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Review Article

New insights into TRAP1 pathway

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Abstract: Tumor Necrosis Factor Receptor-Associated Protein 1 (TRAP1) is a mitochondrial heat shock protein involved in the protection from DNA damages and apoptosis induced by oxidants and several other stress conditions. Despite the well-characterized role in the regulation of mitochondrial integrity, through the interaction with cyclophilin D, a mitochondrial permeability transition pore regulator, several recent studies contributed to draw a more complex "picture" of the TRAP1 pathway: most of these updated functions arise from the identification of novel specific TRAP1 "client" proteins and from the recent discovery of multiple subcellular localizations/functions for this chaperone. This review briefly highlights some general features of TRAP1, and among others its role in cytoprotection, summarizing many different functions, which contribute to its protective role upon several stress inducers. Of note, particular emphasis is given to the recent findings on the regulation of Endoplasmic Reticulum stress and protein quality control by TRAP1, as well as to its role in regulating calcium homeostasis throughout its client protein Sorcin. Starting from the above observations a preliminary "TRAP1 signature" is provided and a new intriguing and interesting field to explore is discussed. Several questions are still open given the complexity of such mechanisms. However, by translating these recent insights at the molecular and cellular levels into personalized individual anticancer treatments, designing novel strategies based on the simultaneous inhibition of multiple tumor-specific pathways, and contemplating subcellular-targeted approaches aimed at reverting drug resistance and improving antitumor activity the struggle to combat cancer become more successful and closer.

Keywords: TRAP1, HSP90, apoptosis, mitochondria, endoplasmic reticulum, stress, cancer, drug resistance

TRAP1: a novel member of the HSP90 family with distinct functions and strong personality

TRAP1 (TNF receptor-associated protein 1) is a member of the HSP90 family, which includes highly conserved molecular chaperones involved in signal transduction, protein folding, protein degradation, and morphologic evolution.

Public databanks report the human *trap1* gene spanning a distance of 59.561 bases on chromosome 16 (single locus on chromosome 16p13 in humans) and containing 18 exons, with 14 potential alternate transcripts. The main transcript is 2.263 bp long and encodes a protein of 704 amino acids of a molecular weight of 80.110 Da, therefore TRAP1 is also indicated as HSP75. This protein contains three major domains: a 59 amino acids N-terminal Mitochondria-Targeting Sequence (MTS), an ATPase domain containing four ATP-binding sites and a C-terminal HSP90-like domain. The protein potentially undergoes several post-translational modifications, including acetylation (N6-acetyllysine at amino acids 87, 332, 382, 424, 466) and phosphorylation (phosphotyrosine 366, phosphoserine 401, phosphothreonine 494).

TRAP1 was firstly identified through a yeast-based two hybrid screening, as a novel protein binding the intracellular domain of the Tumor Necrosis Factor Receptor (TNFR) 1, and thus named TRAP1 (TNF Receptor-Associated Protein 1) [1]. At the same time, another screening identified a novel member of the HSP90 family for its ability to bind Retinoblastoma protein during mitosis and after heat shock [2]. These two proteins later resulted to be identical. This firstly identified TRAP1 mRNA was found ex-

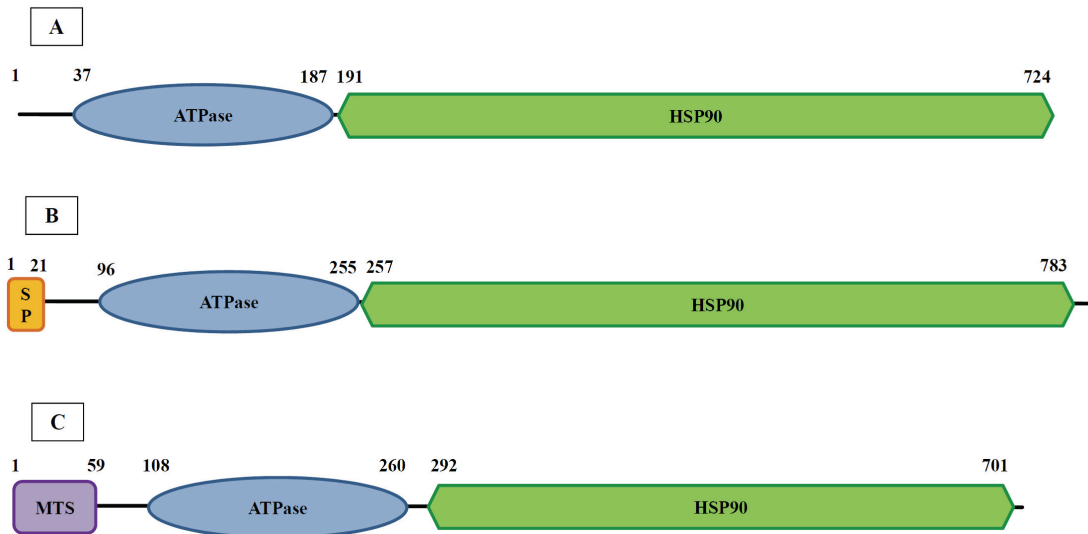

Figure 1. Schematic representation of domain structure similarity among HSP90 protein family members. (A) The two major cytosolic isoforms of HSP90 (HSP90AA and HSP90AB) have an ATPase domain that is necessary for its HSP chaperoning function and a C-terminal domain which terminates with the functional motif MEEVD. (B) The ER-resident HSP90B/Grp94 has a N-terminal signal peptide (SP), which is responsible for its localization in the ER, a middle ATPase domain and a functional C-terminal chaperone domain, which terminates with a motif (KDEL) that prevents secretion from ER. (C) HSP75/TRAP1 has been for a long time considered the mitochondrial paralogue of HSP90. It presents a N-terminal Mitochondria-Targeting Sequence (MTS), an ATPase domain and a C-terminal chaperone domain. Numbers of amino-acid residues are indicated above each domain.

