Molecular chaperone TRAP1 regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis

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Abstract

TRAP1 (TNF receptor-associated protein), a member of the HSP90 chaperone family, is found predominantly in mitochondria. TRAP1 is broadly considered to be an anticancer molecular target. However, current inhibitors cannot distinguish between HSP90 and TRAP1, making their utility as probes of TRAP1-specific function questionable. Some cancers express less TRAP1 than do their normal tissue counterparts, suggesting that TRAP1 function in mitochondria of normal and transformed cells is more complex than previously appreciated. We have used TRAP1-null cells and transient TRAP1 silencing/overexpression to show that TRAP1 regulates a metabolic switch between oxidative phosphorylation and aerobic glycolysis in immortalized mouse fibroblasts and in human tumor cells. TRAP1-deficiency promotes an increase in mitochondrial respiration and fatty acid oxidation, and in cellular accumulation of tricarboxylic acid cycle intermediates, ATP and reactive oxygen species. At the same time, glucose metabolism is suppressed. TRAP1-deficient cells also display strikingly enhanced invasiveness. TRAP1 interaction with and regulation of [...]
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TRAP1 (TNF receptor-associated protein), a member of the HSP90 chaperone family, is found predominantly in mitochondria. TRAP1 is broadly considered to be an anticancer molecular target. However, current inhibitors cannot distinguish between HSP90 and TRAP1, making their utility as probes of TRAP1-specific function questionable. Some cancers express less TRAP1 than do their normal tissue counterparts, suggesting that TRAP1 function in mitochondria of normal and transformed cells is more complex than previously appreciated. We have used TRAP1-null cells and transient TRAP1 silencing/overexpression to show that TRAP1 generates a metabolic switch between oxidative phosphorylation and aerobic glycolysis in immortalized mouse fibroblasts and in human tumor cells. TRAP1-deficiency promotes an increase in mitochondrial respiration and fatty acid oxidation, and in cellular accumulation of tricarboxylic acid cycle intermediates, ATP and reactive oxygen species. At the same time, glucose metabolism is suppressed. TRAP1-deficient cells also display strikingly enhanced invasiveness. TRAP1 interaction with and regulation of mitochondrial c-Src provide a mechanistic basis for these phenotypes. Taken together with the observation that TRAP1 expression is inversely correlated with tumor grade in several cancers, these data suggest that, in some settings, this mitochondrial molecular chaperone may act as a tumor suppressor.

Molecular chaperones help to maintain cellular homeostasis. The heat-shock protein 90 (HSP90) family of molecular chaperones is highly conserved from bacteria to mammals. HSP90 itself is an essential molecular chaperone found in the cytoplasm and nucleus of all eukaryotic cells (1, 2). In multicellular eukaryotes, the HSP90 family includes the mitochondrial chaperone TRAP1 (TNF receptor-associated protein), which shares 50% sequence similarity with HSP90. Although TRAP1 binds and hydrolyzes ATP in an analogous manner to HSP90 (3), its cellular function is less well understood. Thus, although many HSP90-dependent proteins (“clients”) and interacting cochaperones have been described (www.picard.ch/downloads/Hsp90interactors.pdf), the validated list of TRAP1-dependent clients is quite small and TRAP1-interacting cochaperones, if they exist, have yet to be identified (4).

Several studies have suggested that TRAP1 plays a cytoprotective role by buffering reactive oxygen species (ROS)-mediated oxidative stress (5, 6), and others have reported that TRAP1 overexpression attenuates ROS production (7). The antioxidant properties of TRAP1, together with its reported ability to regulate opening of the mitochondrial permeability transition pore (8, 9), may contribute to its antiapoptotic activity (4). For these reasons, TRAP1 has been proposed as an anticancer molecular target, and first-generation inhibitors have shown some anticancer activity in preclinical models (10). However, these inhibitors do not distinguish between HSP90 and TRAP1 (11), and TRAP1 expression in cancer is variable but HSP90 comprises as much as 5% of a cancer cell’s protein complement (12). Indeed, some cancers express less TRAP1 than do their normal tissue counterparts (13). Thus, the functions of TRAP1 in mitochondria of normal and transformed cells are likely more complex than previously appreciated and, in the absence of TRAP1-specific inhibitors, other approaches are necessary to investigate TRAP1-specific cellular effects.

In this study, we have explored the metabolic and phenotypic consequences of TRAP1 gene disruption/knockdown and overexpression in fibroblast cell lines established from adult WT and TRAP1-null mice, and in human tumor cells transiently transfected with either TRAP1-specific siRNA or TRAP1 expression plasmids. We show that loss of TRAP1 results in increased mitochondrial oxygen consumption, elevated levels of tricarboxylic acid (TCA) cycle intermediates, and increased steady-state ATP and ROS levels, with concomitant suppression of aerobic glycolysis, but overexpression of TRAP1 has the opposite effect. Absence of c-Src expression abrogates the ability of TRAP1 to modulate mitochondrial respiration and ATP level, and TRAP1 and c-Src colocalize and interact within mitochondria. Our data suggest that, in some settings, this mitochondrial molecular chaperone may act as a tumor suppressor.

