TRAP1 is involved in BRAF regulation and downstream attenuation of ERK phosphorylation and cell cycle progression: a novel target for BRAF-mutated colorectal tumors

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Abstract

Human BRAF-driven tumors are aggressive malignancies with poor clinical outcome and lack of sensitivity to therapies. TRAP1 is a HSP90 molecular chaperone deregulated in human tumors and responsible for specific features of cancer cells, i.e. protection from apoptosis, drug resistance, metabolic regulation and protein quality control/ubiquitination. The hypothesis that TRAP1 plays a regulatory function on the BRAF pathway, arising from the observation that BRAF levels are decreased upon TRAP1 interference, was tested in human breast and colorectal carcinoma (CRC) in vitro and in vivo. This study shows that TRAP1 is involved in the regulation of BRAF synthesis/ubiquitination, without affecting its stability. Indeed, BRAF synthesis is facilitated in a TRAP1-rich background, while increased ubiquitination occurs upon disruption of the TRAP1 network that correlates with decreased protein levels. Remarkably, BRAF downstream pathway is modulated by TRAP1 regulatory activity: indeed, TRAP1 silencing induces i) ERK phosphorylation attenuation, ii) cell cycle inhibition with cell accumulation in G0-G1 and G2-M transitions, and iii) extensive reprogramming of gene expression. Interestingly, a genome-wide profiling of TRAP1-knock down cells identified cell growth and cell cycle regulation as the most significant biofunctions controlled by the TRAP1 network. It is worth noting that TRAP1 regulation on BRAF is conserved in human CRCs, with the two proteins frequently co-expressed. Finally, the dual HSP90/TRAP1 inhibitor HSP990 showed activity against the TRAP1 network and high cytostatic potential in BRAF-mutated CRC cells. Therefore, this novel TRAP1 function represents an attractive therapeutic window to target dependency of BRAF-driven tumors on TRAP1 translational/quality control machinery.
Introduction

Cancers arising through mutations in the RAS–RAF–MEK–ERK pathway represent approximately 30% of all cancers, 8% bearing BRAF mutations (1, 2), and occurring more frequently in melanomas (40–70%), and thyroid (36–53%), colorectal (5–22%) and low grade ovarian serous (~30%) carcinomas (3). It is worth noting that deregulated BRAF signaling drives the inappropriate activation of the downstream MEK/ERK pathway, thus leading to increased cellular growth, invasion, and metastasis (4), as well as the activation of NF-kB signaling and Bcl-2, which are responsible for apoptosis inhibition (5). In such a context, BRAF-driven tumor cells become addicted to these deregulated signaling pathways, thereby constituting a therapeutic window for tumor-selective targeting (3). This issue is extremely relevant in the clinical setting since human BRAF-driven tumors are frequently characterized by aggressive biological behavior and resistance to apoptosis induced by anticancer therapies (3).

Since HSP90 is the main molecular chaperone responsible for BRAF folding, with specific affinity greater for its mutated form (6, 7), HSP90 targeting is presently evaluated as an antitumor strategy in human BRAF-mutated neoplasms (8). In such a scenario, the characterization of novel mechanisms responsible for the regulation of this oncogene and, thus, the identification of novel molecular targets for BRAF-mutated malignancies represent major clinical challenges, especially considering the lack of effective treatments for these tumors (3). This issue is extremely relevant in BRAF-driven colorectal carcinomas (CRCs), which are characterized by high grade, mucinous histology, poor clinical outcome and poor responsiveness to standard therapies, including BRAF inhibitors and anti-EGFR1 agents (9).

TRAP1 is a molecular chaperone, belonging to the HSP90 chaperone family, which is upregulated in several human malignancies, i.e. colorectal, breast, prostate and lung cancers (10-13). Initial studies demonstrated its involvement in maintaining mitochondrial integrity and regulating the mitochondrial transition pore (MTP), via direct folding/stability regulation on cyclophilin D and, likely, other client proteins critical for MTP opening (11, 14, 15). More recent evidence suggests
that TRAP1 is responsible for additional functions critical to tumor progression, such as reprogramming of tumor cell metabolism (16, 17) and extramitochondrial quality control regulation of specific mitochondrial client proteins, most of which are key regulators of the mitochondrial apoptotic pathway (15, 18, 19, 20). In this context, previous data from our group suggest that TRAP1 interacts with the regulatory protein particle TBP7 in the endoplasmic reticulum (ER), where it is involved in i) quality control of nuclear-encoded mitochondrial proteins through co-translational regulation of their ubiquitination/degradation (18, 20, 21), ii) attenuation of global protein synthesis through activation of GCN2 and PERK kinase pathways, with consequent phosphorylation of eIF2α and attenuation of cap-dependent translation (21), iii) parallel enhancement of IRES-dependent translation, likely favoring synthesis of selective cancer-related genes (21), iv) consequent protection from ER stress with parallel activation of a cytoprotective UPR response (18, 20, 22).

