The transmembrane protein CLPTM1L is overexpressed in non–small cell lung cancer, where it protects tumor cells from genotoxic apoptosis. Here, we show that RNA interference-mediated blockade of CLPTM1L inhibits K-Ras–induced lung tumorigenesis. CLPTM1L expression was required in vitro for morphologic transformation by H-RasV12 or K-RasV12, anchorage-independent growth, and survival of anoikis of lung tumor cells. Mechanistic investigations indicated that CLPTM1L interacts with phosphoinositide 3-kinase and is essential for Ras-induced AKT phosphorylation. Furthermore that the anti-apoptotic protein Bcl-xL is regulated by CLPTM1L independently of AKT activation. Constitutive activation of AKT or Bcl-xL rescued the transformed phenotype in CLPTM1L-depleted cells. The CLPTM1L gene lies within a cancer susceptibility locus at chromosome 5p15.33 defined by genome-wide association studies. The risk genotype at the CLPTM1L locus was associated with high expression of CLPTM1L in normal lung tissue, suggesting that cis-regulation of CLPTM1L may contribute to lung cancer risk. Taken together, our results establish a protumorigenic role for CLPTM1L that is critical for Ras-driven lung cancers, with potential implications for therapy and chemosensitization.

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accumulation of phosphorylated AKT and Bcl-xL, independently. Regulation of AKT activity may be due to an interaction with PI3K catalytic subunits. A robust inhibition of Ras-driven in vitro transformation and in vivo tumorigenesis upon depletion of CLPTM1L establishes this protein as a protumorigenic factor required for oncogenesis by K-Ras. This inhibition of transformation was dependent on inhibition of both AKT phosphorylation and Bcl-xL expression. Our studies strongly implicate protection from apoptosis and regulation of apoptotic effectors as mechanisms for the protumorigenic function of CLPTM1L. Furthermore, association of the risk genotype at 5p with high expression of CLPTM1L suggests that cis-regulation of this gene may contribute to lung cancer risk.

Materials and Methods

Cell culture, knockdown, and overexpression

Human lung adenocarcinoma cell lines (A549 and H838) and Spon 8 mouse lung tumor cell lines were cultured in RPMI-1640 plus 2% FBS (Invitrogen). Beas-2B were cultured in LHC-8 media plus epinephrine (Invitrogen), and NIH3T3 were cultured in Dulbecco’s Modified Eagle Media (DMEM) with 10% FBS (Invitrogen). Cells were transfected with lentiviral short hairpin RNA (shRNA) vectors based on the pLKO.1 vector and designed to specifically target human CLPTM1L transcript (Sigma). Empty vector, scrambled shRNA vector, or vectors targeting CLPTM1L transcript were first packaged in 293T cells (Orbigen) by transfection with helper plasmids using Lipofectamine LTX (Invitrogen) and then transduced into A549 cells with 8 μg/mL polybrene (Sigma). Media were replaced 24 hours after transduction, and cells were split 1:4 48 hours after transduction. At 72 hours posttransduction, cells harboring lentiviral constructs were selected with 1 μg/mL puromycin for 2 to 4 days, until mock-infected cells were dead. Surviving cells were pooled. Mouse cells were transfected and selected similarly with shRNA constructs designed to target mouse CLPTM1L transcript. NIH3T3 cells were transfected using Lipofectamine LTX with pBABE:empty vector or pBABE:H-RasV12, pLKO.1,vector, or pLKO.1:shCLP. shCLP used in NIH3T3 and mouse studies is identical to sh2 in Supplementary Fig. S4. For myrAKT studies, the above described stable cell lines were transfected with pBABEmyrAKT or empty vector and assayed after 48 hours. For Bcl-xL studies, the above-described stable cells were transfected with pSFFV:Bcl-xL plasmid (8749 Addgene) or empty vector and selected with G418 until mock-transfected cells were dead. Cells were plated at 200,000 cells per well on 6-well tissue culture dishes and assayed after 48 hours. Authenticated A549, H838, Beas-2B, and NIH3T3 cells were obtained from American Type Culture Collection (ATCC) within 6 months of experiments. Spon 8 cells were developed from spontaneous and metastatic lung tumors from A/J mice by our laboratory when housed at The Ohio State University in 1996 and are characterized (22, 23). These cells are periodically authenticated based on the molecular profile described in therein, which was conducted within 3 months of their use in experiments.

