Supplemental Information

The Mitochondrial Chaperone TRAP1

Promotes Neoplastic Growth

by Inhibiting Succinate Dehydrogenase

Marco Sciacovelli, Giulia Guzzo, Virginia Morello, Christian Frezza, Liang Zheng, Nazarena Nannini, Fiorella Calabrese, Gabriella Laudiero, Franca Esposito, Matteo Landriscina, Paola Defilippi, Paolo Bernardi, and Andrea Rasola
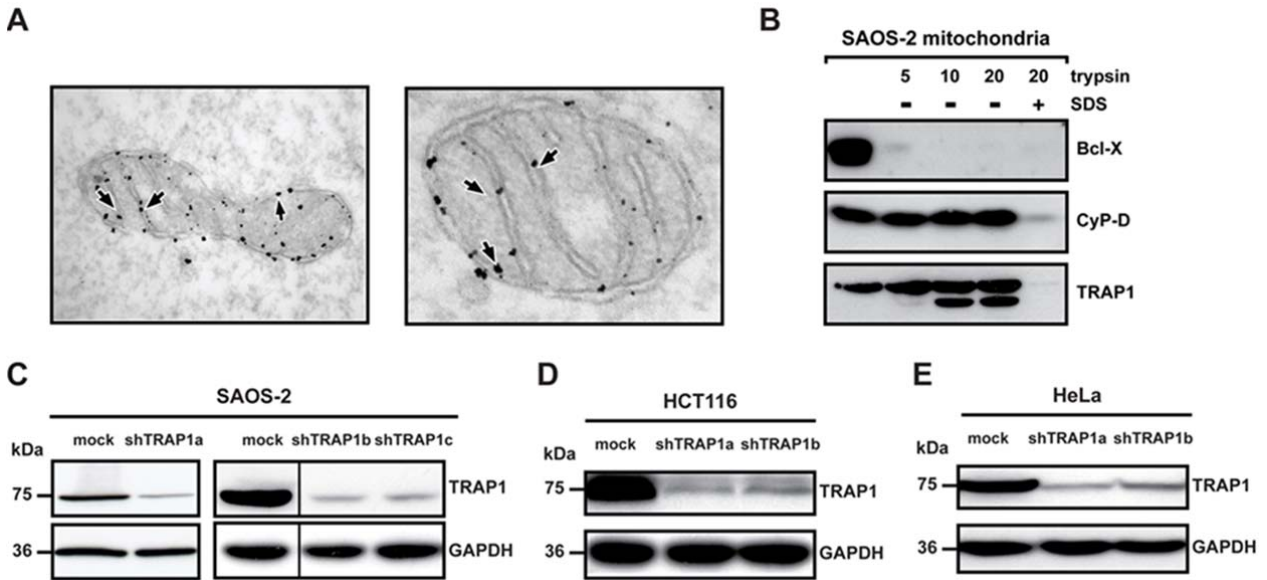
Figure S1. Mitochondrial Localization of TRAP1 and Inhibition of its Expression in Several Tumor Cell Types, Related to Figure 1