Significance

TNF receptor-associated protein (TRAP1) is found predominantly in mitochondria. A possible direct impact of TRAP1 on mitochondrial metabolism remains unexplored. We used TRAP1-null cells and transient TRAP1 silencing/overexpression to show that TRAP1 regulates a metabolic switch between oxidative phosphorylation and aerobic glycolysis in immortalized mouse fibroblasts and in human tumor cells. TRAP1 deficiency promotes increased mitochondrial respiration, fatty acid oxidation, tricarboxylic acid cycle intermediates, ATP and reactive oxygen species, while concomitantly suppressing glucose metabolism. TRAP1 deficiency also results in strikingly enhanced cell motility and invasiveness. TRAP1 interaction with and regulation of mitochondrial c-Src provide a mechanistic basis for these phenotypes.


The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1220659110/-/DCSupplemental.
are thus consistent with a model in which TRAP1 regulates the previously reported ability of mitochondrial c-Src to stimulate oxidative phosphorylation (14, 15). Reduced/absent TRAP1 expression also correlates with increased cell motility/invasiveness that is sensitive to c-Src inhibition and ROS buffering strategies. These findings highlight a previously unrecognized physiological role for TRAP1 in regulating the metabolic balance between oxidative phosphorylation and aerobic glycolysis, and they support an indirect role for TRAP1 in suppressing c-Src- and ROS-dependent cell invasion.

**Results**

**TRAP1 Deficiency Is Associated with Increased Mitochondrial Respiration and Decreased Glycolysis.** We established fibroblast cell lines (termed MAFs, murine adult fibroblasts) from adult TRAP1KO/– (null, hereafter referred to as KO) and WT mice to explore the metabolic consequences of TRAP1 knockout. We confirmed the absence of TRAP1 protein expression in two independently derived MAF cell lines (Fig. 1A). Because TRAP1 is primarily localized to mitochondria, we first asked whether TRAP1 knock-out affects mitochondrial mass. Using flow cytometric analysis of Mitotracker Green-labeled cells, we saw no increase in signal in KO cells compared with WT (Fig. 1A). In addition, assessment of mitochondrial membrane potential revealed no difference between WT and TRAP1 KO MAFs (Fig. 1B).

To assess the possible impact of TRAP1 loss on mitochondrial function, we measured mitochondrial respiration by determining the cellular oxygen consumption rate (OCR) in WT and TRAP1 KO cells using an extracellular flux analyzer. KO cells displayed a higher basal OCR and a significantly higher maximum respiratory capacity [e.g., OCR determined after treatment with the ATP synthase inhibitor oligomycin and the chemical uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP); see Methods] compared with WT, and this was accompanied by significantly decreased glycolysis (extracellular acidification rate, ECAR, is an indicator of glycolysis; see Methods) (Fig. 1B). Importantly, oxygen consumption in KO cells was reduced to the level of WT upon stable reintroduction of exogenous TRAP1, supporting the likelihood that the metabolic disparity between TRAP1 WT and KO cells is TRAP1-dependent (Fig. S1C). To confirm these data from intact cells, we assessed the basal OCR of mitochondrial preparations isolated from cells grown in DMEM (containing 4.5 g/L glucose and Glutamax; see Methods). After normalization to total mitochondrial protein, the OCR of mitochondria prepared from KO cells was significantly increased compared with the OCR of mitochondria prepared from WT cells (Fig. 1C). These data support the hypothesis that the mitochondria of TRAP1 KO cells consume a significantly greater amount of oxygen compared with WT, suggesting a preference for oxidative phosphorylation over aerobic glycolysis.

In agreement with this possibility, metabolomic analysis of WT and TRAP1 KO cell lines revealed marked reduction in the steady-state levels of several glycolytic metabolites, but the levels of several TCA cycle metabolites (e.g., α-ketoglutarate and citrate) were increased (Fig. 1D). Also increased was the anaplerotic substrate propionylcarnitine, a metabolic precursor of propionyl CoA, which in turn is converted to oxaloacetate to replenish TCA cycle intermediates (16–19). Fatty acid oxidation is also significantly increased in TRAP1 KO MAFs compared with WT cells (Fig. 1E), as is the NAD+/NADH ratio (2.5-fold higher in TRAP1 KO vs. WT cells, P = 0.02), consistent with increased oxygen consumption and suggesting increased metabolic flux through the TCA cycle of TRAP1 KO cells that is independent of glucose metabolism (Fig. 1D and Fig. S1D).

**ATP Levels and Mitochondrial Complex IV Activity Are Elevated in TRAP1-Deficient Cells.** Because mitochondrial respiration is a more efficient generator of ATP than is glycolysis, we asked whether TRAP1 KO cells produce more ATP compared with WT cells (Fig. 1F). Indeed, steady-state ATP levels in both TRAP1 KO cell lines were significantly higher than in either WT cell line. Metabolomic analysis independently confirmed the increased steady-state ATP concentration in TRAP1 KO cells (Fig. 1D). To determine whether changes in ATP production in the KO cells are a consequence of TRAP1 deficiency, we reexpressed TRAP1 (GFP-tagged) in KO cells. The mean ATP level of the transfected population (estimated transfection efficiency of 60–70% based on GFP expression) was significantly reduced compared with that of empty (GFP) plasmid-transfected TRAP1 KO cells (Fig. 1G).