pressed at different levels in skeletal muscle, liver, heart, brain, kidney, pancreas, lung and placenta [1]. Although these initial reports attributed cytoplasmic/nuclear localization to TRAP1, it was afterwards considered mostly or exclusively mitochondrial, mostly in the matrix [3, 4]. Nevertheless, TRAP1 was found in a number of non-mitochondrial locations, including pancreatic zymogen granules, insulin secretory granules, cardiac sarcomeres, and nuclei of pancreatic and heart cells, and on the cell surface of blood vessel endothelial cells [4]. In later studies, TRAP1 was found abundantly expressed in various tumor cell types, conversely present at very low levels in mitochondria isolated from normal mouse tissues, while absent in the cytosol of tumor or normal cells [5]. By immunohistochemistry, TRAP1 was found to be intensely expressed in pancreas, breast, colon and lung adenocarcinomas, whereas normal matched epithelia contained very low levels of this chaperone. Analogously HSP90, mainly a cytoplasmic HSP, was found in the intermembrane space and matrix of tumor mitochondria [5]. Since the beginning, many similarities were found between TRAP1 and other HSP90 protein family members which share 34% sequence identity and an overall homology of about 60% [1] (**Figure 1**).

TRAP1 activity is affected by the HSP90 inhibitors, geldanamycin and radicicol [3], forming with them a tight homodimer [6]. However, it was immediately clear that TRAP1 has different functional properties: it is unable to bind the two typical HSP90 co-chaperones p23 and Hop, it has no effect on the HSP90-dependent reconstitution of hormone binding to the progesterone receptor *in vitro*, nor could it substitute HSP90 in promoting the maturation of the receptor to its hormone binding state [3]. Noteworthy, its ATPase cycle exhibits differences compared to the known HSP90 ATPase cycles [6]. However, this ATPase activity is similar to the *E. coli* mismatch repair enzyme MutL, an ATPase belonging to a superfamily that includes the DNA topoisomerase II, whose ATPase activity is stimulated by DNA and likely acts as a switch to coordinate DNA mismatch repair [7].

Nowadays, more than 10 years after the original features attributed to TRAP1, poorly understood and not deeply investigated at those times, a novel view of the TRAP1 pathway is arising. Al-

though previous available evidences already suggested an independent role of TRAP1, not shared by HSP90, in terms of subcellular localization and consequent related selective functions, either inside or outside mitochondria, only recently new insights into the TRAP1 pathway have been provided. This has been made possible by several recent studies which contributed to draw a more complex “picture” of TRAP1 pathway, based both on the identification of specific TRAP1 client proteins and dependent on its mitochondrial/extramitochondrial localization.

New TRAP1 client proteins

TRAP1/TBP7 in protein quality control

To investigate for new TRAP1 functions, among other putative partners identified by LC-MS/MS analysis by our group use of mass spectrometry, our attention focused on a component of the 19S proteasome regulatory subunit, the AAA-ATPase TBP7 (S6-ATPase 4/Rpt3). The identification of novel pathways for protein quality control, most likely altered in tumor cells, in which TRAP1 could be involved, moved our interest towards a further characterization of this interaction.

In our view, one of the most striking findings of this study was the observation that TRAP1 and TBP7 do not interact in mitochondria, but they both are located in the endoplasmic reticulum (ER), specifically on the outer side of this compartment. Indeed, these two proteins directly interact in the ER, as demonstrated by coimmunoprecipitation and microscopy experiments and are close enough to FRET, as demonstrated by FRET analysis of ER fractions [8]. This is the first demonstration of an extramitochondrial function of TRAP1, whereas previous studies revealed non-mitochondrial subcellular localization without identifying a specific role. Given the ER localization of TRAP1 and TBP7, it became even more important to assess the involvement of these two proteins in ER homeostasis. To this aim, the observation that colon carcinoma cells, in which the expression of TRAP1 was stably knocked down by Sh-RNAs (Sh-TRAP1 stable clones), are characterized by an increased expression of Grp78/BiP, the major ER chaperone and marker of ER stress conditions, upon stress induction, sounds very appropriate; interestingly, a rescue of this phenotype, which yielded a lower Grp78/BiP expression, was observed upon the transfection of TRAP1 expression vector. Since molecular chaperones are often involved in regulation of protein ubiquitination, and given the confirmed interaction of TRAP1 with the regulatory proteasome subunit TBP7, the test of a protein quality control function exerted by these two chaperones through the regulation of cellular ubiquitination became unavoidable. Indeed, the analysis of cellular lysates from TRAP1 stably interfered clones (sh-RNAs) showed higher amounts of ubiquitinated proteins than control cells, and this phenotype was reverted by re-addition of TRAP1 expression vector. The same effect was observed in TBP7 interfered cells [8].

A driving force in the identification of new specific TRAP1 functions was the generation of a collection of TRAP1 mutants shown in **Figure 2**. One of the most important findings that emerged from the design of these “domain-deletion” mutants was the observation that the removal of the first 59 amino acids from the amino terminus of TRAP1 protein, containing a putative MTS as already suggested by Felts et al [3], is sufficient to yield a TRAP1 protein unable to enter mitochondria [8]. Other advantages of having the use of these molecular tools was the assessment of compartment-specific TRAP1 functions: in fact, only the above-described cytosolic deletion mutant of TRAP1, still able to bind TBP7, was able to rescue the high-ubiquitin phenotype in sh-TRAP1 cells. By contrast, the generation of another mutant in which one of the putative TBP7 binding sites was deleted, yielding a TRAP1 protein thus unable to bind the proteasome protein but still able to enter mitochondria, allowed to demonstrate that the direct interaction between TRAP1 and TBP7 is necessary to control cellular ubiquitination [8].