Starting from our unpublished observation showing decreased BRAF protein levels in TRAP1-knock down (KD) cells and considering that i) HSP90 chaperones are involved in the regulation of BRAF stability (6, 7), ii) TRAP1 is a HSP90 chaperone and key regulator of several process driving tumor progression (11, 16, 17, 18, 22), and iii) BRAF is an intracellular kinase controlling tumor cell proliferation, migration and metastasis (3), we studied the relationship between the TRAP1 network and the BRAF signaling pathway. Herein, we report that, in addition to HSP90 folding regulation, BRAF is under additional control by TRAP1 translational/quality control machinery and that this regulation is relevant to BRAF-dependent control on ERK signaling and cell cycle progression.

Materials and Methods

Tumor specimens and cell cultures. Specimens from 41 BRAF-wild type (wt) CRCs and corresponding normal, non-infiltrated peritumoral mucosa were obtained from the General Surgery Unit of the University of Foggia. Express written informed consent to use biological specimens for
investigational procedures was obtained from all patients. Human breast carcinoma (BC) MCF7 and CRC HCT116, HT29, COLO320 and COLO205 cells were purchased from American Type Culture Collection (ATCC). Cell lines were routinely monitored in our laboratory by microscopic morphology, while cell line authentication was verified by STR profiling in July 2013, according to ATCC product description.

**RNA extraction and Real Time RT-PCR analysis.** Total RNA was extracted using the TRIzol Reagent (Invitrogen). For first strand synthesis of cDNA, 1 µg of RNA was used in a 20 µl reaction mixture utilizing a Transcriptor First Strand cDNA Synthesis Kit (Roche). For Real Time PCR analysis, 0.5 ng of cDNA sample was amplified using the LightCycler 480 SYBR Green I Master (Roche) in a Light Cycler 480 (Roche). Primers are reported in Supplementary Methods. Reaction conditions were as follows: pre-incubation at 95 °C for 5 min, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C, 10 s at 72 °C. GAPDH was chosen as an internal control.

**Immunoblot analysis.** Total cell lysates were obtained by homogenization of cell pellets and tissue samples in a 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 leupetin and 0.2 % (w/v) deoxycholate) for 2 min at 4°C and further sonication for 30 sec on ice. Mitochondria were purified by Qproteome Mitochondria Isolation kit (Qiagen) according to manufacturer protocol. Immunoblot analysis was performed as previously reported (23). Primary antibodies are reported in Supplementary Methods. Where indicated, protein levels were quantified by densitometric analysis using the Quantity One 4.5 software (BioRad Laboratories GmbH).

**Pulse-chase assay.** Pulse–chase analysis was performed as described elsewhere (24), with some modifications. In brief, 24h after transfection with cDNAs encoding for BRAF wild type and V600E mutant tagged with Myc epitope, scramble and shTRAP1 HCT116 cells were incubated for 1h in a cysteine/methionine-free medium containing 100 µCi/ml 35S-labeled cysteine/methionine (GE Healthcare). After labeling, cells were washed once with culture medium containing 10-fold
excess of unlabeled methionine and cysteine (5mM each) then further incubated in the same medium for the indicated time periods. Cells were collected at the indicated time points with immunoprecipitates (IPs) performed on total protein lysates by using anti-Myc antibody. The IPs were separated by 10% SDS-PAGE, transferred onto a PVDF membrane (Millipore) and analyzed by autoradiography, after which the same filters were probed by immunoblot analysis.

Radio-labeled amino acid incorporation assay was performed by incubating cells for 1, 3 and 6h in a cysteine/methionine-free medium containing 100 μCi/ml 35S-labeled cysteine/methionine as described above.

**Cell cycle analysis.** Cells were incubated in a culture medium supplemented with 20 μM 5-bromo-2’-deoxyuridine (BrdU) for 20 min and harvested. Subsequent to incubation in a solution containing 3N HCl for 30 min at room temperature to obtain DNA denaturation, cell pellets were further incubated in the presence of anti-BrdU FITC (Becton Dickinson) for 1h at room temperature in the dark. After washing with PBS, cells were further incubated with 6 μg/ml propidium iodate (PI) for 20 min and then evaluated using the FACsCalibur™ (Becton Dickinson).

