sorcin induces the upregulation of MDR1 in HeLa cells (15); by contrast, a direct correlation between Sorcin and multidrug resistance overexpression has also been reported in human gastric cancer cell lines and vincristine-resistant gastric cancer cells (16–18). Finally, recent studies aimed at evaluating changes in gene expression profile in oral squamous cell carcinoma and non–small cell lung cancer identified a cDNA homologous to Sorcin expressed only in tumors, apparently responsible for drug resistance and poor prognosis (19, 20).

This study shows for the first time that TRAP1 and a Sorcin isoform with an electrophoretic motility of 18 kDa interact in mitochondria, and that the result of this interaction may serve as an additional or alternative indirect route by which both proteins can regulate apoptosis and chemoresistance in colorectal carcinoma cells.

Materials and Methods

Chemicals, cell cultures, constructs, short hairpin RNAs and small interfering RNAs

l-OHP, FU, and IRI and all reagents were purchased by Sigma-Aldrich unless otherwise specified. MG-132 was purchased by Merck Chemicals Ltd-Calbiochem; shepherdin and control scrambled peptides were synthesized by PRIMM as reported in ref. (7).

Human Saos-2 osteosarcoma and HCT-116 colon carcinoma cells were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM containing 10% fetal bovine serum, 1.5 mmol/L glutamine, and 100 U/mL penicillin and streptomycin. Cell lines are routinely monitored in our laboratory by microscopic morphology check. Authentication of cell lines has been checked before starting this study, <1 year ago, through standardized techniques by evaluating, respectively, the haplotype in Saos-2 cells (HLA A2, A3, Bw16, and Bw47) and the presence of a mutation in codon 13 of the *ras* proto-oncogene in HCT-116 cells, according to ATCC product description.

Full-length TRAP1 cDNA was cloned in pRc-CMV vector (Invitrogen; ref. 21). cDNAs for 22- and 18-kDa sorcin isoforms were cloned in pRc-CMV or p-cDNA 3.1 vectors each carrying HA or Myc epitopes at the COOH terminus, respectively. Small interfering RNAs (siRNA) of TRAP1 and Sorcin were purchased from Qiagen. For knockdown experiments, siRNAs were diluted to a final concentration of 20 nmol/L and transfected according to the manufacturer's protocol. TRAP1-stable interference was achieved by transfecting HCT-116 cells with TRAP1 (*TGCTGTTGACAGTGAGCGACCCGGTCCCTGTACTCAGAAATAGTGAAGCCACAGATGTATTTCTGAGTACAGGGACCGGGCTGCCTACTGCCTCGGA*) or scrambled (sequence containing no homology to known mammalian genes) short hairpin RNAs (shRNA) (Open Biosystem).

Fluorescence microscopy

The analysis of immunofluorescence was performed with a confocal laser scanner microscopy Zeiss 510 LSM (Carl Zeiss Microimaging GmbH), equipped with Argon ionic laser (Carl Zeiss Microimaging GmbH) whose λ was set up to 488 nm, a HeNe laser whose λ was set up to 546 nm, and an immersion oil objective, 63 ×/1.4 f.

Cotransfection experiment in cultured HCT116 cells were with 18-kDa Sorcin and mitochondria-targeted red fluorescence protein (p-mitoRFP) expression vectors.

Proteomic analysis of TRAP1 coimmunoprecipitation complexes

Lysis and immunoprecipitation procedures. TRAP1 complexes were isolated from Saos-2 osteosarcoma cell total extracts by affinity purification. Stable cells expressing TRAP1-HA were lysed and then incubated onto anti-HA agarose–conjugated antibody overnight under gentle stirring. Beads were collected by centrifugation (3,000 rpm for 5 minutes*), extensively washed with lysis buffer supplemented with 150 mmol/L NaCl to eliminate nonspecific bound proteins. Elution of the desired protein complexes was performed by competition with HA peptide in elution buffer. The eluted proteins were precipitated in methanol/chloroform and then loaded onto a 10% SDS-PAGE. The gel was stained with colloidal Coomassie blue (Thermo Scientific). Protein bands were excised from the gel, reduced, alkylated, and digested with trypsin (22).

Mass spectrometry analysis and protein identification

Peptide mixtures extracted from the gel were analyzed by nanochromatography tandem mass spectrometry (MS/MS) on a CHIP MS Ion Trap XCT Ultra equipped with a capillary 1100 high-performance liquid chromatography system and a chip cube (Agilent Technologies). Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400–2,000 *m/z*) followed by MS/MS scans of the three most abundant ions in each MS scan. Raw data from nanochromatography

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MS/MS analyses were used to query a nonredundant protein database using the in-house MASCOT software (Matrix Science).

Cytotoxicity assays

Apoptosis was evaluated by cytofluorimetric analysis of Annexin V and 7-amino-actinomycin D–positive cells by using the FITC-Annexin V/7-amino-actinomycin D kit (Beckman Coulter). Stained cells were analyzed by "EPICS XL" Flow Cytometer (Beckman Coulter). Ten thousand events were collected per sample. Positive staining for Annexin V as well as double staining for Annexin V and 7-amino-actinomycin D were interpreted as signs of, respectively, early and late phases of apoptosis (23). In specific experiments, HCT-116 wild-type (WT) or HCT sh-TRAP1 cells were transiently cotransfected with pRc-CMV (mock) or 18-kDa Sorcin constructs and pEGFP-F (Clontech) and, 24 hours later, treated with 10 μmol/L, FU, IRI or l-OHP. Drug-induced apoptosis was evaluated by propidium iodide labeling. The transfection vector pEGFR-F, which encodes for a farnesylated-enhanced green fluorescent protein (EGFP-F), was used as a repoter vector both to monitor transfection efficiency and as a cotransfection marker. Experiments were performed at least thrice using three replicates for each drug concentration.

Immunoblot analysis

Total cell lysates were obtained by homogenization of cell pellets in cold lysis buffer [20 mmol/L Tris (pH 7.5) containing 300 mmol/L sucrose, 60 mmol/L KC1, 15 mmol/L NaC1, 5% (v/v) glycerol, 2 mmol/L EDTA, 1% (v/v) Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 0.2% (w/v) deoxycholate] for 1 minute at 4°C and further sonication for an additional 30 seconds at 4°C. Mitochondria were purified using the Qproteome Mitochondria Isolation kit (Qiagen) according to the manufacturer's protocol. Mitochondrial subfractionation was performed according to Tang and colleagues (24). Briefly, 150 μg of mitochondria prepared from HCT-116 cells were swollen with hypotonic medium to break the outer mitochondrial membrane, keeping intact the inner mitochondrial membrane. The swollen mitochondria were pelleted by centrifugation; the supernatant contained the soluble proteins released from the inner mitochondrial membrane; and the pellet was the mitoplast fraction. Immunoblot analysis was performed as previously reported (2). Specific proteins were detected by using the following mouse monoclonal antibodies from Santa Cruz Biotechnology: anti-TRAP1 (sc-13557), anti-Sorcin (sc-100859), anti-cMyc (sc-40), anti-COX4 (sc-58348), anti–cytochrome *c* (sc-13156), anti-tubulin (sc-8035), and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-69778). The rabbit polyclonal anti–Mn-SOD was purchased from Upstate Biotechnology, and anti–F1-ATPase (sc-16690) was from Santa Cruz Biotechnology. Anti-Sorcin polyclonal antibody was a kind gift from Prof. E. Chiancone, University of Rome "La Sapienza", Rome, Italy.

Semiquantitative reverse transcription-PCR analysis

Total RNA was extracted using the Trizol Reagent (Invitrogen). The following primers used were as follows: TRAP1, forward 5′-*GACGCACCGCTCAACAT*-3′, reverse 5′-*CACATCAAACATGGACGGTTT*-3′; Sorcin 22 kDa (transcript b) forward 5′-*CGCAGTCTGCAGCATGGCG*-3′, reverse 5′-*AAGCCGGCAAGTCTCCAGGT*-3′; Sorcin 18 kDa (transcript a) forward 5′-*GGCCACTCTGCAAGAAGGCA*-3′, reverse 5′-*TCCGGGAGCCCCTCCATACT*-3′; GAPDH, forward 5′-*CAAGGCTGAGAACGGGAA*-3′, reverse 5′-*GCATCGCCCCACTTGATTTT*-3′. Reaction conditions were 95°C for 10 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 5 minutes at 72°C, and 7 minutes at 72°C.

Statistical analysis

The paired Student's *t* test was used to establish the statistical significance between different levels of apoptotic cell death in cell lines overexpressing 18-kDa Sorcin or TRAP1, or in which Sorcin and/or TRAP1 expression had been knocked down compared with the respective mock-transfected control. Statistically significant values ($P < 0.05$) are reported in the Tables.

Results

Sorcin is a mitochondrial TRAP1 interactor

A "fishing for partners" strategy combined with mass spectrometric procedures was carried out to identify TRAP1 protein partners specifically interacting with the bait. Previous studies from our group showed that TRAP1 gene expression increased upon adaptation of human osteosarcoma cells to chronic mild oxidizing conditions and a phenotype resistant to H_2O_2– or cisplatin-induced DNA damage and apoptosis was generated upon transfection with TRAP1 (21). Therefore, Saos-2 osteosarcoma cells were stably transfected with the HA-TRAP1 construct or the empty vector as control. Total protein lysates were incubated with anti-HA–conjugated agarose beads and the retained samples were eluted by competition with HA peptide. The sample and the control were fractionated by 10% SDS-PAGE. The entire lanes from the gel were cut into slices, and each gel slice was submitted to the identification procedure (25). The resulting peptide mixtures were directly analyzed by mass spectrometry (liquid chromatography/tandem mass spectrometry, LC-MS/MS) and identified by the MASCOT protein database search. Proteins identified in the control and the sample lanes were discarded, whereas those proteins solely identified in the sample and absent in the control were selected as putative TRAP1 interactors. Among the putative TRAP1 binding partners, several mitochondrial proteins, mainly components of the membrane carrier family, were identified. Other interactors include cytoskeleton proteins as tubulin and some actin isoforms, as well as proteins involved in cell remodeling, as previously suggested by Kubota and colleagues (8), protein biosynthesis, posttranslational modifications and cell cycle regulation, as previously described (26). We focused our attention on a protein of ~18 kDa identified as Sorcin by LC-MS/MS analysis. No trace of this protein could be observed in

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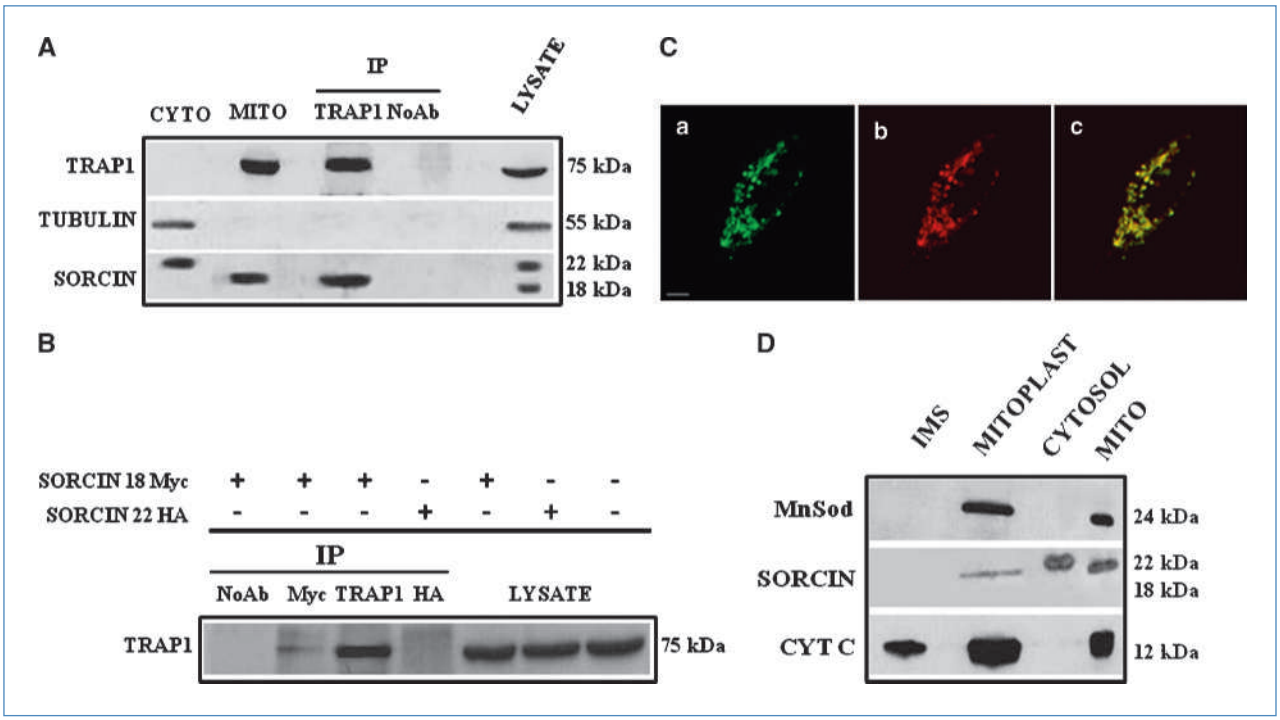

Figure 1. TRAP1 interacts with Sorcin within the mitochondria. A, total cell lysates from HCT-116 cells were fractionated in the cytosolic (CYTO) and mitochondrial (MITO) fractions, separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-TRAP1, anti-Sorcin, and anti-tubulin antibodies. No Ab, total cellular extracts incubated with protein A/G-Sepharose without antibodies; IP, immunoprecipitation with the corresponding antibodies. B, HCT-116 were transfected with expression vectors specific for the 22- or 18-kDa Sorcin isoforms carrying, respectively, HA or Myc epitopes at the COOH terminus. Total cell lysates, and TRAP1 and Sorcin immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-TRAP1 antibodies. C, cotransfection experiment in HCT116 cells with pcDNA 3.1 Myc plasmid expressing 18-kDa sorcin cDNA (a) or mitochondria-targeted red fluorescence protein (mitoRFP) plasmid (p-mitoRFP; b). Fluorescence microscopy shows that the chimeric sorcin protein (a) colocalizes with the fluorescent protein tag of mitochondria (b). c, merge. Scale bar, 2 μm. D, mitochondria prepared from HCT-116 cells were swollen with hypotonic medium to break the outer mitochondrial membrane, keeping the inner mitochondrial membrane intact as described in Materials and Methods. The swollen mitochondria were pelleted by centrifugation; the supernatant contained the soluble proteins released from the IMS and the pellet the mitoplast fraction. The purity of fractions was assessed by using the listed antibodies specific for the submitochondrial compartments.

any gel slice from the control lane. Interestingly, the Sorcin identified showed an electrophoretic mobility corresponding to a molecular mass slightly lower (18 kDa) than that reported in the Swiss Prot databank (22 kDa).

Our group is involved in the study of molecular mechanisms involved in chemoresistance of colorectal cancers (3). Therefore, TRAP1/Sorcin interaction was confirmed in HCT-116 (Figs. 1A and 2A) and HT-29 (data not shown) colon carcinoma cells by coimmunoprecipitation experiments using either anti-TRAP1 (Fig. 1A) or anti-Sorcin (Fig. 2A) antibodies. As shown in Fig. 1A, Western blot analysis using anti-Sorcin antibodies revealed two specific bands in total lysates from HCT-116 cells. Because TRAP1 is almost completely localized in mitochondria, a subfractionation of total lysates was performed and revealed that the 22-kDa Sorcin band is present only in the cytosolic fraction, whereas the lower molecular weight (LMW) protein with an electrophoretic motility of ~18 kDa is localized in the mitochondria (Fig. 1A). Interestingly, we observed that TRAP1 interacts specifically with the 18-kDa Sorcin isoform (Fig. 1A). Accordingly, coimmunoprecipitation analyses using mitochondrial lysates confirmed the specific interaction between TRAP1 and the LMW Sorcin (Supplementary Fig. S1). The identification of the 18-kDa Sorcin as specific to the mitochondrial compartment and the finding that it interacts with TRAP1 in mitochondria were both unexpected because Sorcin has been previously described as a cytosolic protein, with no details on its specific organelle localization. The finding of two Sorcin isoforms with different electrophoretic mobilities and the specific interaction of TRAP1 with the LMW isoform was further confirmed by immunoblot analysis of immunoprecipitates from HCT-116 cells transfected with either 22- or 18-kDa Sorcin expression vectors (Fig. 1B). These results agree with quantitative reverse transcription-PCR (RT-PCR) analyses on RNA purified from HCT-116 cells showing the presence of two independent transcripts encoding for two Sorcin isoforms, namely variants a and b (data not shown), as also reported in the BLAST databases (Supplementary Fig. S2). Finally, fluorescence microscopy analysis shows that the LMW sorcin isoform (a) colocalizes with mitoRFP (b) in mitochondria (Fig. 1C). To further characterize the submitochondrial compartments involved in the interaction, we purified mitochondrial subfractions and showed that TRAP1/Sorcin binding occurs in the mitoplast fraction,

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which was separated upon hypotonic shock of purified mitochondria (Fig. 1D; ref. 24). These results agree with the submitochondrial localization of TRAP1.

The interaction between Sorcin and TRAP1 is specific and requires the NH_2-terminal domain of TRAP1

The binding specificity between the two proteins was confirmed using protein lysates from HCT-116 cells in which TRAP1 expression was notably decreased by shRNAs (sh-TRAP1). Indeed, in these cells, coimmunoprecipitation analysis failed to detect the interaction between TRAP1 and LMW Sorcin (compare coimmunoprecipitation analyses in Fig. 2A and B) due to the very low intracellular TRAP1 levels after RNA interference (compare TRAP1 protein levels in the lanes of total lysates in Fig. 2A and B). As previously shown, TRAP1 has a mitochondrial targeting sequence located at the NH_2 terminus of the protein (26). To define which TRAP1 domain was involved in TRAP1/Sorcin interaction, we performed the same coimmunoprecipitation experiments with protein lysates obtained from HCT-116 cells transfected either with a mutant of TRAP1 in which the first 100 amino acids at the NH_2-terminal end were fused to the myc tag (Fig. 2C), or with the ΔN mutant lacking the NH_2-terminal domain of TRAP1 and carrying an HA epitope at the COOH terminus (Fig. 2D). The results indicate that Sorcin binding requires the NH_2 terminus of TRAP1 protein (Fig. 2C) and that the interaction between these two proteins is lacking when the first 100 amino acids are deleted (Fig. 2D). This also agrees with the cytosolic localization of this TRAP1 deletion mutant (data not shown).