Which proteins are screened for quality control by this ER-located custom office made by TRAP1 and TBP7? Given the observation that TRAP1 and TBP7 do not affect overall protein stability, we checked for the levels of specific TRAP1 mitochondrial substrates: the 18kDa Sorcin isoform (whose identification and features are widely described below in this review) and the β-subunit of F1ATPase. This analysis allowed us to conclude that the levels of both proteins are decreased, due to their elevated ubiquitination levels when TRAP1, TBP7 or both are silenced. Remarkably, as better characterized in *in vivo*

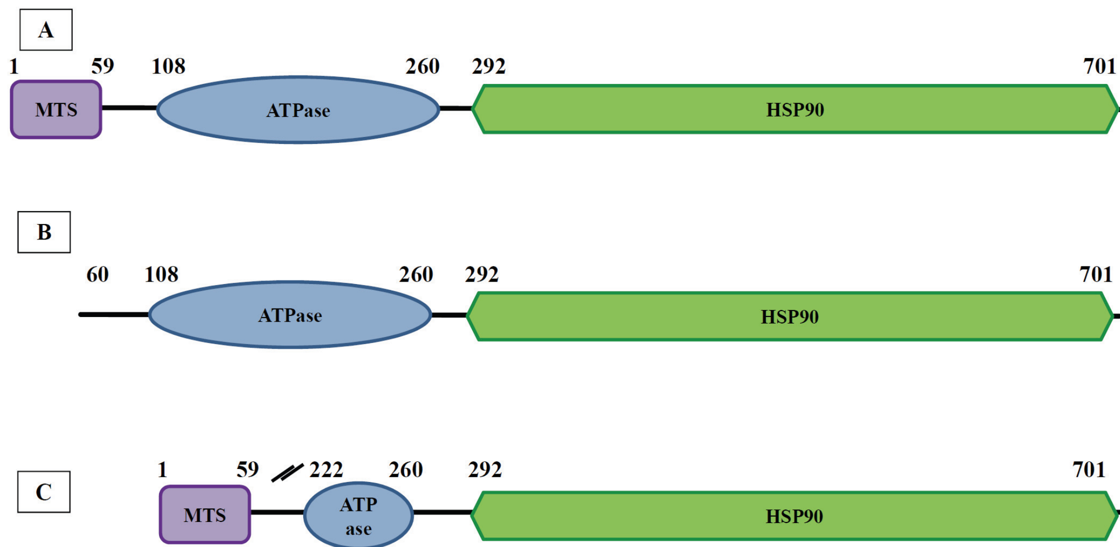

Figure 2. Schematic representation of TRAP1 mutants. (A) Full-length TRAP1 has a N-terminal 59 amino-acid long Mitochondria Targeting Sequence (MTS), an ATPase domain and a C-terminal chaperone domain. (B) Δ1-59 TRAP1 is a protein of 645 amino-acids including amino-acids 60-704 of full-length TRAP1. (C) Δ101-221 TRAP1 mutant lacks of part of the ATPase domain, but it still has the transit peptide which is able to target the protein to mitochondria. Numbers of amino-acid residues are indicated above each domain.

studies, in pathological conditions in which TRAP1 levels are strongly increased, a concomitant upregulation of Sorcin and F1ATPase was observed in human colorectal tumors [8]. These data strongly support the hypothesis that TRAP1 and TBP7 exert a protein quality control at the ER-mitochondria interface, monitoring specific mitochondria-destined proteins: when these neo-synthesized proteins are damaged, they are not imported into mitochondria, but are sequestered by TRAP1 to be repaired; whereas, if this attempt fails, the substrates are ubiquitinated, recognized by the regulatory subunits of proteasome to which TBP7 belongs, and delivered to the proteolytic core for degradation (**Figure 3**).

The elimination of misfolded proteins represents an important mechanism to maintain cell viability. Interestingly, disorders of protein folding and degradation are emerging as fundamental mechanisms in the pathogenesis of many diseases, in particular tumor progression [9]. In this context, the identification of a regulatory proteasome subunit particle as TRAP1-interacting protein appears a strong prerequisite to further analyze the contribution of this novel pathway to cancer biology. 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 clearance of misfolded proteins, the characterization of this interaction in the ER could elucidate the molecular pathways that link Protein Quality Control, Unfolded Protein Response (UPR) and cell survival.

TRAP1/Sorcin in the regulation of ER stress and calcium homeostasis

Another new TRAP1-interacting protein investigated by our group is Sorcin, a Ca^{2+}-binding protein widely distributed among mammalian tissues, such as skeletal and cardiac muscle, kidney, and brain [10]. Sorcin behaves as a Ca^{2+}-sensor, being involved in the regulation of Ca^{2+} homeostasis and excitation-contraction coupling in the heart [11, 12]. These Sorcin functions are mainly dependent on its ability to modulate the activity of the ryanodine receptor RyRs, the Na^{+}/Ca^{2+} exchanger NCX and the voltage-dependent L-type Ca^{2+} channel [10, 13, 14], thus regulating Ca^{2+} compartmentalization within the cell.

