

Anticancer Activity of the Cholesterol Exporter *ABCA1* Gene

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SUMMARY

The *ABCA1* protein mediates the transfer of cellular cholesterol across the plasma membrane to apolipoprotein A-I. Loss-of-function mutations in the *ABCA1* gene induce Tangier disease and familial hypoalphalipoproteinemia, both cardiovascular conditions characterized by abnormally low levels of serum cholesterol, increased cholesterol in macrophages, and subsequent formation of vascular plaque. Increased intracellular cholesterol levels are also frequently found in cancer cells. Here, we demonstrate anticancer activity of *ABCA1* efflux function, which is compromised following inhibition of *ABCA1* gene expression by oncogenic mutations or cancer-specific *ABCA1* loss-of-function mutations. In concert with elevated cholesterol synthesis found in cancer cells, *ABCA1* deficiency allows for increased mitochondrial cholesterol, inhibits release of mitochondrial cell death-promoting molecules, and thus facilitates cancer cell survival, suggesting that elevated mitochondrial cholesterol is essential to the cancer phenotype.

INTRODUCTION

Current interest in cancer cell metabolism originates from several recent discoveries indicating that elevated glycolytic activity found in various types of cancer, commonly known as the Warburg effect (Warburg, 1930), is an essential feature of the cancer phenotype (Christofk et al., 2008; Fantin et al., 2006; Weinberg et al., 2010). Similarly, a causal role for increased cholesterol in malignant cell transformation has long been debated, perhaps first suggested by work in the early 20th century indicating accelerated tumor growth following cholesterol injection into xenografts (Robertson and Burnett, 1913). Genetic evidence for a causal link between multistep oncogenesis and the control of cholesterol homeostasis, however, has ever been lacking.

Aberrant regulation of cholesterol homeostasis has been associated with multiple types of cancer. Numerous studies have shown increased levels of cholesterol in tumors as compared to normal tissue (Dessí et al., 1992, 1994; Kolanjiappan et al., 2003; Rudling and Collins, 1996; Schaffner, 1981; Yoshioka

et al., 2000). Moreover, low serum cholesterol levels have been associated with tumor presence in cancer patients, suggesting cholesterol may accumulate in tumor tissue (Benn et al., 2011; Jacobs et al., 1992; Strasak et al., 2009). Multiple paths to increasing intracellular cholesterol have been observed in cancer cells. These include upregulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR) activity, the rate-limiting step of the cholesterol synthesis pathway (Caruso et al., 1999, 2002; Notamicola et al., 2004), loss of feedback inhibition of HMG-CoAR by cholesterol (Gregg et al., 1986; Hentosh et al., 2001; Siperstein, 1995), increased uptake of extracellular cholesterol through the low-density lipoprotein receptor (Graziani et al., 2002; Schimanski et al., 2009; Tatidis et al., 2002), and decreased expression of the cholesterol exporter termed ATP binding cassette transporter A1 (*ABCA1*) (Basso et al., 2005; Ki et al., 2007; Moustafa et al., 2004; Schimanski et al., 2009), all together suggesting that cholesterol levels in cancer cells can be modulated by any of these interconnected processes.

In vivo evidence to support a cancer-promoting role of cholesterol is limited to pharmacological approaches. Various statins, which inhibit cholesterol synthesis by blocking activity of HMG-CoAR, have been shown to reduce tumor growth in xenograft models (Gao et al., 2010; Huang et al., 2010; Kochuparambil et al., 2011). Moreover, pharmacological inhibition of squalene synthase, the first committed step in cholesterol synthesis, lowered resistance to the chemotherapeutic agent doxorubicin in a xenograft model of liver cancer, albeit without significant effect on tumor growth, when used on its own (Montero et al., 2008). Furthermore, liver X receptor (LXR) agonists, which are known to induce *ABCA1* expression, inhibited tumor growth and progression to androgen independence in a xenograft model of prostate cancer (Chuu et al., 2006). The antitumor effects of these compounds, however, may involve a variety of mechanisms, as for example, statins inhibit GTPases such as Ras and Rho family proteins via blocking protein prenylation and/or farnesylation (Demierre et al., 2005) and LXR agonists induce cell cycle arrest through upregulation of p27 (Chuu and Lin, 2010).

Efforts to identify causal relationships between oncogenic mutations and associated metabolic or cholesterol dysregulation have been limited, presumably for two reasons: (1) most cancer mutations have been identified in genes regulating cellular signaling pathways (Pickeral et al., 2000), and (2) analysis of regulatory processes downstream of oncogenic mutations has been difficult due to the complexity of change associated with malignant transformation. Only recently have several genetic

approaches been developed readily capable of identifying non-mutant genes essential to cell transformation (nonmutant drivers) (Luo et al., 2009; McMurray et al., 2008; Schlabach et al., 2008; Shaffer et al., 2008; Zender et al., 2008). We developed a strategy to identify such genes based on the notion that malignant cell transformation is a highly cooperative process (Hahn et al., 1999; Land et al., 1983) and involves synergistic regulation of nonmutant genes and proteins downstream of oncogenic mutations (Lloyd et al., 1997; Sewing et al., 1997; Xia and Land, 2007). In fact, we were able to demonstrate that genes regulated synergistically by multiple cancer gene mutations, i.e., “cooperation response genes” (CRGs), are highly enriched for nonmutant drivers of the cancer phenotype (McMurray et al., 2008). Notably, CRGs contain a large fraction of genes involved in the control of cell metabolism (McMurray et al., 2008), thus pinpointing potential causal linkages between oncogenic mutations and the regulation of cancer cell metabolism.