(A) Immuno-electron microscopy inspection shows most of TRAP1 along the inner mitochondrial membranes (arrows) of HCT116 colorectal carcinoma cells. (B) Trypsin treatment of isolated mitochondria from osteosarcoma SAOS-2 cells shows that TRAP1 is partially cleaved at the highest trypsin concentration, but it displays a pattern similar to that of the matrix protein cyclophilin D (CyP-D), thus indicating that most of TRAP1 is found in the internal mitochondrial compartments. Mitochondria (70 μg per point) were treated for 1 h with the reported quantities of trypsin (in micrograms) and analyzed by Western immunoblot. Where indicated, 0.1% SDS was added before trypsin to achieve complete digestion. Blots were probed for Bcl-X as a marker of the outer mitochondrial membrane and for CyP-D as a matrix marker. (C-E) Western immunoblot showing TRAP1 expression level in human osteosarcoma SAOS-2 cells (C), in human colorectal carcinoma HCT116 cells (D), and in human cervix carcinoma HeLa cells (E) stably transfected with a scrambled shRNA (mock) or with different TRAP1 shRNAs dubbed shTRAP1a, shTRAP1b and shTRAP1c. GAPDH is shown as a loading control.
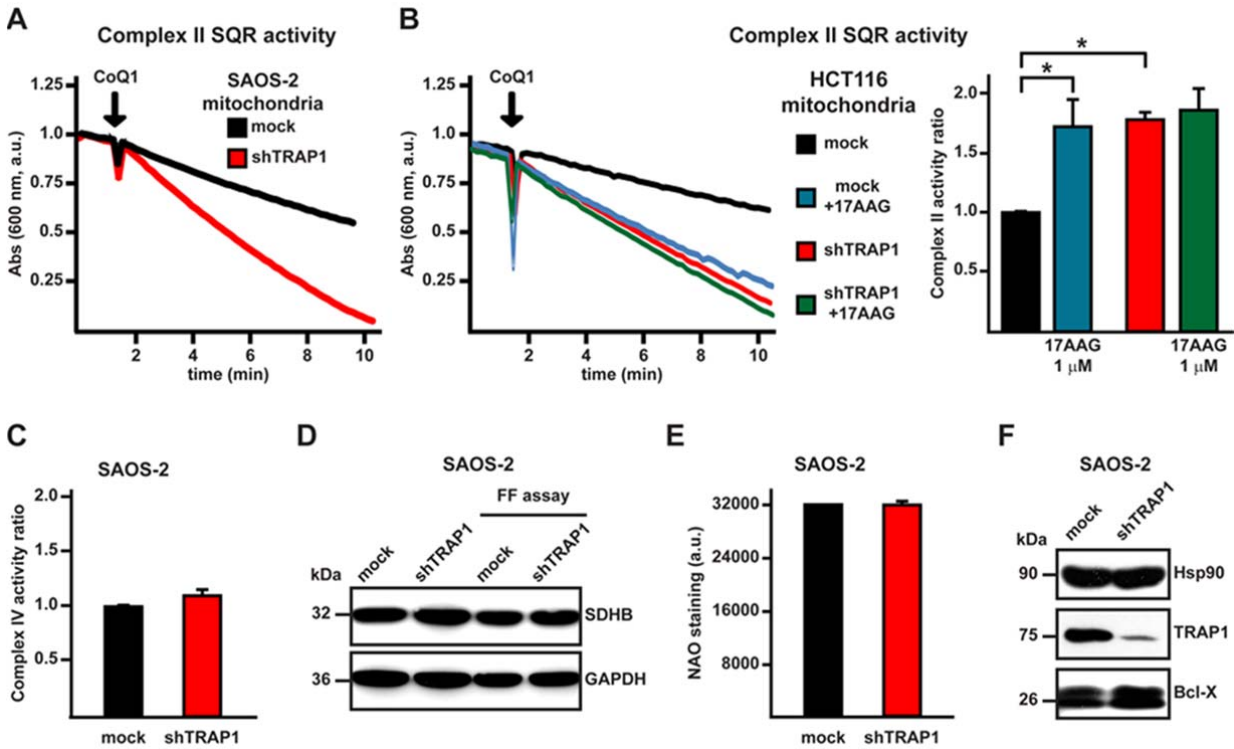
Figure S2. TRAP1 Expression Inhibits ETC Complex II without Affecting ETC Complex IV Activity, SDH Protein Expression Level, or Mitochondrial Mass, Related to Figure 4

(A, B), analysis of the succinate:coenzyme Q reductase (SQR) enzymatic activity of complex II. In (A), representative spectrophotometric traces performed on SAOS-2 mitochondria are reported. In (B), analysis of the succinate:coenzyme Q reductase (SQR) enzymatic activity of complex II on HCT116 mitochondria. In the left part, representative spectrophotometric traces are reported; in the right part, analysis was performed as in Figure 3D. The TRAP1 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) was added 5 min before starting recordings. In (C), spectrophotometric analysis of the COX enzymatic activity was performed on SAOS-2 cells. Mock indicates SAOS-2 cells transfected with a scrambled shRNA; shTRAP1 indicates SAOS-2 cells transfected with a TRAP1 shRNA. (D) Western immunoblot showing that no changes in SDHB protein expression occur with or without TRAP1 and during the focus-forming assay (FF samples were obtained at the 15th experimental day). GAPDH was used as a loading control. (E) N-acrydine