Several lines of evidences suggest that Sorcin is up-regulated in human malignancies where it is involved in cytoprotective functions and drug resistance. In this view, the choice of selecting this TRAP1 interactor is relevant for further elu-

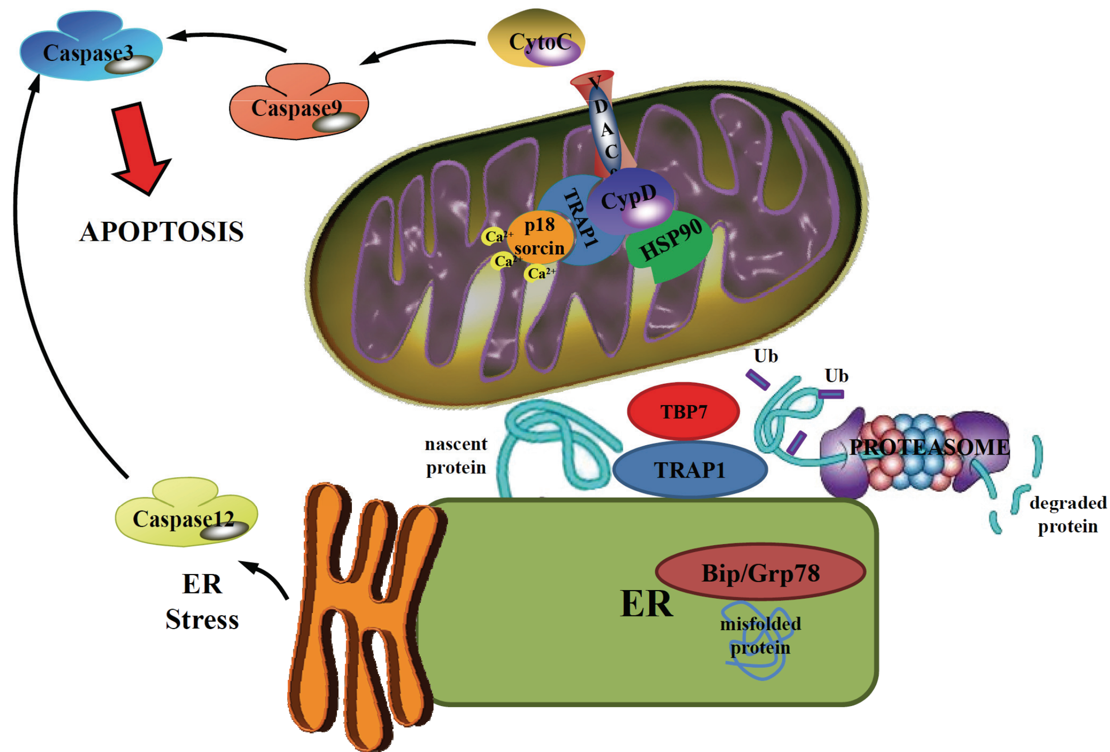

Figure 3. The regulatory function of TRAP1 in the crosstalk between mitochondrial and endoplasmic reticulum. TRAP1 is characterized by different subcellular localizations/functions. The majority of the protein is localized in mitochondria where it is part of a supramolecular complex with HSP90 and cyclophilin D, responsible for regulating mitochondria integrity and apoptotic cell death by controlling the mitochondrial permeability transition pore, the cytochrome c release and the caspase cascade. The 18kDa sorcin isoform is part of this complex, being a TRAP1 client protein involved in the regulation of mitochondrial Ca^{2+} homeostasis. TRAP1 is also localized on the outside of the endoplasmic reticulum, at the interface with mitochondria, where proteasomes are also present. In this context, TRAP1 is in a multiprotein complex with the 19S proteasome regulatory subunit TBP7, and is responsible for the quality control and intracellular protein ubiquitination of mitochondria-destined proteins. This TRAP1/TBP7 regulatory function is relevant in judging whether a protein can be repaired and reach the final mitochondrial destination or, if damaged, needs to be degraded through the ubiquitin-*proteasome system*.

cidating TRAP1 role in these processes. Indeed, Sorcin was originally purified from a vincristine-resistant HOB1 lymphoma cell line [15], while Sorcin overexpression has also been associated with resistance to vincristine in gastric cancer cells [16], gemcitabine in non-small cell lung cancer (NSCLC) [17] and CHOP regimen in diffuse large B-cell lymphoma [18]. Several studies suggest a correlation between the expression of Sorcin and the multidrug resistance (MDR)1/P-glycoprotein: it has been demonstrated that Sorcin knock-down induces the up-regulation of MDR1 in HeLa cells [19]; by contrast, a direct correlation between Sorcin and MDR1 expression and a role of Sorcin in inducing a multidrug resistant phenotype has been observed in human leukemia cells and in gastric carcinoma cells [20, 21]. Finally, reports evaluating changes in gene expression profile in oral squamous cell carcinoma, NSCLC and acute myeloid leukemia showed that Sorcin may be responsible for drug resistance and poor prognosis [17, 22, 23].

The potential involvement of Sorcin in cytoprotective functions is consistent with the evidence that the 18kDa Sorcin isoform, whose expression is restricted to mitochondria, is a TRAP1 client protein [8, 24]. Indeed, the Ca^{2+}-dependent interaction between TRAP1 and 18kDa Sorcin is required for Sorcin antiapoptotic activity, its mitochondrial localization/stability and seems crucial for TRAP1 regulation of cell survival [24]. Remarkably, as above un-

derlined, TRAP1 regulates, together with TBP7, the ubiquitination of the 18kDa Sorcin isoform, since Sorcin expression levels are decreased upon TRAP1 interference as a consequence of increased ubiquitination [8]. The 22kDa isoform of Sorcin, which is the most abundant cellular isoform and is not a TRAP1 interacting protein [24], is up-regulated in about 50% of human colorectal carcinomas. Indeed, 22kDa Sorcin is an ER-resident protein up-regulated in conditions of ER stress and responsible for enhancing the accumulation of Ca^{2+} in the ER, thus preventing ER stress. Its silencing favors the activation of caspase-12 and Grp78/BiP in response to stress and induces apoptosis through the mitochondrial pathway [25].