The presence among CRGs of the ATP-binding cassette transporter A1/cholesterol exporter (*Abca1*) gene, a key player in cholesterol metabolism, prompted us to investigate the role of ABCA1 in regulating both intracellular cholesterol levels and the cancer phenotype. The ABCA1 protein mediates the transfer of cellular cholesterol across the plasma membrane to apolipoprotein A-I (ApoA1), the major apolipoprotein component of high-density lipoprotein (HDL) (Attie, 2007). Loss-of-function mutations in the *ABCA1* gene induce Tangier disease (TD) and familial hypoalphalipoproteinemia, both cardiovascular conditions characterized by abnormally low levels of HDL-bound serum cholesterol and excess cholesterol in macrophages followed by vascular plaque (Singaraja et al., 2006). Based on our previous identification of *Abca1* as a synergistically downregulated CRG (McMurray et al., 2008) and its well-characterized function as cholesterol exporter, we hypothesized that loss of ABCA1 efflux function promotes carcinogenesis presumably via increasing intracellular cholesterol levels. Similarly, we wanted to explore whether somatic mutations in the *ABCA1* gene found in human colon cancers following genomic DNA sequencing (Sjöblom et al., 2006) cause defects in ABCA1 efflux function and thus may facilitate malignant cell transformation.

RESULTS

ABCA1-Mediated Tumor Inhibition

Cooperation response genes (CRGs) were identified by virtue of their synergistic regulation by mutant p53^{175H} (mp53) and activated H-Ras^{V12} (Ras) at the level of polysomal RNA abundance in young adult mouse colon (YAMC) cells (McMurray et al., 2008). The list of CRGs includes the gene encoding the cholesterol exporter ABCA1, which is synergistically downregulated both at RNA (McMurray et al., 2008) and protein (Figure 1A) levels in cells expressing mp53 and Ras (mp53/Ras cells), as compared to cells expressing either mp53 or Ras alone.

Gene perturbation experiments indicate that ABCA1 has strong antitumor activity. Ectopic ABCA1 reexpression in mp53/Ras cells to levels approximating those found in the parental YAMC cells, i.e., ABCA1 reconstitution, (Figure 1B) caused a marked reduction in the size of tumors forming after implantation of the genetically modified cells into the flanks of

immunocompromised mice (Figure 1C). ABCA1-associated tumor inhibition is further indicated by the loss of ectopic *Abca1* expression in the resulting tumors. *Abca1* mRNA levels were found 3-fold lower in tumor tissue than in cells prior to implantation (Figure S1A), presumably due to rapid selection from the retrovirally transduced polyclonal cell population expressing ectopic *Abca1* at various levels. Consistent with this interpretation, ectopic expression of genes not affecting tumor growth was observed to remain relatively stable following implantation into mice (example shown in Figures S1B and S1C). Taken together, these results indicate a causal role for ABCA1 loss of function in malignant cell transformation. As ABCA1-reconstituted cells were rapidly excluded from tumors, analysis of mechanisms underlying ABCA1-mediated tumor inhibition in tumor tissue was not feasible. We observed, however, that ABCA1-reconstituted mp53/Ras cells showed increased rates of nonapoptotic cell death/necrosis in vitro, as compared to unperturbed controls, when cells were cultivated at a low cell density and in the absence of serum and other survival factors (Figure 1D). Under similar conditions proliferation rates remained unaffected (data not shown). This suggests that loss of ABCA1 expression, as found in mp53/Ras cells contributes to the relative cell death resistance characteristic of cancer cells.

ABCA1 loss of function in cardiovascular pathogenesis is associated with diminished cholesterol export activity and thus increased intracellular cholesterol (Singaraja et al., 2006). A similar scenario may result from suppression of ABCA1 expression in malignant cell transformation induced by cooperating oncogenic mutations. In fact, perturbations of the cholesterol synthesis pathway mimic the effects of ABCA1 reconstitution. Several steps downstream of HMG-CoAR, in the cholesterol biosynthetic pathway, lanosterol cyclase (LC) produces the first sterol intermediate, and pharmacological inhibition of this enzyme has been demonstrated to block cholesterol synthesis (Dollis and Schuber, 1994). As we show here, both a pharmacological LC inhibitor, and genetic perturbation of LC protein via small hairpin RNA (shRNA)-mediated knockdown (Figures S1D–S1F) induce cell death as observed for ABCA1 reconstitution (Figure 1D). Moreover, tumors arising after implantation of LC knockdown cells into mice efficiently escape from LC knockdown (Figure S1G), consistent with a tumor-inhibitory effect of LC knockdown and presumably due to negative selection.

ABCA1 Lowers Mitochondrial Cholesterol and Promotes Cytochrome c Release

Due to its role as a cholesterol exporter, reconstitution of ABCA1 expression in mp53/Ras cells up to levels found in untransformed parental YAMC cells (Figure 1B) is predicted to induce depletion of cellular cholesterol stores. Increased cholesterol in cancer cells has been found specifically in mitochondria (Feo et al., 1973; Feo et al., 1975; Montero et al., 2008). This led us to measure cholesterol levels in mitochondrial fractions, where we observed significantly lower levels of cholesterol in samples from ABCA1-reconstituted mp53/Ras cells as compared to controls (Figures 2A and S2A). Comparison of cholesterol levels in total cell membranes, however, indicated no significant overall differences between ABCA1-reconstituted and control mp53/Ras cells (data not shown).