orange (NAO) cytofluorimetric analyses indicate that no change in mitochondrial mass occur between SAOS-2 mock and shTRAP1 cells. (F) Western immunoblot comparing Hsp90 and TRAP1 expression level in mock and shTRAP1 SAOS-2 cells. As Hsp90 protein levels were identical in mock and shTRAP1 cells, the selective effect of 17-AAG on mock cells can be reasonably ascribed to its TRAP1 inhibition. Blots were probed with an anti-Bcl-X antibody to check for mitochondrial protein load. All along the Figure, bar graphs report mean±SD values (n≥3). Asterisks indicate a significant difference (p<0.01 with a Student's *t* test analysis). Cells are dubbed as in previous figures.
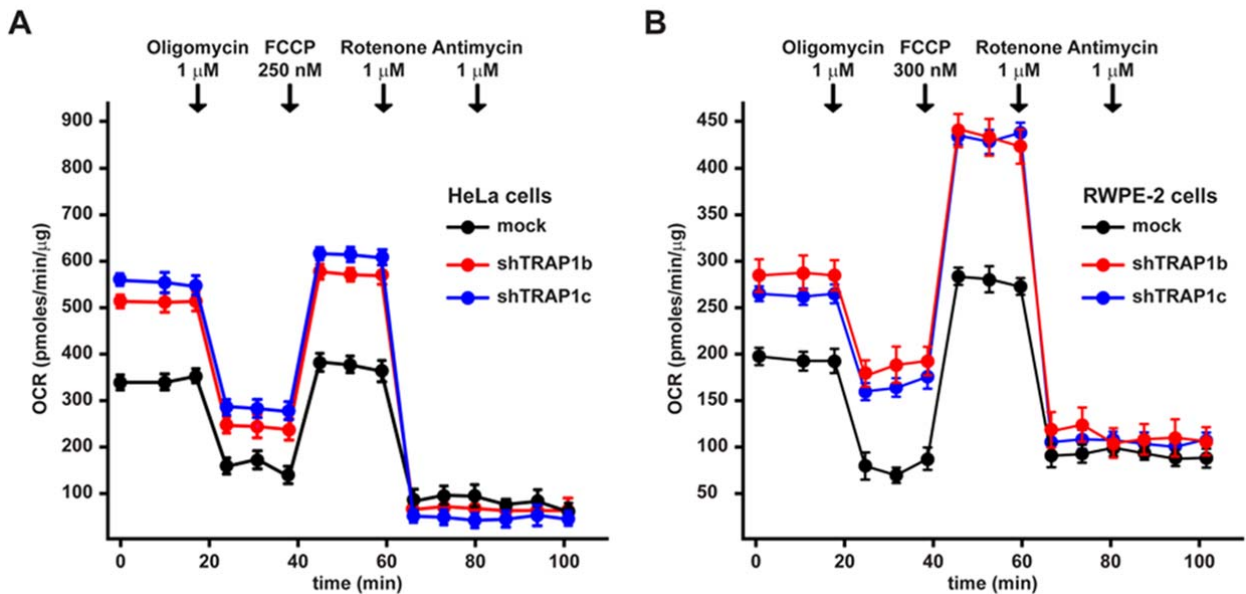

Figure S3. TRAP1 Decreases Cell Oxygen Consumption Rate in Cancer Cell Models, Related to Figure 5