**Results**

**TRAP1 regulates BRAF synthesis/ubiquitination at translational levels.**

The role of TRAP1 in regulating BRAF protein levels was evaluated in BRAF-wt scramble and shTRAP1 colon HCT116 and breast MCF7 carcinoma cells (Figure 1A), as well as colon BRAF-wt COLO320 (Figure 1B) and BRAF-V600E HT29 carcinoma cells (Figure 1C) upon transient TRAP1 silencing. Interestingly, while decreased BRAF protein expression was observed upon TRAP1 silencing, HSP90 expression levels remained unchanged throughout all those experimental conditions (Figure 1A-C).

To determine whether TRAP1 control on BRAF was at the transcriptional level, BRAF mRNA levels were analyzed by quantitative RT-PCR in TRAP1-silenced cells compared to their scramble controls. The unchanged mRNA levels (Figure 1D) along with the awareness that TRAP1 is a...
member of the HSP90 molecular chaperone family, known to regulate BRAF protein folding and stability (6), prompted us to evaluate long term BRAF protein stability by pulse/chase experiments in TRAP1-interfered HCT116 cells (Figure 2A). Indeed, the half-lives of BRAF-wt and BRAF-V600E proteins were comparable for up to 24h after the pulse in both cells types (Figure 2A), thus suggesting that TRAP1 does not influence stability of BRAF within this time frame.

Previous observations by our group suggest that TRAP1 is involved in co-translational control of cancer cells through i) attenuation of Cap-dependent translation (18, 21), ii) preferential synthesis of selective cancer-specific genes (21), and iii) co-translational ubiquitination/degradation of selective stress-protecting client proteins (18, 21). Since BRAF stability is not affected by TRAP1, we tested the hypothesis that BRAF is regulated at the translational level by measuring radiolabeled amino acid incorporation in scramble/shTRAP1 HCT116 cells. Results show that scramble cells incorporate more radiolabeled amino acids into both BRAF-wt and BRAF-V600E proteins over time, compared to shTRAP1 cells (Figure 2B). Since BRAF stability is unaffected by TRAP1, we further evaluated whether the reduced protein levels of BRAF in sh/siTRAP1 cell lines were dependent on increased ubiquitination favored by the lack of TRAP1 quality control. Accordingly, BRAF was immunoprecipitated from scramble and shTRAP1 HCT116 cells (Figure 3A) and HT29 cells after TRAP1 transient silencing (Figure 3B). Ubiquitin immunoblot analysis of BRAF IPs showed increased levels of protein ubiquitination in TRAP1-interfered cells despite overall reduction of total BRAF (Figure 3A-B). In parallel experiments, HCT116 cells were transfected with a TBP7 deletion mutant unable to bind TRAP1, which behaves as dominant negative over the TRAP1/TBP7 ubiquitination control (18, 25). In line with data obtained upon TRAP1 silencing, ubiquitin immunoblot analysis of BRAF IPs showed increased ubiquitin-conjugated proteins in cells transfected with the ΔTBP7 dominant negative mutant (Figure 3C). Finally, to support further the hypothesis that TRAP1 modulates BRAF synthesis/ubiquitination in the ER, shTRAP1 HCT116 cells were transfected with full length TRAP1 (Figure 3D) or the Δl-59TRAP1 deletion mutant (Figure 3E), which lacks the mitochondrial targeting sequence and is thus unable to enter into
mitochondria, while still capable of binding TBP7, modulating protein synthesis and protecting from ER stress (18, 20). Of note, either full length TRAP1 (Figure 3D) or the ER-associated Δ1-59TRAP1 deletion mutant (Figure 3E) rescued BRAF levels in shTRAP1 cells. Taken as a whole, these observations suggest that TRAP1 control on BRAF occurs in the extramitochondrial compartment and that BRAF is among the genes whose synthesis is selectively enhanced in a TRAP1-rich background, while its ubiquitination is enhanced in a TRAP1-low background.

**BRAF downregulation upon TRAP1 silencing correlates with attenuation of ERK signaling and cell cycle progression.**

Since BRAF is responsible for the downstream activation of MAPK pathway (3), we evaluated ERK1/2 phosphorylation in a TRAP1-low background. In fact, shTRAP1 MCF7 (Figure 4A) and siTRAP1 COLO320 (Supplementary Figure S1A) showed reduced activation of ERK1/2 phosphorylation upon serum stimulation compared to control scramble cells. The close relationship between BRAF control by TRAP1 and activation of MAPK signaling was further investigated by transfecting BRAF-wt and BRAF-V600E constructs in both scramble and shTRAP1 MCF7 cells: accordingly, reduced expression of BRAF-wt and BRAF-V600E and ERK1/2 phosphorylation was observed in TRAP1-silenced cells (Supplementary Figure S1B). Furthermore, as for the rescue of BRAF levels (Figure 3E), re-expression of the Δ1-59TRAP1 deletion mutant in shTRAP1 HCT116 cells analogously restored ERK phosphorylation in parallel with BRAF re-expression (Figure 4B).