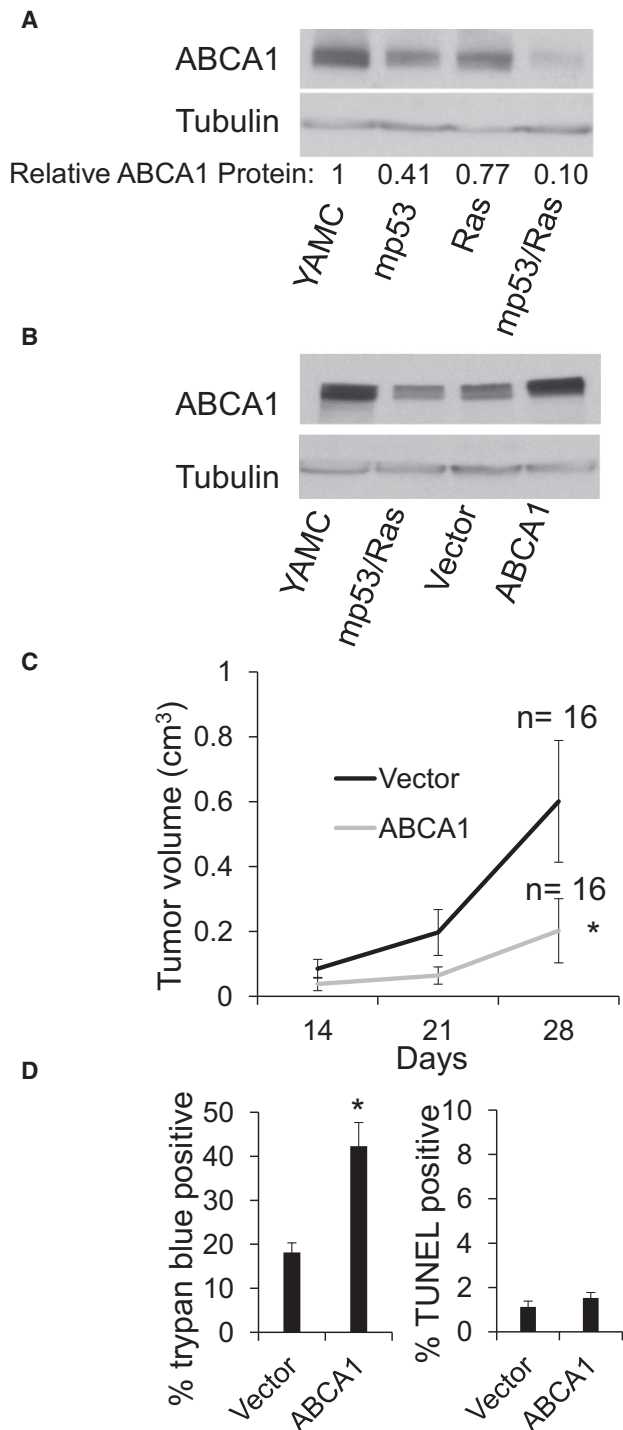


Figure 1. Reconstitution of ABCA1 Expression Inhibits Tumor Formation

(A) Western blot showing murine ABCA1 protein expression in YAMC, mp53, Ras, and mp53/Ras cells.

(B) Western blot showing stable re-expression of ectopic murine ABCA1 in mp53/Ras cells to levels found in YAMC parental cells. Tubulin served as loading control for western blots.

(C) Vector control or ABCA1-reconstituted mp53/Ras cells were subcutaneously injected in the flanks of nude mice and tumor volumes were measured at

indicated times after injection. Values represent average of 16 tumors and error bars indicate standard deviation at each time point. * $p < 0.001$, by Student's *t* test.

Elevated cholesterol levels as found in the membranes of cancer cell mitochondria decrease membrane fluidity (Colell et al., 2003; Montero et al., 2008). This, in turn, inhibits mitochondrial permeability transition (MPT) and the associated matrix swelling and release from mitochondria of cell-death-promoting molecules such as cytochrome *c* (Colell et al., 2003; Montero et al., 2008), presumably supporting cancer cell survival. Conversely, lowering mitochondrial cholesterol in cancer cells, as in the case of increased ABCA1 expression, would be predicted to increase sensitivity to MPT in response to stress. We thus hypothesized that ABCA1-associated tumor inhibition may be linked to its ability to lower mitochondrial cholesterol. Consistent with this idea, mitochondria from ABCA1-reconstituted mp53/Ras cells (Figure 1B) released more cytochrome *c* (Figure 2C) and were more sensitive to matrix swelling (Figure 2E) in response to Ca²⁺ than mitochondria from controls. Notably, we also could show that the ABCA1-mediated sensitization of both cytochrome *c* release and matrix swelling is dependent on the associated decrease in mitochondrial cholesterol. This is demonstrated by the inhibition of ABCA1-induced cytochrome *c* release and matrix swelling, following restoration of cholesterol in isolated mitochondria to levels found in controls (Figures 2A, 2C, and 2E).

Independent support that lowering mitochondrial cholesterol is sufficient to enable mitochondrial cytochrome *c* release in response to stress comes from experiments targeting the cholesterol synthesis pathway via knockdown of LC in mp53/Ras cells (Figure S1F). This perturbation not only induces a reduction in mitochondrial cholesterol (Figures 2B and S2A), but also increased mitochondrial sensitivity to calcium-induced cytochrome *c* release (Figure 2D) and matrix swelling (Figure 2F), to levels similar to those seen in ABCA1-reconstituted cells (Figures 2A, 2C, and 2E). Moreover, restoration of cholesterol in mitochondria from LC knockdown cells reversed this sensitization (Figures 2B, 2D, and 2F). In this context it is relevant to mention that cell death was not detected in any cells used for preparation of mitochondria, as these cells were cultured at higher cell densities than cells used for measuring the impact of ABCA1 reconstitution and LC knockdown on cell survival. Our data thus suggest reduction of mitochondrial cholesterol as a causal factor in ABCA1-mediated matrix swelling and cytochrome *c* release.

In addition to lowering mitochondrial cholesterol, ABCA1 has been associated with decreased lipid raft cholesterol (without detectable alterations in overall levels of plasma membrane cholesterol) and reduced Akt activity (Landry et al., 2006). A reduction in Akt-dependent survival signaling thus may also

indicated times after injection. Values represent average of 16 tumors and error bars indicate standard deviation at each time point. * $p < 0.001$, by Student's *t* test.

(D) Cell death of vector control and ABCA1-reconstituted mp53/Ras cells (ABCA1). Cell death was measured by trypan blue exclusion (left panel), with no indication for DNA fragmentation/apoptosis, as measured by TUNEL staining (right panel). Values represent the average of three independent experiments. Error bars indicate standard deviation. * $p < 0.01$ versus vector control, by Student's *t* test.

See also Figure S1.