Mitochondrial complex IV (cytochrome c oxidase) is the last enzyme in the respiratory electron transport chain, converting molecular oxygen to water while providing the electrochemical potential used by ATP synthase (complex V) to produce ATP. We examined whether complex IV enzymatic activity was increased in TRAP1 KO cells compared with WT, and found that both independently derived TRAP1 KO MAF cell lines displayed elevated complex IV activity compared with WT cells (Fig. S1E). Based on the slopes of the absorbance curves (WT = 0.373, KO1 = 0.906, KO2 = 0.657), complex IV activity is increased approximately twofold in TRAP1 KO cells compared with WT. These data are consistent with the hypothesis that loss of TRAP1 expression deregulates mitochondrial respiration and ATP production.

We extended these results by investigating the impact of transient TRAP1 deficiency or overexpression on mitochondrial respiration and ATP levels in human tumor cells (HeLa and HCT116 cell lines). Using HeLa cells, we verified the predominant mitochondrial localization of transiently expressed TRAP1-Flag and TRAP1-GFP proteins (Fig. S1F) and the efficiency of siRNA-mediated silencing of endogenous TRAP1 (Fig. S1G). In agreement with the data we obtained from TRAP1 WT and KO MAFs, transient TRAP1 knockdown in HeLa cells resulted in a nearly twofold increase in the OCR of isolated mitochondria (Fig. 1H). TRAP1 silencing also significantly increased the OCR and steady-state ATP level in HCT116 cells (Fig. S1H-J). Also in accord with our data from mouse fibroblasts, TRAP1 overexpression significantly reduced the OCR and cellular ATP level of HeLa cell mitochondria (Fig. 1I and J) and the OCR in HCT116 mitochondria (Fig. S1K).

How does TRAP1 affect the balance between mitochondrial respiration and aerobic glycolysis? One of the key regulatory steps in commitment to glycolysis is conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by phosphofructokinase (PFK), because this reaction is irreversible. High cellular ATP levels allosterically inhibit PFK activity (20). PFK is also inhibited by abundant citrate, another indicator of a cell’s high energy state (21, 22). Because TRAP1 deficiency causes a high energy state (elevated ATP and citrate), we queried whether TRAP1 KO MAFs contained reduced levels of fructose 1,6-bisphosphate. Indeed, fructose 1,6-bisphosphate levels were reduced by more than 90% in TRAP1 KO fibroblasts compared with WT cells (Fig. 1K). However, when mitochondrial ATP synthase was poisoned with oligomycin for 30 min, long enough for ATP levels to decline, both WT and TRAP1 KO cells were equally capable of producing ATP by glycolysis (Fig. 1L and M, respectively), supporting the hypothesis that the reduced glycolysis characteristic of TRAP1-deficient cells is an indirect and reversible consequence of these cells’ high energy state caused by deregulated mitochondrial respiration.

**ROS Are Elevated in TRAP1-Deficient Cells.** Because the mitochondrial electron transport chain is a significant source of cellular ROS, we determined whether the increased mitochondrial respiration occurring as a consequence of TRAP1 deficiency leads to elevated ROS. In agreement with previous reports (5–7), we observed a strong inverse correlation between oxidation of