Since ERK signaling is known to induce cell cycle progression (26), we questioned the ability of TRAP1 silencing to block cell proliferation. Indeed, TRAP1-interfered MCF7 cells decreased the percentage of cells in the S-phase of the cell cycle, with parallel up-regulation of cells in G0-G1 and G2-M transitions (Figure 4C and Supplementary Figure S1C). It is worth noting the ability of TRAP1 to inhibit ERK phosphorylation and progression through the S phase, observed upon transient TRAP1 silencing in BRAF-wt HCT116 (Figure 4D) and COLO320 (Supplementary Figure 1D), as well as in BRAF-V600E HT29 (Figure 4E) cells, thus reinforcing the relevance of TRAP1 control of BRAF function.
TRAP1 silencing results in extensive reprogramming of gene expression involving genes responsible for cell cycle progression.

Since RAF/ERK signaling is responsible for the transcriptional regulation of several genes involved in the progression of the cell cycle (27), we performed full genome gene expression profiling of scramble and shTRAP1 HCT116 cells which allowed for the identification of 504 genes significantly modulated in TRAP1-silenced cells (p < 0.001), 246 up- and 258 down-regulated (Supplementary Table S1). Gene ontology (GO) analysis identified several GO categories linked to genes modulated in our data set, and, among others, cell cycle, cell proliferation and growth (Supplementary Figure S2A). We used the IPA to identify the biofunctions deregulated in TRAP1-silenced cells: it is noteworthy that cell growth and proliferation and cell cycle regulation are among the top 5 predicted biofunctions (Figure 5A). As these results were confirmed by the FIDEA platform (Supplementary Figure S2B), we selected 9 down-regulated genes with a cutoff value of <1.5, associated to cell cycle progression and identified by both bioinformatic platforms (Table 1). In agreement with the role of TRAP1 in regulating cell cycle progression, these genes are all responsible for the regulation of G0-G1 and/or G2-M transitions (28-35), with most of them activated by ERK signaling (36). In order to validate microarray data, the expressions of 8 of these genes were analyzed by quantitative PCR and immunoblot analyses in scramble/shTRAP1 HCT116 cells (Figure 5 and Supplementary Figure S3, respectively): in actual fact, 6 of these genes were confirmed to be significantly down-regulated upon TRAP1 stable silencing by quantitative PCR (Figure 5B). GAS6 was not further evaluated, since its expression levels were undetectable in the majority of our cell models. The relevance of the results shown in Figure 5B was further supported by analogous down-regulation of the same genes in HCT116 cells upon transient TRAP1 (Figure 5C) or BRAF (Figure 5D) silencing, as well as by down-regulation of 5 of these genes in BRAF-V600E HT29 cells silenced for TRAP1 (Supplementary Figure S2C). Conversely, high BRAF and pERK levels and increased expression of the same previously validated genes were found in HCT116 cells transfected with TRAP1 cDNA (Figure 5E). Taken as a whole, these data suggest
that the reprogramming of gene expression induced by TRAP1 silencing is at least partially dependent on TRAP1 regulation of RAF/ERK signaling. Immunoblot analysis confirmed the down-regulation of CCNE1, PBK, TFDP1, and NEK6 in HCT116 and HT29 cells upon TRAP1 silencing (Supplementary Figure S3A), as well as the up-regulation of the same genes upon TRAP1 up-regulation (Supplementary Figure S3B). To further study the contribution of the above genes to TRAP1-dependent regulation of cells growth, cell cycle distribution was further evaluated in HCT116 cells silenced for these genes. The independent silencing of each of these genes induced a significant attenuation of S phase, with arrest of cells in G2-M phase upon TFDP1 and PBK silencing, and arrest in G0-G1 phase upon CCNE1 and NEK6 interference. Of note, the simultaneous silencing of all these genes significantly inhibited S phase with cell cycle arrest in both G0-G1 and G2-M phases, thus reproducing the phenotype obtained upon TRAP1 silencing (Supplementary Figure S3C).

**TRAP1 and BRAF are co-expressed in human CRCs and human BRAF V600E CRC cells are highly sensitive to TRAP1 inhibitions.**

Since TRAP1 is responsible for the regulation of BRAF synthesis/ubiquitination in CRC cell lines (Figures 2-3), the relevance of this mechanism was further evaluated in human malignancies. Accordingly, BRAF protein expression was assessed in a cohort of 41 BRAF-wt CRCs previously characterized for TRAP1 expression and subdivided in TRAP1-positive (21 tumors) and TRAP1-negative (20 tumors) (Figure 6A). Interestingly, a statistically significant co-expression between the two proteins was observed (chi square test, $p=0.018$; Figure 6B and Supplementary Table S2), thus suggesting that TRAP1-dependent control on BRAF is conserved in human CRCs.