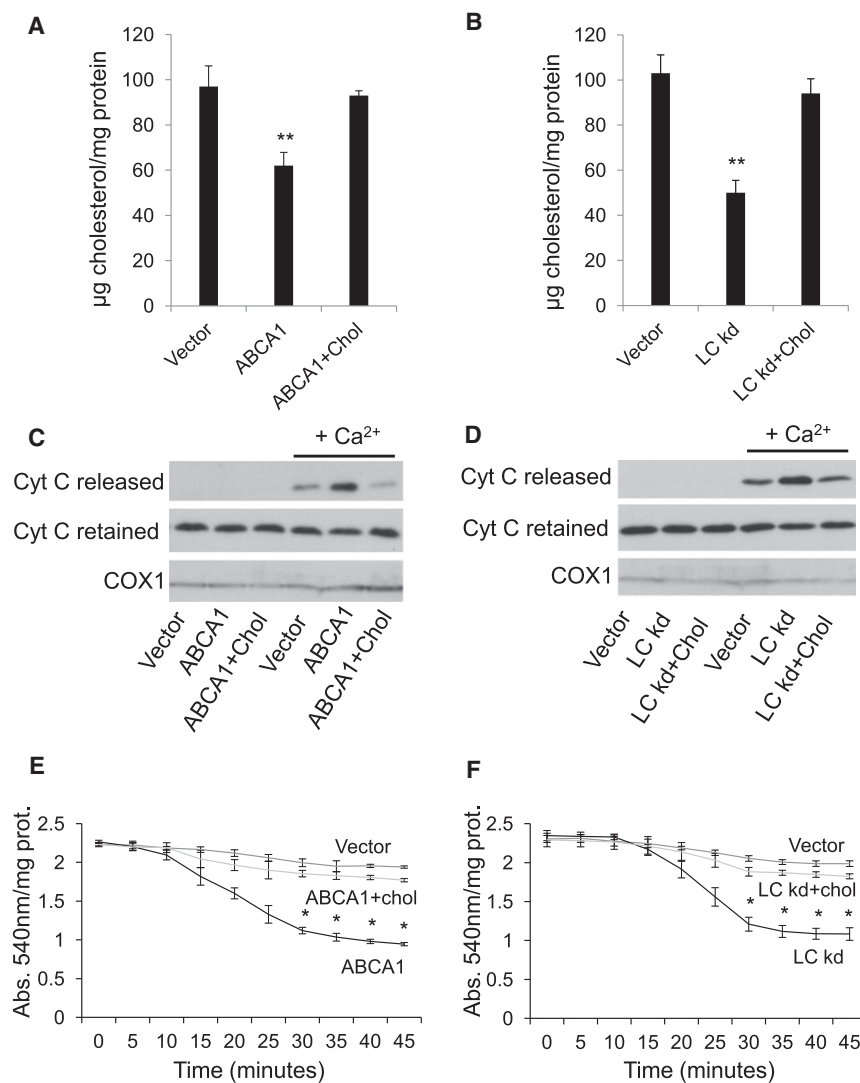


Figure 2. ABCA1 Reconstitution and Knockdown of LC Decrease Mitochondrial Cholesterol and Increase Sensitivity to Calcium-Induced Cytochrome c Release and Matrix Swelling

(A and B) Cholesterol in mitochondria from vector control, ABCA1-reconstituted (ABCA1), and lanosterol cyclase knockdown (LC kd) mp53/Ras cells and following cholesterol restoration (ABCA1+Chol and LC kd+Chol). Cholesterol levels determined by Amplex Red cholesterol assay and normalized to total mitochondrial protein. Indicated values represent average from three independent experiments and (B) two independent LC shRNA constructs. Error bars indicate SD, ** $p < 0.01$ versus vector by Student's t test.

(C and D) Calcium-induced release of cytochrome c (Cyt C) from mitochondria of cells indicated in (A) and (B). Cyt C was measured by western blot, with cytochrome oxidase I (COX1) serving as loading control. Blots shown are representative of three independent experiments.

(E and F) Calcium-induced swelling of mitochondria from cell lines indicated in (A) and (B). Mitochondrial swelling was assessed by measuring changes in absorbance at 540 nm. Error bars indicate standard deviation, * $p < 0.001$ versus vector by Student's t test.

See also [Figure S2](#).

contribute to the anticancer activity of ABCA1. We found, however, no evidence for decreased AKT activity in response to ABCA1 reconstitution in mp53/Ras cells, as indicated by unaltered phosphorylation at AKT residues Ser473 and Thr308 (data not shown). This may be due to the presence of activated Ras in the mp53/Ras cells, a potent activator of AKT ([Repasky et al., 2004](#)). AKT inhibition thus appears unlikely to contribute to ABCA1-associated tumor inhibition in mp53/Ras cells. Moreover, in our system we were unable to confirm reports linking increased cholesterol to elevated endoplasmic reticulum stress and the associated unfolded protein response ([Tabas, 2010](#)) (data not shown).

Our data described above indicate that mitochondrial cholesterol in cancerous cells is controlled effectively by both cholesterol biosynthesis and the cholesterol exporter ABCA1. Consistent with this idea, elevated mitochondrial cholesterol in mp53/Ras cells is accompanied not only by reduced ABCA1 expression ([Figure 1A](#)), but also by increased cholesterol synthesis rates, as compared to YAMC, Ras, or mp53 cells ([Fig-](#)

[ure S2B](#)). Correspondingly, levels of mitochondrial cholesterol in mp53/Ras cells are uniquely higher than in YAMC, Ras, or mp53 cells ([Figures S2C and S2D](#)). As one might predict in such a context, decreasing ABCA1 expression in cells in which cholesterol synthesis is not fully activated, i.e., reduction of ABCA1 levels in Ras and mp53 cells to levels found in mp53/Ras cells ([Figure S2E](#)), did not increase mitochondrial cholesterol ([Figure S2B](#)), presumably because of insufficient supply. Moreover, mitochondrial cholesterol is also not increased by an additional reduction of ABCA1 expression in malignant mp53/Ras cells ([Figure S2D](#)), suggesting saturation of mitochondrial cholesterol in these cells. Notably, in either context tumor formation capacity was not affected ([Figures S2F and S2G](#)), presumably because mitochondrial cholesterol levels remained unaltered. Taken together, it thus seems reasonable to suggest that ABCA1 functions to restrict tumor growth via maintaining mitochondrial cholesterol at levels incompatible with the cancer phenotype, particularly in the presence of elevated rates of cholesterol synthesis that would otherwise drive up mitochondrial cholesterol.