(A, B) Representative traces of oxygen consumption rate (OCR) experiments performed on monolayers of living human cervix carcinoma HeLa cells (A), or of human epithelial prostate RWPE-2 cells transformed by *v*-Ki-Ras expression (B). Subsequent additions of the ATP synthase inhibitor oligomycin, of the uncoupler FCCP, of the ETC complex I inhibitor rotenone and of the ETC complex III inhibitor antimycin A were carried out. Mock indicates cells stably transfected with a scrambled shRNA; shTRAP1 indicates cells stably transfected with a TRAP1 shRNA.
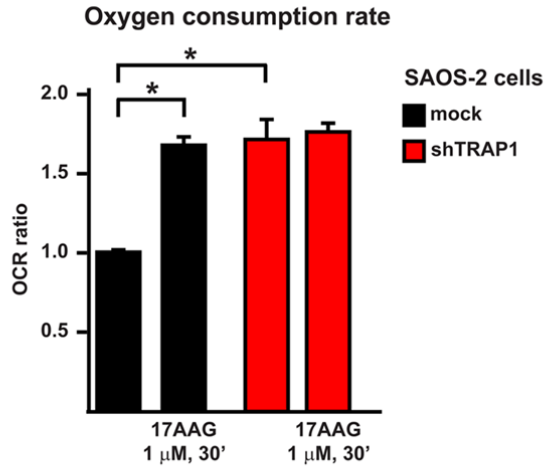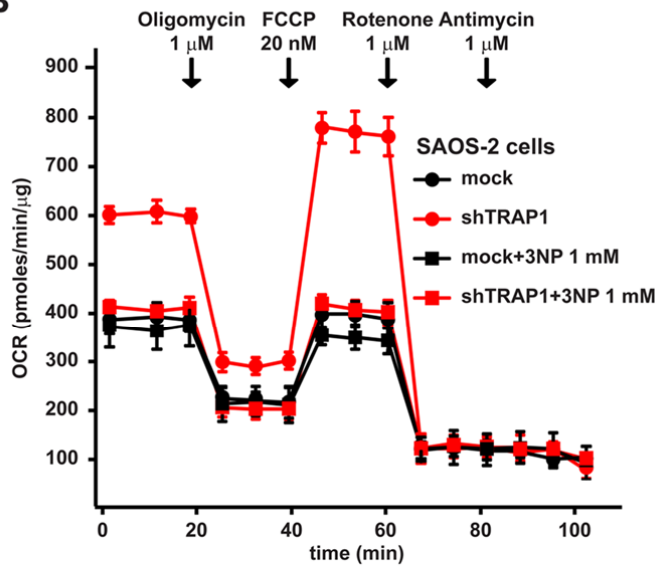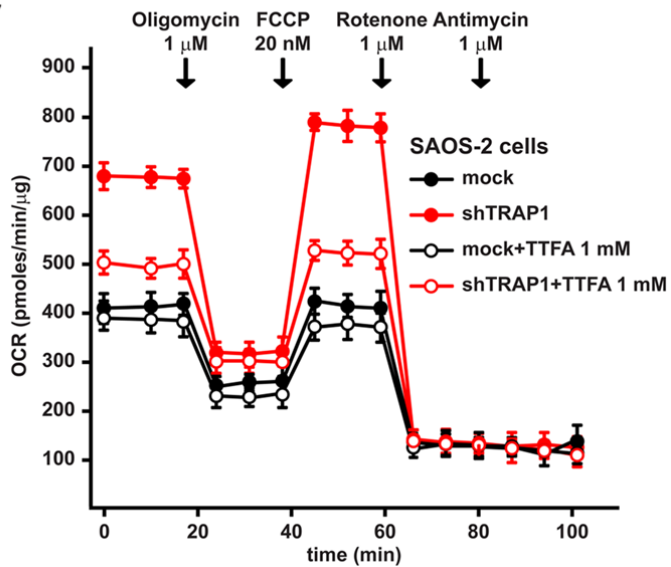
A

Oxygen consumption rate

B

C

Figure S4. The OCR Is Increased by TRAP1 Inhibition in Mock Cells and Downregulated by SDH Inhibition in shTRAP1 Cells, Related to Figure 5

(A) Effect of the TRAP1 inhibitor 17-AAG on the OCR of SAOS-2 cells before oligomycin addition; data are shown as fold increase with respect to untreated mock cells. 17-AAG was pre-incubated within cell monolayers for 30 minutes. (B, C) Representative traces of OCR experiments. Subsequent additions of the ATP synthase inhibitor oligomycin, of the uncoupler FCCP, of the ETC complex I inhibitor rotenone and of the ETC complex III inhibitor antimycin A were carried out. The SDH inhibitors 3-nitro propionic acid (3-NP; B) and thenoyltrifluoroacetone (TTFA; C) were added to cell monolayers immediately before starting the experiments.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

Human SAOS-2 osteosarcoma cells, human HCT-116 colorectal carcinoma cells, human HeLa cervix carcinoma cells, and MEF cells obtained from C57BL/6J mice through SV40-immortalization were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2%-10% fetal bovine serum (Invitrogen); RWPE prostate epithelial cells were grown in keratinocyte medium (Gibco) supplemented with EGF and bovine pituitary extract; 100 units/ml penicillin and 100 μg/ml streptomycin were added to all media, and cells were kept in a humidified atmosphere of 5% CO_2/95% air at 37°C. Hypoxic treatments were achieved by incubating cells in an InVivo2 300 hypoxic chamber (Ruskinn Technology). TRAP1 stable interference of human cells was achieved by transfecting cells with a panel of TRAP1 shRNAs from Sigma: a) CCGGCAGAGCACTCACCCTACTATGCTCGAGCATAGTAGGGTGAGTGCTCTGTTTTTG;

b) CCGGGCGCTCATCAAGAAGCTGAATCTCGAGATTCAGCTTCTTGATGAGCGCTTTTTG;

c) CCGGTGGTTCTGGAGTGTTTGAAATCTCGAGATTTCAAACACTCCAGAACCATTTTTG.