Finally, the issue of TRAP1 targeting was evaluated as a novel therapeutic strategy in human BRAF-mutated CRC cells, known to be addicted on the RAF/RAS/ERK pathway for their proliferative potential (3). This approach was tested at the preclinical level by using two ATPase inhibitors, HSP990 and AUY922, both of which are described to bind the ATPase domain of TRAP1 in addition to HSP90 (37, 38). Interestingly, only HSP990 exhibited significant activity
against TRAP1 and TRAP1 translational regulatory apparatus in our experimental models, Indeed, HSP990 i) downregulated specific TRAP1 interactors such as the 18kDa isoform of sorcin (15) (Supplementary Figure S4A) and eEF1G, eIF4A, eIF4E, members of the translational machinery involved in TRAP1-dependent modulation of protein synthesis (21) (Supplementary Figure S4B), ii) inhibited eI2Fα phosphorylation (Figure 6C), a master regulator of protein synthesis whose phosphorylation status decreased upon TRAP1 silencing, as shown in our previous studies (21), and iii) induced increased levels of protein ubiquitination (Figure 6C). Remarkably, short exposure of HT29 cells to HSP990 increased BRAF ubiquitination (Figure 6D), while prolonged exposure to the dual TRAP1/HSP90 inhibitor yielded a significant down-regulation of BRAF expression and ERK phosphorylation, more evident in BRAF-V600E CRC cell lines (Figure 6E). Conversely, AUY922 exhibited minimal degrading activity against BRAF, while still being active on HSP90, as demonstrated by AKT degradation (Figure 6E). Consistently, HSP990 showed high cytostatic activity in human BRAF V600E HT29 cells, and was active in inhibiting either cell cycle progression (Figure 6F) or cell proliferation (Supplementary Figure 4C) at concentrations 10 times higher than AUY922 (Figure 6F). By contrast, HSP990 induced significant levels of apoptosis only at concentrations above 100nM, being AUY922 inactive in inducing cell death in this concentration range (Supplementary Figure 4D). These data strongly suggest that TRAP1 translational machinery deserves evaluation as a novel molecular target in human BRAF-mutated CRCs.

Discussion

BRAF is one of the top12 mutant genes, showing around 8% of mutation frequency in human tumors (39). Likewise, human BRAF-driven malignancies are highly aggressive tumors, resistant to anticancer therapies and, frequently, with a poor outcome (3). Thus, novel targeted therapeutic strategies are urgently needed in order to impact on molecular mechanisms driving tumor progression and responsible for the aggressive behavior of these malignancies.
This study provides a strong molecular rationale to candidate TRAP1 network as a novel target in colorectal BRAF-driven tumors. Indeed, starting from the well-known observation that HSP90 is the molecular chaperone responsible for BRAF stability and folding (6), as well as from our unpublished data suggesting that BRAF is down-regulated in TRAP1-silenced cells, here we show that BRAF is under the control of TRAP1 translational quality control machinery, as an additional, non redundant mechanism responsible for regulating BRAF activity/synthesis in tumor cells. Seen in this light, while HSP90 is responsible for direct folding regulation on BRAF (6), our data suggest that TRAP1 regulates BRAF synthesis/ubiquitination at translational levels. A number of observations support this conclusion: i) BRAF protein levels are significantly attenuated in TRAP1-silenced cells with no effect on its mRNA levels and long term stability, ii) BRAF protein synthesis is facilitated in a TRAP1-high background, iii) BRAF ubiquitination is increased upon TRAP1 silencing and after disruption of the ER-associated TRAP1/TBP7 quality control network, and iv) BRAF protein levels are restored under the re-expression of a TRAP1 mutant whose activity is restricted to ER and retains TRAP1 quality control function (18, 20).

To our knowledge, this is the first evidence that TRAP1 quality control function is not restricted to nuclear-encoded mitochondria destined proteins (18, 20). Indeed, recent studies by our group demonstrated that TRAP1 is responsible for the attenuation of cap-dependent translation, favoring the IRES-dependent version, and that, upon TRAP1 silencing, specific TRAP1 client proteins (i.e., 18kDa Sorcin and F1ATPase) are down-regulated at the protein level through co-translational ubiquitination (18, 21). Critical for this extramitochondrial function of TRAP1 is the interaction with TBP7, an AAA-ATPase of the 19S proteasomal subunit (18). Remarkably, TRAP1 and TBP7 are both upregulated in human colorectal tumors, thus suggesting the relevance of this pathway in vivo (18). In such a perspective, the enrichment of the list of TRAP1 regulated proteins, which remarkably includes a key oncogene driving tumor progression in human malignancies, further highlights the relevance of the TRAP1 quality control pathway in favoring and maintaining the malignant phenotype in human tumors. Indeed, the molecular characterization of human CRCs
allowed us to demonstrate that TRAP1 is co-expressed with BRAF (Figure 6) and other TRAP1 client proteins, beyond TBP7 (18, 21), thus suggesting both that selected human malignancies are likely to be dependent on TRAP1 translational quality control network for their survival, proliferation and migration (18, 21), and that this mechanism probably favors their growth indefinitely and protects from apoptotic cell death even under unfavorable environments. In such a scenario, TRAP1 network is emerging as a key element in the ER protein quality control apparatus and as a relevant component of the adaptive stress response pathway of cancer cells aimed at maintaining proteostasis and, consequently, gaining a survival advantage (40).