Reciprocal regulation of TRAP1 and Sorcin

We then sought to characterize the regulation that occurs between the two proteins upon their binding. To this aim, we measured the levels of each protein by overexpressing or downregulating either Sorcin and/or TRAP1, respectively. Figure 3A shows that a decreased expression of the LMW Sorcin isoform is detected in mitochondria of sh-TRAP1 HCT-116 colon carcinoma cells compared with WT cells. Because TRAP1 has chaperone activity and is the only member of the HSP90 family with mitochondrial localization (26), and considering that Sorcin is devoid of a canonical mitochondrial localization sequence, we hypothesized that TRAP1 might be involved in Sorcin transport to mitochondria. With this aim, we showed that the transfection of TRAP1 expression vectors in cells with very low amount of TRAP1 (sh-TRAP1) restores mitochondrial levels of LMW Sorcin to those observed in WT cells (Fig. 3A). The fact that the "low Sorcin" phenotype in sh-TRAP1 cells is rescued upon TRAP1 transfection suggests an involvement of TRAP1 in the mitochondrial transport of the LMW Sorcin isoform. As a control, interference of either HSP90 or HSP70 by specific siRNAs did not affect the levels of Sorcin (data not shown). Conversely, TRAP1 protein levels decrease in colorectal carcinoma cells upon

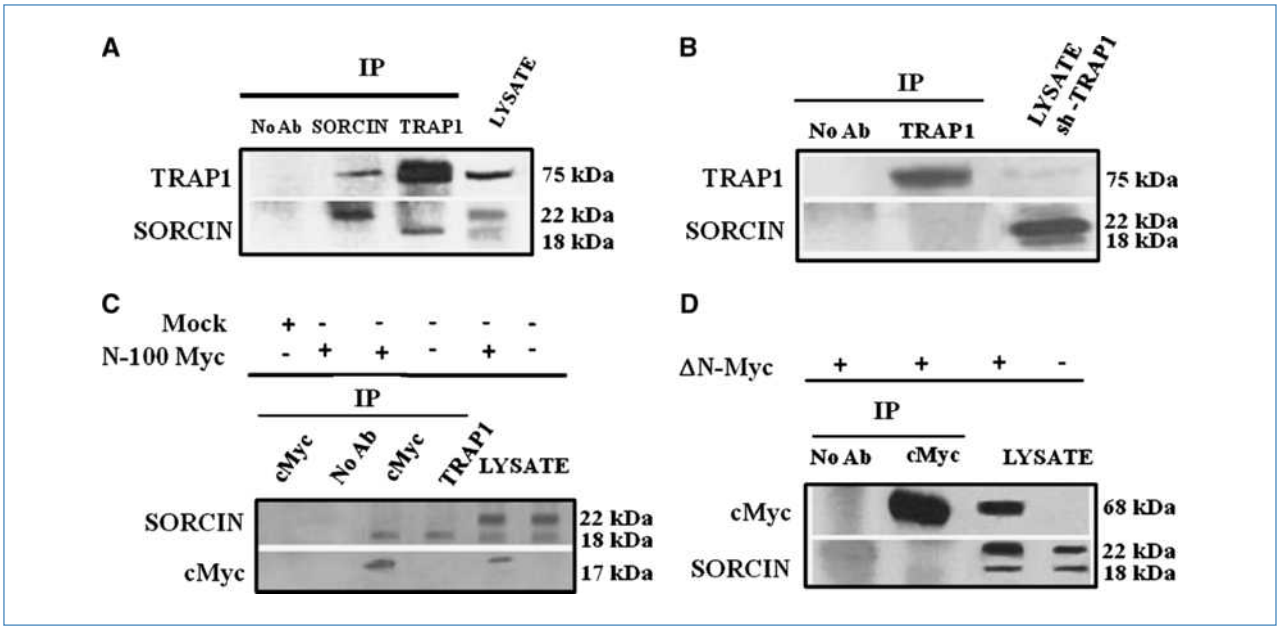

Figure 2. Characterization of TRAP1 domains involved in the interaction with Sorcin. Protein lysates from different cells (see below) were immunoprecipitated with the indicated antibodies, analyzed by SDS-PAGE, and immunoblotted with the listed antibodies. No Ab, total cellular extracts incubated with protein A/G-Sepharose without antibodies; IP, immunoprecipitation with the corresponding antibodies. A, WT HCT-116 cells. B, HCT-116 cells in which TRAP1 expression was stably decreased by shRNA (sh-TRAP1). C, HCT-116 cells were transfected with a mutant TRAP1 expression vector containing cDNA sequences coding for amino acids 1 to 100 at the NH_2 end, fused to cMyc epitope at the COOH terminus (N100-Myc). D, HCT-116 cells were transfected with a mutant TRAP1 construct lacking 100 amino acids at the NH_2 end and containing cDNA sequences coding for amino acids 101 to 604 fused to cMyc epitope at the COOH terminus (ΔN-Myc).

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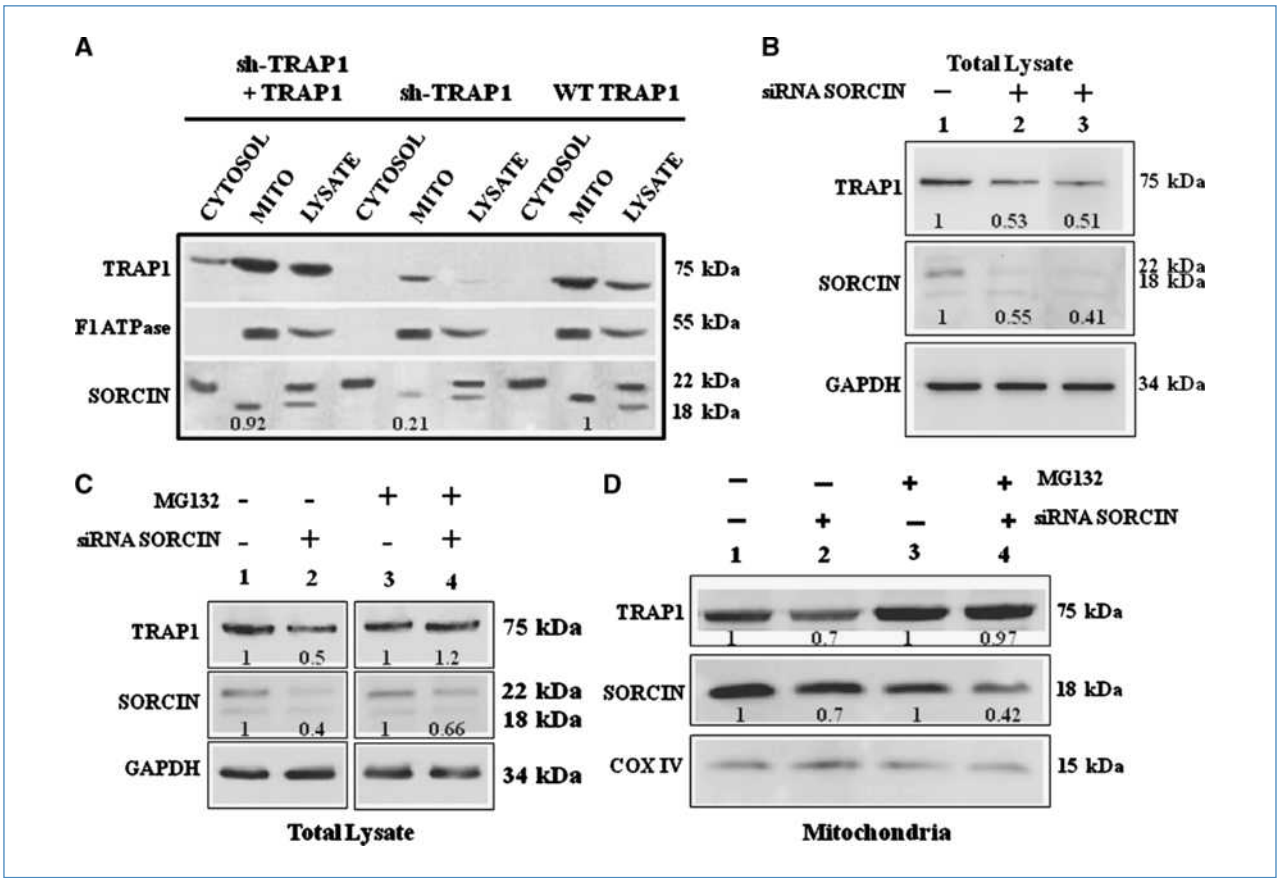

Figure 3. TRAP1/Sorcin reciprocal regulation. A, total cell lysates from WT HCT-116, sh-TRAP1, or sh-TRAP1 cells transfected with TRAP1 full-length construct were fractionated in the cytosolic (CYTOSOL) and mitochondrial (MITO) fractions as described in Materials and Methods, separated by SDS-PAGE, and immunoblotted with mouse monoclonal anti-TRAP1 and anti-Sorcin antibodies, and rabbit polyclonal anti-F1 ATPase antibodies. Note that the densitometric band intensities of LMW Sorcin in mitochondria of indicated cell populations are 1 (WT), 0.21 (sh-TRAP1 cells), and 0.92 (sh-TRAP1+TRAP1), respectively. Three independent experiments were performed with similar results. B, total cell lysates from HCT-116 cells transfected with nonspecific siRNA as a control (line 1) or two independent siRNAs of Sorcin (lines 2 and 3) were separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-TRAP1, rabbit polyclonal anti-Sorcin, and mouse monoclonal anti-GAPDH antibodies. Note that densitometric band intensities for TRAP1 or 22-kDa Sorcin are indicated by numbers by assuming protein levels of the controls (WT cells) are equal to 1. Three independent experiments were performed with similar results. C and D, TRAP1 and Sorcin immunoblot analysis of total cell lysates (C) and mitochondrial fractions (D) from HCT-116 cells transfected with siRNA-negative control (lines 1 and 3) or siRNA of Sorcin (lines 2 and 4), and cultured in the presence of the proteasome inhibitor MG-132 (250 nmol/L) for 48 h (lines 3 and 4), or the inhibitor-free solvent (lines 1 and 2). Note that densitometric band intensities for TRAP1 or 18-kDa Sorcin are indicated by numbers by assuming protein levels of the respective control (mock-transfected cells) are equal to 1. Data represent the mean of three independent experiments.

transient downregulation of Sorcin expression by specific siRNAs (Fig. 3B). These results show a reciprocal regulation between TRAP1 and Sorcin. Previous data suggest that this calcium-binding protein could be involved in the regulation of the gene expression of MDR1 in HeLa cells (15). The subcellular fractionation analysis by Western blot shown in Fig. 3C and D indeed suggests that Sorcin's functional role could be to contribute to TRAP1 protein stability. In fact, the decreased TRAP1 expression observed upon Sorcin interference (Fig. 3C, lane 2) is prevented by the pretreatment of colon carcinoma cells with the proteasome inhibitor MG132 (Fig. 3C, lane 4). The same results were obtained using purified mitochondrial lysates (Fig. 3D). In agreement with posttranslational regulatory mechanisms, we confirmed that mRNA levels of TRAP1 were not affected by Sorcin interference, thus excluding transcriptional regulation (Supplementary Fig. S3).

TRAP1/Sorcin interaction is prevented upon Ca^{2+} chelation

It is known that Sorcin may modulate the cytoplasmic release of Ca^{2+}, mainly from smooth endoplasmic reticulum in neurons (27). We therefore evaluated whether TRAP1/Sorcin interaction could be modulated by chelating free Ca^{2+} with ethylene glycol tetraacetic acid. As shown in Supplementary Fig. S4, binding between the two proteins is greatly decreased in the presence of 0.1 mmol/L ethylene glycol tetraacetic acid, being completely abolished at a higher concentration (0.5 mmol/L) of the Ca^{2+} chelator. This suggests a regulatory role of Ca^{2+} in TRAP1/Sorcin binding.

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TRAP1 and Sorcin are both implicated in multidrug resistance and are coupregulated in human colorectal carcinomas

We previously showed the role of TRAP1 in the resistance of colon carcinoma cells to FU-, l-OHP–, and IRI-induced apoptosis (3), and data are available on Sorcin involvement in multidrug resistance in gastric cancer cell lines (16). Here, we confirm an analogous role of Sorcin in drug resistance in HCT-116 colon carcinoma cells exposed to the same three drugs. Indeed, HCT-116 were cultured for 48 hours in the presence of FU, IRI, or l-OHP, and evaluated for the rate of apoptotic cell death. Cell sensitivity to each drug is expressed as a ratio between drug-induced and vehicle-induced apoptosis (Table 1). Interestingly, protection by the 18-kDa Sorcin isoform toward the programmed cell death induced by FU, l-OHP, and IRI is observed (Table 1). To study the functional effects of TRAP1/Sorcin interaction on the protection from apoptosis, we evaluated the rates of apoptotic cell death in colorectal carcinoma HCT-116 cells treated with l-OHP upon transient (siRNA) or stable (shRNA) downregulation of TRAP1 and/or Sorcin gene expression. As shown in Table 2, the silencing of either TRAP1 or Sorcin by siRNA enhanced drug-induced apoptosis. However, TRAP1/Sorcin double knockdown did not induce any additional effect on cell death (Table 2), thus suggesting that the two proteins concomitantly contribute to cytoprotection by interacting and working together in a common pathway. Accordingly, in parallel experiments, drug-induced cell death was evaluated in sh-TRAP1 HCT-116 cells transfected with the 18-kDa Sorcin isoform (Table 1). Interestingly, although HCT-116 cells overexpressing the 18-kDa Sorcin isoform are characterized by a reduced sensitivity to FU, l-OHP, and IRI, the downregulation of TRAP1 abolishes this apoptosis-resistant phenotype, further supporting the hypothesis that Sorcin and TRAP1 cooperate in protecting from cell death and that the cytoprotective activity of Sorcin requires TRAP1 to be functioning (Table 1).

It was recently shown that shepherdin, a mitochondria-directed peptidomimetic, induces programmed cell death by disrupting a cytoprotective pathway involving TRAP1, selectively active in tumor cell mitochondria (7). We have previously shown that, indeed, this network is activated in colon carcinoma cells (3). Here, we show that Sorcin interference by siRNAs does not increase sensitivity to shepherdin-induced apoptosis either in the presence or absence of TRAP1 (Supplementary Table S1). Again, these results suggest an interdependent activity on the part of both proteins, which interact to protect cells from mitochondrial apoptosis.

Based on the evidence that TRAP1 and Sorcin cooperate in inducing an apoptosis-resistant phenotype in colorectal carcinoma cells, we sought to evaluate TRAP1 and Sorcin expression in human colorectal carcinomas. Interestingly, we observed a concomitant upregulation of both Sorcin isoforms and TRAP1 protein levels in 44% of our series of 41 human colorectal carcinomas, and a parallel increase in TRAP1 and Sorcin mRNA expression levels evaluated by semiquantitative PCR analyses. Figure 4 shows the protein and mRNA levels of TRAP1 and Sorcin in three tumor specimens and in the respective normal noninfiltrated mucosas, chosen as representative analytic results of our series. A parallel upregulation of TRAP1 and Sorcin was observed in colorectal carcinoma cells resistant to FU, l-OHP, and IRI (data not shown).

Table 1. Rates of apoptotic cell death in colorectal carcinoma cells transiently transfected with pRc-CMV (mock) or 18-kDa Sorcin constructs and pEGFP-F vector treated with FU, l-OHP, or IRI

	Apoptosis (% ± SD)	Ratio (±SD)	Apoptosis (% ± SD)	Ratio (±SD)	*P*
	Mock		18-kDa Sorcin		
HCT-116 cells					
Vehicle	1.9 ± 0.2		2.4 ± 0.3		
10 μmol/L FU	9.6 ± 0.9	5.0 ± 1.2	3.3 ± 0.4	1.4 ± 0.4	0.008*
10 μmol/L l-OHP	10.5 ± 1.1	5.5 ± 1.3	1.9 ± 0.6	0.8 ± 0.4	0.008*
10 μmol/L IRI	12.7 ± 1.2	6.7 ± 1.5	3.4 ± 0.5	1.4 ± 0.5	0.004*
HCT-116 shRNA TRAP1					
Vehicle	2.2 ± 0.3		2.6 ± 0.2		
10 μmol/L FU	21.5 ± 0.9	9.7 ± 2.1	24.3 ± 0.5	9.3 ± 1.0	n.s.*
10 μmol/L l-OHP	24.9 ± 0.8	11.3 ± 2.2	25.1 ± 0.4	9.6 ± 1.0	n.s.*
10 μmol/L IRI	28.3 ± 0.9	12.9 ± 2.5	31.4 ± 0.7	12.1 ± 1.3	n.s.*

NOTE: Ratios are calculated between rates of apoptosis in drug- and vehicle-treated cells. *P* values indicate the statistical significance between the ratios of apoptosis in Sorcin- and mock-transfected cells. Apoptotic rates are calculated based only on sorted pEGFP-F–positive cells.
Abbreviation: n.s., not significant.
*Compared with mock-transfected cells.