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Fig. 1. TRAP1 negatively regulates mitochondrial respiration and ATP level. (A) TRAP1 protein expression of WT and TRAP1 KO MAFs was analyzed by Western blot; α-tubulin was used as loading control. (B) OCR and ECAR of WT and TRAP1 KO MAF cells were monitored by using the Seahorse Bioscience Extra Cellular Flux Analyzer in real time. (C) Basal OCR of isolated mitochondria of WT and TRAP1 KO cells was assessed using the Seahorse Bioscience Flux Analyzer (mean ± SD, n = 3 per group). (D) Metabolomic analysis of compounds related to glycolysis and mitochondrial respiration, and ATP levels, in WT (two independent clones) and TRAP1 KO (two independent clones) cells. The median value of each metabolite was calculated from six replicates. The fold-change relative to WT2 is indicated by the color of each box (see also Fig. S1E). Metabolite names shown in italic bold font indicate significantly altered metabolites between WT and TRAP1 KO cells (P < 0.05). (E) Fatty acid oxidation in WT and TRAP1 KO cells was assessed using the Seahorse Bioscience Flux Analyzer (mean ± SD, n = 5 per group). ATP levels were assessed in two independent WT and TRAP1 KO clones (mean ± SD, n = 3 per group). ATP level in KO MAFs was assessed after transduction with TRAP1-GFP or empty-GFP plasmid (mean ± SD, n = 3 per group). ATP level in WT MAFs transfected with empty-GFP plasmid is shown for comparison. (F) Isolated mitochondrial OCR of HeLa cells 72 h after transfection with control (CTR) or TRAP1 siRNA was assessed using the Seahorse Bioscience Flux Analyzer (mean ± SD, n = 3 per group). OCR and ECAR were measured in HeLa MAFs transfected with TRAP1-Flag or TRAP1-GFP plasmids was assessed using the Seahorse Bioscience Flux Analyzer (mean ± SD, n = 3 per group). ATP level in WT MAFs transfected with empty-GFP plasmid is shown for comparison. (H) Fructose 1,6-bisphosphate levels were determined in two WT and two TRAP1 KO fibroblast clones by metabolomic analysis (Methods). Values are normalized by cell number. Mean ± SD is shown; for each group, n = 6. (I) ATP levels were assessed in HeLa cells 24 h after transfection with TRAP1-GFP or empty plasmid. Transfection did not affect cell viability (mean ± SD, n = 3 per group). (K) ATP levels were assessed in HeLa cells 24 h after transfection with TRAP1-Flag or TRAP1-GFP plasmids was assessed using the Seahorse Bioscience Flux Analyzer (mean ± SD, n = 3 per group). Twenty-four hours later, medium was aspirated, cells were washed twice in PBS, and either complete or defined medium (lacking glutamine, pyruvate, galactose, and glucose) was added. Galactose (10 mM) or glucose (10 mM) were added to defined media where shown. After 3 h, oligomycin (10 μg/mL, final) was added to inactivate mitochondrial ATP synthase and ATP levels were measured after 30 min. Data were normalized to cell number. Mean ± SD is shown; for each group, n = 6. ATP levels were measured after 30 min. Data were normalized to cell number. Mean ± SD is shown; for each group, n = 6. (M) TRAP1 KO MAFs were treated and analyzed as in L.
CM-h2DCFDA, an indicator of total cellular ROS, and TRAP1 expression in both mouse fibroblasts and HCT116 cells (Fig. 2A–C). Furthermore, mitochondrial superoxide assessed with MitoSOX Red reagent was also significantly increased in HCT116 cells upon TRAP1 silencing (Fig. 2D and E). Finally, lipid peroxidation was significantly increased in TRAP1 KO MAFs compared with WT MAFs (Fig. 2F), consistent with the likelihood that TRAP1 KO cells are constitutively exposed to elevated oxidative stress. Interestingly, taurine, hypotaurine, and propionylcarnitine have all been reported to protect cells from oxidative stress (23–29). Thus, the elevated levels of these metabolites in TRAP1 KO cells (Fig. 1D) may reflect up-regulation, albeit limited, of alternative buffering mechanisms to compensate for loss of TRAP1 expression.

**TRAP1 Interacts with Mitochondrial c-Src and Suppresses Its Activity.** How does TRAP1 regulate mitochondrial respiration? Because TRAP1 is a member of the HSP90 chaperone family, whose clientele is rich in cytosolic and nuclear kinases (2) (www.picard.ch/downloads/Hsp90interactors.pdf), we hypothesized that the impact of TRAP1 on mitochondrial respiration may be mediated, at least in part, by interaction with/regulation of one or more mitochondrial kinases. Unexpectedly, we found TRAP1 to be tyrosine phosphorylated (Fig. 3A, Left), which caused us to examine c-Src, a kinase reported to be present in mitochondria (14, 15, 30–33). We probed TRAP1 immunoprecipitates for c-Src association and we identified c-Src as a TRAP1-interacting protein in HCT116 cells (Fig. 3A, Center) and in HeLa cells (Fig. 3G). Using reciprocal immunoprecipitations, we validated the interaction between endogenous c-Src and endogenous TRAP1 in HCT116 cells (Fig. 3B). In agreement with previous reports, we identified both TRAP1 and c-Src in mitochondrial preparations whose purity was confirmed by reciprocal blotting for VDAC (voltage-dependent anion channel, a mitochondrial marker) and β-actin (a cytosolic protein) (Fig. 3C). Analysis of subfractionated HeLa cell mitochondrial preparations demonstrated TRAP1 localization to the inner mitochondrial membrane and mitochondrial matrix (Fig. S2A), the same location described for mitochondrial c-Src (14). We confirmed colocalization of c-Src and TRAP1 in isolated HeLa cell mitochondria by demonstrating their similar sensitivity to proteinase K (Fig. S2B). Similar to HSP60, which is localized to the mitochondrial matrix (34), full proteinase K sensitivity of both TRAP1 and c-Src requires detergent permeabilization of isolated mitochondria (Table S1).

To query the functional significance of the c-Src–TRAP1 interaction, we measured the intensity of mitochondrial c-Src Tyr-416 phosphorylation, an indicator of c-Src activation state, in WT and TRAP1 KO fibroblasts. We observed that c-Src Tyr-416 phosphorylation was markedly increased in TRAP1 KO mitochondria compared with mitochondria of WT cells, although mitochondrial c-Src levels were equivalent (Fig. 3D). Importantly, Tyr-416 phosphorylation of cytosolic c-Src in TRAP1 KO cells was negligible, supporting the specificity of the impact of TRAP1 expression on mitochondrial c-Src activation. Mitochondrial c-Src Tyr-416 phosphorylation was significantly reduced following TRAP1 overexpression in HCT116 cells and in TRAP1 KO fibroblasts (Fig. 3E and F, and Fig. S2C).