In order to re-express TRAP1 in interfered cells in a shRNA-insensitive way, shTRAP1 cells obtained with sequence b), which is highly divergent between human and mouse genes, were transfected with a mouse TRAP1 cDNA (Origene). HIF1α stable interference was achieved with the following HIF1α shRNAs from Sigma: CCGGCCAGTTATGATTGTGAAGTTACTCGAGTAACTTCACAATCATAACTGGTTTTT;

CCGGTGCTCTTTGTGGTTGGATCTACTCGAGTAGATCCAACCACAAAGAGCATTTT;

HIF1β interference was achieved with the following shRNAs from Sigma:

CCGGGCCTACACTCTCCAACACAATCTCGAGATTGTGTTGGAGAGTGTAGGCTTTT;

CCGGCCTTTGTCTTTCTGTGTACTTCTCGAGAAGTACACAGAAAGACAAAGGTTTTT;

CCGGCATTGTCCAGAGGGCTATTAACTCGAGTTAATAGCCCTCTGGACAATGTTTTT;

scrambled shRNAs were always used as negative controls. Stable interfered cells were selected in 0.8 µg/ml puromycin (Sigma). TRAP1 mutant lacking the mitochondrial import sequence (ΔN TRAP1) was a Δ1–59-Myc construct generated as in (Amoroso et al., 2012). MEF cells were transfected with Lipofectamine 2000 (Invitrogen) and selected in G418 (0.5 mg/ml; Sigma) for the expression of TRAP1 cDNA (cloned in a pCMV6 vector, Origene). The rate of cell growth was measured with a Scepter^{TM} cell counter (Millipore).

Tissue Samples

Specimens from both tumor and normal, non-infiltrated peritumoral mucosa were obtained from patients with colorectal carcinoma during surgical cancer removal, after an expressed written informed consent to use biological specimens for investigational procedures was obtained from all patients. Samples were cut into 125 mm^3 pieces and one specimen was fixed in formalin to confirm the histopathological diagnosis, while the others were frozen in liquid nitrogen for further analyses. TRAP1 expression was evaluated by Western immunoblot followed by densitometric analysis, and its level, which was considered as induced when the ratio between tumor and non-infiltrated surrounding mucosa was ≥3 (Costantino et al., 2009), increased in all samples from metastatic neoplasias and in the majority of initial, non-metastatic tumors . For ETC complex II evaluations, at least three samples with increased TRAP1 expression and three samples without any TRAP1 expression changes were analyzed.

In Vitro Tumorigenesis Assays

For the focus-forming assay, 10^6 cells were plated in 10 cm Petri dishes (BD Falcon) in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal bovine serum (Gibco). When cells reached sub-confluence, serum concentration was decreased to values ranging between 0.5%

and 2%, which did not induce cell death *per se* (data not shown), and changed every 4th day. Mitochondria utilized for the determination of ETC complex enzymatic activities or cell lysates used for Western immunoblot assays were obtained at the 15th day after serum decrease, *i.e.* 1-2 days before cells that did not form foci started a massive death process (see below for sample preparation). 25 days after serum decrease foci appeared as thick masses and cords of cells. Plates were washed in PBS, fixed in methanol for 30 min and foci colored with GIEMSA solution for 1 h. After washing in deionized water, size and number of foci were analyzed with an Image Analyzer custom software (Rasola et al., 2007). For the soft agar assay, cells were grown in 6 cm Petri dishes covered by a bottom layer composed by DMEM medium mixed with low melting point agarose (Promega) at a final concentration of 1.0%, and by a top layer of DMEM medium supplemented with 0.5% serum and mixed with low melting point agarose at a final concentration of 0.6%. Cells ($3x10^5$) were added during the preparation of the upper layer, where they remained embedded. Dishes were then maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C for three weeks, adding medium (DMEM 0.5% serum) on the top of the two layers every 7th day. At the 25th day, dishes were washed in PBS and colonies were stained with Crystal Violet 0.005% and analyzed with Image Analyzer software.

In Vivo Tumorigenesis Assays

Experiments were performed in 5-week-old female CD1 nude mice (Charles River Laboratories) treated in accordance with the European Community guidelines. Twelve mice were injected subcutaneously bilaterally in the flanks with $1.5x10^7$ SAOS-2 mock or shTRAP1 cells in 200 μl of serum-free sterile PBS. In a subset of animals, tumor growth was favored by injecting cells in PBS mixed with Matrigel in a 1:1 ratio. Tumor growth was evaluated on alternate days by calliper measuring. After three weeks, mice were sacrificed and tumors stored at -80°C or fixed in formaldehyde and maintained in 70% ethanol for immunohistochemical analyses.