Based on these evidences, it is reasonable to hypothesize that both Sorcin isoforms are involved in regulating Ca^{2+} homeostasis in separate cell compartments and that this function is relevant for their antiapoptotic activities in tumor cells. While the 22kDa Sorcin isoform is one of the several ER stress proteins involved in the control of Ca^{2+} levels in the ER, preventing ER stress and the subsequent apoptotic events, it is likely that 18kDa Sorcin is critical in controlling Ca^{2+} homeostasis in mitochondria, thus contributing to the regulation of the opening of the mitochondrial transition pore (MTP). This hypothesis sheds some light on the relevance of the TRAP1/Sorcin interaction and the role of the 18kDa Sorcin isoform in the TRAP1 cytoprotective pathway. Indeed, TRAP1 chaperone's activity may be crucial for 18kDa Sorcin protein folding [8, 24], contributing to Sorcin-induced regulation of Ca^{2+} homeostasis in mitochondria and participating in the Ca^{2+}-dependent MTP regulation. This observation may be relevant in the perspective of evaluating TRAP1 network as a potential molecular target to revert drug resistance. Indeed, the modulation of Ca^{2+} homeostasis is a crucial step in the cell response to stress conditions and in favoring the multidrug resistance (MDR) phenotype in human tumors [26]. In order to revert the MDR phenotype, previous studies evaluated verapamil, an agent blocking Ca^{2+} influx through L-type Ca^{2+} channels, in several human tumor cell models, and suggested that such a strategy may antagonize P-glycoprotein-mediated MDR *in vitro*, but it provided uncertain results in clinical trials [27]. Thus, the evidence that TRAP1 regulates Ca^{2+} homeostasis in mitochondria throughout Sorcin may form the basis for an innovative therapeutic strategy to induce apoptosis and likely counteract the MDR phenotype by modulating Ca^{2+} metabolism within mitochondria.

Finally, it is intriguing to speculate that TRAP1 and Sorcin are both involved in the cross-talk between mitochondria and ER stress response pathways, being components of a coordinated adaptive response of tumor cells to counteract ER stress conditions and apoptotic signaling. This hypothesis is supported by the following observations: i) the dual localization of TRAP1 and Sorcin isoforms in mitochondria and ER, ii) their involvement in the regulation of Ca^{2+} homeostasis [24, 25] and the unfolded protein response induced by ER stress [8, 28, 29], and iii) the observation that, in human colorectal carcinomas, TRAP1 expression significantly correlates with the protein levels of 22kDa Sorcin [25].

TRAP1: the cytoprotective chaperone

Among the first and widely characterized function of the chaperone TRAP1 is its role in protecting from oxidative stress and reactive oxygen species. Few years ago, concomitantly, several groups characterized the protective role of TRAP1 against oxidative stress induced by several agents [30-32]. Our group demonstrated, by a differential screening between Saos-2 osteosarcoma cells chronically adapted to grow in mild oxidizing conditions induced by the GSH-depleting agent diethylmaleate and the untreated counterpart, that the expression of TRAP1 mRNA and protein is highly increased in oxidant-adapted cells. In turn, these TRAP1-positive cells also exhibit a phenotype resistant to H_2O_2- or cisplatin-induced DNA damage and apoptosis [30], which is strongly dependent from an antioxidant function by this chaperone: in fact, TRAP1 stable transfectants contain higher levels of reduced glutathione [30]. Accordingly, Im et al described the effect of an iron chelator (DFO), an inducer of mitochondrial dysfunction, on TRAP1 expression levels: indeed, decreased TRAP1 levels, with an increase in ROS concentration, was demonstrated in a normal hepatic cell line treated with DFO; interestingly, this high level of oxidative stress was reverted upon TRAP1 overexpression in the same cell model [31]. These results suggest that TRAP1 might play a role in protecting mitochondria against damaging stimuli via a decrease of ROS generation. In the same year, other Authors

described the function of Granzyme M (GzmM)-mediated apoptosis in Natural Killer cells [32]: their findings provided a demonstration that GzmM causes a TRAP1-dependent mitochondrial swelling and loss of mitochondrial transmembrane potential, initiating ROS generation and cytochrome *c* release. In fact, an antagonistic function toward ROS damages by TRAP1 was provided, in terms of cell protection by GzmM-mediated apoptosis. Surprisingly and remarkably, TRAP1 was identified as a substrate of this cytotoxin and its cleavage by GzmM resulted in ROS accumulation; accordingly, TRAP1 silencing, through RNA interference, increased ROS accumulation, whereas TRAP1 overexpression attenuated ROS production [32].

It has also been reported that the Ser/Thr kinase PINK1, whose altered function is implicated in the ethiopathogenesis of some autosomal recessive forms of Parkinson's Disease, phosphorylates TRAP1 as downstream effector to prevent oxidative stress-induced apoptosis and the consequent neurodegeneration [33]. These findings contributed to raise the interest on TRAP1 as a possible target in neurological diseases. Several studies support thee relevance of TRAP1 regulation in neuronal systems. PINK1's effects on the activity of heme oxygenase (HO)-1 in SH-SY5Y neuronal cells were very recently analyzed [34]: this study demonstrated that HO-1 induction upon oxidant treatment was impaired by the expression of a recombinant PINK1 mutant. In addition, the same group demonstrated that TRAP1 knockdown in SH-SY5Y cells which, as above stated, is a substrate of PINK1 kinase, also inhibited HO-1 expression by oxidative stress and that the upregulation of TRAP1 expression following H_2O_2 treatment was inhibited by the addition of a recombinant PINK1 mutant. Interestingly, the HO-1 induction by H_2O_2 treatment was Akt- and ERK-dependent. In fact, ERK and Akt phosphorylation were inhibited in cells expressing the PINK1 mutant and by TRAP1 knockdown. These results suggest a novel pathway by which the defect of PINK1 inhibits the oxidative stress-induced HO-1 production, and this effect seems to be mediated by TRAP1 [34]. Furthermore, Xu et al. showed that the overexpression of TRAP1 in rat brain protects from ischemia, improves mitochondrial function as shown by protection of complex IV activity, reduces generation of ROS detected by hydroethidine fluorescence, reduces lipid peroxidation detected by 4-hydroxy -2-nonenol immunoreactivity, and preserves ATP levels [35].