We next investigated whether c-Src phosphorylation status affected its interaction with TRAP1. After treating TRAP1 and c-Src cotransfected cells with the c-Src inhibitor dasatinib, we observed that mitochondrial c-Src autophosphorylation was abolished (Fig. S2D). However, immunoprecipitation of either c-Src or TRAP1 from HCT116 and HeLa cells revealed that interaction of the two proteins was markedly enhanced following c-Src inactivation, suggesting that TRAP1 preferentially binds the inactive form of c-Src (Fig. 3G and Fig. S2E). Because c-Src overexpression induced dasatinib-sensitive TRAP1 tyrosine phosphorylation (Fig. 3G), we speculate that c-Src-mediated TRAP1 phosphorylation disrupts TRAP1/c-Src interaction and TRAP1 binding to c-Src inhibits its kinase activity.

**TRAP1 and Mitochondrial c-Src Have Opposing Effects on Mitochondrial Respiration.** Based on these data and on previous reports that mitochondrial c-Src stimulates complex IV activity and enhances oxidative phosphorylation (14, 15), we speculated that the effect of TRAP1 on mitochondrial respiration may depend on its regulation of mitochondrial c-Src. To investigate this possibility, we confirmed that c-Src overexpression significantly increased the OCR of isolated HeLa cell mitochondria, but...
simultaneous overexpression of TRAP1 partially reversed this effect (Fig. 4A). To confirm the importance of mitochondrial c-Src in this process, we assessed the OCR in HEK293 cells transiently transfected with various mitochondrially targeted c-Src constructs (WT, constitutively active, kinase-dead) (15), in the presence or absence of cotransfected TRAP1 (Fig. S3A and B). Both WT and constitutively active mitochondrially targeted c-Src, but not kinase-dead c-Src, increased OCR in HEK293 compared with transfection with empty plasmid. Importantly, cotransfection of TRAP1 with either WT or constitutively active mitochondrially targeted c-Src completely reversed the increase in OCR. c-Src overexpression also increased the steady-state ATP level in HeLa cells (Fig. S3C). Consistent with these data, both the OCR and ATP level of stably v-Src–transformed 3T3 cells was significantly greater than that of nontransformed 3T3 (Fig. S3 D and E). Next, we observed that the c-Src inhibitor dasatinib caused a significant reduction in the OCR of both WT and TRAP1 KO fibroblasts (Fig. 4B). Taken together, these data are consistent with the hypothesis that mitochondrial c-Src and TRAP1 exert opposing effects on cellular respiration and that the increased OCR seen in TRAP1 KO cells is at least partially a consequence of deregulated mitochondrial c-Src.

To provide further support for this hypothesis, we evaluated the impact of decreased TRAP1 expression on ATP level and mitochondrial OCR in Src family kinase-deficient (Src−/− Fyn−/−, SFY) murine embryo fibroblasts (MEFs), and in c-Src–restored (SFY-Src) MEFs. We used siRNA to efficiently silence TRAP1 expression in both SFY and SFY-Src MEFs (Fig. S3 F and G). In contrast to our previous data, TRAP1 knockdown failed to increase either OCR or ATP level in parental SFY cells (Fig. 4 C and D). However, both OCR and ATP level in SFY-Src cells were significantly increased upon TRAP1 knockdown (Fig. 4 E and F). These findings strongly suggest that the impact of TRAP1 on mitochondrial respiration requires and is mediated by modulation of mitochondrial c-Src.
TRAP1 Deficiency Potentiates Cell Invasion. Because elevated ROS are reported to stimulate cell invasion (35, 36), we examined whether TRAP1 expression affected this phenotype. Unlike WT cells, TRAP1 KO MAFs displayed a strikingly enhanced ability to invade through Matrigel-coated membranes, and this activity was sensitive to both the c-Src inhibitor dasatinib and the ROS scavenging agent ascorbic acid (Fig. 5A). Importantly, ascorbic acid dose-dependently reduced, and the oxidizing agent hydrogen peroxide dose-dependently increased c-Src autophosphorylation (Fig. S4A and B), suggesting that the elevated ROS level in TRAP1-deficient cells may contribute to c-Src activation and enhanced cell invasion. TRAP1 silencing in several cell lines, including HCT116, HEK293, and Caki-1, similarly resulted in increased cell invasion (Fig. 5B, F, and G), but TRAP1 overexpression in HeLa cells significantly decreased this activity (Fig. 5C–E).

TRAP1 Expression Correlates Inversely with Tumor Stage in Cervical, Bladder and Clear Cell Renal Cell Cancer. Although TRAP1 is reported to be up-regulated in some cancers, its expression is diminished in others (8, 37–39). The impact of reduced TRAP1 expression on in vitro cell invasion raises the possibility that certain more aggressive, metastatic, or late-stage cancers may express less TRAP1 than less advanced tumors. Using meta-analysis of mRNA expression profiles available in publicly accessible databases (Methods), we confirmed that TRAP1 expression displays a significant inverse correlation with tumor stage in cervical and bladder cancer (Fig. S5A and B). Immunohistochemical analysis of TRAP1 protein expression in normal bladder urothelium and bladder cancer was consistent with these findings (Fig. S5C). Furthermore, comparison of TRAP1 protein expression in normal kidney, normal liver, and several clear cell renal cell cancer specimens also demonstrated greater immunoreactivity in normal tissue compared with adjacent tumor (Fig. S5D), and evaluation of the intensity of TRAP1 immunoreactivity in localized vs. advanced clear cell renal cell carcinoma specimens (12 cases each) revealed a significant inverse correlation with tumor stage (Table 1).