Cytofluorimetric Analyses

Flow cytometry recordings were performed to determine cell death, as described (Fassetta et al., 2006; Gramaglia et al., 2004), and mitochondrial mass. Briefly, cells were incubated at 37°C in 135 mM NaCl, 10 mM HEPES, 5 mM $CaCl_2$ with FITC-conjugated Annexin-V (Boehringer Mannheim) and propidium iodide (PI, 1 μg/ml; Sigma), to detect phosphatidyl-serine exposure on the cell surface (increased FITC-conjugated Annexin-V staining) and loss of plasma membrane integrity (PI permeability and staining). N-acrydine orange (NAO 200 nM, Invitrogen), which binds to cardiolipin in mitochondrial membranes, was utilized to evaluate mitochondrial mass. Samples were analyzed on a FACS Canto II flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using FACSDiva software.

Mitochondria Purification and Quantification

Mitochondria were isolated after cell disruption with a glass-Teflon or electrical potter (Sigma) in a buffer composed by 250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA-Tris, pH 7.4. Nuclei and plasma membrane fractions were separated by a first mild centrifugation (700g, 10 min); mitochondria were then spinned down at 7000g, 10 min, and washed twice (7000g, 10 min each). All procedures were carried out at 4°C. In order to define submitochondrial protein localization, isolated mitochondria were digested with trypsin at different concentrations at 4°C for 1h. Where indicated, 0.1% SDS was added before trypsin. Trypsin was then inactivated with a protease inhibitor cocktail (Sigma).

Western Immunoblots, Immunoprecipitations and Crosslinkings

For Western immunoblots analyses, cells kept in standard culture conditions or in focus-forming assays were lysed at 4°C in a buffer composed by 140 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10% glycerol, 1% Triton X-100, in the presence of phosphatase and protease inhibitors

(Sigma). Lysates were then cleared with a centrifugation at 13000g for 30 min at 4°C, and proteins were quantified using a BCA Protein Assay Kit (Thermo Scientific-Pierce).

Protein immunoprecipitations were carried out on 3 mg of total cellular extracts. Lysates were pre-cleared with an incubation with protein A-Sepharose (Sigma) for 1 h at 4°C and then incubated in agitation for 18 h at 4°C with the antibody conjugated to fresh protein A-Sepharose beads. Where indicated, an anti mouse IgG was added as a negative isotype control. Beads were then washed several times in lysis buffer.

For the crosslinking assays, isolated mitochondria were suspended in PBS buffer and incubated with dimethyl 3,3′-dithiobis-propionimidate (DTBP, Sigma, 1 mM), a membrane-permeable, homo-bifunctional reagent that reacts with the primary amines of two interacting proteins at an average distance of about 8 Å (Giorgio et al., 2009), for 15 minutes at room temperature and then spinned at 7000 rcf for 5 minutes. Pellet was then lysed as above, and lysates were ultracentrifuged at 100000 rcf for 25 minutes at 4°C prior to TRAP1 immunoprecipitation.

Proteins extracted from total cell lysates or from immunoprecipitations were then boiled for 5 min in Laemmli sample buffer, separated in reducing conditions on SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham) following standard methods. Primary antibodies were incubated 16 h at 4°C, and horseradish peroxidase-conjugated secondary antibodies were added for 1 h. Proteins were visualized by enhanced chemiluminescence (Millipore). Anti human TRAP1 (sc-13557), anti SDHA (sc-166947), anti SDHB (sc-59688) mouse monoclonal antibodies and anti actin (sc-1615) goat polyclonal antibody were all from Santa Cruz; anti HIF1α (610959) and anti human and mouse TRAP1 (612344) mouse monoclonal antibodies were from Becton Dickinson; anti GAPDH (MAB374) mouse monoclonal antibody was from Chemicon; anti caspase-3 (8G10), rabbit monoclonal antibody, anti Bcl-X (54H6) and anti Hydroxy-HIF1α (Pro564; D43B5) rabbit polyclonal antibodies were from Cell Signaling; anti Hydroxy-HIF1α (Pro402; 07-1585) rabbit polyclonal antibody was from Millipore; anti Cyp-D (AP1035)

mouse monoclonal antibody was from Calbiochem; the anti mouse IgG was from Thermofisher; anti COXII (12C4F12) mouse monoclonal antibody was from MitoSciences (Total OXPHOS Human Antibody Cocktail, MS601); anti Hsp90 (610418) mouse monoclonal antibody was from BD Transduction Laboratories; mouse monoclonal anti Complex II imunocapture antibody was from MitoSciences; mouse monoclonal anti HIF1β antibody (ab2771) was from Abcam; mouse monoclonal anti HIF2α antibody (NB100-132) was from Novus Biologicals; mouse monoclonal anti pimonidazole-protein adducts (HypoxyprobeTM-1 Mab1) was from Chemicon.