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Table 2. Rates of apoptotic cell death in colorectal carcinoma HCT-116 cells treated with l-OHP upon transient (siRNA) or stable (shRNA) downregulation of TRAP1 and/or Sorcin gene expression

	Apoptosis (% ± SD)	Ratio (± SD)	*P*
siRNA-negative control			
Control	2.6 ± 0.2		
10 μmol/L l-OHP	5.9 ± 0.4	2.3 ± 0.3	
30 μmol/L l-OHP	23.6 ± 1.2	9.1 ± 1.2	
siRNA Sorcin			
Control	1.1 ± 0.1		
10 μmol/L l-OHP	7.5 ± 0.5	6.8 ± 1.2	0.003
30 μmol/L l-OHP	19.0 ± 1.4	17.3 ± 3.1	0.013
siRNA TRAP1			
Control	1.6 ± 0.2		
10 μmol/L l-OHP	21.3 ± 1.4	13.3 ± 2.9	0.003
30 μmol/L l-OHP	28.0 ± 1.2	17.5 ± 3.3	0.014
siRNA TRAP1/Sorcin			
Control	1.7 ± 0.3		
10 μmol/L l-OHP	15.9 ± 1.1	9.3 ± 2.8	n.s.*
30 μmol/L l-OHP	28.6 ± 1.3	16.8 ± 4.5	n.s.*
shRNA TRAP1			
Control	2.4 ± 0.2		
10 μmol/L l-OHP	20.2 ± 1.2	8.4 ± 1.3	0.001
30 μmol/L l-OHP	27.3 ± 0.7	11.4 ± 1.3	0.007
shRNA TRAP1/siRNA Sorcin			
Control	2.6 ± 0.2		
10 μmol/L l-OHP	17.7 ± 1.0	6.8 ± 1.0	n.s.†
30 μmol/L l-OHP	32.4 ± 1.5	12.5 ± 1.6	n.s.†

NOTE: Ratios are calculated between rates of apoptosis in drug- and vehicle-treated cells. *P* values indicate the statistical significance between the ratios of apoptosis induced by l-OHP in transfected cells and the siRNA-negative control.

Abbreviation: n.s., not significant.

*Compared with either siRNA Sorcin or siRNA TRAP1-transfected cells.

†Compared with shRNA TRAP1 transfected cells.

Discussion

This article provides evidence of a new mitochondrial network involving TRAP1 and the Ca^{2+}-binding protein Sorcin. Using mass spectrometry analysis of TRAP1 coimmunoprecipitating proteins, we identified Sorcin as a novel TRAP1 interactor. To our knowledge, this is the first time that Sorcin had been found to associate to a mitochondrial protein. This finding prompted us to investigate the subcellular localization of Sorcin because none of the previous data described the organelle compartmentalization of this protein. Cell fractionation experiments clearly indicate the occurrence of two main Sorcin isoforms: the 22-kDa isoform, quantitatively the major Sorcin band, is found in the cytosol, whereas the LMW 18-kDa isoform is specifically localized in the mitochondrial fraction. According to this finding, we show that TRAP1 interacts with the LMW Sorcin isoform within mitochondria, more specifically in mitoplasts (Fig. 1).

Sorcin is a Ca^{2+}-sensitive protein, interacting with many important regulatory proteins involved in Ca^{2+} homeostasis. Consistent with these features, the existence of a functional role of Ca^{2+} in the interaction between Sorcin and TRAP1 is preliminarily shown by the interference of Sorcin mitochondrial localization upon Ca^{2+} depletion (see Supplementary Fig. S2). Although the role of Ca^{2+} in mitochondria is widely shown, little is known about the role of Ca^{2+} in TRAP1 signaling. Because Sorcin is highly sensitive to the modulation of Ca^{2+} levels, the interaction with TRAP1 and the consequent import of Sorcin into mitochondria, in which the homeostasis of calcium is strictly controlled (28), might contribute to defending the Ca^{2+}-sensitive protein against perturbations of Ca^{2+} levels, most frequently observed in the cytosolic compartment. The selective binding of TRAP1 to LMW Sorcin could also be dependent on the same subcellular compartmentalization of both proteins. However, more in-depth analysis is required to characterize the molecular mechanisms involved in TRAP1/LMW Sorcin interaction (i.e., the effects of different protein structure/conformation of Sorcin isoforms on the interaction with TRAP1).

The interaction of Sorcin with a mitochondrial chaperone led us to investigate how the Ca^{2+}-binding protein, which has no canonical mitochondria-targeting sequence, is imported into these organelles and to analyze whether Sorcin might be translocated into the mitochondrial compartment through a mechanism involving its interaction with TRAP1. Consistently with this assumption, we found that the localization of Sorcin in the mitochondria of human cells is dependent on the presence and abundance of TRAP1 (Fig. 3A). In fact, the levels of 18-kDa Sorcin decrease upon TRAP1 knockdown by shRNAs, whereas a rescue of this phenotype is achieved after transfection of colon carcinoma cells with TRAP1 expression vectors. Conversely, Sorcin interference might affect TRAP1 protein stability through posttranslational mechanisms, as suggested by preliminary experiments with the proteasome inhibitor MG132 (Fig. 3B–D). Although further investigation is necessary to clarify the mechanism involved in the regulation of TRAP1 expression by Sorcin in this regard,

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it is important to underline that our findings are in line with previous studies indicating that the suppression of Sorcin induced a >3-fold increase in the expression of MDR1 (15), thus indicating a role by this protein in regulatory mechanisms of gene expression.

Apoptosis is the essential response induced in tumor cells by most chemotherapeutic agents. Several mechanisms are involved in resistance to chemotherapeutic agents in tumor cells (29). Among others, we recently studied, at a preclinical level, the role of TRAP1 in inducing a chemoresistant phenotype in human colorectal carcinoma (3), whereas a possible involvement of this mitochondrial chaperone in ovarian cancer has been suggested (30). Other studies have identified TRAP1 as a novel mitochondrial survival factor differentially expressed in localized and metastatic prostate cancer compared with normal prostate. Targeting this pathway could be explored as novel molecular therapy in patients with advanced prostate tumors (4). Sorcin has been implicated in chemoresistance and cancer as well. In fact, high levels of this protein have been reported in cancer cell lines and vincristine-resistant gastric cancer cell lines (16–18). Furthermore, Sorcin expression might be responsible for drug resistance and poor prognosis in non–small cell lung tumors (19, 20). The results reported here suggest that the previously described role of Sorcin in chemoresistance and cell survival may be dependent on its interaction with TRAP1 and involvement in TRAP1 mitochondrial antiapoptotic network.

To our knowledge, this study is the first evidence of a role played by Sorcin in resistance to FU, IRI, and l-OHP, three antiblastic agents that represent the backbone of human colorectal carcinoma therapy (31). Interestingly, RNAi knockdown of Sorcin and/or TRAP1 sensitizes human HCT-116 colon cancer cells to anticancer compounds that induce apoptosis, suggesting that targeting the TRAP1/Sorcin network directly in mitochondria may represent a novel therapeutic strategy for colorectal tumors. Indeed, it is very well established that regimens combining FU with IRI or l-OHP are equally effective as first-line treatment for advanced colorectal carcinoma (31), but achieve a response rate of 30% to 50% and an overall survival of 14 to 20 months (32). Thus, the development of novel strategies able to counteract multidrug resistance to improve the efficacy of systemic chemotherapy is a major issue in the clinical management of colorectal cancer patients. In such a perspective, our results suggest that the TRAP1/Sorcin pathway deserves to be further evaluated as a potential molecular target in human colorectal carcinoma for the clinical development of mitochondria-targeting agents. Indeed, a derivative of geldanamycin, 17AAG, is at present under clinical investigation in cancer patients due to its ability to block the HSP90/TRAP1 pathway, by binding the regulatory pocket in the NH_2-terminal domain of HSP90 (33), whereas other small molecules (e.g., shepherdin, gamitrinibs) have been designed to selectively accumulate in the mitochondria of human tumor cells targeting the HSP90/TRAP1 network and acting as ATPase antagonists (34). Finally, the clinical validation of novel biomarkers predictive of resistance/sensitivity to specific chemotherapy regimens is urgently required in human tumors. Thus, the novel preliminary finding of a parallel upregulation of TRAP1 and Sorcin in human colorectal cancers highlights the existence of a functional link between the two

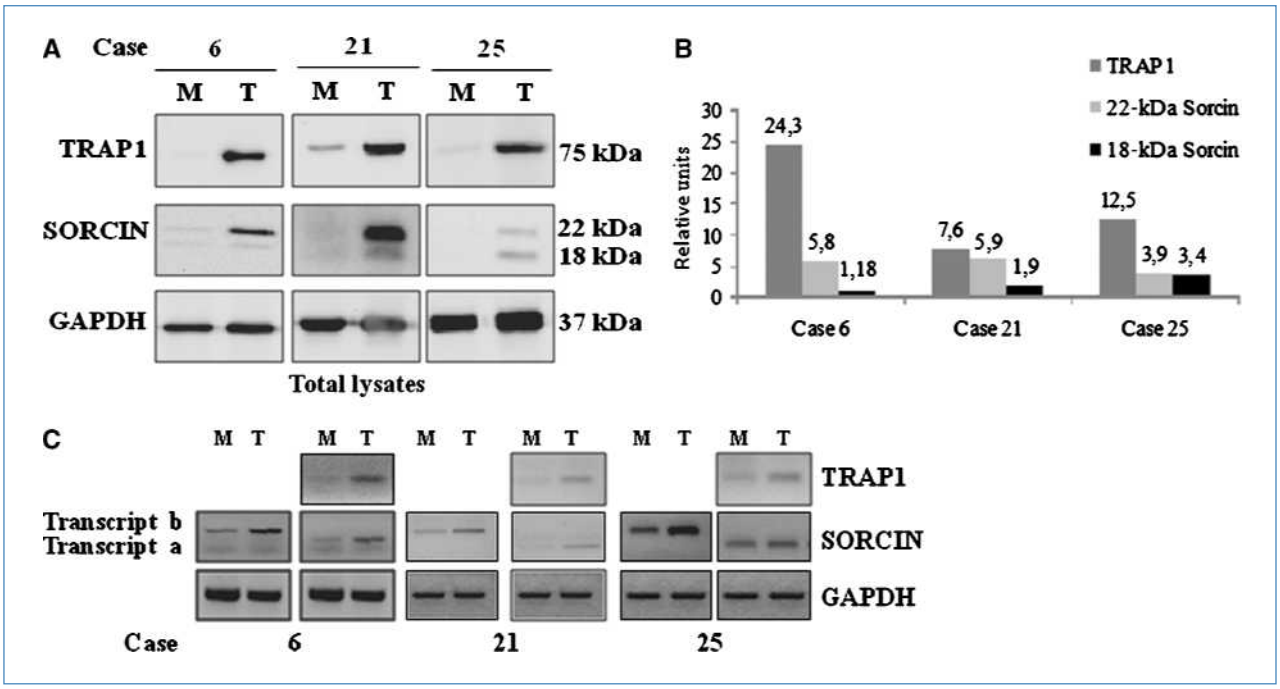

Figure 4. TRAP1 and Sorcin protein and mRNA levels in human colorectal carcinomas. A and C, protein (A) and mRNA (C) levels of TRAP1, and 18 and 22-kDa Sorcin in three colorectal tumors (T), and the corresponding noninfiltrated peritumoral mucosas (M) analyzed by immunoblot (A) and semiquantitative RT-PCR (C). B, densitometric analysis of TRAP1 and 18- and 22-kDa Sorcin isoforms in the immunoblot analysis reported in A. All the values are expressed as fold increases, by assuming protein levels of the controls (M) are equal to 1.

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proteins, suggesting the opportunity to further characterize these two polypeptides as candidate biomarkers to predict the response of colon cancer patients to antiblastic treatments and personalize individual therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The ribonuclease/angiogenin inhibitor is also present in mitochondria and nuclei

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ABSTRACT

The data presented here show for the first time that the protein known as "ribonuclease (RNase) inhibitor" (RI or RNH1) is present not only in the cell cytosol, but also in mitochondria, the central organelles in cell redox homeostasis. This finding directly correlates with the reported ability of RI to protect the cell from oxidative stress, with its sensitivity to oxidation and reactivity as a reactive oxygen species scavenger. While this study was carried out we also surprisingly discovered the presence of RI in the cell nucleus. We deem that these data open new views in the investigation on the cellular role(s) of the RI.

Structured summary:
RI physically interacts with **ATP synthase sub Alpha mitochondrial, Stress 70 Protein, Mitochondrial, Calcium-binding mitochondrial carrier protein Aralar 2 ADP/ATP Translocase 2 Elongation factor Tu** and **Cytochrome b-c1 complex Sub 2** by anti bait coimmunoprecipitation (View interaction)
PARP and **RI** colocalize by cosedimentation (View interaction)

1. Introduction

The label of "ribonuclease (RNase) inhibitor" (RI, also termed RNH1) to a ubiquitous protein of about 50 kDa, acidic, very abundant in the cell cytosol, with a high content of Cys residues, consisting of 15 Leu repeats (LRR), has been for more than half a century certainly correct. RI inhibits the great majority of the extracellular RNases from the vertebrate superfamily, bound by RI with very high affinity and catalytically neutralized. The structure of RI, free or complexed to an RNase, has been determined [1,2]. Extensive reviews have been published on RI structure and function [3,4].

However, the physiological role of this protein has not been conclusively defined. It has been proposed that the role of RI in the cell is that of a "sentry", to protect its cytosolic RNA from extracellular RNases that may enter that compartment [5]. This hypothesis is based on the following evidence: RI resistant RNases, such as bovine seminal RNase [6] and onconase [7], are cytotoxic; non-cytotoxic RNases, strongly inhibited by RI, become cytotoxic when they are engineered into relatively RI-resistant RNases [8]; cells manipulated to increase the RI levels become more resistant to RNase cytotoxicity [5,8,9].

In contrast with the "sentry" hypothesis, it has been reported that the removal of RI from cells, obtained through RNA silencing, does not render these cells sensitive to non-cytotoxic RNases [10]. Moreover, cytotoxic RNases have been described [11] that bind RI with high affinity [12]. On the other hand, data have been accumulating on a different research line. Hofsteenge and co-workers have early reported that in cultured cells an oxidative insult with H_2O_2, a general oxidant, or with diamide, a thiol specific oxidant, engenders a severe cellular loss of RI as a protein [13]. More recently, it has been reported that cells over-expressing RI become more resistant to H_2O_2 stress [14], and that RI has a strong scavenging activity on reactive oxygen species (ROS) [15]. These data, and those from a parallel investigation of the effects of oxidants and antioxidants on normal and RI deprived cells, has led to the proposal that RI plays the physiological role of a defensive system against oxidative stress [16]. Furthermore, in cells stressed with arsenite

Abbreviations: RI, RNase inhibitor; RNase, ribonuclease; ROS, reactive oxygen species; PBS, Phosphate Buffered Saline; PBS GS, PBS containing 0.2% Gelatin and 0.075% Saponin; MPTP, mitochondrial permeability transition pore; ANT, ADP/ATP Translocase; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecylsulfate; TRITC, tetramethylrhodamine isothiocyanate

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as an oxidant RI inhibits the action of angiogenin, leading to inactivation of tRNAs and translational arrest [17,18].

The data presented here, collected through biochemical and mass spectrometric analyses, as well as inspections by immunofluorescence at the subcellular level, support the hypothesis of a key physiological role of RI in redox homeostasis. They show for the first time that RI is present and reactive as a ROS scavenger not only in the cell cytosol, but also in mitochondria, the organelles central in the cell redox homeostasis. Furthermore, they have enabled us to illustrate how RI is surprisingly abundant also in the cell nucleus.

These data and conclusions do not provide a complete, exhaustive picture of the physiological significance of RI in the cell, but certainly open the way to completely novel approaches to the investigation of the cellular role of the RI.

2. Materials and methods

2.1. Cell culture and extracts

HeLa and HCT cells were cultured in DMEM supplemented with 10% bovine fetal serum in standard conditions. Cell viability was assessed with the Trypan Blue exclusion method. Cell extracts were prepared in 1% NP-40 in Phosphate Buffered Saline (PBS) with protease and phosphatase inhibitors (Roche). Surnatants were collected after centrifugation at 18 000×*g* for 30 min 4 °C, then stored at −80 °C. In some experiments, cells were transfected at 80% confluence with Metafectene–Pro (Biointex) coated plasmid DNA, and incubation lasted o/n to allow expression of the recombinant protein. Cytosolic and nuclear extracts were prepared as described [19]. Cytosolic and mitochondrial fractions were prepared with the Qproteome Mitochondria Isolation Kit (Qiagen). Protein concentration was measured by the Bradford method (Bio-Rad).

2.2. Immunoprecipitation and mass spectrometry analyses

The sequence encoding human RI was amplified with GoTaq DNA polymerase (Promega) using the following primers:

5′-GGGGGAATTCAATGAGCCTGGACATCC-3′ (forward);
5′-GGGGGGTCGACTCAGGAGATGACCCTC-3′ (reverse);

bearing EcoRI and SalI restriction sites, respectively, at 5′ ends, and the plasmid pTRP-cRI as a template [20]. The amplified fragment was then directionally inserted into the EcoRI/SalI sites of p3xFlag-CMV-7.1 to facilitate selection of immune complexes with M2 Anti-Flag Affinity Gel (SIGMA). Lysates of HeLa cells transfected with p3xFlag-CMV-7.1 or the recombinant p3xFlag-RI (about 2 mg) were challenged with M2 Anti-Flag Affinity Gel, the immune complexes were separated in 10% polyacrylamide gel electrophoresis in sodium dodecyl-sulfate (SDS–PAGE) and stained with colloidal Coomassie Blue (Invitrogen). Relevant protein bands and the corresponding gel slices from control immunoprecipitation were excised and processed for liquid chromatography and tandem mass spectrometry (LC/MS/MS) analyses, performed on an LC/MSD Trap XCT Ultra equipped with a 1100 nanoHPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). Raw data were used to query a non-redundant protein database using in-house MASCOT software (Matrix Science, Boston, MA).

2.3. Western blotting

Cell lysates were separated in 8% or 10% SDS–PAGE, then blotted on PVDF membranes (Bio-Rad) using HEP-1 electroblot apparatus (OWL) at 2.5 mA/cm^2. After blocking, membranes were incubated o/n with relevant primary antibodies, then washed and incubated with secondary peroxidase-linked antibodies. Light emission in the presence of Immobilon Western Chemiluminescent HRP Substrate (Millipore) was detected with the ChemiDoc system (Bio-Rad).