Based on our data summarized above, we hypothesized that elevated mitochondrial cholesterol can promote increased death resistance in cancer cells and thus malignant cell transformation. Such a view is supported by work of others showing that RNA interference knockdown of squalene synthase, the enzyme catalyzing the first committed step of cholesterol synthesis, leads to

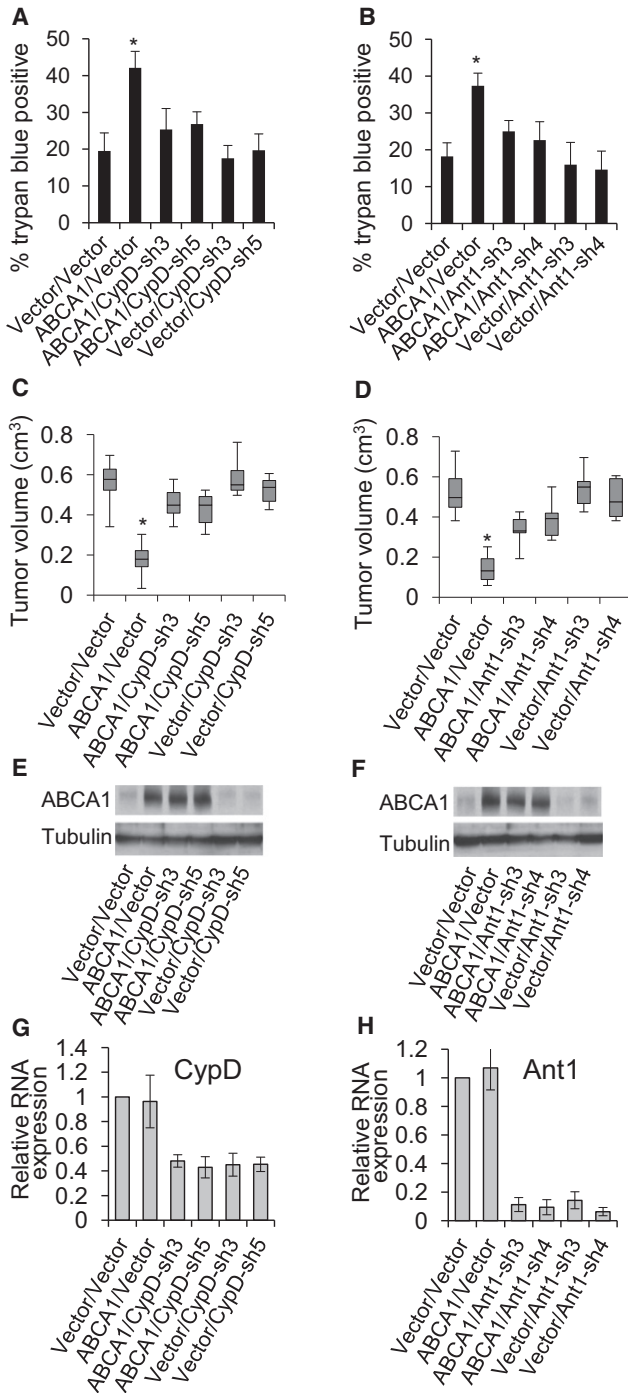


Figure 3. Suppression of *CypD* or *Ant1* Restores Death Resistance of ABCA1-Reconstituted mp53/Ras Cells In Vitro and Rescues Tumor Growth In Vivo

(A and B) Cell death of the indicated derivatives of mp53/Ras cells: vector control (Vector/Vector), ABCA1-reconstituted (ABCA1/Vector), ABCA1-reconstituted plus cyclophilin D knockdown (ABCA1/CypD-sh3 and -sh5) or Adenine Nucleotide Translocator 1 knockdown (ABCA1/Ant1-sh3 and -sh4), cyclophilin D knockdown (Vector/CypD-sh3 or -sh5), and Adenine Nucleotide Translocator 1 knockdown (Vector/Ant1-sh3 or -sh4). Cell death was measured by trypan blue exclusion. Values represent the average of three

decreased cell proliferation and survival of prostate cancer cells in vitro (Bresselmans et al., 2007), and that shRNA-mediated knockdown of the steroidogenic acute regulatory protein (StAR), a mitochondrial protein that transports cholesterol into mitochondria, sensitized hepatocellular carcinoma cells to chemotherapeutic agents in vitro (Montero et al., 2008). Nevertheless, direct demonstration of a causal role of metabolites, such as cholesterol, in intact cells cannot be achieved by genetic manipulation. We thus further examined the validity of our hypothesis by testing several independent predictions in a variety of biological contexts.

ABCA1 Promotes Mitochondria-Mediated Cell Death in Cancer Cells

ABCA1 promotes reduction in mitochondrial cholesterol, MPT-associated cytochrome c release, and non-apoptotic/necrotic cell death in response to cell stress, suggesting that ABCA1-mediated cell death depends on cholesterol for modulation of mitochondrial function. We tested this prediction in two steps, first by examining whether ABCA1-induced cell death requires MPT pore components and second by testing whether reduction of mitochondrial cholesterol levels by independent means can mimic the effects of ABCA1 reexpression. Mitochondria-dependent cell death occurs following MPT, during which mitochondrial proteins form membrane pores that enable the release of molecules such as cytochrome c, which can initiate both apoptotic and nonapoptotic cell death. (Kroemer et al., 2007; Nakagawa et al., 2005). Cyclophilin D (CypD) and the adenine nucleotide translocator (Ant1) are two mitochondrial proteins that play key roles in initiating MPT (Baines et al., 2005; Lee et al., 2009; Nakagawa et al., 2005). To test whether CypD or Ant1 are required for the increased death sensitivity of ABCA1-positive mp53/Ras cells and ABCA1 anticancer activity, we lowered expression of *CypD* or *Ant1* via shRNA-mediated knockdown. We found that both *CypD* and *Ant1* are essential for ABCA1-mediated death sensitization and tumor inhibition (Figure 3), suggesting that the antitumor activity of ABCA1 requires mitochondrial function. In addition, reduction of mitochondrial cholesterol following knockdown of LC in mp53/Ras cells not only induces cytochrome c release (Figure 2), but also facilitates cell death in a *CypD*- and *Ant1*-dependent manner (Figure S3) indistinguishable from ABCA1-positive cells. Taken together, these data support the idea that ABCA1-induced reduction of mitochondrial cholesterol sensitizes cancer cells to stimuli or stresses promoting cell death.

independent experiments, and error bars indicate SD. *p < 0.01 versus all other cell lines, by Student's t test.