This working hypothesis is consistent with the evidence that TRAP1 regulation on BRAF synthesis/ubiquitination affects specific features of BRAF-driven tumor cells, i.e., the downstream ERK signaling pathway, cell cycle progression and gene expression. Indeed, TRAP1 silencing results in the attenuation of ERK phosphorylation and progression of cell cycle with accumulation of cells in G0-G1 and/or G2-M checkpoints.

Furthermore, the whole-genome expression analysis of shTRAP1 CRC cells provides evidence that the attenuation of RAF/ERK signaling correlates with wide reprogramming of gene expression affecting several functions central to tumor progression, such as regulation of cell metabolism and apoptosis, organization of cell components and location, cell communication and regulation of multicellular and multiorganism processes. While several of these predicted functions are consistent with TRAP1 literature (10, 11, 14-18, 41, 42), it is worth noting that analysis of our dataset highlights regulation of cell cycle progression as the top biofunction, identifying several cell cycle-regulatory genes whose expression is reduced, most of them under the control of ERK signaling (26). Furthermore, 6 of these genes were significantly downregulated in CRC cell lines in conditions of TRAP1 or BRAF silencing and up-regulated in parallel to BRAF and ERK phosphorylation on TRAP1 transfection, thus supporting the role of TRAP1 in controlling a network of genes involved in the regulation of cell proliferation through its modulation on RAS/ERK...
signaling (Supplementary Figure S5). Remarkably, the silencing of 4 of these genes reproduced the inhibitory phenotype of cell cycle progression obtained upon TRAP1 interference.

These findings are consistent with the initial identification of TRAP1 as a molecular chaperone interacting with Rb protein during mitosis and after heat shock (41) and are in agreement with microarray data by Liu et al, showing that TRAP1 may control cell proliferation by promoting genes encoding for G protein coupled receptors, and cell adhesion and Rho-kinase pathway proteins (36). Furthermore, recent observations suggest that TRAP1 knockdown reduces cell growth and clonogenic cell survival in lung carcinoma cells (13), while TRAP1 upregulation increases ERK phosphorylation in mice fibroblast (43). Finally, the relevance of this mechanism in tumor cell growth is strongly supported by our previous observation that TRAP1 KD cells are not tumorigenic either in vitro or in vivo (17).

These results provide a strong molecular rationale for evaluating TRAP1 quality control machinery as a novel target in BRAF-driven tumors with a view towards the development of specific therapeutic strategies to inhibit a mechanism relevant for maintenance of the malignant phenotype. Indeed, the co-expression between TRAP1 and BRAF and other TRAP1 client proteins in human CRCs (18, 20, 21) supports the relevance of TRAP1 co-translational regulation in human pathology. Furthermore, here we demonstrate that HSP990, an HSP90 inhibitor previously hypothesized as inhibiting TRAP1 based on in vitro binding data (38), showed the capacity to downregulate specific TRAP1-interacting proteins (21), inhibit eIF2α, a master regulator of protein synthesis (21), and enhance BRAF ubiquitination, thus opening new perspectives in the development of TRAP1-targeted therapies. Indeed, HSP990 induced a significant down-regulation of BRAF protein expression in CRC cell lines and showed a potent cytostatic activity in BRAF-mutated CRC cells, providing evidence for the concept that TRAP1-dependent translational/quality control machinery represents a potentially attractive target to develop novel anticancer inhibitors, opening a new window for a compartmentalized (ER versus mitochondria) and network-directed therapeutic strategy in BRAF-driven CRCs.
In this perspective, the evidence that BRAF protein expression is under the control of TRAP1 quality control machinery ideally candidates human BRAF-driven tumors for TRAP1-directed therapy. It follows that new studies are needed to design and develop specific anti-TRAP1 inhibitors in order to target TRAP1 function directly in the ER, most likely with a reduced toxicity profile compared to HSP90 inhibitors presently under preclinical and clinical development (38).