Discussion

Since Toft and colleagues first cloned TRAP1 in 2000 (40), detailed analysis of the function of this mitochondria-localized molecular chaperone has remained incomplete. Some studies have reported abundant expression of TRAP1 in various cancer tissues but not in normal tissues, and these investigators have suggested that TRAP1 contributes to oncogenesis (41, 42). However, others have found the expression of TRAP1 in both normal and cancer tissues to be more variable (13, 39). Using data from publically available gene-expression databases, as well as archived tumor tissues, we have demonstrated here an inverse correlation between TRAP1 expression and tumor stage in cervical, bladder, and clear cell renal cell carcinoma. Intriguingly, cervical carcinoma is among those cancers whose predominant energy metabolism is via oxidative phosphorylation, not glycolysis (43). Our findings suggest a reevaluation of the assumption that TRAP1 is uniformly pro-oncogenic, and they support a more nuanced role for this mitochondrial chaperone in regulating cellular metabolism and impacting tumorigenesis. In this study, we have identified TRAP1 to be a negative regulator of mitochondrial respiration able to modulate the balance between oxidative phosphorylation and aerobic glycolysis, and we have provided evidence that TRAP1 interaction with, and modulation of the activity of mitochondrial c-Src is essential for this function.
As a consequence of reduced or absent TRAP1 expression mitochondrial respiration is deregulated, establishing a high energy state characterized by elevated \( \alpha \)-ketoglutarate, citrate, and ATP. Aerobic glycolysis is suppressed as a byproduct of this altered metabolic landscape, likely because of, in part, allosteric inhibition of the glycolytic enzyme phosphofructokinase. These data are in general agreement with a recent study showing that TRAP1 silencing in a glioblastoma cell line resulted in a variety of metabolic changes suggestive of reduced glycolysis, including reduced lactic acid secretion (44). Suppression of glycolysis concomitant with enhanced fatty acid oxidation and increased steady-state levels of several TCA cycle intermediates, including oxaloacetate, strongly suggest that TRAP1-deficient cells use one or more anaplerotic mechanisms to sustain their mitochondrial metabolism. Intriguingly, a nearly identical metabolic phenotype has been described for castrate-resistant prostate cancer (45), and a recent study suggests that oxidative phosphorylation may play a prominent role in advanced melanoma (46). Although evaluation of TRAP1-specific inhibitors warrants further exploration as a novel approach to cancer therapy, our findings would suggest that TRAP1 inhibition alone is unlikely to be a viable treatment strategy for cancers that use oxidative phosphorylation.

Although our data were obtained by manipulating TRAP1 protein expression, the impact of TRAP1 on mitochondrial respiration is mediated, at least in part, by its interaction with, and regulation of mitochondrial c-Src. Miyazaki et al. first reported that c-Src is located within mitochondria and affects energy metabolism by phosphorylating complex IV (14). Later studies confirmed that c-Src is present in the inner mitochondrial membrane/matrix (15). Although the mechanism by which TRAP1 regulates mitochondrial c-Src is not yet clarified, the cytosolic c-Src–HSP90 interaction provides a model to address this question. Cytoplasmic c-Src is a well-studied HSP90 client protein and its interaction with HSP90 is required for Src maturation (47). However, we have shown previously that pharmacological disruption of HSP90 association with mature c-Src induces a transient but distinct increase in c-Src activity (48, 49). Release of enzymatically competent c-Src from HSP90 relieves its auto-inhibition and permits phosphorylation of Tyr-416 in the activation loop. In the present study, we observed a similar increase in mitochondrial c-Src Tyr-416 phosphorylation upon TRAP1 suppression or gene disruption, concomitant with increased mitochondrial respiration and ATP production. Furthermore, TRAP1 interacted more efficiently with the inactive

### Table 1. Comparison of TRAP1 protein expression in localized and advanced clear cell renal cell carcinoma

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<th>TRAP1 staining intensity</th>
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<tr>
<td>Clear cell renal cell cancer/localized disease (n = 12)</td>
<td>1</td>
<td>3</td>
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<td>Clear cell renal cell cancer/advanced disease (n = 12)</td>
<td>10</td>
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For the \( \chi^2 \) test (comparing localized disease to advanced disease): \( P = 0.0004 \). TRAP1 protein expression in tumor tissue was assessed by immunocytochemistry (see Methods) and is compared with that of normal renal tissue (designated “−”). See also Fig. S5.
form of c-Src, while also being a c-Src substrate (directly or indirectly). Thus, we propose that regulated association/dissocia-
tion of TRAP1 and c-Src, perhaps via TRAP1 phosphorylation,
modulates the activity of mitochondrial c-Src which, in turn,
affects the rate of mitochondrial respiration.