(C and D) Box plots indicating the volumes of tumors 4 weeks after subcutaneous implantation of the mp53/Ras cell derivatives indicated in (A, B). Each box shows the range from the first to third quartile of the tumor volumes. The line in the box indicates the median tumor volume. The bars represent the largest and smallest tumors. *p < 0.01 versus all other cell lines, by Student's t test.

(E and F) Western blots showing expression of ABCA1. Tubulin serves as a loading control.

(G and H) Expression of *CypD* and *Ant1* was measured by real-time quantitative PCR. Error bars indicate SD.

See also Figure S3.

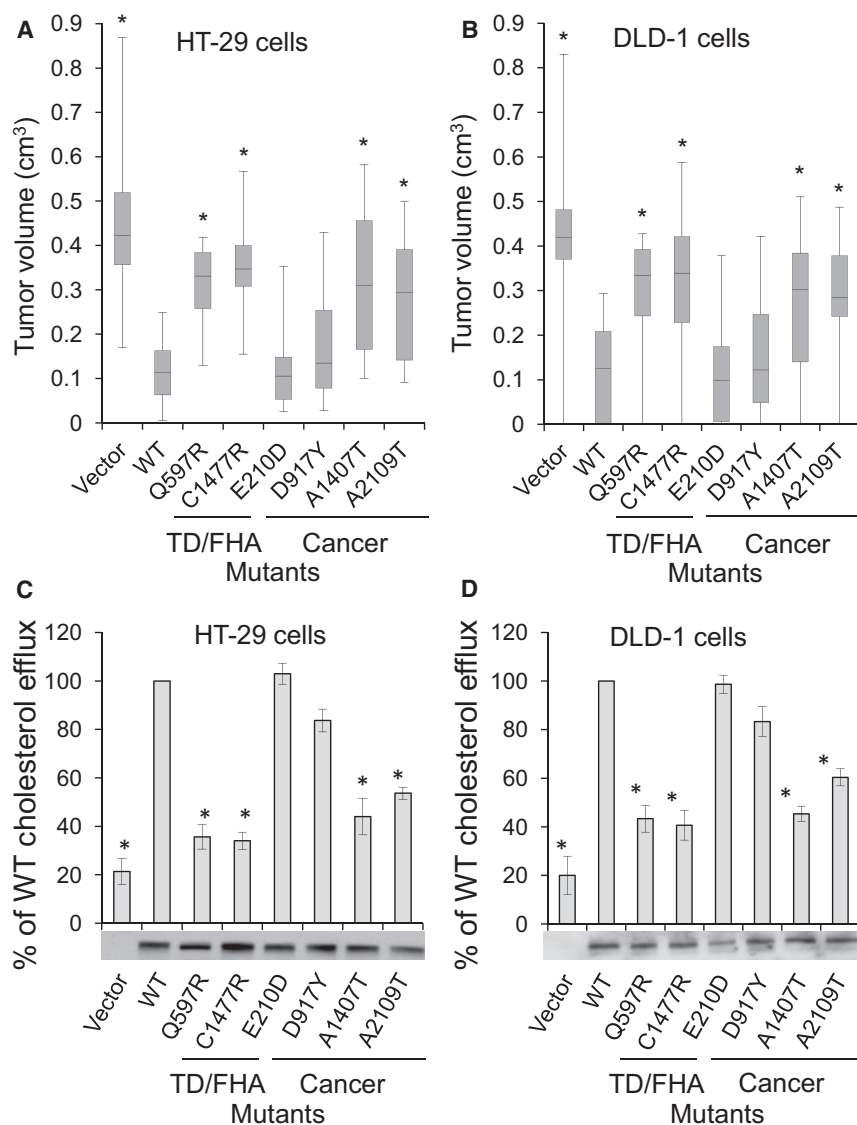


Figure 4. Loss of ABCA1 Efflux Function Correlates with Reduced Tumor Suppression

(A and B) Box plots indicating the volumes of tumors 5 weeks after subcutaneous implantation of the indicated derivatives of human colon cancer cell lines: (A) HT-29 and (B) DLD-1 expressing wild-type human ABCA1 (hABCA1 WT) or the indicated ABCA1 mutants. Each box shows the range from the first to third quartile of the tumor volumes. The line in the box indicates the median tumor volume. The bars represent the largest and smallest tumors. * $p < 0.01$ versus WT hABCA1 expressing cell line, by Student's t test.

(C and D) Cholesterol efflux capacities of hABCA1 mutants expressed in (A) HT-29 and (B) DLD-1 cells relative to hABCA1 wild-type. Values indicate percentage of wild-type efflux and represent the average of three independent experiments. Error bars indicate SD. * $p < 0.01$ versus WT hABCA1 expressing cell line, by Student's t test. Images below each graph are western blots showing representative protein expression of WT and mutant hABCA1 in cell lines used in (A)–(D).

See also Figure S4.

cells when implanted subcutaneously into immunocompromised mice and increased cholesterol efflux capacity in both cell types (Figure 4). In contrast, both TD/FHA mutants, although expressed to similar levels as wild-type ABCA1, did not decrease tumor formation, and as expected were deficient for cholesterol efflux (Figure 4), thus supporting a causal link between ABCA1's anti-tumor activity and its efflux capacity.