This issue is extremely relevant in human BRAF-mutated CRCs, characterized by an aggressive biological and clinical behavior and a dismal prognosis (5). These malignancies represent, at present, a real clinical challenge as they are insensitive to both traditional chemotherapeutics and new anti-EGFR agents (44). While recent findings suggest that only the combination of a triple chemotherapy regimen (i.e., 5-fluorouracil, irinotecan and oxaliplatin) with the anti-VEGF agent, bevacizumab, may impact on the progression of BRAF-mutated metastatic CRCs (45), our data provide a strong rationale for the evaluation of TRAP1 targeting agents in these tumors.

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**Figure Legends**

**Figure 1. TRAP1 modulates BRAF protein levels.** A-C. Total cell lysates from scramble and shTRAP1 HCT116 and MCF7 cells (A), or BRAF-wt COLO320 (B) and BRAF-V600E HT29 (C) cells transfected with negative or TRAP1 siRNA were separated by SDS–PAGE and immunoblotted with indicated antibodies. D. TRAP1 and BRAF mRNA levels in shTRAP1 HCT116 and MCF7 cells and in siTRAP1 COLO320 cells reported as relative units compared to their scramble controls. *p<0.0001.

**Figure 2. TRAP1 regulates BRAF expression at translational level without affecting its long term stability.** A. Scramble and shTRAP1 HCT116 cells transfected with BRAF-wt or BRAF-V600E constructs were pulse labeled with 100 μCi/mL ^35^S Met/^35^S Cys for 1h and chased for the
indicated times. IPs were analyzed by autoradiography and immunoblot analysis with anti-MYC antibodies. B. Scramble and shTRAP1 HCT116 cells were transfected with MYC-tagged BRAF-wt or BRAF-V600E cDNAs. After 24h, cells were labeled with 100 μCi/mL $^{35}$S Met/$^{35}$S Cys for 1, 3 and 6h and immediately harvested. IPs were analyzed by autoradiography and immunoblot analysis with anti-MYC antibodies. A-B. Densitometric band intensities, each normalized to the respective IP, were calculated by assuming protein levels of the control (scramble cells at time 0) equal 1 and represents the mean values of 3 independent experiments (±S.D.).

Figure 3. ER-associated TRAP1 is responsible for the quality control/ubiquitination of BRAF. A-B. BRAF IPs were obtained from total cells lysates of scramble and shTRAP1 HCT116 cells transfected with MYC-tagged ubiquitin vector (Ub-MYC) (A) and HT29 cells transfected with negative or TRAP1 siRNA (B), both incubated with 10μM MG132 for 2h before cell lysis. IPs were separated by SDS–PAGE and immunobotted with indicated antibodies. Inputs: Total lysates from previously described cell lines were separated by SDS–PAGE and immunobotted with indicated antibodies. C. HCT116 cells were co-transfected with BRAF-wt or BRAF-V600E constructs and the ΔTBP7 deletion mutant. Total lysates were quantified with an equal amount of each lysate immunoprecipitated with anti-MYC antibodies. IPs were separated by SDS–PAGE and immunobotted with indicated antibodies. Input: Total cell lysates from cells treated as in B were separated by SDS–PAGE and immunobotted with indicated antibodies. A-C. Densitometric band intensities are reported as ratios between ubiquitinated BRAF bands and total BRAF in IPs. D-E. Total cell lysates from scramble and shTRAP1 HCT116 cells transfected with full length TRAP1 cDNA (C) or the Δ1-59TRAP1 mutant (D) were separated by SDS–PAGE and immunobotted with indicated antibodies. Densitometric band intensities were calculated by assuming protein levels of scramble cells as equal to 1.

Figure 4. TRAP1 silencing results in reduced ERK phosphorylation and cell cycle progression. A. Serum-starved scramble and shTRAP1 MCF7 cells were serum stimulated for 1 and 3h. Total cell lysates were separated by SDS–PAGE and immunobotted with indicated
antibodies. B. Total cell lysates from scramble and shTRAP1 HCT116 cells transfected with the Δ1-59TRAP1 mutant were separated by SDS–PAGE and immunoblotted with indicated antibodies. C. Graphs of cytofluorimetric analysis of cell cycle distribution in MCF7 cells transfected with negative or TRAP1 siRNA. D-E. Cell cycle distribution in HCT116 (D) and HT29 (E) cells transfected with negative or TRAP1 siRNA expressed as mean values from 3 independent experiments. D-E. Inserts: Total cell lysates from HCT116 (D) and HT29 (E) cells transfected with negative or TRAP1 siRNA were separated by SDS–PAGE and immunoblotted with indicated antibodies.