2.4. Antibodies

Antibodies used in these analyses were: anti-RI rabbit polyclonal antibodies, a kind gift of Dr. Guo-Fu Hu, Department of Pathology, Harvard Medical School, Boston, USA; an alternative anti-RI monoclonal antibody (anti-RNH1 M07, Abnova); anti-PARP-1 C2-10 monoclonal antibody (Enzo) directed to poly-ADP-ribose polymerase as nuclear marker. Antibodies anti-GAPDH, directed to glyceraldehyde-P dehydrogenase as cytosolic marker, 6C5 anti-tubulin, COX4 monoclonal antibody, and goat polyclonal antibody anti-B23 as a nucleolar marker, were from Santa Cruz Biotechnology. Alexa Fluor 488 goat anti-rabbit (Molecular Probes). Peroxidase-linked secondary antibodies were anti-mouse IgG (Fc specific) or anti-rabbit IgG goat polyclonal antibodies (Sigma).

2.5. Immunofluorescence and confocal microscopy

Cells growing on glass cover slips (in medium containing or not 250 nM Mitotracker Red as a mitochondrial dye, according to the manufacturer's protocol) were fixed for 20 min in PBS containing 4% paraformaldehyde (Sigma), washed two times in 50 mM NH_4Cl, then with PBS GS (PBS containing 0.2% Gelatin and 0.075% Saponin, from Sigma) and incubated in humidified atmosphere with the relevant antibodies at room temperature. Cells were then washed with PBS GS and incubated in humidified atmosphere at room temperature with the secondary antibodies and with tetramethylrhodamine isothiocyanate (TRITC)–phalloidin as a dye specific for microfilaments (Sigma), as indicated. As a negative control, cells were also stained with secondary antibodies alone (not shown). Nuclei were stained with DNA intercalants Hoechst 33258 or DRAQ5 (Bio status, Alexis Corporation), as indicated. Cells were finally mounted in 50% glycerol in PBS and analyzed with a Zeiss LSM 510 Meta confocal microscope. The lambda of the argon ion laser was set at 488 nm, those of the two HeNe lasers were set one at 546, the other at 633 nm. Fluorescence emission was revealed by BP 505–530 band pass filter for Alexa Fluor 488, by BP 560–615 band pass filter for TRITC–phalloidin or Mitotracker Red and by 615 long pass filter for DRAQ5. Nuclei stained with Hoechst 33258 were acquired as non-confocal images by mean of a light detector and 352/461 nm filter (UV light). Double- and triple staining immunofluorescence images were acquired separately in the green, red and infra red channels and then saved in TIFF format to prevent the loss of information. They had been acquired with a resolution of 1024 × 1024 pixels with the confocal pinhole set to one Airey unit.

3. Results

3.1. Searching for proteins interacting with RI

We have designed co-immunoprecipitation experiments to identify proteins interacting with RI in the cellular environment, as a new approach to the understanding of its biological role. To this end, lysates from HeLa cells transfected with the recombinant vector p3xFlag-RI or control vectors, were challenged with anti-Flag M2 affinity Gel, then bound proteins were analyzed by SDS–PAGE and stained with Coomassie Blue. The outcome revealed, in addition to Flag-RI, a number of protein bands absent in the control

Table 1
Mitochondrial proteins specifically interacting with RI, the RNase/angiogenin inhibitor in HeLa cell lysates as identified by mass spectrometry. Peptides corresponding to proteins listed in the table were totally absent in control immunoprecipitation.

Swiss-Prot #	Protein	MW	Peptides
P38646	Stress 70 Protein Mitochondrial	73 680	8
Q9UJS0	Calcium-binding mitochondrial carrier protein Aralar 2	74 176	10
P25705	ATP synthase sub Alpha mitochondrial	59 751	12
P49411	Elongation factor Tu	49 542	13
P22695	Cytochrome b-c1 complex Sub 2	48 443	5
P05141	ADP/ATP Translocase 2	32 895	9

sample; the most prominent ones were subjected to LC/MS/MS mass spectrometry analysis. The results (see Fig. S1) show that a relevant group of interacting proteins belongs to the mitochondrial inner membrane or matrix (see Table 1), suggesting that, in addition to the cytosolic compartments, RI appears to reside also inside mitochondria.

3.2. RI is localized in mitochondria

We then tried a direct biochemical approach to detect the presence of RI in mitochondria. The mitochondrial and cytosolic fractions of transfected HeLa and HCT cells were analyzed by Western blotting to identify 3xFlag-RI. The results (Fig. 1A and B) show that a substantial amount of recombinant RI is associated to the mitochondrial fractions. In addition, endogenous RI is revealed by specific antibodies in the mitochondrial fractions of untransfected cells (Fig. 1C), while an unrelated control antiserum was unable to produce the signal (not shown).

To confirm this finding, we performed an immunofluorescence confocal analysis of HeLa cells. The results (see Fig. 2) show that endogenous RI largely colocalizes with mitochondria stained with Mitotracker Red. As expected, RI is also present in the cytosol and, surprisingly, also in the nuclear matrix.

3.3. Evidence of RI localization in the nucleus

We have validated the presence of RI in the nucleus by probing HeLa cells nuclear extracts and cytosolic lysates with polyclonal anti-RI antibodies. The results of Western blottings, shown in Fig. 3, confirm the presence of RI in the cell nucleus. The signal generated by a monoclonal anti-RI antibody in confocal immunofluorescence analyses (see Fig. 4A–C) is consistent with the results shown in Figs. 2, 3 and 4D–G, in which the RI nuclear localization is revealed by specific polyclonal antibodies. In confocal immunofluorescence analyses, both antibodies show the same very intense and diffuse staining pattern of the nucleus, with the exception of regions corresponding to nucleolar areas, identified by an antibody

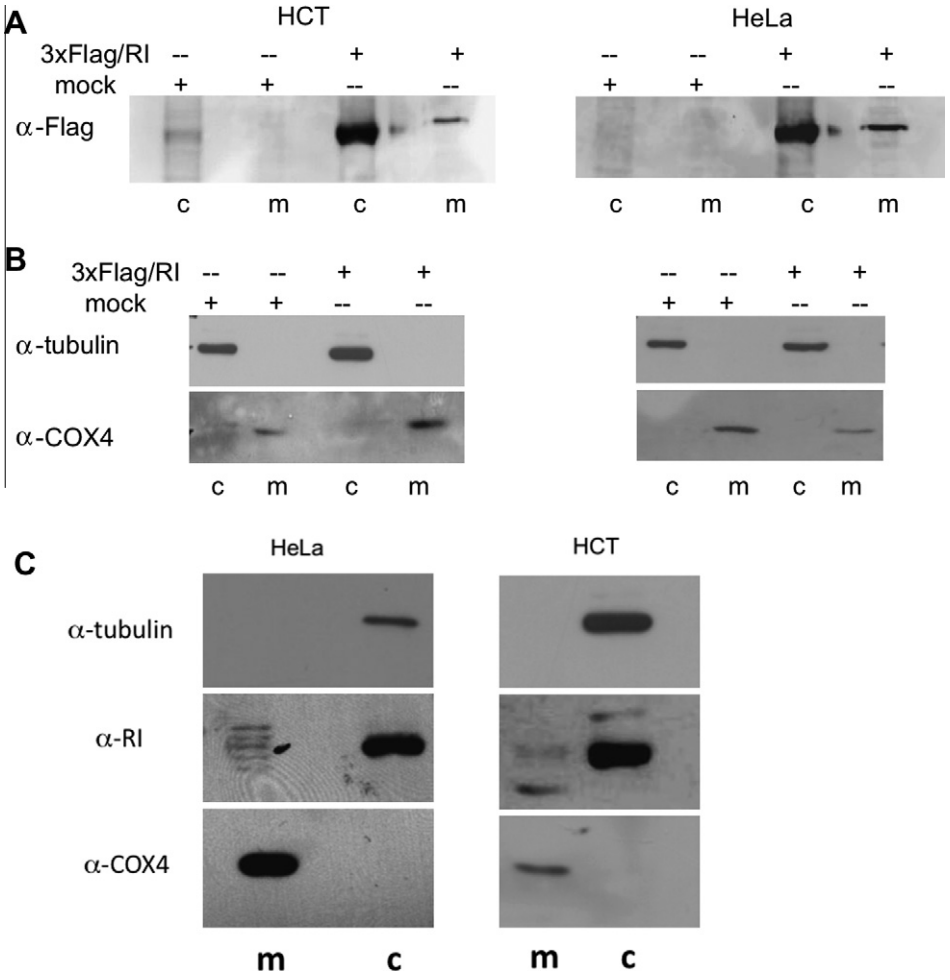

Fig. 1. Subcellular localization of RI in HCT and HeLa cells transfected with p3xFlag or control vector (mock). (A) Recombinant RI, revealed by SDS–PAGE 10% followed by Western blotting with anti-Flag antibody (α-Flag), is detected not only in the cytosolic (c) but also in the mitochondrial (m) fractions. (B) The same lysates were blotted with anti-tubulin (α-tubulin) and anti-COX4 (α-COX4) antibodies as markers for cytosolic and mitochondrial fractions, respectively. (C) The subcellular localization of endogenous RI in HCT and HeLa cells is shown, revealed by Western blotting with anti-RI polyclonal antibodies. Filters were re-blotted with anti-tubulin (α-tubulin) and anti-COX4 (α-COX4) antibodies. 40 μg of each lysate were analyzed.

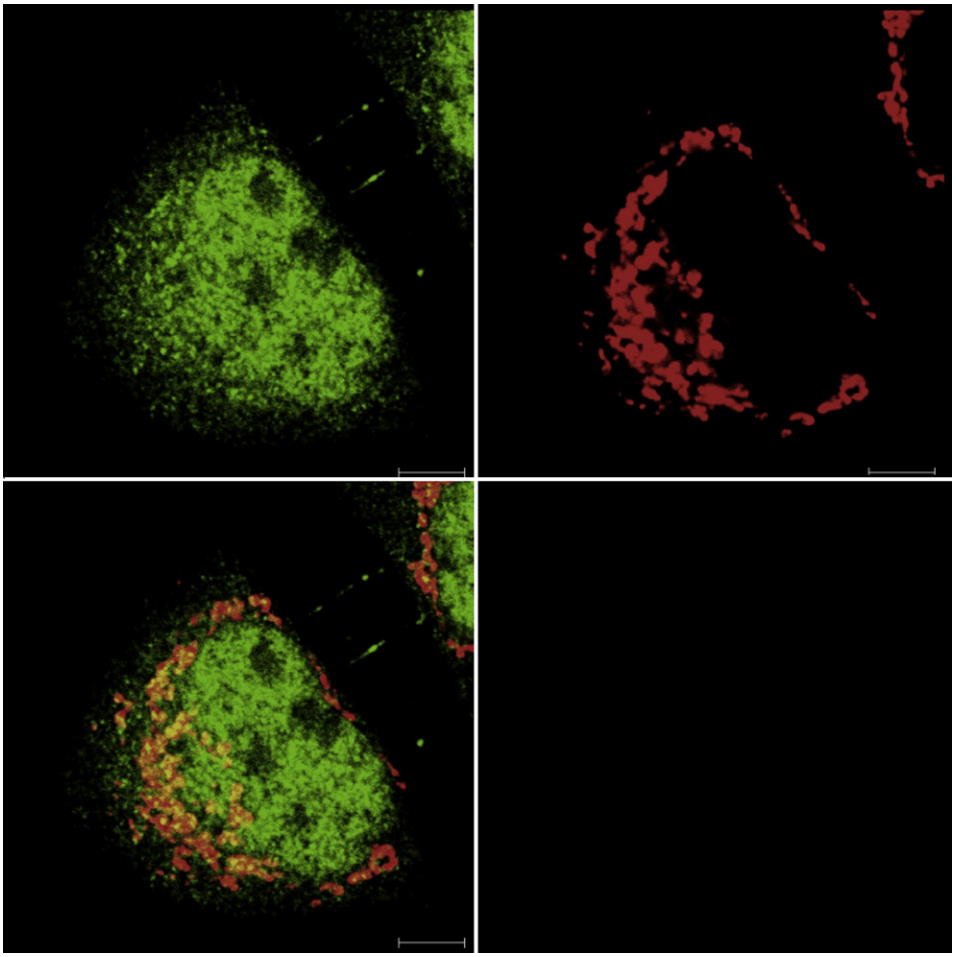

Fig. 2. Confocal microscopy showing significant mitochondrial localization of endogenous RI in HeLa cells. Red: mitochondria stained with Mitotracker Red; green: α-RI followed by Alexa fluor 488-conjugated goat anti-rabbit IgG antibody; yellow: merge. Bars: 5 μm.

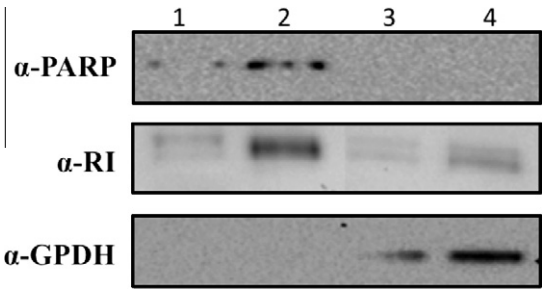

Fig. 3. Evidence of RI localization in the nucleus. Western blot analyses with anti-RI polyclonal antibodies. Lanes 1 and 2: 2.5 or 5 μg of HeLa cells nuclear extracts; in lanes 3 and 4: 2.5 or 5 μg of cytosolic lysates. The blot was re-probed with anti PARP and anti-GAPDH antibodies as markers for nuclear and cytosolic extracts, respectively.

against the nucleolar marker phosphoprotein B23 (data not shown).

4. Discussion

The result of our work uncover novel, previously unnoticed subcellular localizations of RI. We have established that RI, known as a cytosolic protein, is also present in the cell nucleus and mitochondria. Association of RI with proteins of the mitochondrial inner membrane and matrix place this protein in a major environment of ROS production. The ability of RI to protect genome integrity and cell viability from oxidative stress injury is documented in the literature, and correlates well with the sensitivity to oxidation and the in vitro activity as ROS scavenger of the protein [14–16].

Singh et al. have shown that apoptosis induced in HeLa cells by H_2O_2 induced oxidative stress, depends on ROS generated in mitochondria [21]. HeLa cells on the other hand show increased resistance to H_2O_2 induced mortality when RI is overexpressed [14]. The evidence reported here provides a new direct basis, through experimental methodologies distinct from the others so far proposed on the role of RI as a ROS scavenger. Interesting to note protein ADP/ATP Translocase (ANT), co-immunoprecipitated with RI, is a regulative component of the mitochondrial permeability transition pore (MPTP), which plays a pivotal role in mitochondrial induced cell death [21]. Oxidative stress promotes glutathione-mediated oxidation of ANT cysteine residues located in matrix-facing loops, resulting in Ca^{++} binding of the protein and activation of MPTP [22]. Thus, we are tempting to speculate that ANT-associated RI could act as a shield against ROS induced, GSH mediated oxidation of the nucleotide transporter. Oxidation of RI leads to the formation of intramolecular disulfide bridges with an all-or-none pattern, and to its rapid degradation in the cell [13]. We have observed some degradation of endogenous RI in mitochondrial lysates (see Fig. 1C), a finding consistent with the hypothesis that RI is easily oxidized in mitochondria due to the ROS rich environment.

The identification of RI in the nucleus is surprising; its role in this cellular compartment is unknown. In immunoprecipitation

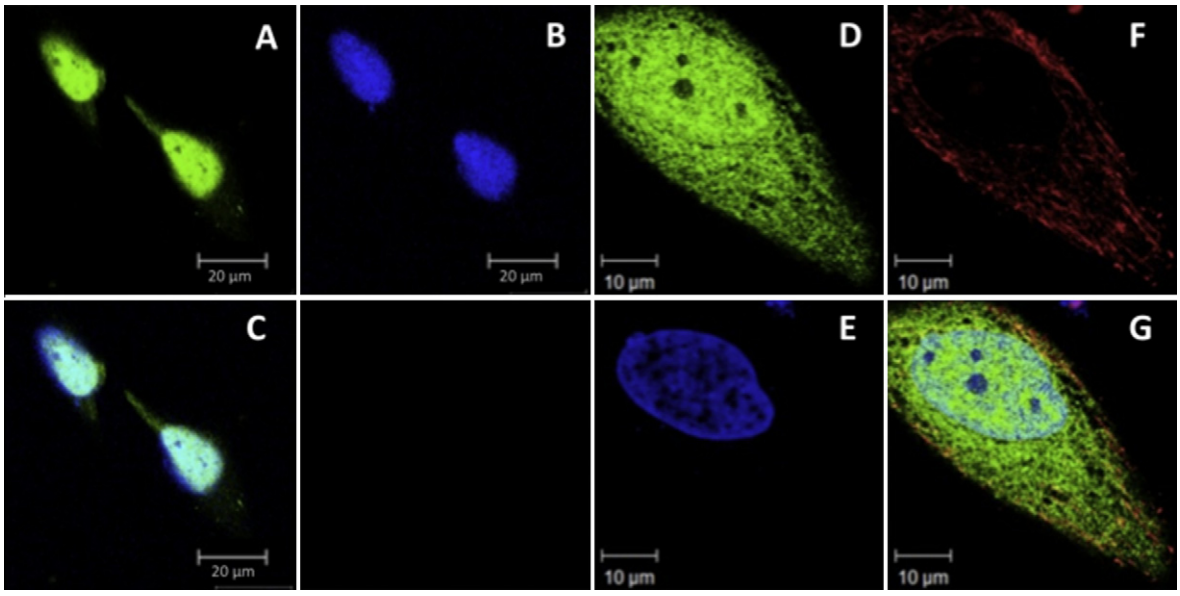

Fig. 4. Confocal microscopic analysis of HeLa cells. (A) Green fluorescence associated with monoclonal anti-RI antibody. (B) Blue fluorescence associated with Hoechst staining, acquired as non-confocal images. (C) Merge between (A) and (B). RI is evident in the nuclear matrix, absent in nucleoli. Bars: 20 μm. (D) Green fluorescence associated with polyclonal anti-RI antibodies. (E) Nuclear blue fluorescence after staining with DRAQ5. (F) Red fluorescence after staining with TRITC-conjugated phalloidin. (G) Merge between (D), (E) and (F). Bars: 10 μm.

assays we failed to identify nuclear proteins associated with RI, except the nuclear pore membrane glycoprotein 210 (accession number Q8TEM1). This finding is in line with the observation that the recombinant 3xFlagRI, perhaps due to the extension of 28 residues at the amino end, does not enter the nucleus (not shown). While the protein appears abundant in the nuclear matrix, it seems definitely excluded from nucleoli. It can be assumed that in the reducing nuclear environment RI is in the thiol form, thus able to bind and inhibit RNases, such as angiogenin. As angiogenin accumulates in HeLa nucleoli, where it plays a key role in rRNA biogenesis [23], the absence of RI, a powerful inhibitor of the enzyme, from the nucleolar areas, would be consistent with this role of angiogenin.