Notably, somatic mutations in the ABCA1 gene have been discovered following genomic sequencing of human colon cancer samples. In addition to the well-known cancer genes (i.e., p53, Apc,

Cholesterol Efflux Function Linked to ABCA1 Antitumor Activity

Further evidence substantiating a causal link between ABCA1-mediated tumor inhibition and mitochondrial cholesterol depletion comes from testing the dependence of ABCA1 anticancer activity on its efflux capacity. To this end, we utilized two missense mutations of human ABCA1 proteins (Q597R and C1477R) linked to TD and familial hypoalphalipoproteinemia (FHA), which were previously characterized to have diminished cholesterol efflux activity (Singaraja et al., 2006). Functionality of these ABCA1 mutants in terms of tumor inhibition and cholesterol efflux was tested in human cancer cells lacking endogenous ABCA1 expression, as preliminary experiments revealed that human ABCA1 is inactive in murine cells, indicating a cross-species incompatibility (data not shown). We thus first confirmed that ectopically expressed wild-type human ABCA1 inhibited tumor growth of HT-29 and DLD-1 human colon cancer

and Ras), ABCA1 placed in the top group of genes mutated in human colon cancers at low frequency, with four separately occurring single base pair substitutions identified (Sjöblom et al., 2006). Given the correlation between tumor inhibition and efflux activity of ABCA1 described above, we sought to test whether the ABCA1 colon cancer mutations affect efflux and anti-tumor activity similar to the TD/FHA mutants. We thus engineered each of these four colon-cancer-associated mutations individually into a human ABCA1 cDNA to test their effects on ABCA1 functionality. Two of the mutants (A1407T and A2109T) demonstrated a marked reduction in both cholesterol efflux and anti-tumor activity, while the other two mutants (E210D and D917Y) showed activity indistinguishable from the wild-type (wt) ABCA1 cDNA in both assays and in both human cancer cell lines used (Figure 4). Our data thus identify ABCA1 human colon cancer mutations A1407T and A2109T as loss-of-function mutations disabling both cholesterol efflux and anti-tumor activity.

Additional evidence to support the conclusion that efflux-deficient *ABCA1* mutants lose antitumor activity is provided by *ABCA1* expression analysis in tissue samples pre- and postimplantation into mice. While expression of wt *ABCA1* is strongly reduced following transplantation (see also Figure S1A for murine *Abca1*), expression of loss-of-function *ABCA1* mutants A1407T and A2109T remains relatively unchanged, presumably because their antitumor activity is strongly diminished (Figure S4).

In summary, our data reveal a close correlation between cholesterol efflux capacity, reduction of mitochondrial cholesterol in presence of elevated cholesterol synthesis rates, and antitumor activity of *ABCA1*. We also demonstrate a loss-of-function phenotype for somatic mutations identified in human colon cancer and thus suggest a role of *ABCA1* as a tumor suppressor, albeit specifically in the context of increased cholesterol synthesis rates competent to raise levels of mitochondrial cholesterol.

DISCUSSION

Here, we demonstrate an anticancer function of the cholesterol exporter *ABCA1* in human cancer cells. Our data link malignant cell transformation to defective cholesterol efflux, either following suppression of *ABCA1* gene expression in response to cooperating oncogenic mutations or loss-of-function mutation. *ABCA1* deficiency allows for elevated mitochondrial cholesterol and ultimately supports cancer cell survival. We suggest that this involves increased retention of cell death-promoting molecules in mitochondria, presumably due to decreased membrane fluidity and inhibition of MPT (Colell et al., 2003; Montero et al., 2008). Conversely, *ABCA1* antitumor activity requires efflux function and appears to be mediated by reduced mitochondrial cholesterol combined with an increased potential for release of cell death promoting molecules, such as cytochrome *c*, from mitochondria.

ABCA1 loss-of-function mutations play a causal role in TD and FHA, cardiovascular conditions characterized by abnormally low levels of plasma HDL cholesterol (HDL-C) (Rust et al., 1999). Missense mutations identified in the *ABCA1* gene of TD/FHA patients reduce efflux function, leading to a diminished ability of *ABCA1* to export excess intracellular cholesterol to apolipoprotein A1, forming HDL-C particles (Singaraja et al., 2006). While TD/FHA has not been linked to cancer, possibly due to the relatively low incidence of TD/FHA, low serum HDL levels, a characteristic feature of TD/FHA, correlate with higher incidence of multiple cancers (Liu et al., 2011; McGrowder et al., 2011; Van Hemelrijck et al., 2011). Our finding that multiple independent *ABCA1* missense mutations found in TD/FHA patients and human colon cancers show loss of both cholesterol export function and antitumor activity, supports an anticancer function of *ABCA1* in human malignancy. In addition our data strongly argue in favor of a causal link between export and anticancer functions.

Genome-scale DNA sequencing of human cancer samples has revealed tumor-associated genetic alterations, thus identifying numerous candidate oncogenes or tumor suppressor genes (Beroukhi et al., 2010; Cancer Genome Atlas Research

Network, 2008; Ding et al., 2008; Sjöblom et al., 2006). Specifically, sequencing DNA from human colon cancer specimen identified, among others, four independently occurring somatic mutations in the *ABCA1* gene, albeit with lower frequency than the well-known oncogenic mutations in genes such as *Apc*, *p53*, and *Ras* (Sjöblom et al., 2006). Here we demonstrate that two of four mutations identified in colon cancer samples are loss-of-function mutations. The two other *ABCA1* mutations, in contrast, had no significant effects on *ABCA1*-mediated efflux or tumor inhibition, and thus may represent functionally neutral passenger mutations serendipitously coselected with other cancer-relevant driver mutations (Forrest and Cavet, 2007; Getz et al., 2007; Parmigiani et al., 2007; Rubin and Green, 2007). Alternatively, these mutations may exhibit a subtle and rather context-dependent loss-of-function phenotype, e.g., dependence on intermolecular interactions, that remains undetected in our experimental models.

Diminished cholesterol efflux can be presumed a likely consequence of somatic *ABCA1* loss-of-function mutations in human colon cancers, although the mutations were found to be heterozygous (Sjöblom et al., 2006). While TD/FHA patients exhibiting almost complete absence of plasma HDL-C are typically homozygous for *ABCA1* loss-of-function mutations, heterozygosity of *ABCA1* is sufficient to produce intermediate HDL-C deficiencies (Singaraja et al., 2006). This suggests haploinsufficiency for *ABCA1* function, thus rendering cholesterol export particularly vulnerable not only to gene mutation but also to partial suppression of *ABCA1* gene expression, particularly in scenarios where this will lead to increased mitochondrial cholesterol. In fact, inhibition of *ABCA1* expression has been observed in human colon, breast, lymphoblastic, and liver cancer specimen (Basso et al., 2005; Ki et al., 2007; Moustafa et al., 2004; Schimanski et al., 2009), indicating decreased *ABCA1* efflux activity in a variety of human cancers.