All these evidences depict a role for TRAP1 in protecting from apoptosis, but this is not only realized through the control of ROS levels. TRAP1 also directly interacts with members of the MTP, inhibiting its opening and the consequent cytocrome *c* release upon apoptotic stimuli. This hypothesis was initially provided by Altieri and co-workers, one of the leading groups in the study of TRAP1, who demonstrated that molecular chaperones like HSP60 directly associate with cyclophilin D (CypD), a regulator of the MTP. This interaction occurs in a multichaperone complex comprising HSP60, HSP90 and TRAP1, whose expression is selectively upregulated in tumor, but not in normal mitochondria [36, 37]. Further evidence supported the involvement of TRAP1, along with HSP90, in the mitochondrial pathway which antagonizes the proapoptotic activity of CypD, responsible for the maintenance of mitochondrial integrity, thus favoring cell survival [5]. TRAP1 overexpression confers resistance to pro-apoptotic drugs, whereas shRNA-mediated knockdown of TRAP1 enhances a death pathway which is inhibitable by the MTP inhibitor cyclosporin A [5]. These observations suggest that TRAP1 and HSP90 both act as MTP inhibitors themselves, and can be therefore considered promising targets for mitochondria-directed antitumor agents [38].

The role of TRAP1 in regulating the MTP in hypoxic conditions was also studied by Xiang et al in different metabolic conditions: these Authors demonstrated that hypoxia causes increased cell death, as well as mitochondrial membrane potential; TRAP1 overexpression prevents hypoxia induced damage in cardiomyocytes, whereas its silencing induces an increase in cell death and decreases both cell viability and mitochondrial membrane potential under normoxic and hypoxic conditions. Furthermore, cell damage induced by TRAP1's knockdown was prevented by the MTP inhibitor, cyclosporin A. These findings led to the important concluding remark that hypoxia induces an increase in TRAP1 expression in cardiomyocytes, and that TRAP1 plays a protective role by regulating the opening of the MTP [39].

TRAP1 cytoprotection is also mediated by other functions

In recent years, many other groups have been contributing to highlighting TRAP1 signaling,

confirming that the main roles of this chaperone are in tumor progression, protection from oxidative damage and cell survival, but also suggesting other novel environments responsible for TRAP1 cytoprotective activity. In 2009, Kubota et al. investigated the role of TNF-α/TNFR1 signaling in cell adhesion properties in the brain. TRAP1 works synergistically with TNFR1 to modulate the expression of the cell adhesion molecule N-cadherin, while altering inter-cellular adhesion of neuronal cells through STAT3 phosphorylation status. These observations prompted the Authors to speculate that TRAP1 could be involved in the signal transduction pathway modulating N-cadherin expression, thus inducing morphological changes at synapses. Interestingly, this feature plays a key role in the cognitive function, again suggesting a remarkable, but not yet extensively investigated field of study, i.e. the role of TRAP1 in brain and in regulating neurological diseases. Intriguingly, the involvement of TRAP1 in cell adhesion opens an attractive scenario for a role of this protein in the processes of cell invasion and motility, both characteristic of tumorigenesis and metastatic spread [40]. Starting from these findings, other groups supported a role of TRAP1 in favoring metastatic cancers: in fact, an antibody response to galectin-8, TARP and TRAP1 was observed in metastatic castration-resistant prostate cancer patients treated with a GM-CSF-secreting cellular immunotherapy [41]. These preliminary findings suggest that antibody induction to tumor-associated antigens may represent a possible biomarker for treatment response to GM-CSF secreting cellular immunotherapy in prostate cancer patients. Almost concomitantly, other Authors demonstrated that TRAP1 regulates genes involved in cell cycle and metastases [42]. The aim of this study was to identify the pathways regulated by TRAP1. TRAP1 was silenced by siRNA in A549 cells and re-expressed by stable transfection in MDA231 cells. In TRAP1 negative cells there are higher levels of genes involved in cell motility and metastatic spread, suggesting that TRAP1 could activate proliferation, whilst inhibiting metastatic spread in tumor cells. Finally, as reported below in this review article, Leav et al. demonstrated that the cytoprotective mitochondrial chaperone TRAP1 can be considered a novel molecular target in localized and metastatic prostate cancer [43].

The most recent function attributed to TRAP1 is its new role in ER stress protection. Two important articles contributed to highlight molecular mechanisms involving TRAP1 in protection from this biological stress. In fact, TRAP1 expression plays a key role in protection from cell death caused by the mitochondrial apoptotic machinery in response to strong ER stress. Recently, Altieri's group has shown that selective targeting of HSP90 chaperones in mitochondria of human tumor cells triggered compensatory autophagy and an UPR based on the upregulation of CCAAT enhancer binding protein (C/EBP) transcription factors. These processes led to inhibition of NF-κB-dependent gene expression, enhanced tumor cell apoptosis initiated by death receptor ligation, and inhibited intracranial glioblastoma growth in mice without detectable toxicity [29]. At the same time other results have shown that mitochondrial TRAP1 influences the activation of the ER stress-induced UPR pathway, protecting cells not only from mitochondrial stress but also from ER stress-induced apoptosis [28]. Almost simultaneously, our group "revealed" for TRAP1 an extra-mitochondrial localization and an involvement in platinum-induced protection in the ER [8]. Altogether, these findings contribute to unravel initial observations of the early 90s on TRAP1's "eclectic" functions in different subcellular compartments and through the interaction with different partners.