In conclusion, the observations reported in this study offer new evidence supporting the role of RI as a scavenger of ROS, and raise intriguing new questions about its significance and interactions in the nucleus, thus opening new lines of investigation for an advancement in the knowledge of RI functions in cell biology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.034.

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TRAP1 and the proteasome regulatory particle TBP7/Rpt3 interact in the endoplasmic reticulum and control cellular ubiquitination of specific mitochondrial proteins

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Tumor necrosis factor receptor-associated protein-1 (TRAP1) is a mitochondrial (MITO) antiapoptotic heat-shock protein. The information available on the TRAP1 pathway describes just a few well-characterized functions of this protein in mitochondria. However, our group's use of mass-spectrometric analysis identified TBP7, an AAA-ATPase of the 19S proteasomal subunit, as a putative TRAP1-interacting protein. Surprisingly, TRAP1 and TBP7 colocalize in the endoplasmic reticulum (ER), as demonstrated by biochemical and confocal/electron microscopic analyses, and interact directly, as confirmed by fluorescence resonance energy transfer analysis. This is the first demonstration of TRAP1's presence in this cellular compartment. TRAP1 silencing by short-hairpin RNAs, in cells exposed to thapsigargin-induced ER stress, correlates with upregulation of BiP/Grp78, thus suggesting a role of TRAP1 in the refolding of damaged proteins and in ER stress protection. Consistently, TRAP1 and/or TBP7 interference enhanced stress-induced cell death and increased intracellular protein ubiquitination. These experiments led us to hypothesize an involvement of TRAP1 in protein quality control for mistargeted/misfolded mitochondria-destined proteins, through interaction with the regulatory proteasome protein TBP7. Remarkably, expression of specific MITO proteins decreased upon TRAP1 interference as a consequence of increased ubiquitination. The proposed TRAP1 network has an impact *in vivo*, as it is conserved in human colorectal cancers, is controlled by ER-localized TRAP1 interacting with TBP7 and provides a novel model of the ER–mitochondria crosstalk.
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Tumor necrosis factor receptor-associated protein-1 (TRAP1) was initially identified as a TNF-receptor-associated protein and is a member of the heat-shock protein-90 (HSP90) chaperone family.[1,2] Through an mRNA-differential display analysis between oxidant-adapted and control osteosarcoma cells, our group identified, among other proteins, TRAP1, whose expression was highly induced upon oxidant adaptation.[3] Furthermore, TRAP1 showed antioxidant and antiapoptotic functions,[4] while an involvement of this mitochondrial (MITO) chaperone in the multi-drug resistance of human colorectal carcinoma (CRC) cells was also established.[5]

Little is known about TRAP1 signal transduction: the first most important finding on TRAP1 function came from studies by the Altieri's group, which identified TRAP1 as a member of a cytoprotective network selectively active in the mitochondria of tumor tissues.[6] The same group has recently proposed TRAP1 as a novel molecular target in localized and metastatic prostate cancer,[7] and is now involved in a promising preclinical characterization of mitochondria-targeted small-molecule HSP90 inhibitors.[8,9] Besides some well-characterized TRAP1 functions in mitochondria, during preparation of this manuscript it was reported that interference by HSP90 chaperones triggers an unfolded protein response (UPR) and activates autophagy in the mitochondria of tumor cells.[10] A putative role of TRAP1 in endoplasmic reticulum (ER) stress control was concomitantly suggested by Takemoto *et al.*,[11] even though no evidence regarding the mechanisms involved was provided in this study.

A proteomic analysis of TRAP1 co-immunoprecipitation (co-IP) complexes was performed in our laboratory, in order to

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Keywords: TRAP1; TBP7; mitochondria/ER crosstalk; protein quality control; ubiquitination; apoptosis

Abbreviations: TRAP1, tumor necrosis factor receptor-associated protein-1; UPR, unfolded protein response; ER, endoplasmic reticulum; MS, mass spectrometry; FRET, fluorescence resonance energy transfer; EM, electron microscopy; CRC, colorectal carcinoma; TG, thapsigargin; Ub, ubiquitin; MTS, mitochondrial targeting sequence; UPS, ubiquitin–proteasome system; shRNA, short-hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FITC, fluorescein isothiocyanate; MITO, mitochondrial; CYTO, cytosolic; S, supernatant; P, pellet; PM, post-mitochondrial

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further characterize the TRAP1 network and evaluate protein interactors relevant for its roles. Among several other proteins, a novel MITO isoform of Sorcin, a calcium-binding protein, was identified as a new TRAP1 'ligand' and a cytoprotective function against apoptosis induced by anti-blastic agents was recently demonstrated for this protein by our group.[12] In the present paper, we characterize another new interaction of TRAP1 with TBP7/ATPase-4/Rpt3, an S6 ATPase protein of the proteasome regulatory subunit.[13,14] TBP7 was first identified as a novel synphilin-1-interacting protein,[13] so a functional role in Parkinson's disease was proposed for this protein. However, not many novel results became available subsequently on TBP7 function.

Altogether, (i) the absence of TBP7 in mitochondria; (ii) its still uncharacterized function as a regulatory protein; and (iii) its association with TRAP1 prompted us to analyze the sub-cellular localization of TRAP1/TBP7 interaction and to investigate its functional role. Several studies have described a function of HSP in the control of gene expression,[15,16] and recent evidence demonstrated the importance of 19S ATPases in the transcription machinery, as well as their additional regulatory mechanisms in mammalian transcription.[17] Therefore, we hypothesized that the TRAP1/TBP7 interaction might have a role in protein quality control and cellular ubiquitination. Moreover, the finding that the two proteins directly interact in the ER further supports our hypothesis, as it is known that misfolding of proteins is tightly controlled by a large number of molecular chaperones and, if the quality control fails, they are ubiquitinated and degraded by the proteasome.[18] This paper, for the first time, describes the presence of TRAP1 on the outer side of the ER and shows the functional role that TRAP1 has in the quality control of proteins destined to the mitochondria, and in the regulation of intracellular protein ubiquitination, through interaction with TBP7.

Results

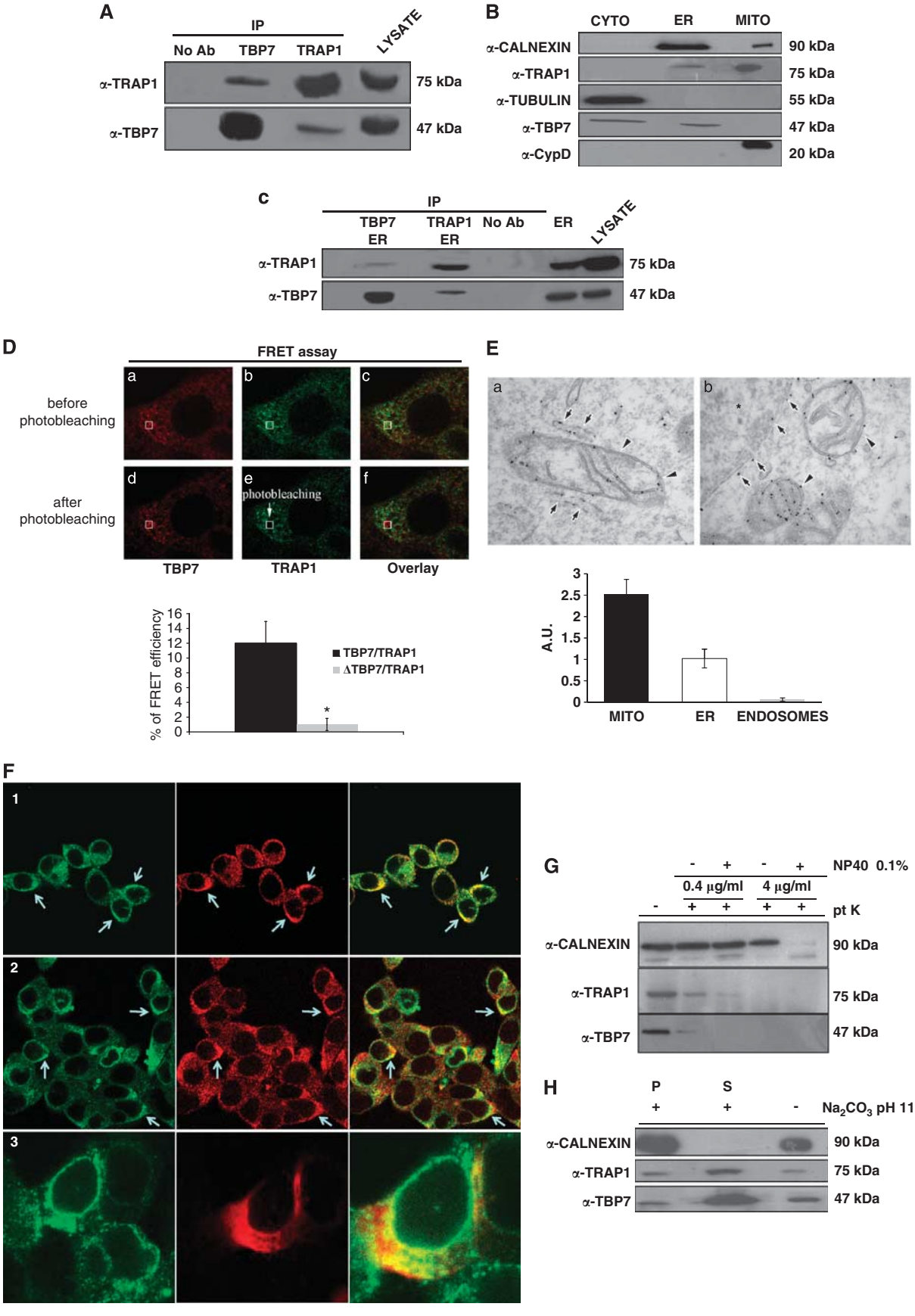
TRAP1 and TBP7 colocalize and directly interact in the ER. The 'fishing for partners' strategy combined with mass spectrometric procedures performed to identify TRAP1 protein partners has already been described elsewhere.[12]

Among the putative TRAP1-binding partners, we focused our attention on a protein of about 50 kDa identified by our LC-MS/MS analysis as S6/TBP7/ATPase-4/Rpt3.[19] To confirm that this proteasome subunit was indeed a TRAP1-interacting protein, we performed western blot (WB) and co-IP analyses in total extracts from HCT116 colon carcinoma cells (Figure 1A). As TRAP1 is localized in mitochondria, sub-fractionation of HCT116 cellular extracts was performed. Surprisingly, WB analysis of sub-cellular compartments failed to demonstrate the presence of the regulatory protein in mitochondria, but identified TBP7 both in the cytosol and microsomal fraction (Figure 1B). Co-IP analyses from ER fraction confirmed the TRAP1/TBP7 interaction in the ER (Figure 1C).

The TRAP1/TBP7 molecular interaction was further investigated by using a fluorescence resonance energy transfer (FRET) approach in fixed cells (Figure 1D).[20] In cells co-expressing wild-type TRAP1 and TBP7 we found about 12% of FRET efficiency (Figure 1D) indicating that TRAP1 and TBP7 are close enough to allow energy transfer. Interestingly we found FRET exclusively when we bleached ER regions, which we selected on the basis of their morphological features (one example in Figure 1D, a–f). Thus, these data indicate that TRAP1 and TBP7 directly interact with each other and this interaction occurs specifically in the ER compartments. Furthermore, according to the biochemical data described below in the paper (Supplementary Figure 1 and Figure 4H), we found no FRET between TRAP1 and a mutant form of TBP7 (Figure 1D).

As it had never been reported previously, we aimed to further confirm the novel localization of this MITO chaperone in the ER through electron microscopy (EM) and confocal microscopy analyses (Figures 1E and F). To further evaluate whether TRAP1 is associated with the ER membranes, cells stably expressing TRAP-HA vectors were prepared for immuno-EM. Labeling with an anti-HA antibody revealed significant amounts of TRAP1 in mitochondria (Figure 1E, a and b, arrowheads), as has been reported previously.[6] In

Figure 1 TRAP1 and TBP7 interact and colocalize in the ER. (**A**) Total HCT116 lysates were harvested and immunoprecipitated using anti-TRAP1 and anti-TBP7 antibodies as described under Materials and Methods, separated by SDS-PAGE and immunoblotted using the indicated mouse monoclonal anti-TRAP1 and mouse monoclonal anti-TBP7 antibodies. No Ab, total cellular extracts incubated with A/G plus agarose beads without antibody; IP, immunoprecipitation using the corresponding antibodies. (**B**) Total HCT116 lysates were fractionated into MITO, CYTO and microsomal (ER) fractions as described under Materials and Methods, separated by SDS-PAGE and immunoblotted using mouse monoclonal anti-TRAP1 and mouse monoclonal anti-TBP7 antibodies. The purity of the fractions was assessed by using mouse monoclonal anti-tubulin, goat polyclonal anti-CypD, rabbit polyclonal anti-calnexin antibodies specific for the single subcellular compartments. (**C**) TRAP1 and TBP7 co-IP analysis on the microsomal fraction (ER), obtained as described under Materials and Methods. WB of immunoprecipitates was performed by using the indicated antibodies. (**D**) TRAP1/TBP7 direct interaction. FRET was measured by using the acceptor photo-bleaching technique as described under Materials and Methods. The images show the signal of TBP7 (red) and TRAP1 (green) before (a–c) and after photo-bleaching (d–f). The selected ROI for bleaching was indicated. Energy transfer efficiency was measured in cells transiently co-transfected with TRAP1 and either TBP7 or its mutant form (ΔTBP7-Flag), and is expressed in % as mean of three independent experiments. Error bars: $\pm$ S.D.; $^*P<0.0001$. (**E**) ER Distribution of TRAP1 in HCT116 cells (EM). Cells expressing TRAP-HA vector were fixed and prepared for immuno-EM (see Materials and Methods). Labeling with the anti-HA antibody revealed significant amount of TRAP1 in mitochondria (a, b, arrowheads). In addition, TRAP1 was distributed throughout the elongated membrane profiles (a, arrows) that on the basis of their ultrastructural features (such as attached ribosomes) can be attributed to the rough ER compartment, and detected along the nuclear envelope (b, arrows). The density of immuno-gold labeling (in arbitrary units; average $\pm$ S.D.) in mitochondria (MITO), ER and endosomes (as a negative control) is reported in the lower histogram. (**F**) ER TRAP1/TBP7 colocalization (confocal microscopy). Immunofluorescence shows colocalization of TBP7 with TRAP1 and with the ER protein calnexin. In Panel-1, a double immunofluorescent staining is shown for TRAP1 (green) and TBP7 (red). In Panel-2, a double immunofluorescent staining is shown for calnexin (green) and TBP7 (red). In cells expressing the Myc-tagged TRAP1 construct (red) the protein co-distributes to a great extent with endogenous calnexin (green, Panel-3). (**G** and **H**) Biochemical characterization of TRAP1/TBP7 'topology' in the ER. WB of HCT116 microsomal fractions treated with 0.4 μg/ml or 4 μg/ml proteinase-K (pt K) $\pm$ 1% NP-40 for 20 min on ice (**G**) or with 100 mM Na_2CO_3 (pH 11.3) for 30 min (**H**) as described under Materials and Methods. Specific proteins were revealed using the indicated antibodies. (**H**): S, supernatant; P, pellet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addition, we found that TRAP1 was distributed throughout the elongated membrane profiles (Figure 1E, a, arrows) that on the basis of their ultrastructural features (such as attached ribosomes) can be attributed to the rough ER compartment. Moreover, gold particles – indicating TRAP1 molecules – were detected along the nuclear envelope (Figure 1E, b, arrows), considered to be part of the ER membrane network. A careful examination of the density of immuno-gold labeling (in arbitrary units; average ± S.D.) over different intracellular membranes allowed us to demonstrate that indeed TRAP1 was enriched in mitochondria (2.53 ± 0.34) as it has already been reported. However, significant labeling density was also calculated for ER membranes (1.02 ± 0.22) as shown in Figure 1E. Taken together, the EM observations are in line with the above results showing association of TRAP1 with the ER (Figures 1A–C). Accordingly, immunofluorescence confocal microscopy analysis showed colocalization of TBP7 with TRAP1 and with the ER protein calnexin, thus confirming ER localization further (Figure 1F).

Altogether, these findings demonstrate that the interaction between TRAP1 and TBP7 occurs in the ER, but are unable to reveal a detailed localization in this context. To further evaluate the 'topology' of TRAP1 and TBP7 in the ER, a biochemical assay based on protease digestion was performed. Figure 1G shows that both proteins are sensitive to a proteinase-K digestion, whereas calnexin, a well-known ER-resident protein, is undigested. These approaches allowed us to demonstrate that TRAP1 and TBP7 are located on the outside of the ER. Moreover, alkaline treatment of ER fractions to remove peripheral membrane proteins allowed us to demonstrate that both TRAP1 and TBP7 are loosely associated to ER membranes (Figure 1H).