In addition to loss of cholesterol export activity, *ABCA1* mutations linked to TD/FHA, including those utilized in the current study, also show deficiency in phospholipid efflux (Singaraja et al., 2006). As a key component of HDL particles, phospholipids play an important role in the removal of cellular cholesterol (Attie, 2007) and thus diminished phospholipid efflux may exacerbate defects in cholesterol efflux. Although we cannot exclude the possibility that *ABCA1* loss-of-function effects are in part mediated by metabolites such as phospholipids, extensive experimentation described above indicates that lowering cholesterol synthesis closely mimics the effects of *ABCA1* suppression or loss of function, thus suggesting cholesterol as a key driver of the observed effects.

Cholesterol lowering drugs, such as statins, in use for their benefits in cardiovascular disease have been evaluated for their efficacy in prevention and treatment of cancer. A variety of retrospective studies have revealed association between long-term statin use and lower cancer incidence (Cauley et al., 2003; Farwell et al., 2008; Karp et al., 2008; Khurana et al., 2007; Poynter et al., 2005; Shannon et al., 2005), as well as improved outcome (Gutt et al., 2010; Kawata et al., 2001; Nowakowski et al., 2010; Siddiqui et al., 2009). In addition, when combined with chemotherapy, statins significantly augment efficacy of therapy in patients with acute myelogenous leukemia (Kornblau

et al., 2007), while little to no anticancer effects of statins have been observed when used in patients as single agents (Holstein et al., 2006; Knox et al., 2005; Thibault et al., 1996). Therapeutic efficacy of statins in combination with chemotherapy may be due to the capability of statins to sensitize cancer cells to chemotherapy-induced cell death (Kornblau et al., 2007). This chemosensitization is consistent with the antitumor mechanism of lowering cholesterol proposed here and by others (Montero et al., 2008), where decreased cholesterol in mitochondria leads to increased sensitivity to stimuli inducing cell death, presumably by facilitating MPT.

The in vivo genetic evidence provided here reveals anticancer activity of the cholesterol exporter *ABCA1*, which can be disabled by *ABCA1* loss-of-function mutations or suppression of *ABCA1* gene expression in response to cooperating oncogenic mutations. Moreover, we suggest that loss of *ABCA1*-mediated efflux is a key step in allowing the accumulation of mitochondrial cholesterol levels that support cancer survival. Taken together, our data thus support a causal role for elevated mitochondrial cholesterol in cancer cells and provide a mechanistic rationale for cholesterol targeting as a potentially fruitful approach to augment cancer intervention and/or prevention strategies.

EXPERIMENTAL PROCEDURES

Cells

Young adult mouse colon (YAMC) cells (Whitehead et al., 1993), and YAMC cells expressing p53^{175H} (mp53), HRas^{V12} (Ras), and both p53^{175H} and HRas^{V12} (mp53/Ras) cells were maintained as previously described (McMurray et al., 2008; Xia and Land, 2007). DLD-1 human colon cancer cells were provided by Dr. J. Filmus and HT-29 human colon cancer cells were purchased from ATCC. Both human cell lines were cultured at 37°C in DMEM media (Invitrogen) supplemented with 10% FBS (Hyclone) and 2.5 µg/ml genamycin (Invitrogen). See Extended Experimental Procedures for derivation of genetically perturbed cells.

Xenograft Assay

Tumor formation was assayed as previously described (McMurray et al., 2008). See Extended Experimental Procedures for detailed description.

Cell Death Assays

Prior to cell death assay, mp53/Ras cells were grown at 39°C for at least 48 hr. To assess resistance to cell death, mp53/Ras cells were seeded at low density (2,265 cells/cm²) in standard RPMI growth media. Sixteen hours later, cells were washed with PBS and then given RPMI media devoid of serum and ITS. Media given to *ABCA1*-reconstituted and matched vector control mp53/Ras cells was also supplemented with 20 µg/ml apolipoprotein A1 (Meridian Life Science) to lower intracellular cholesterol by inducing cholesterol efflux. These cells were given a second dose of 20 µg/ml apolipoprotein A1 48 hr later. Cell death was measured 72 hr after initial starvation by trypan blue exclusion assay as described in product manual (Invitrogen), or fixed in 4% paraformaldehyde and TUNEL stained (Roche). The amount of TUNEL-positive cells was quantified via fluorescence-activated cell sorting analysis.

Intracellular Cholesterol Measurements

Prior to measuring intracellular cholesterol, cells were grown at 39°C for at least 48 hr followed by growth in RPMI media, devoid of serum and ITS, for 72 hr. Media given to *ABCA1*-reconstituted, *ABCA1*-knockdown, and matched vector control cells during this 72 hr time period was also supplemented with 20 µg/ml apolipoprotein A1 to induce cholesterol efflux. A second dose of 20 µg/ml apolipoprotein A1 was given after 48 hr. Intracellular cholesterol levels were determined using the Amplex Red Cholesterol Assay Kit

(Invitrogen) or thin layer chromatography. See Extended Experimental Procedures for more detailed description of cholesterol measurements.

Mitochondrial Cholesterol Loading, Matrix Swelling, and Cytochrome c Release Assays

Cholesterol levels were restored in cholesterol-depleted mitochondria of *ABCA1*-reconstituted and lanosterol cyclase-knockdown mp53/Ras cells as described previously (Montero et al., 2008). Sensitivity to calcium-induced mitochondrial matrix swelling and cytochrome c release was then determined as previously described (Montero et al., 2008).

Cholesterol Efflux Assay

Cholesterol efflux was measured as previously described (Singaraja et al., 2006). Briefly, cells were given 1 µCi/ml [³H]cholesterol (Perkin-Elmer) for 16 hr, followed by washing with PBS containing 0.2% fatty acid free BSA (Sigma). Cells were then incubated for 24 hr in serum free DMEM containing 0.2% fatty acid free BSA and 20 µg/ml Apolipoprotein A1. Following incubation, media containing effluxed cholesterol was collected and centrifuged at 10,000 × g for 5 min to remove debris. Radioactivity was quantified by liquid scintillation and normalized to total protein. Efflux rate was calculated as a percentage of efflux by wild-type *ABCA1* and indicated values represent the average of three independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.08.011>.

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