A number of studies have reported that TRAP1 protects mi-
tochondria from oxidative stress, and that TRAP1 expression and ROS levels are inversely correlated, although the molecular mechanisms underlying this observation have remained to be elucidated. Based on our data, we propose that the elevated ROS generation characteristic of TRAP1-deficient cells is a con-
sequence of deregulated mitochondrial respiration. ROS play important roles in a variety of signaling pathways. For ex-
ample, multiple protein tyrosine phosphatases are reversibly inac-
tivated by oxidation, and this in turn affects the activity of cytosolic
kinases, including c-Src. Deregulated cytosolic c-Src activity
promotes cell motility and invasion (50), and TRAP1 knockout or transient suppression dramatically enhances cell invasiveness,
both in mouse fibroblasts and in a variety of human cell lines.
Importantly, this phenotype is sensitive to c-Src inhibition and
ROS neutralization, supporting a direct link between TRAP1
deficiency, elevated ROS, and c-Src activation in mediating this
process. The impact of TRAP1 on cellular bioenergetics may also
contribute to this phenotype. Interestingly, a recent study
reported that transient TRAP1 silencing in cancer cells was
associated with up-regulation of a number of cell motility and
metastasis-associated genes, and TRAP1 overexpression was
associated with increased expression of genes associated with cell
proliferation (13). The indirect transcriptional impact of TRAP1
expression requires further exploration, but taken together with
our findings these data reinforce the reciprocal relationship
between TRAP1 expression and cell motility/invasion, at both the
transcriptional and posttranslational level.

Although glucose metabolism is markedly down-regulated in
TRAP1 KO cells, these cells are able to use glycolysis to replenish
their ATP stores when mitochondrial ATP synthase is inhibited
for a long enough period to significantly affect the cellular ATP
level. Recent studies have shown that utilization of alternative
carbon sources affects tumor evolution, and the role of oxidative
phosphorylation in cancer metabolism has not been fully appre-
ciated (46, 51–54). Furthermore, the ability of normal as well as
cancer cells to rapidly adjust their metabolic behavior in response
to environmental conditions and cellular requirements is essential
to survival and the metabolic requirements of proliferating and
nonproliferating cells are distinct, as are the metabolic demands
underlying tumor initiation, metastasis, and growth at distant sites
(55, 56). For these reasons, optimal growth/survival of all normal
and most cancer cells would benefit greatly from the ability to use
either mitochondrial respiration or glycolysis as conditions war-
rant. Identification of the mitochondrial chaperone TRAP1 as
a key modulator of this metabolic switch provides unique mech-
animistic insight into its molecular regulation.

Methods

Cell Culture and Reagents. HCT 116, HeLa, COS-7, SFY, SFY-Src, and NIH 3T3 cells were
derived from the American Type Culture Collection. These cells were
cultured as directed by the provider. pE8c-transected NIH/3T3 cells (3T3αcV)
were obtained from O. Sartor (Tulane University, New Orleans, LA). Contin-
uously growing adult fibroblast cell lines (see below) were cultured in DMEM
with GlutaMAX supplemented with 10% (vol/vol) FBS and 100 U·mL⁻¹ peni-
cillin/100 U·mL⁻¹ streptomycin in a humidified incubator with 5% CO
and 95% (vol/vol) O. Dasatinib was obtained from Bristol Myers Squibb. H2O2,
ascorbic acid, oligomycin and FCCP and were obtained from Sigma. TRAP1
antibody was from Cell Signaling Technologies. Antibodies recognizing c-Src, phospho-Src
(Tyr416), VDAC, and β-actin were from Cell Signaling Technology. Antibody
recognizing GFP was from Santa Cruz Biotechnology. TOM70 and α-tubulin
antibodies were obtained from Thermo Scientific and Calbiochem/Millipore, respectively.

Establishment of Immortalized Fibroblasts from Adult WT and TRAP1 KO Mice. Mice
with a gene trap disruption of the Trap1 gene were used to derive
TRAP1-null cells. A piece of the ear of adult TRAP1−/− and TRAP1+/− mice was
cut and incubated overnight in a 3-cm dish with RPMI-1640 supple-
mented with 30% (vol/vol) NCS, 2 mM l-glutamine, 100 U·mL⁻¹ penicillin/100
U·mL⁻¹ streptomycin, and 1 mg/mL proteinase K. The next day, the resultant
single cell suspension was centrifuged and transferred to fresh medium.
After 1 wk, RPMI-1640 medium was replaced by DMEM with GlutaMAX,
supplemented with 10% FBS and 100 U·mL⁻¹ penicillin/100 U·mL⁻¹ strepto-
mycin. Cells were continuously passaged until they became spontaneously
immortalized. Two independently derived TRAP1−/− (WT) and TRAP1+/− (KO)
MAF cell lines were established. Where only one cell line of each type is
examined, it is WT1 or KO1, respectively.