TRAP1 interference sensitizes CRC cells to TG-induced ER stress. We therefore investigated the functional role of the TRAP1/TBP7 interaction. Several data suggest that ER stress, worsened by the high ROS concentrations in this sub-cellular compartment, is controlled by networks of molecular chaperones.[21] Considering the previously described protective role of TRAP1 against several stresses, including oxidative stress through its antioxidant functions,[4,22] we hypothesized that the TRAP1/TBP7 interaction constituted an additional control to check the state/folding of proteins damaged in the ER. In order to induce protein misfolding in the ER, we induced ER stress by using thapsigargin (TG), a well-known agent that mobilizes Ca^{2+} from the ER.[23] As a marker of ER stress we analyzed the expression of BiP/Grp78, a major ER chaperone protein essential for protein quality control in the ER, as well as a central regulator of the UPR.[24] Figure 2 shows that BiP mRNA expression is strongly increased, upon TG treatment, in TRAP1-interfered cells as compared with the respective control (Figures 2a and b). Interestingly, a rescue of this phenotype is achieved upon TRAP1 re-addition (Figure 2b). These results demonstrate that TRAP1 knockdown sensitizes cells to TG-induced ER stress, thus suggesting an involvement of this protein in ER stress response, as reported recently.[10,11]

We and other groups have previously demonstrated the protective roles of TRAP1 against cell death induced by

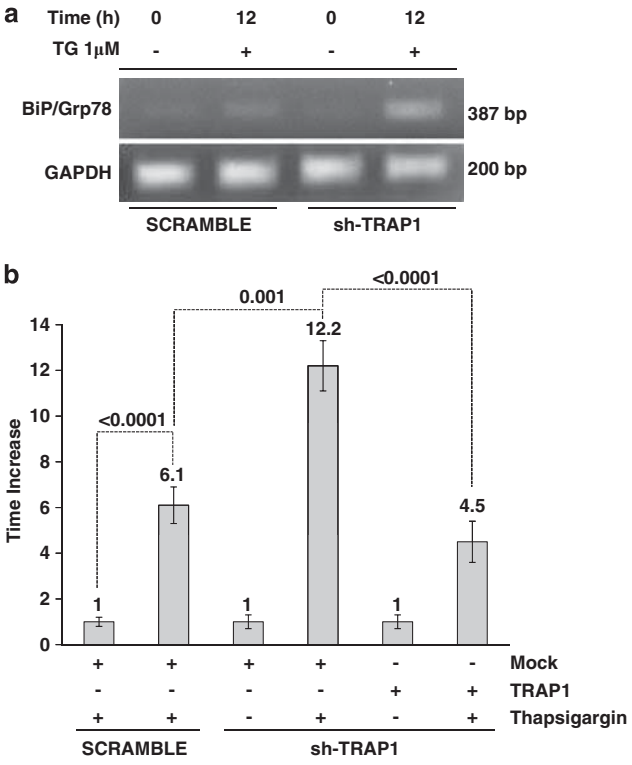


Figure 2 BiP mRNA levels in sh-TRAP1 stable clones. (**a**) Semi-quantitative RT-PCR analysis of BiP/Grp78 mRNA expression in sh-TRAP1 stable clones with respect to scrambled transfectants after 12-h treatment with 1 μM TG. As control, the levels of GAPDH transcript were analyzed. (**b**) Real-time RT-PCR analysis of BiP/Grp78 mRNA expression in scrambled and sh-TRAP1 HCT116 cells exposed to 1 μM TG for 12 h and in sh-TRAP1 HCT116 cells transfected with TRAP1 cDNA before treatment with TG. The *P*-values indicate the statistical significance between different BiP/Grp78 levels under the indicated conditions

several agents.[4,6] Therefore, we addressed whether TRAP1-interfered cells become more sensitive to apoptosis, as a possible and expected consequence of increased ER stress, and whether TBP7 might have a role in this process. Table 1 shows that TRAP1 and TBP7 interference sensitized HCT116 cells to TG-induced apoptosis. Noteworthy, a similar result was observed upon cell treatment with oxaliplatin, a genotoxic agent (Table 1). Taken together, all these observations confirm the well-known role of TRAP1 in the protection against stress-induced cell death and highlight a new role for regulatory proteasome proteins in apoptotic control.

TRAP1/TBP7 control of intracellular protein ubiquitination. We then hypothesized that, if the level of protein damage upon ER stress is too severe to be counteracted/repaired by TRAP1 refolding, increased protein degradation might occur, and increased ubiquitination levels of intracellular proteins could be observed. Indeed, tight regulation of protein ubiquitination by TRAP1/TBP7 does occur: in fact, a low-ubiquitin (Ub) signal is observed in TRAP1-containing cells, whereas TRAP1 interference strongly increases general protein ubiquitination levels (Figure 3).

The control of ubiquitination of intracellular proteins by TRAP1/TBP7 is a general phenomenon as it is present in total

Table 1 Rates of apoptotic cell death in CRC HCT116 cells treated with 1 μM TG for 24 h or with 10μM oxaliplatin (l-OHP) for 48 h upon transient (siRNA) or stable (shRNA) downregulation of TRAP1 or TBP7

	Apoptosis (% ± S.D.)		
	Vehicle	Ratio (± S.D.)	*P*-value
Scramble			
Vehicle	3.5 ± 0.3		
TG	5.0 ± 0.2	1.4 ± 0.2	
l-OHP	11.2 ± 0.4	3.2 ± 0.4	
siRNA TRAP1			
Vehicle	4.7 ± 0.2		
TG	21.5 ± .0.4	4.6 ± 0.3	<0.0001
l-OHP	55.5 ± 0.6	11.8 ± 0.7	<0.0001
shRNA TRAP1			
Vehicle	3.5 ± 0.4		
TG	24.2 ± .0.5	6.9 ± 1.1	0.001
l-OHP	31.8 ± 0.4	9.1 ± 1.3	0.002
siRNA TBP7			
Vehicle	5.2 ± 0.2		
TG	15.7 ± 0.3	3.0 ± 0.2	<0.0001
l-OHP	55.1 ± 0.6	10.6 ± 0.5	<0.0001

Ratios are calculated between rates of apoptosis in drug- and vehicle-treated cells. The *P*-values indicate the statistical significance between the ratios of apoptosis in siRNA-transfected cells and the respective scrambled controls.

extracts (Figure 3a). Interestingly, both TRAP1 and TBP7 seem to have a critical role in the regulation of protein ubiquitination. In fact, upon transfection of TRAP1 expression vectors in sh-TRAP1 stable transfectants a rescue of the high-Ub 'phenotype' is observed (Figure 3a). Sub-cellular fractionation allowed us to demonstrate that this regulatory role is more evident in the post-mitochondrial (PM) fraction (cytosol + microsomes), where TRAP1/TBP7 control is particularly necessary given the abundance of proteins translated, but often damaged. Conversely, the same regulation is not observed in MITO extracts even after re-transfection of TRAP1, likely because TBP7 is absent in these organelles (Figure 3b). These results confirm that TRAP1 function in the regulation of protein ubiquitination requires the presence of TBP7. Additionally, increased cellular levels of ubiquitinated proteins, very similar to those obtained in cells transiently transfected with TRAP1 siRNAs, were observed upon TBP7 siRNA transfection (Figure 3c), thus confirming the role of TBP7 in this regulation. Altogether, these results demonstrate that the TRAP1/TBP7 interaction is a useful and important checkpoint in which these two proteins concomitantly work to judge whether a protein can be repaired and reach the final destination or, if the damage is too severe, it needs to be degraded. Furthermore, despite the block of proteasome activity by MG132 treatment, the experiments shown in Figure 3 demonstrate that the regulation of protein ubiquitination by TRAP1 is not due to inhibition of proteasome function, as it is observed also in the absence of the inhibitory drug (Figure 3a). Indeed, this finding was confirmed by assaying the proteasome's activity *in vitro* using fluorescent substrates and extracts from scrambled- and TRAP1- or TBP7-interfered cells. The results shown in Figure 3d demonstrate that neither TRAP1 nor TBP7 interference inhibits the proteasome's function.

More importantly we asked whether the control of protein ubiquitination by TRAP1/TBP7 requires TRAP1 MITO localization. To address this issue we generated the Δ1–59 deletion mutant of TRAP1 (in which the first 59 aa containing the MITO targeting sequences (MTS[2]) were removed from the N-terminus) yielding a TRAP1 mutant defective for MITO import, but still able to bind to TBP7 (Supplementary Figure 1 and Figures 4a and b). Interestingly, transfection of this mutant in sh-TRAP1 stable clones rescued the heavy Ub levels present in TRAP1-interfered cells (Figure 4c). Conversely, another TRAP1 deletion mutant (Δ101–221) was generated that keeps the MTS (Supplementary Figure 1) and, thus, is able to localize into mitochondria, but is unable to bind to TBP7, which, as shown previously, is absent in the MITO fraction. Transfection of the Δ101–221 mutant in sh-TRAP1 cells showed no changes in protein ubiquitination levels (Figures 4d–f). These experiments provide proof of concept that the relevant amount of TRAP1 present in the ER fractions (see the EM qualitative/quantitative analyses shown in Figure 1e) is indeed involved in the regulation of protein ubiquitination through its binding to TBP7, whereas MITO TRAP1 does not influence Ub levels, according to the phenotype generated by the Δ101–221 TRAP1 deletion mutant. Finally, we demonstrated that transfection of the TRAP1 Δ1–59 mutant decreased the BiP/Grp78 mRNA levels present in sh-TRAP1 clones upon TG-induced ER stress. Figure 4g shows that a rescue of BiP/Grp78 mRNA levels is obtained upon transfection of the Δ1–59 mutant, whereas the Δ101–221 TRAP1 mutant with MITO localization not only is unable to counteract ER stress, but even further increased BiP/Grp78 levels.

Strikingly, a TBP7 deletion mutant (Supplementary Figure 1), unable to bind to TRAP1 (Figure 4h), yields, upon transfection in HCT116 cells (scrambled), a strong Ub pattern undistinguishable from the phenotype of sh-TRAP1 stable transfectants (Figure 4i), likely acting as dominant negative over the endogenous TBP7 protein's function.

Quality control of specific mitochondria-destined proteins by TRAP1/TBP7. Once we demonstrated the contribution of TRAP1/TBP7 to the regulation of ER stress/apoptosis, with the consequent modulation of intracellular protein ubiquitination, we hypothesized that TRAP1 could be involved in a general control of protein stability. To this aim, pulse–chase experiments were performed in scrambled and sh-TRAP1 stable transfectants. However, a comparable pattern of degraded proteins is still observed 72 h after Met/Cys chase (Figure 5a). This unchanged overall protein stability led us to hypothesize that TRAP1 could be involved in the control of protein folding/stability for selective proteins, likely those directed to mitochondria. To evaluate this hypothesis, the levels of F1ATPase, a nuclear-encoded MITO protein and a potential TRAP1 interactor as suggested by MS analysis,[12] were analyzed in cells in which TRAP1 expression was lowered by short-hairpin RNA (shRNA) interference. Interestingly, protein levels decreased in TRAP1 stably interfered cells (Figure 5b and Supplementary Figure 2), whereas a rescue was obtained upon re-addition of the Δ1–59 TRAP1 mutant but not by the Δ101–221 TRAP1 mutant (Supplementary Figure 3). These findings confirm that the regulation of the observed

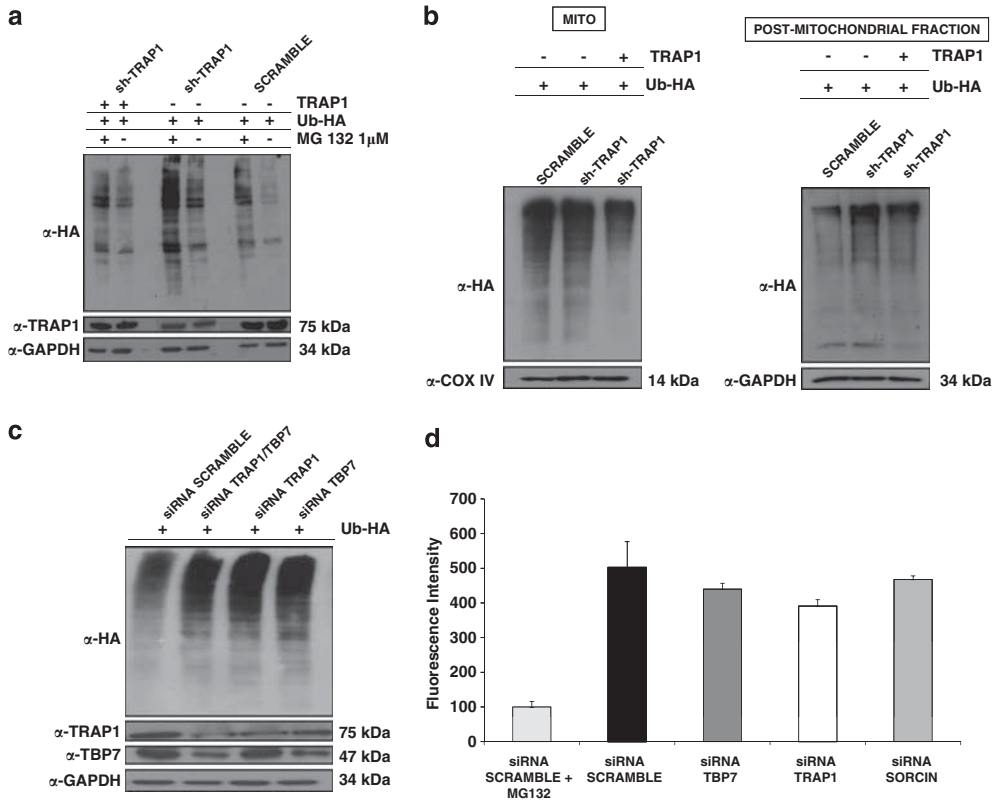
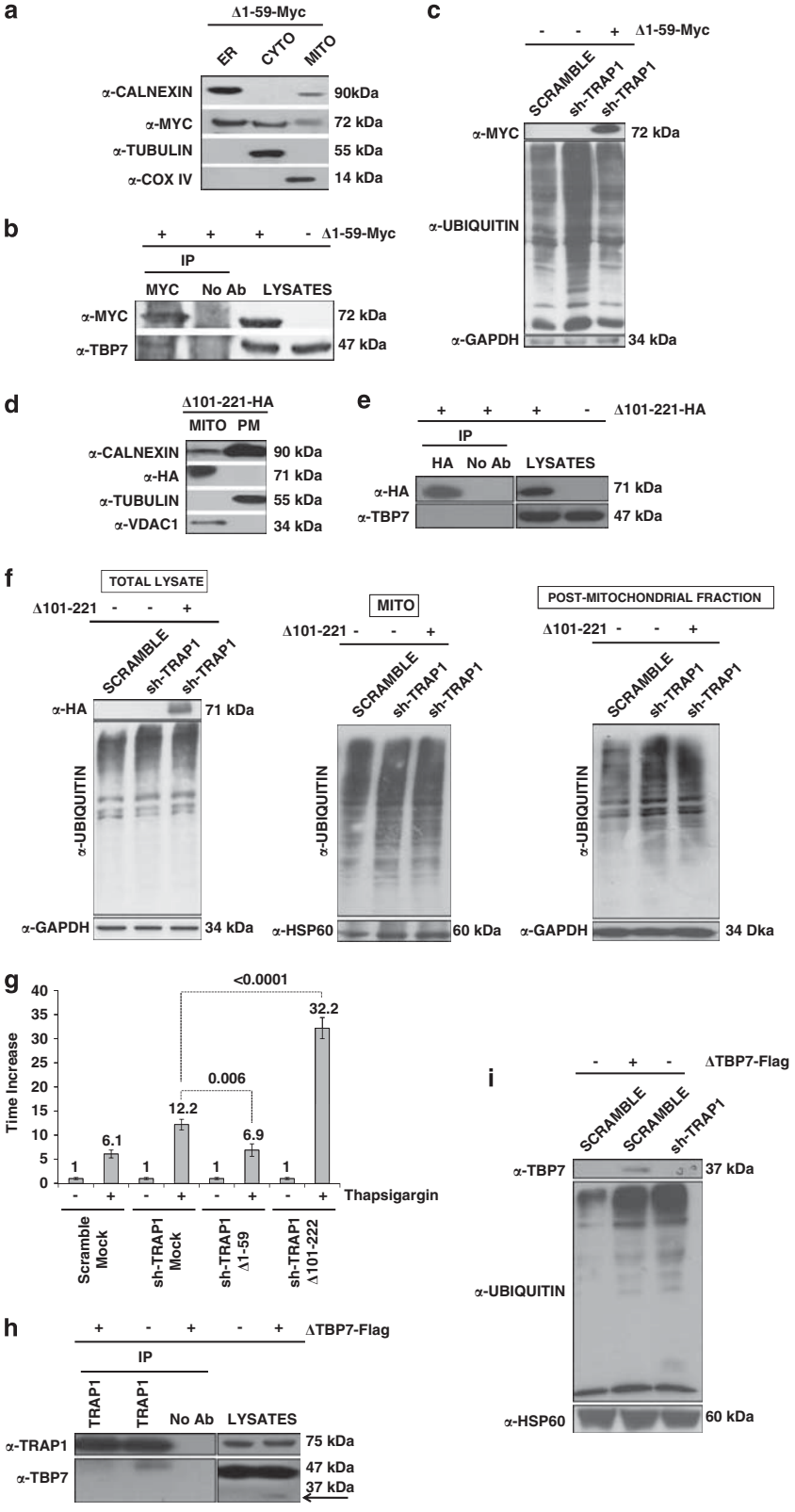