Finally a total uninvestigated, and in our opinion very important feature of TRAP1, came from RNA pull down assays, mass spectrometry, and RNP immunoprecipitation assay experiments, demonstrating that TRAP1 specifically interacts with the 3′-UTR of the mRNA coding for the mitochondrial ribosomal protein S12 [44]. These data suggest other possible mechanisms used by TRAP1 to regulate mitochondrial physiology.

TRAP1 as a biomarker/molecular target in human malignancies

Several studies evaluated TRAP1 expression in human malignancies with the intent to validate its up-regulation as a molecular event critical for initiating/maintaining the malignant phenotype.

In support to a TRAP1 role in cancer, studies based on oligonucleotides microarray demonstrated that TRAP1-positive cells contain high levels of cell proliferation promoting genes, whilst TRAP1-negative cells higher levels of

genes involved in cell motility and metastatic spread [42]. Pathway map analysis is consistent with the first evidence of TRAP1 binding the TNFR1, since TRAP1 appears to control cell cycle activity through its pathway. In such a perspective, the evidence that TRAP1 is up-regulated in tumor cells, while is expressed at very low levels, and sometimes is undetectable, in normal tissues represents an attractive opportunity for considering TRAP1 network a molecular target to design cancer-specific agents. Indeed, TRAP1 was initially described to be up-regulated in platin-resistant ovarian cancer cells [45-47] and in human nasopharingeal carcinomas [48]. More recently, Altieri's group demonstrated that TRAP1 is abundantly and ubiquitously expressed in human high-grade prostatic intraepithelial neoplasia, Gleason grades 3 through 5 prostatic adenocarcinomas, and metastatic prostate cancers, but largely undetectable in normal prostate or benign prostatic hyperplasia *in vivo* [43]. Of note, targeting TRAP1/HSP90 pathway with a novel class of mitochondria-directed HSP90 family inhibitors caused rapid and complete killing of androgen-dependent or -independent prostate cancer cells, but not of non-transformed prostatic epithelial BPH-1 cells, whereas reintroduction of TRAP1 in BPH-1 cells conferred sensitivity to anti-HSP90 induced cell death [43]. Furthermore, studies from our group allowed to demonstrate that TRAP1 protein expression is increased in about 60% of human colorectal carcinomas and in drug-resistant colorectal cancer cells [49]. Interestingly, TRAP1 seems to be critical in regulating the fate of its client proteins (i.e., 18kDa Sorcin and F1ATPase) in human colorectal carcinomas *in vivo*. Remarkably, in our study, all colorectal carcinomas overexpressing TRAP1 were characterized by the concomitant up-regulation of the 18kDa Sorcin isoform, and the majority of these Sorcin- and TRAP1-positive tumors exhibited the up-regulation of F1ATPase. By contrast, colon cancer specimens characterized by low expression of both 18kDa Sorcin and TRAP1 did not exhibit any increase in F1ATPase expression [8]. These observations suggest that TRAP1/TBP7 pathway is conserved in human colorectal tumors, providing new insights into the quality control/ubiquitination of proteins in human cancer. Consistently with a role of the mitochondria/ER stress response pathways in protecting tumor cells from apoptosis induced by cytotoxic agents, TRAP1 up-regulation in human colorectal cancer cells is responsible for a multi-drug resistant phenotype since its overexpression induces resistance to 5-fluorouracil, oxaliplatin and irinotecan, three agents widely used in the treatment of human colorectal cancer [49].

These observations strongly stimulated efforts aimed at designing/evaluating novel inhibitors of the HSP90 chaperone family able to induce massive tumor cell death, counteract multi-drug resistance and improve the efficacy of systemic chemotherapy. 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 N-terminal domain of HSP90 [50]. However, 17AAG showed gastrointestinal toxicity and uncertain antitumor activity [50]. The evidence that TRAP1 knockdown triggers Cyp D-dependent apoptosis gave a strong impulse to the development of specific TRAP1/HSP90 antagonists, delivered selectively to mitochondria in order to maximize the pro-apoptotic activity against cancer cells and minimize systemic toxicity toward normal cells. In such a perspective, a cell-permeable peptidomimetic, shepherdin, was designed based on the binding interface between the molecular chaperone HSP90 and the antiapoptotic regulator, survivin. Shepherdin was demonstrated to selectively accumulate in mitochondria due to the antennapedia sequence, interact with the ATP pocket of HSP90, destabilize its client proteins, and induce massive death of tumor cells by apoptotic and nonapoptotic mechanisms [5, 51]. Systemic administration of shepherdin in nude mice revealed significant inhibition of human tumor growth without significant toxicity [51]. The inhibition of TRAP1/HSP90 ATPase activity by shepherdin was also demonstrated to increase the sensitivity to apoptosis in oxaliplatin- and irinotecan-resistant colon cancer cells exposed to the resistant cytotoxic agent [49]. Altogether, these evidences suggest that disabling the HSP90/TRAP1 antiapoptotic network directly in mitochondria causes collapse of mitochondrial function, selective tumor cell death and enhancement/restoration of the activity of cytotoxics, thus validating the hypothesis that mitochondria-directed antagonists may represent a novel class of potent anticancer agents [52].

In further support to this view, gamitrinib, another inhibitor of HSP90 chaperone family, was

designed by fusing the benzoquinone ansamycin backbone structure derived from 17AAG with a mitochondrial targeting moiety [53]. Gamitrinibs thus represent the first class of fully synthetic, combinatorial small molecules, targeting the HSP90 chaperones to mitochondria. Gamitrinibs were shown to accumulate in the mitochondria of human tumor cell lines, to inhibit HSP90 chaperones activity behaving as ATPase antagonists, and exhibited a "mitochondriotoxic" mechanism of action, causing rapid tumor cell death and inhibiting the growth of xenografted human tumor cell lines in mice. Importantly, gamitrinibs, like shepherdin, were non toxic to normal cells or tissues and did not affect HSP90 homeostasis in cellular compartments other than mitochondria [53].