Blue Native PAGE

BN-PAGE experiments were performed on mitochondria isolated as described. ETC complexes and super-complexes were extracted from 200 μg of mitochondria in a buffer composed by 1M aminocaproic acid, 50 mM Bis Tris pH 7, in the presence of 1% digitonin at 4°C for 2 min. After extraction mitochondria were spinned at 100000 *rcf* for 30 min and supernatants were collected and loaded on polyacrylamide 3-12% Bis Tris pre-cast gradient gels (Invitrogen) after addition of sample buffer added of G250 (Invitrogen). Bands were then visualized with a 18 h Coomassie Blue staining. Bands corresponding to ETC complex II and IV were cut and run on a normal SDS PAGE, in order to separate single protein components and to identify them by Western immunoblotting. To perform immunoblotting directly on the BN-PAGE, this was transferred using a NuPAGE transfer buffer (Invitrogen) and then treated following standard procedures.

ETC Complex II and IV Activity Assays

To measure the enzymatic activity of respiratory chain complex II, cells or biopsies were homogenized with an electric potter (Sigma) in a buffer composed by 250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA-Tris, pH 7.4, Percoll 10%, protease and phosphatase inhibitors and mitochondria isolated as described above. Mitochondrial enriched fractions (40 μg per trace) were

used for spectrophotometric recordings (600 nm, 30°C) of the reduction of 2-6 dichlorophenolindophenol (DCPIP). Mitochondria are preincubated for 10 min at 30°C in a buffer composed by potassium phosphate 25 mM pH 7.2, sodium succinate 20 mM, alamethicin 5 μM. After the pre-incubation time, sodium azide (500 μM), antimycin A (1 μM), rotenone (1 μM) and DCPIP (50 μM) were added for 1 min to the medium. Reaction started after the addition of an intermediate electron acceptor (Coenzyme Q1, 6.5 μM). Each measurement of ETC complex II activity was normalized for citrate synthase (CS) activity. To measure CS activity, citrate formation is determined with a spectrophotometer as an increase in absorbance at 420 nm, 37°C. Reaction buffer was composed by 100 mM Tris-HCl pH 8, 100 μM DTNB, 300 μM Acetyl -CoA, 500 μM Oxaloacetate.

To measure the enzymatic activity of respiratory chain Complex IV, cells were subjected to three cycles of freezing and thawing in liquid nitrogen. These cellular homogenates were used for spectrophotometric recordings (550 nm, 37°C) of the oxidation of reduced cytochrome c, (ε=18.5 mM^{-1} cm^{-1}) in a buffer composed by potassium phosphate 100 mM pH 7, water and lauryl-maltoside 10% p/v. The reaction started by the addition of cellular homogenates and the decrease in absorbance was followed for 3 min. Reduced cytochrome c was prepared immediately before use by adding a few grains of sodium dithionite. Each measurement of ETC complex IV activity was normalized for protein amount and for CS activity, as above.

Oxygen Consumption Rate Experiments

The rate of oxygen consumption was assessed in real-time with the XF24 Extracellular Flux Analyzer (Seahorse Biosciences), which allows to measure OCR changes after up to four sequential additions of compounds. Cells (5x10^4/well) were plated the day before the experiment in a DMEM/10% serum medium; experiments were carried out on confluent monolayers. Before starting measurements, cells were placed in a running DMEM medium (supplemented with 25 mM

glucose, 2mM glutamine, 1mM sodium Pyruvate, and without serum) and pre-incubated for 1h at 37°C in atmospheric CO_2. OCR values were then normalized for the protein content of each sample. An accurate titration with the uncoupler FCCP was performed for each cell type, in order to utilize the FCCP concentration (20-300 nM, depending on the cell type) that maximally increases OCR. To exclude that OCR differences between mock and shTRAP1 cells were caused by changes in succinate availability inside mitochondria, the cell-permeable analogue methyl-succinate was used, and no appreciable difference was recorded (not shown).