Figure 3 Ub levels in HCT116 cells. (**a**) Total cell lysates from sh-TRAP1 and scrambled HCT116 stable clones were transfected with either an HA-tagged Ub vector (Ub-HA) or with TRAP1 expression vectors; treated with 1 μM MG132 for 24 h; harvested 48 h after transfection; and subjected to immunoblot using rabbit polyclonal anti-HA antibodies. The same filter was re-probed using mouse monoclonal anti-GAPDH antibodies for normalization of cell lysates. Three independent experiments were performed, with similar results. (**b**) Sub-cellular fractionation was obtained from sh-TRAP1 and scrambled HCT116 stable transfectants treated as described in panel **a**. The extracts from the PM fraction (microsomes + CYTO fraction) and mitochondria (MITO, see Materials and Methods) were separated by SDS-PAGE and immunoblotted using a rabbit polyclonal anti-HA antibody to detect Ub levels. The purity of fractions was verified by using mouse monoclonal anti-COX IV or mouse monoclonal anti-GAPDH antibodies. Three independent experiments were performed, with similar results. (**c**) HCT116 cells were co-transfected with a Ub-HA vector and an siRNA negative control (scramble), or with siRNAs specific for TRAP1, TBP7, or both (as indicated) and total cell lysates were harvested after 48 h from transfection. Total lysates were subjected to SDS-PAGE and immunoblotted using rabbit polyclonal anti-HA antibodies to detect total Ub levels. The same filter was re-probed using mouse monoclonal anti-GAPDH antibodies for normalization of cell lysates, and using mouse monoclonal anti-TRAP1 and mouse monoclonal anti-TBP7 antibodies. (**d**) Proteasome activity is not affected by TRAP1 and TBP7 silencing. Total cellular extracts were prepared after 48 h of transfection with specific siRNA for TRAP1, TBP7 or Sorcin, as control, or with an siRNA negative control (scramble), and incubated in the presence of assay buffer and the fluorogenic substrate Suc-LLVY-AMC, as described under Materials and Methods. Samples were analyzed in triplicate using an excitation wavelength of 360 nm and an emission wavelength of 450 nm to detect chymotryptic proteasome activity. The data represent the mean of three independent experiments

Figure 4 The TRAP1/TBP7 interaction in the ER is required for control of protein ubiquitination and ER stress. (**a** and **d**) Sub-cellular localization of Δ1–59-Myc/Δ101–221-HA mutants. HCT116 cells were transfected with the Δ1–59-Myc (**a**) or Δ101–221-HA (**d**) TRAP1 mutants; sub-fractionated into MITO, CYTO and microsomal (ER) fractions (**a**), or MITO and PM (cytosol + microsomes) fractions (**d**), as described under Materials and Methods; separated by SDS-PAGE; and immunoblotted using the indicated antibodies to verify the expression of mutants and the purity of fractions. For details on procedures for generation of the mutants see Materials and Methods. (**b**, **e**) Interaction between Δ1–59-Myc/Δ101–221-HA mutants and TBP7. HCT116 cells were transfected with Δ1–59-Myc (**b**) or Δ101–221-HA (**e**) TRAP1 mutants, harvested and immunoprecipitated using anti-Myc or anti-HA antibodies as described under Materials and Methods. Immunoprecipitates were separated by SDS-PAGE and immunoblotted using the indicated antibodies. No Ab, total cellular extracts incubated with A/G plus agarose beads without antibody; IP, immunoprecipitation with the corresponding antibodies. Three independent experiments were performed, with similar results. (**c**) Ubiquitination levels upon transfection of the Δ1–59-Myc TRAP1 deletion mutant. Total lysates from HCT116 scrambled, sh-TRAP1 stable clones and sh-TRAP1 cells transfected with the Δ1–59-Myc TRAP1 mutant were subjected to immunoblot analysis using mouse monoclonal anti-Ub antibodies to detect total ubiquitination levels and with an anti-GAPDH antibody for normalization of cell lysates. Three independent experiments were performed, with similar results. (**f**) Ubiquitination levels upon transfection of the Δ101–221-HA TRAP1 deletion mutant. HCT116 scramble, sh-TRAP1 and sh-TRAP1 cells transfected with the Δ101–221-HA TRAP1 mutant were sub-fractionated in PM (microsomes + CYTO fraction) and MITO fractions as described under Materials and Methods. Total lysates from the same cells were used as controls (left panel). Protein lysates were subjected to immunoblot analysis using mouse monoclonal anti-Ub antibodies to detect total ubiquitination levels. The purity of fractions was verified using mouse monoclonal anti-GAPDH (left and right panels) and mouse-monoclonal anti-COX IV (middle panel) antibodies. Three independent experiments were performed, with similar results. (**g**) Real-time RT-PCR analysis of BiP/Grp78 mRNA expression in scrambled and sh-TRAP1 HCT116 cells exposed to 1 μM TG for 12 h (same as in Figure 2b) and in sh-TRAP1 HCT116 cells transfected with the Δ1–59-Myc or Δ101–221-HA TRAP1 mutant, as indicated, before treatment with TG. The *P*-values indicate the statistical significance between the BiP/Grp78 levels under the indicated conditions. (**h**) Interaction between TRAP1 and the ΔTBP7-Flag deletion mutant. HCT116 cells were transfected with the ΔTBP7-Flag deletion mutant, harvested and immunoprecipitated using anti-TRAP1 antibodies as described under Materials and Methods. Immunoprecipitates were separated by SDS-PAGE and immunoblotted using the indicated antibodies. No Ab, total cellular extracts incubated with A/G plus agarose beads without antibody; IP, immunoprecipitation with the corresponding antibodies. Three independent experiments were performed, with similar results. The arrow indicates the ΔTBP7-Flag mutant band. (**i**) Ubiquitination levels upon transfection of the ΔTBP7-Flag deletion mutant. Total lysates from HCT116 scrambled cells transfected with ΔTBP7-Flag mutant were subjected to immunoblot analysis using mouse monoclonal anti-Ub antibodies to detect total ubiquitination levels and with mouse monoclonal anti-HSP60 antibodies for normalization of cell lysates. Three independent experiments were performed, with similar results

phenomena occurs in the cytosolic (CYTO) compartment and support our model. Accordingly, the protein levels of p18 Sorcin, another MITO protein, recently identified by our group as a novel MITO Sorcin isoform interacting with TRAP1,[12] decreased upon TRAP1 interference (Figure 5b, arrow). Of note, under the same experimental conditions, no differences were observed in the protein levels of the higher mobility p22 Sorcin isoform, which shares high homology with p18 Sorcin,



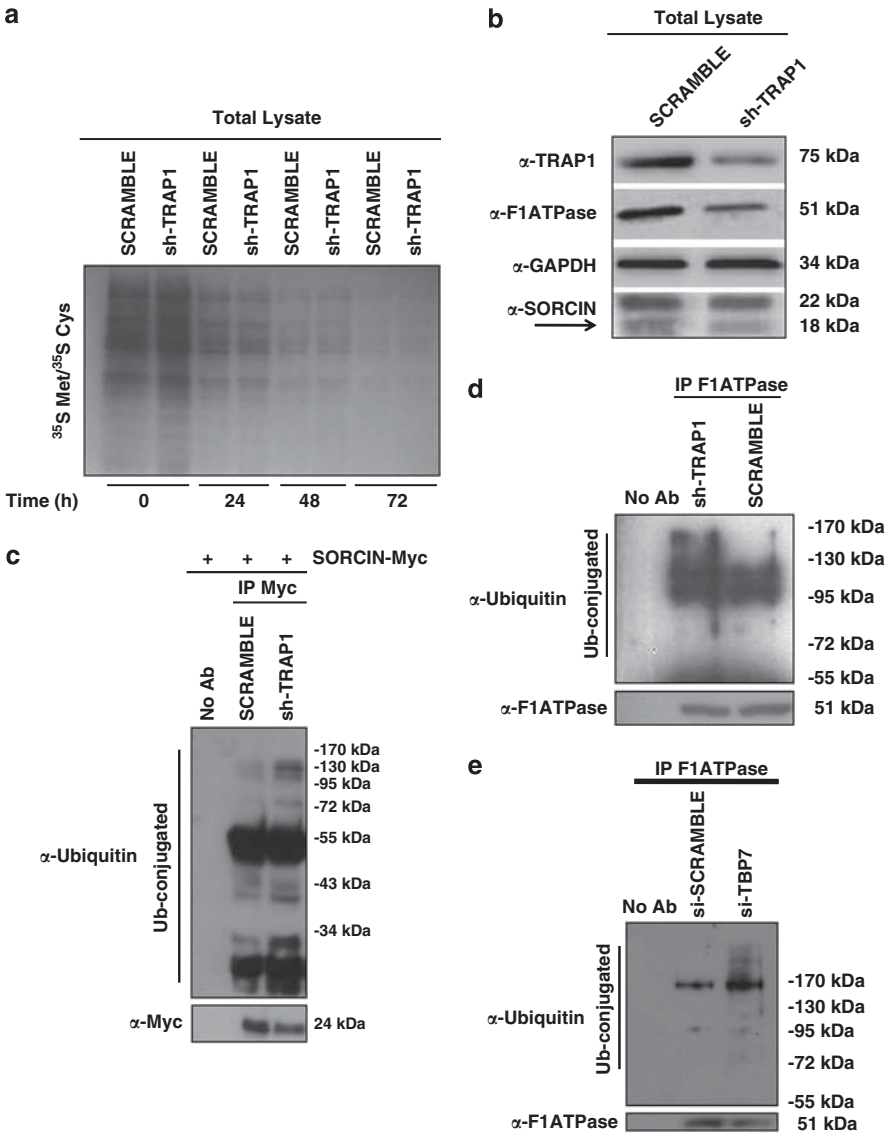


Figure 5 Control of intracellular protein stability and ubiquitination pattern of p18 Sorcin and F1ATPase. (**a**) Pulse–chase analysis of total lysates of scrambled and sh-TRAP1 HCT116 cells. HCT116 cells were incubated in cysteine/methionine-free medium for 1 h followed by incubation in cysteine/methionine-free medium containing 50 μCi/ml ^{35}S-labeled cysteine/methionine (^{35}S Met/^{35}S Cys) for 1 h. After labeling, cells were washed once with culture medium containing 10-fold excess of unlabeled methionine and cysteine (5 mM each) and incubated further in the same medium for the indicated time periods. Cells were collected at the indicated time points and total lysates subjected to SDS-PAGE were analyzed by autoradiography. (**b**) Total lysates of scrambled and sh-TRAP1 HCT116 cells were subjected to SDS-PAGE and immunoblotted using rabbit polyclonal anti-Sorcin, mouse monoclonal anti-TRAP1, goat polyclonal anti-F1ATPase antibodies. The same filter was re-probed using mouse monoclonal anti-GAPDH antibodies for normalization of cell lysates. The arrow indicates the MITO 18-kDa Sorcin isoform band. (**c**) Scrambled and sh-TRAP1 HCT116 clones were transfected with an expression vector containing the cDNA of p18 Sorcin fused to a c-Myc epitope at the C-terminus (Sorcin-Myc) and treated with 1 μM MG132 for 24 h before harvesting. Lysates were immunoprecipitated using mouse monoclonal anti-Myc antibodies and analyzed by immunoblot analysis using mouse monoclonal anti-Ub antibodies. The membrane was re-probed using an anti-Myc antibody to control transfection efficiency. (**d**) Scrambled and sh-TRAP1 HCT116 clones were treated with 1 μM MG132 for 24 h before harvesting, immunoprecipitated with an-anti F1ATPase antibody, subjected to SDS-PAGE and immunoblotted using mouse monoclonal anti-Ub and goat polyclonal anti-F1ATPase antibodies. Three independent experiments were performed with similar results. (**e**) HCT116 cells were co-transfected with a Ub-HA vector and an siRNA negative control (scramble), or with siRNAs specific for TBP7; treated with 1 μM MG132 for 24 h before harvesting; immunoprecipitated using a goat polyclonal anti-F1ATPase antibody; subjected to SDS-PAGE; and immunoblotted using mouse monoclonal anti-Ub and goat polyclonal anti-F1ATPase antibodies. Three independent experiments were performed, with similar results

but is not a MITO protein, neither is a TRAP1 'partner' (Figure 5b).[12] Therefore, we hypothesize that the decreased expressions of F1ATPase and p18 Sorcin in the mitochondria of TRAP1-interferred cells were dependent on increased ubiquitination. To this aim, the respective ubiquitination levels in scrambled- and TRAP1-interfered cells were analyzed. Figures 5c and d show that both proteins are more ubiquitinated in sh-TRAP1 transfectants. Accordingly, increased levels of ubiquitinated F1ATPase were induced upon TBP7 interference (Figure 5e).

Finally, we evaluated whether the TRAP1/TBP7 interaction and the relative effects on protein levels would have an impact

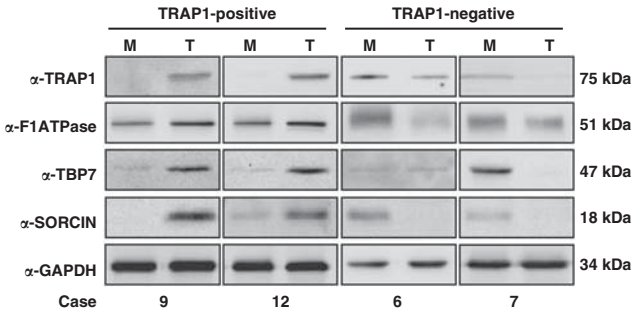


Figure 6 TRAP1, TBP7, F1ATPase and Sorcin expression in human CRCs. Total cell lysates from four human CRCs (T) and the respective non-infiltrated peritumoral mucosas (M) were separated by SDS-PAGE and immunoblotted using rabbit polyclonal anti-Sorcin, mouse monoclonal anti-TRAP1, mouse monoclonal anti-TBP7 and goat polyclonal anti-F1ATPase. The same filter was re-probed using mouse monoclonal anti-GAPDH antibodies for normalization of cell lysates. For details on case numbers, refer to Supplementary Figure 1

in vivo. To this aim, we analyzed our tissue collection of CRCs, previously characterized for TRAP1 and Sorcin expression by immunoblot analysis.[12] We observed that all CRCs overexpressing the 18-kDa Sorcin isoform (11/59 cases) shared the concomitant upregulation of TRAP1. Therefore, we analyzed for TRAP1, Sorcin, F1ATPase and TBP7 expression the 11 Sorcin-overexpressing tumors and, as controls, other 14 CRC specimens. Figure 6 reports the immunoblot analysis of the four proteins in four tumor samples representative of our tumor collection, whereas the expression profile of the four genes in 25 CRCs is reported in Supplementary Table 1. Remarkably, the majority of the p18 Sorcin- and TRAP1-positive tumors showed upregulation of TBP7 (9/11 cases) and F1ATPase (8/11 cases). By contrast, among eight tumors with low expression of TRAP1, all showed low levels of TBP7 and p18 Sorcin and 7/8 showed low expression of F1ATPase. χ^2-Test demonstrated a positive statistical correlation between the expression levels of TRAP1 and those of Sorcin, F1ATPase and TBP7 (Supplementary Table 1).

Discussion

TRAP1 was identified by our group as one of the proteins involved in and important for the homeostasis of osteosarcoma cells adapted to mild oxidative stress.[4] The control of the protein folding environment in sub-cellular organelles, such as mitochondria, is important for adaptive homeostasis and may participate in human diseases, but the regulators of this process are still largely elusive. During the preparation of this manuscript, Altieri *et al.*[10] demonstrated that selective targeting of HSP90 chaperones in mitochondria of human tumor cells triggered compensatory autophagy, and an organelle UPR enhanced tumor cell apoptosis initiated by death receptor ligation, and inhibited tumor growth in mice without detectable toxicity. These results reveal a novel role of HSP90 chaperones in the regulation of the protein folding environment in mitochondria of tumor cells.

Starting from the above observations and in agreement with Altieri's results, here we demonstrate an additional role of TRAP1 in protein quality control, acting on the outside of the ER. This TRAP1 function requires TBP7, a protein of the 19S proteasome regulatory subunit. As demonstrated by mass spectra analysis, this AAA-ATPase is a novel TRAP1-interacting protein whose role is determinant in the quality control of proteins. In fact, the interference of either TRAP1 or TBP7 proteins resulted both in the induction of apoptosis in response to both TG-induced ER stress or oxaliplatin treatment, and in increased intracellular protein ubiquitination, which was selectively rescued by re-addition of TRAP1. Interestingly, TRAP1/TBP7 regulation of cellular ubiquitination is independent of modulation of proteasome function, as an *in vitro* assay of proteasome activity shows that TRAP1/TBP7 interference does not affect proteasome functionality. The regulatory networks that control the protein folding machinery in mitochondria are still largely elusive. In this regard, the demonstration that regulation of protein quality control by TRAP1 is directed toward MITO proteins, expands the currently rather limited list of MITO proteasome targets. It is reasonable to hypothesize that multiple chaperone networks control the misfolding of proteins addressed to different sub-cellular compartments. In this study, an additional 'pre'-screening for proteins directed to mitochondria is characterized: if the protein is highly damaged and not successfully refolded by the chaperone machineries inside or outside the ER, including TRAP1, it will be identified by the regulatory proteasome protein, and targeted for degradation. TRAP1-containing supra-molecular complexes might be present just outside the mitochondria, and in the cellular compartment of the tight ER–mitochondria interface (Hayashi and Su[25] and Figure 1e), where proteasomes have also been identified[26] and ensure that among all the proteins translated in the ER, only undamaged proteins could enter the mitochondria. Very recently, Takemoto *et al.*[11] suggested that the MITO chaperone TRAP1 regulates the UPR in the ER, even though its presence in the ER has not been reported. Therefore, the demonstration that TRAP1 is present also on the external side of the ER is an important achievement strongly supporting its role in protection against stress.

A still unsolved question is the mechanistic link between TRAP1-dependent MITO adaptive response to stress and regulation of gene expression. Interestingly, some analyses have demonstrated the importance of 19S ATPases in the assembly of the transcription machinery.[17] Alternatively, it could be hypothesized that MITO chaperones may regulate gene expression by modulating ER stress. In support of this hypothesis, our results suggest that TRAP1 may be involved in the ER stress response. Furthermore, an interaction between TRAP1 and Sorcin, a calcium-binding protein, was characterized by our group,[12] which might contribute to a regulatory role of the TRAP1 and TRAP1-interacting proteins in ER stress induced by perturbation of calcium homeostasis (Maddalena *et al.*, unpublished data) and protein quality control.