Figure 5. TRAP1 silencing results in a wide reprogramming of gene expression. A. Top predicted biofunctions (p<0.02) associated to genes downregulated upon TRAP1 silencing in HCT116 cells. Bar graph represents –log (p-value) for each biofunction. Biofunction prediction from IPA software. B. Real Time PCR validation of gene expression of 8 genes significantly down-regulated in shTRAP1 HCT116 cells compared to scramble cells (*p=0.002; **p=0.001; °p=0.006; °°p<0.0001). Insert: TRAP1 expression evaluated by immunoblot in scramble (1) and shTRAP1 (2) HCT116 cells. C-E. Real time gene expression analysis of previously validated genes in HCT116 cells upon TRAP1 (C) or BRAF (D) transient interference or TRAP1 upregulation (E). Statistical significance C. *p<0.0001; **p=0.008; °p=0.004; °°p=0.001). D. *p=0.002; **p=0.007; °p=0.003; °°p<0.0001). E. *p<0.0001; **p=0.009. Inserts: C-D. TRAP1 (C) and BRAF (D) expression evaluated by immunoblot in scramble (1) and siTRAP1 or siBRAF (2) HCT116 cells. E. Total cell lysates from HCT116 cells transfected with pMock or TRAP1 cDNA were separated by SDS–PAGE and immunoblotted with indicated antibodies.

Figure 6. TRAP1 and BRAF are co-expressed in human CRCs and TRAP1 inhibition results in a significant cytostatic effect on human BRAF V600E CRC cells. A. Total cell lysates from 10 human CRCs (T) and the respective non-infiltrated peritumoral mucosas (M) (5 TRAP1-positive and 5 TRAP1-negative tumors) were separated by SDS-PAGE and immunoblotted with indicated antibodies. B. Distribution of CRCs according to TRAP1 and BRAF protein levels. C. Total cell...
lysates from HT29 cells treated with indicated concentrations of AUY922 and HSP990 for 24h were separated by SDS–PAGE and immunoblotted with indicated antibodies. D. Total lysates from HT29 cells treated with 300 nM HSP990 for 30 and 60 min were quantified with an equal amount of each lysate immunoprecipitated with anti-BRAF antibodies. IPs were separated by SDS–PAGE and immunoblotted with indicated antibodies. Input: Total lysates from HT29 cells treated as described in D were separated by SDS–PAGE and immunoblotted with indicated antibodies. E. Total lysates from HCT116, COLO205 and HT29 cells treated for 24h with 70, 150 or 300nM HSP990 (upper panel) or AUY922 (lower panel) were separated by SDS–PAGE and immunoblotted with indicated antibodies. F. Cell cycle distribution in HT29 cells treated with indicated concentrations of AUY922 and HSP990 for 24h.
Table 1. Genes downregulated in shTRAP1 HCT116 cells and involved in cell cycle regulation as obtained from IPA and FIDEA analysis. Statistically significant genes with a cut-off value <1.5, identified by both bioinformatic platforms are reported.

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<tr>
<th>Entrez</th>
<th>HGNC gene symbols</th>
<th>Gene Description</th>
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<tr>
<td>79866</td>
<td>BORA</td>
<td>Bora, aurora kinase A activator</td>
<td>-2.13</td>
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<tr>
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<td>ID2</td>
<td>Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein</td>
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<td>7027</td>
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<td>Transcription factor Dp-1</td>
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<td>Cell division cycle 16 homolog (S. cerevisiae)</td>
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<tr>
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<td>PDZ binding kinase</td>
<td>-1.61</td>
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<tr>
<td>387893</td>
<td>SETD8</td>
<td>SET domain containing (lysine methyltransferase) 8</td>
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<td>10783</td>
<td>NEK6</td>
<td>NIMA (never in mitosis gene a)-related kinase 6</td>
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<tr>
<td>2621</td>
<td>GAS6</td>
<td>growth arrest-specific 6</td>
<td>-1.51</td>
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Figure 1

A. Western blot analysis of HSP90, BRAF, TRAP1, and GAPDH in HCT 116 cells treated with SCRAMBLE or shTRAP1.

B. Western blot analysis of HSP90, BRAF, TRAP1, and GAPDH in COLO320 cells treated with siTRAP1, siNeg, or HCT 116 cells treated with shTRAP1.

C. Western blot analysis of HSP90, BRAF, TRAP1, and GAPDH in HT29 cells treated with siTRAP1, siNeg, or MCF7 cells treated with shTRAP1.

D. Bar graph showing the relative units of TRAP1 and BRAF in HCT116, MCF7, and Colo320 cells treated with siTRAP1 or shTRAP1.
TRAP1 is involved in BRAF regulation and downstream attenuation of ERK phosphorylation and cell cycle progression: a novel target for BRAF-mutated colorectal tumors

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