Immunohistochemical Analyses

Four thick serial sections of paraffin-embedded tumor samples were stained with haematoxylin-eosin (H&E) and Azan-Mallory. For immunohistochemistry analysis with TRAP1, HIF1α and MIB1/Ki67, briefly after dewaxing and hydration, sections were incubated in EDTA buffer at pH 8.0 or in citrate buffer 5 mM at pH 6.0, respectively, in a microwave oven for antigen retrieval. Afterward, sections were treated with ULTRA V Block for 5 min (Thermo Scientific) and incubated for 60 min with the primary antibody (anti-HIF1α monoclonal antibody, 1:50, was from BD Biosciences and anti- MIB-1/Ki67 GTX73546 monoclonal antibody, 1:100, was from GeneTex). Sections were subsequently incubated with rabbit Primary Antibody Amplifier Quanto for 10 min (Thermo Scientific). Then, after the buffer wash step, sections were treated with HRP Polymer Quanto for 10 min. Immunoreactivity was visualized with 3-30-diaminobenzidine (DAKO).

Immunoelectron Microscopy Analyses

To perform immuno-electron microscopy experiments, cells were fixed with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde, antibody-labeled using the gold-enhanced protocol, embedded in Epon-812, and cut. EM images were acquired from thin sections using a FEI Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (FEI).

Determination of Intracellular Succinate Level

To determine the intracellular succinate level, cells were first washed three times in PBS at room temperature and then quickly scraped in a buffer composed by 30% acetonitrile, 50% methanol and 20% water on ice. Lysates were collected and frozen in a cold solution with methanol and dry ice for 5 min in order to favor the metabolic quenching needed for metabolites extraction. The insoluble material was immediately spun down in a cooled centrifuge at 16000 g for 15 min at 0°C and the supernatant was collected for subsequent analysis. Metabolites were separated using a liquid chromatography (LC) system and analyzed by mass spectrometry (MS). A ZIC-HILIC column (4.6 mm×150 mm, guard column 4.6 mm×10 mm, Merck, Germany) was used for LC separation using formic acid, water acetonitrile as component of the mobile phase. Results were normalized to protein concentration, measured from parallel cell culture using BCA kit as described above.

Intracellular ATP Determination

Intracellular ATP was quantified by the luciferin/luciferase method using the ATP determination kit by Invitrogen/Molecular Probes following manufacturer's instructions. Cells were kept for two hours in the different experimental conditions, washed in PBS, lysed in boiling water to avoid ATPase activity. 2.5 μg of proteins were analyzed in a 100 μl final volume. Each experiment was performed in triplicate.

SUPPLEMENTAL REFERENCES

Amoroso, M.R., Matassa, D.S., Laudiero, G., Egorova, A.V., Polishchuk, R.S., Maddalena, F., Piscazzi, A., Paladino, S., Sarnataro, D., Garbi, C., *et al.* (2012). TRAP1 and the proteasome regulatory particle TBP7/Rpt3 interact in the endoplasmic reticulum and control cellular ubiquitination of specific mitochondrial proteins. Cell Death Differ. *19*, 592-604.

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

Fassetta, M., D'Alessandro, L., Coltella, N., Di Renzo, M.F., and Rasola, A. (2006). Hepatocyte growth factor installs a survival platform for colorectal cancer cell invasive growth and overcomes p38 MAPK-mediated apoptosis. Cell. Signal. *18*, 1967-1976.

Giorgio, V., Bisetto, E., Soriano, M.E., Dabbeni-Sala, F., Basso, E., Petronilli, V., Forte, M.A., Bernardi, P., and Lippe, G. (2009). Cyclophilin D modulates mitochondrial F0F1-ATP synthase by interacting with the lateral stalk of the complex. J. Biol. Chem. *284*, 33982-33988.

Gramaglia, D., Gentile, A., Battaglia, M., Ranzato, L., Petronilli, V., Fassetta, M., Bernardi, P., and Rasola, A. (2004). Apoptosis to necrosis switching downstream of apoptosome formation requires inhibition of both glycolysis and oxidative phosphorylation in a BCL-X(L)- and PKB/AKT-independent fashion. Cell Death Differ. *11*, 342-353.

Rasola, A., Fassetta, M., De Bacco, F., D'Alessandro, L., Gramaglia, D., Di Renzo, M.F., and Comoglio, P.M. (2007). A positive feedback loop between hepatocyte growth factor receptor and beta-catenin sustains colorectal cancer cell invasive growth. Oncogene *26*, 1078-1087.