We did not observe any strong differences in MITO protein ubiquitination upon modulation of TRAP1 levels. Quality control of MITO proteins must be monitored by molecular chaperones.[27] Only very small numbers of MITO proteins are currently known to be degraded in a proteasome-dependent manner,[28] so a more focused effort to identify such additional substrates might dramatically expand the list of MITO proteasome targets. Our results demonstrate that expression of p18 Sorcin and F1-ATPase is decreased upon TRAP1 interference as a consequence of their increased

ubiquitination. The identification of TRAP1/TBP7-specific 'substrates' strongly contributes to the complex study of MITO protein quality control. One reason for ubiquitination of MITO proteins may be that when mitochondria-destined proteins are mistargeted or misfolded, they are identified as aberrant and recognized by the Ub–proteasome system (UPS) for removal. This may even support a role of the CYTO UPS in controlling the levels and/or the quality of proteins destined for mitochondria. In addition, the presence of TRAP1 on the outer side of ER and the absence of TBP7 in mitochondria suggest that TRAP1 functions in these latter organelles are not directly linked to ubiquitination control, whereas this control is present in a different compartment and requires interaction with TBP7. In support of this model are results of transfection experiments using newly generated TRAP1 mutants, either able to interact with TBP7 and localized in the ER/cytosol fractions (Δ1–59) or unable to bind to TBP7 but imported into mitochondria (Δ101–221). Indeed, only the MITO import-defective Δ1–59 mutant, and not the MITO Δ101–221 deletion mutant, rescues the strong Ub levels in sh-TRAP1-interfered cells. These findings provide proof of concept that protein quality control depends on the CYTO interaction between TRAP1 and TBP7. Consistent with these results is the observation that again only the Δ1–59 TRAP1 mutant rescues the decreased levels of TRAP1-regulated proteins (Supplementary Figure 3), as well as the levels of BiP/Grp78 mRNA upon TG-induced ER stress (Figure 4g). All these observations are in agreement with still unidentified proteasome members in mitochondria, even though several proteases, ATPases and Ub ligases have been identified.[29] Furthermore, a functional interplay between MITO and proteasome activity has been demonstrated, thus suggesting that both systems are interdependent.[30]

Remarkably, the finding that the proposed TRAP1 network is conserved in CRCs is consistent with our model and provides new insights into the quality control/stability/ubiquitination of proteins in human cancer, a still highly debated issue. Indeed, the proteotoxic stress generated by accumulation of misfolded proteins and the consequent heat-shock response is currently under evaluation as a potential anticancer treatment target, as many tumor cells show constitutive proteotoxic stress and dependence on heat-shock response because of their rapid rates of proliferation and translation.[31] Interestingly, bortezomib, a reversible inhibitor of the 26S proteasome, is at present a valuable option for the first-line treatment of multiple myeloma.[32] Thus, characterization of TRAP1, a chaperone upregulated in about 60% of human CRCs,[5] as a protein involved in quality control and in protection against apoptosis in cancer cells provides a strong rationale for considering this network as a novel molecular target for treatment of human CRC.

In summary, a new crosstalk between ER and mitochondria is suggested and summarized in the working model, as shown in Figure 7. Our study demonstrates for the first time that

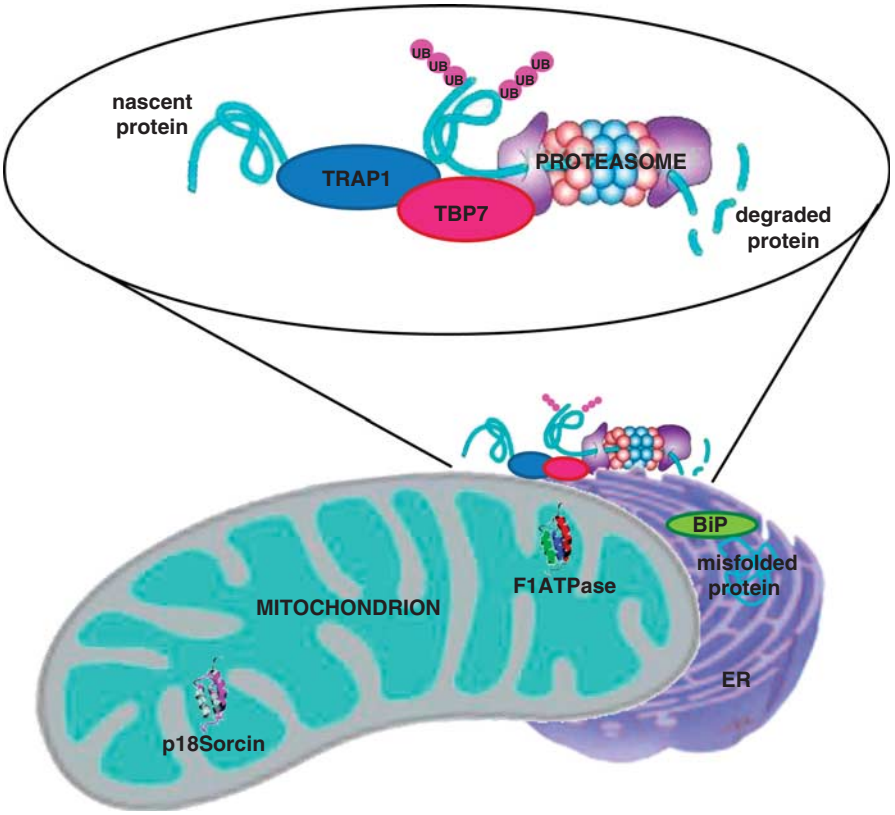


Figure 7 Crosstalk between ER and mitochondria, and MITO protein quality control. TRAP1 forms a supra-molecular complex with TBP7 on the outside of the ER, in a cellular compartment of tight ER–mitochondria contact sites, where proteasomes are also present. This TRAP1/TBP7 complex is involved in the control of protein stability and intracellular protein ubiquitination of mitochondria-destined proteins. These two proteins, each with independent but related functions, help to judge whether a protein can be repaired and reach the final MITO destination or, if damaged, needs to be degraded through the Ub–proteasome system

TRAP1 is also present in the ER of cancer cells where it is involved in the quality control and intracellular protein ubiquitination of mitochondria-destined proteins, through direct interaction (as demonstrated by the FRET analysis shown in Figure 1d) with TBP7, one of the proteins present in the regulatory proteasome subunit. Thus, a 'customs office' could be hypothesized at the ER/mitochondria interface, with TRAP1 and TBP7 being the officers at this important checkpoint. These two officers, each with independent but related functions, help to judge whether a protein can be repaired and reach its final MITO destination or, if the damage is too severe, it needs to be degraded.

Materials and Methods

Cell culture, plasmid generation and transfection procedures. HCT116 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) under standard conditions. Full-length TRAP1 and Sorcin expression vectors were obtained as described previously.[12]

Mutant Δ1–59-Myc was generated by using the following primers: Δ1–59-myc, forward: 5′-*ATTAGAATTCATGAGCACGCAGACCGCCGAGG*-3′, reverse: 3′-*ATTACTCGAGGTGTCGCTCCAGGGCCTTGA*-5′. PCR-amplified fragments were gel-purified and cloned in-frame into the pcDNA 3.1 plasmid (Invitrogen, San Giuliano Milanese, Italy) at the *Eco*RI and *Xho*I restriction sites.

Mutant Δ101–221-HA was generated by using the following primers: TRAP1-HA, forward: 5′-atta*GCGGCCGCGCAGCCAACATGGCGCGCGAGCCTGCGGG*-3′, reverse: 5′-atta*TCTAGATTAAGCGTAATCTGGAACATCATATGGGTATCAGT GTCGCTCCAGGGCCTTGA*-3′; and Δ101–221-HA, forward: 5′-atta*CCGCGGT CGGCAGCCCCGGGGAGCCT*-3′, reverse: 5′-atta*CCGCGGAAACACCTCTTTTTC TGAGT*-3′. The PCR products obtained with the primers TRAP1-HA forward and Δ101–221-HA reverse were cloned in the pRc-CMV vector (Invitrogen); the PCR product obtained with the primers ΔATPase-HA forward and TRAP1-HA reverse was subcloned in the same plasmid. All clones were sequenced to confirm identity and PCR fidelity. The plasmid pCMV5L/S6 (TBP7-HA) was a gift from Dr Simon Dawson (University of Nottingham).

Mutant ΔTBP7-FLAG was generated by excising a fragment from the full-length TBP7 expression vector by using *Eco*RI and *Bam*HI restriction endonucleases. The fragment was gel-purified and cloned into the corresponding sites of the expression vector p3x-FLAG.

Transient transfection of DNA plasmids was performed with the Polyfect Transfection reagent (Qiagen, Milan, Italy). siRNAs of TRAP1 and TBP7 were purchased from Qiagen (cat. no. S100301469 for TBP7 and cat. no. SI00115150 for TRAP1). For knockdown experiments, siRNAs were diluted to a final concentration of 20 nmol/l and transfected according to the manufacturer's protocol. For control experiments, cells were transfected with a similar amount of scrambled siRNA (Qiagen; cat. no. SI03650318). Transient transfections of siRNAs were performed by using the HiPerFect Transfection Reagent (Qiagen). TRAP1-stable interference was achieved by transfecting HCT116 cells with TRAP1 (TGCTGTTGACAGT GAGCGACCCGGTCCCTGTACTCAGAAATAGTGAAGCCACAGATGTATTTCTG AGTACAGGGACCGGGCTGCCTACTGCCTCGGA) or scrambled (sequence containing no homology to known mammalian genes) shRNAs (Open Biosystems, Huntsville, AL, USA).

Cell extracts, purification and treatments. Total cell lysates were obtained by homogenization of cell pellets and tumor specimens in cold lysis buffer (20 mM Tris (pH 7.5), containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% (v/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2% (w/v) deoxycholate) for 1 min at 4 °C and further sonication for 30 s at 4 °C. For ER stress induction, cells were treated overnight with 1 μM TG (Sigma-Aldrich, Milan, Italy) before harvesting.

Mitochondria and ER were purified by using the Qproteome Mitochondria Isolation kit (Qiagen) according to the manufacturer's protocol and as described elsewhere.[12] Briefly, HCT116 cells were washed and suspended in lysis buffer, which selectively disrupts the plasma membrane without solubilizing it, resulting in the isolation of CYTO proteins. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria and ER, remained intact and were pelleted by centrifugation. The resulting pellet was resuspended in disruption buffer, repeatedly passed through a narrow-gauge needle (to ensure complete cell disruption) and centrifuged to pellet nuclei, cell debris and unbroken cells. The supernatant (containing mitochondria and the microsomal fraction) was re-centrifuged to pellet mitochondria. The resulting supernatant (microsomal fraction) was treated with proteinase-K for 20 min on ice ± NP-40 (Igepal; Sigma-Aldrich) according to Hassink *et al*.[33] or with 0.1 M Na_2CO_3 (pH 11.3) for 30 min to remove peripheral ER membrane proteins.[34]

WB analysis and antibodies. Equal amounts of protein from cell lysates and tumor specimens were subjected to 10% (v/v) SDS-PAGE and transferred to a PVDF membrane (Millipore, Temecula, CA, USA). The membrane was blocked with 5% (w/v) skimmed milk and incubated with the primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (GE Healthcare, Waukesha, WI, USA). The following antibodies from Santa Cruz Biotechnology (Segrate, Italy) were used for WB analysis and immunoprecipitation: anti-TRAP1 (sc-13557), anti-Sorcin (sc-100859), anti-TBP7 (sc-166003), anti-cMyc (sc-40), anti-CypD (sc-82570), anti-VDAC1 (sc-8830), anti-HSP60 (sc-1052), anti-Ub (sc-8017), anti-COX4 (sc-58348), anti-F1ATPase (ATP5B subunit; sc-58619), anti-tubulin (sc-8035), anti-HA (sc-805) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-69778). A rabbit polyclonal anti-calnexin antibody (BD Biosciences, Milan, Italy) was also used.

RNA extraction and semi-quantitative and real-time RT-PCR analysis. Total RNA from cell pellets and tumor specimens was extracted by using the TRIzol Reagent (Invitrogen). For first-strand synthesis of cDNA, 3 μg of RNA were used in a 20-μl reaction mixture by using a cDNA Superscript II (Invitrogen). For real-time PCR analysis, 1 μl of cDNA sample was amplified by using the Platinum SYBR Green qPCR Supermix UDG (Invitrogen) in an iCycler iQ Real-Time Detection System (Bio-Rad Laboratories GmbH, Segrate, Italy). The following primers were used: BiP/Grp78, forward: 5′-*CGTGGATGACCCGTCTGTG*-3′, reverse: 5′-*cTGCCGTAGGCTCGTTGATG*-3′ (PCR product 308 bp); and GAPDH, forward: 5′-*CAAGGCTGAGAACGGGAA*-3′, reverse: 5′-*GCATCGCCC CACTTGATTTT*-3′ (PCR product 90 bp). Primers were designed to be intron-spanning. The reaction conditions were 50 °C for 2 min; 95 °C for 2 min; followed by 45 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. GAPDH was chosen as an internal control.

For semi-quantitative RT-PCR, the RNA obtained by scrambled and sh-TRAP1 HCT116 cells was retro-transcribed and amplified using specific primers for BiP/Grp78 and GAPDH by using the Superscript III-One STEP kit (Invitrogen), according to the manufacturer's instructions. The following primers were used to amplify the corresponding transcripts: GAPDH, forward: 5′-*GAAGGTGAAGGTCG GAGTC*-3′, reverse 5′-*GAAGATGGTGATGGGATTTC*-3′; and BiP/Grp78, forward: 5′-*CTGGGTACATTTGATCTGACTGG*-3′, reverse: 5′-*GCATCCTGGTGGCTTTCC AGCCATTC*-3′. The primers for BiP/Grp78 were a gift from Professor P Remondelli (University of Salerno, Italy).

Apoptosis assay. HCT116 cells were subjected to downregulation of TRAP1 and TBP7 expression by siRNA transfection. Apoptosis was evaluated by cytofluorimetric analysis of Annexin-V and 7-amino-actinomycin-D (7-AAD)-positive cells using the fluorescein isothiocyanate (FITC)–Annexin-V/7-AAD kit (Beckman Coulter, Milan, Italy). Stained cells were analyzed by using the 'EPICS XL' Flow Cytometer (Beckman Coulter). Ten thousand events were collected per sample. Positive staining for Annexin-V as well as double staining for Annexin-V and 7-AAD were interpreted as signs of, respectively, early and late phases of apoptosis.[35] Experiments were performed three times using three replicates for each experimental condition.

Immunofluorescence, confocal microscopy and EM analysis. HCT116 cells were fixed with 0.1 M phosphate buffer containing 4% (w/v) paraformaldehyde for 15 min, then blocked and permeabilized with 5% (w/v) BSA, 0.1% (v/v) Triton X-100, 10% (v/v) FBS in PBS for 20 min at RT before staining with primary antibodies (for TRAP1, CALNEXIN and TBP7) and the corresponding secondary TEXAS RED/FITC-conjugated antibodies. Immunofluorescence was analyzed by confocal laser-scanning microscopy using Zeiss 510 LSM (Carl Zeiss Microimaging, Göttingen, Germany), equipped with an Argon ionic laser (Carl Zeiss Microimaging) whose wavelength was set up to 488 nm; a He–Ne laser whose wavelength was set up to 546 nm; and an oil-immersion × 63/1.4 f objective. For immuno-EM analysis, cells were fixed with a mixture of 4% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde; labeled with a monoclonal antibody against HA by

using the gold-enhance protocol; embedded in Epon-812; and cut as described previously.[36] EM images were acquired from thin sections by using an FEI Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (FEI, Eindhoven, The Netherlands). Thin sections were also used for quantification of gold particles residing within mitochondria by using the AnalySIS software (Soft Imaging Systems GmbH, Munster, Germany).

FRET experiments. FRET was measured by using the acceptor photo-bleaching technique,[20] where, upon irreversible photo-bleaching, the donor fluorescence increase was recorded. Cells on coverslips were fixed; immunostained with specific anti-TBP7 and anti-TRAP1 antibodies, and secondary antibodies conjugated, respectively, to Cy3 and Cy5; and mounted in PBS/glycerol (1 : 1). Images were collected using a laser-scanning confocal microscope (Zeiss LSM 510 Meta) equipped with a planapo $\times 63$ oil-immersion (NA 1.4) objective lens. Laser lines at 543 and 633 nm were used to excite, respectively, the fluorophores Cy3 and Cy5. For Cy5 bleaching, the 633-nm He–Ne laser light with 100% output power was used and pinhole diameters were set to have 1.0-μm optical slices.

FRET measurements were performed by using the LSM software (LSM Zeiss, Göttingen, Germany) after photo-bleaching of a selected squared ROI of 6 μm^2. We calculated the FRET efficiency on the basis of the following equation: $E =$ (Fluorescence intensity of Cy3 after bleaching $-$ Fluorescence intensity of Cy3 before bleaching)/Fluorescence intensity of Cy3 after bleaching.[20]

As control we measured FRET on cells expressing TBP7 alone labeled with Cy3 in order to ensure that photo-bleaching *per se* does not affect the fluorescence of the donor and that photo-conversion does not occur during the photo-bleaching analysis. We calculated the background raised by the photo-bleaching *per se* by bleaching Cy5 in cells negative for this fluorophore. The background value was subtracted from all samples.

Pulse–chase assay. Pulse–chase analysis was performed as described elsewhere.[37] In brief, HCT116 cells were incubated in cysteine/methionine-free medium (Sigma-Aldrich) for 1 h followed by incubation in cysteine/methionine-free medium containing 50 μCi/ml ^{35}S-labeled cysteine/methionine (GE Healthcare) for 1 h. After labeling, cells were washed once with culture medium containing 10-fold excess of unlabeled methionine and cysteine (5 mM each) and incubated further in the same medium for the indicated time periods. Cells were collected at the indicated time points and separated by 10% SDS-PAGE. Proteins were transferred onto a PVDF membrane (Millipore) and analyzed by autoradiography. The same filters were then probed by WB analysis.

Patients. Between May 2008 and May 2011, specimens from both tumor and normal, non-infiltrated peri-tumoral mucosa were obtained from 59 patients with CRC during surgical removal of the neoplasm. Samples were divided into 125-mm^3 pieces; one specimen was fixed in formalin and used for the histopathological diagnosis, whereas the others were immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}$C for immunoblot analysis. Samples were analyzed within 30 days after collection and were thawed only once. Express written informed consent to use biological specimens for investigational procedures was obtained from all patients.

Statistical analysis. χ^2-Test was used to establish statistical correlation between the expression levels of TRAP1 and those of Sorcin, F1ATPase and TBP7 in human CRCs. Statistically significant values ($P < 0.05$) are reported under section Results.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)