The use of gamitrinib to inhibit HSP90 chaperones inside mitochondria provided an important tool to elucidate the function of these chaperones in the regulation of the protein-folding environment in mitochondria of tumor cells [29]. In fact, suboptimal treatment with gamitrinibs induced accumulation of unfolded proteins in mitochondria, with consequent stress-related transcriptional response with up-regulation of multiple chaperones, a hallmark of mitochondrial UPR and compensatory autophagy. Moreover, as a consequence of this transcriptional UPR, a complete suppression of NF-κB-dependent gene expression was observed [29]. Thus, this approach suggests that mitochondria-directed agents targeting HSP90 family chaperones represent novel tools for disabling mitochondria function and induce selective tumor cell death.

Despite these important achievements, several issues are still open about the specificity of these agents and the exact mechanism of their antitumor activity. Indeed, in this context, a very important point is that HSP90 chaperones act on different client proteins [8, 54] and are differently distributed within cellular compartments: among the same family's members TRAP1 is mostly localized in mitochondria, HSP90 mostly in the cytoplasm, Grp78/Bip in the ER. Thus, it is still unclear which is the real target of these mitochondria-directed agents and still unanswered is the question about the need to inhibit single or multiple HSP90 chaperones to obtain a clinically relevant anticancer activity. This issue is also questioned by the evidence that there is a significant diversity in the ATP binding affinity between TRAP1 and HSP90 ATPase domains, being TRAP1 characterized by higher affinity than HSP90 [6], thus suggesting that the single ATPase inhibitor may not be able to target multiple HSP90 chaperones with the same efficiency. Finally, the evidence that TRAP1 is also localized in the ER, where it is involved in the quality control of specific client proteins destined to mitochondria, raises the question about which function of TRAP1 needs to be specifically targeted to induce apoptotic cell death in tumor cells. In such a perspective, the strategy of delivering anti-HSP90 family agents selectively to mitochondria may be still successful, since may target the central TRAP1 regulatory activity on the opening of MTP and the intrinsic apoptotic pathway [5]. However it could also fail to inhibit TRAP1 functions outside the mitochondria, which may also be relevant in favoring drug resistance. Thus, further studies will question the issues of compartmentalization of anti-HSP90 inhibitory strategies and the need for specific TRAP1 inhibitors.

Furthermore, the novel TRAP1 regulatory function in protein quality control, related to TBP7 interaction in the ER, may be striking for its antiapoptotic activity and its 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, since many tumor cells display constitutive proteotoxic stress and dependence on heat shock response due to their rapid rates of proliferation and translation. In breast cancer, recent discoveries show that a cytoplasmic protein quality control pathway is implicated in the suppression of cancer cell growth, suggesting a new role for quality-control mechanisms in suppressing cells with malignant potential [55]. Furthermore, Kajro et al. 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 [56]. These studies raise the unexpected possibility that quality-control pathways may have a role in tumor progression and support the idea that the protein quality control mechanisms in which TRAP1 is involved could represent a new, interesting field to explore, as well as a promising novel molecu-

lar target for the development of anticancer therapies.

Concluding remarks

More than 10 years after the original, poorly understood and not deeply investigated features attributed to TRAP1, a novel view of the TRAP1 pathway is arising. This has been made possible by several recent studies which contributed to drawing a more complex "picture" of TRAP1 network, based on the identification of specific TRAP1 client proteins and the discovery of its multiple subcellular localizations. Despite the wealth of new evidences on TRAP1 regulation of mitochondrial permeability transition pore and mitochondrial integrity, targeting ATPase inhibitors in this organelle may not be sufficient to inhibit other TRAP1 functions analogously important and interconnected to the mitochondrial cytoprotective role. In this view, the recent discovery that TRAP1 is also localized in the ER, where it is involved in the quality control of specific client proteins destined to mitochondria, raises the question about which function of TRAP1 and which cellular environment needs to be specifically targeted to induce the most extensive apoptotic cell death in tumor cells.

Most of the scientists dedicated to TRAP1's study agreed that one of the most important functions of this protein is the protection against programmed cell death and stress conditions induced by several antitumor agents making TRAP1 a good cancer therapeutic target. Given the heterogeneity of this devastating disease, the improvement of patient long-term survival might be achieved by optimizing early detection of aggressive and drug-resistant diseases and translating recent insights at the molecular and cellular levels into personalized individual strategies for treatment. In such a perspective, our studies on TRAP1 and TRAP1 client proteins as novel candidate predictive/prognostic biomarkers: i) will provide proof-of-principle of target modulation, ii) could be used for selecting tumors requiring more aggressive treatments, and iii) will be essential in the development of novel molecular-targeted therapies for different human cancers.

From the above, we postulate the idea that the recently discovered TRAP1 role in ER stress protection and regulation of protein quality control could represent the prerequisite to develop therapeutic strategies aimed at blocking these pathways. ER stress and protein quality control mechanisms in which TRAP1 is involved may therefore represent a new intriguing and interesting field to explore either for their role in tumor progression or as a promising novel molecular target to revert drug resistance. In such a context, the characterization of TRAP1 pathway and the discovery of new client proteins of this chaperone in different subcellular compartments may greatly contribute to the identification of potential molecular tools to improve the activity of anticancer agents. Finally, given the complexity of such mechanisms, an extraordinary effort is required to design novel strategies based on the simultaneous inhibition of multiple tumor-specific pathways, and contemplating different subcellular-targeted approaches with the aim of improving antitumor activity.

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