Plasmids, Gene Transfection, and RNAi. pEGFP-N1-human TRAP1-GFP was
provided by D. Toft (Department of Biochemistry and Molecular
Biology, Mayo Graduate School, Rochester, MN). Conventional molecular
biological techniques were used to generate the pDNA3-human TRAP1-F flag.
Plasmid expressing WT c-Src was purchased from Upstate Biotechnology.
Plasmids expressing mitochondrially targeted c-Src constructs were kind
gifts of Y. Homma (Department of Biomolecular Science, Fukushima Medical
University School of Medicine, Fukushima, Japan) (15). MAFs were tran-
siently transfected with TRAP1-GFP or empty-GFP plasmid using an Amaza
Nucleofector (Lonza), according to the manufacturer’s protocol. Otherwise,
transient transfections were performed using X-tremeGENE 9 Transfection
Reagent (Rego Applied Science). RNA interference was performed with
siRNA specific for TRAP1 (ON-TARGETplus SMARTpool Mouse Trap1 or Human
TRAP1, Thermo Scientific), or control nonspecific siRNA (MISSION siRNA
Negative Control; Sigma) using DharmaFECT Transfection Reagent (Thermo
Scientific) according to the manufacturer’s protocol.

Mitochondrial Respiration, Glycolysis, and Fatty Acid Oxidation. Mitochondrial
respiration, aerobic glycolysis, and fatty acid oxidation were monitored in real
time with the Seahorse Bioscience Extracellular Flux Analyzer (XF96; Seahorse
Bioscience) by measuring the OCR (indicative of respiration) and ECAR (in-
dicative of glycolysis). Further details are provided in SI Methods.

ATP Assay. ATP content of whole cell extracts was determined with a lumi-
nescent ATP detection kit (ATPlite; PerkinElmer) according to the manu-
facturer’s instructions. Results were normalized to cell number.

ROS Determination. To measure total cellular ROS, cells were incubated with
10 μM chloromethyl-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Life
Technologies) for 1 h at 37 °C before being returned to prewarmed media
without phenol red. After 1-h incubation, immunofluorescence was measured
(VICTOR V2; PerkinElmer). Alternatively, mitochondrial ROS (superoxide) was
assessed using the MitoSOX reagent (Life Technologies) and following manu-
facturer’s instructions. Data were normalized to total protein.

Lipid Peroxidation. The end product of lipid peroxidation, malondialdehyde,
was quantified using the TBARS assay kit from Cayman Chemical and following
the manufacturer’s instructions. Further details are provided in SI Methods.

In Vitro Cell Invasion Assay. HeLa cells (20 × 10⁴) or HCT116 cells (10 × 10⁴)
24 h after transfection, or mouse fibroblasts (10 × 10⁴), were seeded in
24-well Matrigel-coated migration chambers (8-μm pores; BD Biosciences)
in serum-free medium. The lower chamber was filled with complete medium
containing 10% FBS. After incubation for 24 h (HeLa cells and fibroblasts) or 48 h
(HCT116 cells), cells remaining in the upper chamber were removed with
cotton swabs. The cells that migrated through the Matrigel and attached to
the lower surface of the inserts were fixed and stained with Diff-Quik (Sie-
men). All experiments were conducted twice in duplicate for each condition.

Immunofluorescence Analysis. TRAP1 WT or KO MAfs were labeled with either
Mitotracker Red (Life Technologies; mitochondrial mass) or JC-1 (Life Technologies;
mitochondrial membrane potential), according to the manufacturer’s instructions.
Data were collected from 10,000 cells per condition by flow cytometry.

Immunohistochemistry. Immunostaining was performed on 5-μm paraffin-
embedded tissues sections using TRAP1 antibody (TRAP1-6; Thermo Scien-
tific). Briefly, the sections were rehydrated, treated in 0.3% hydrogen per-
oxide, and placed in a 550 W microwave oven for 15 min in 10 mM citrate
buffer (pH 6.0). After incubation with 10% goat serum for 30 min, the
sections were incubated with primary antibody overnight at 4 °C. The sec-

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tions were incubated with horseradish peroxidase-labeled secondary anti-body for 30 min at room temperature and color was developed with 3,3-diaminobenzidine. The sections were counterstained with hematoxylin. The study was approved by the Institutional Review Board of Tokyo Medical and Dental University and written informed consent was obtained from each patient whose tissue was evaluated.

Microarray Analysis. Two published microarray datasets (GDS) obtained from the National Center for Biotechnology Information Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo) were used to interpret TRAP1 gene expression in cervical cancer and normal cervix, and in several stages of bladder cancer. Wilcoxon signed-rank test was used to compare TRAP1 gene expression between groups. Box-plots and whiskers present interquartile range and range of all values, respectively.


Metabolic Analysis. Sample preparation and analysis was carried out using established procedures (www.metabolon.com; Metabolon).

Statistics. Statistical analysis was performed using Microsoft Excel and JMP7.0 software. Unpaired Student t test, χ2 test, or Wilcoxon rank-sum test was used to generate the P values for the dataset.

ACKNOWLEDGMENTS. We thank S. Felts and D. Toft (Department of Biochemistry and Molecular Biology, Mayo Graduate School) for TNF receptor-associated protein 1 plasmid and antibody, and Y. Homma (Department of Biomedical Science, Fukushima Medical University School of Medicine) for mitochondrially targeted c-Src plasmids. This work was supported by funds from the Intramural Research Program of the National Cancer Institute, Center for Cancer Research. S.Y. received financial support for overseas study from The Waksman Foundation of Japan.