Reverse transcription quantitative real-time PCR

Patient-matched tumor and tumor-adjacent normal RNA samples were obtained from the Tissue Procurement Core at Washington University in St. Louis under protocol approved by the Institutional Review Board at Washington University in St. Louis School of Medicine. Human Research Protection Office. Written consent was obtained from all patients participating in this tissue bank. RNA was isolated from cell lines using TRIzol reagent and protocols (Invitrogen). Quantitative real-time PCR (qPCR) was carried out using the method as described previously (24). Briefly, 1 μg of total RNA per sample was converted to cDNA using the SuperScript First-Strand Synthesis system for reverse transcription (RT)-PCR (Invitrogen). Quantitative RT-PCR assay was done using the SYBR Green PCR Master Mix (Applied Biosystems). One microliter of cDNA was added to a 25 μL total volume reaction mixture containing water, SYBR Green PCR Master Mix, and primers. Each real-time assay was done in duplicate on a BioRad MyIQ machine. Data were collected and analyzed with Stratagene Mx3000 software. The β-actin gene (Actb) was used as an internal control to compute the relative expression level (ΔCt) for each sample. Primer set efficiency and linearity was calculated, and normalization was conducted in accordance with MIQE guidelines. The fold change of gene expression in tumor tissues as compared with the paired normal tissues was calculated as 2ΔΔCt, where d = ΔCt_tumor − ΔCt_normal.

Western blotting

Cells were lysed with 100 μL of 1× NP40 lysis buffer containing protease inhibitors, sheared 10 times with a 28-gauge needle, spun at 16,000 × g for 30 minutes, normalized by protein concentration as determined by the Bradford method, and the supernatant boiled for 5 min. Twenty microliters of normalized lysate was resolved by SDS-PAGE and immuno-blotting analyzed with indicated antibodies. The following antibodies were used: rabbit anti-CLPTM1L (Novus Biologicals), mouse anti-Actin (Santa Cruz Biotechnology), mouse anti-Bcl2 clone 124 (Dako), rabbit anti-Bax #2774 (Cell Signaling), mouse anti-p53 (Ab-1; Oncogene), Bcl-xL – rabbit Bcl2L1 (Abcam), rabbit anti-H-Ras (Novus Biologicals), mouse anti-K-Ras (BD Transduction Labs), anti-AKT (Cell Signaling), anti-pAKT (Thr308; Cell Signaling), rabbit anti-PIK3C3 (Cell Signaling), and rabbit anti-BAD (Abcam). Quantitation of Western blot analyses of three independent cultures was done using ImageJ software (25).

Comunmunoprecipitation

Antibodies for bait proteins (PIK3C3, PIK3CA, CLPTM1L, and actin, described above) were immobilized covalently using amino-link columns from Pierce Co-Immunoprecipitation Kit (Peirce, Thermo Scientific) according to manufacturer’s protocol. Lysates were obtained, cleared on agarose resin, and immunoprecipitated according to the protocol. Western blotting for PI3K or CLPTM1L was conducted on IP eluates as described above. Immobilized actin antibody was used as irrelevant bait. IP column flow through with no bait antibody was run as an input control.
shRNA/K-RasLSL-G12D/+ mouse model of lung tumorigenesis

Mouse experiments were carried out as described previously (26), with the following modifications. pLKO.1 empty shRNA vector was obtained from Open Biosystems. Short hairpin inserts were designed to specifically target transcripts. Oligos were ordered from IDT. Complimentary oligos were heated to 95°C, cooled to room temperature overnight, and ligated into digested pLKO.1. Vectors were modified by replacing the PGK promoter and puromycin resistance orf with the CMV promoter. Driving expression of CRE-GFP. CMV promoter was subcloned from pLenti CMV GFP Puro plasmid #17448 (Addgene) and CRE-GFP was subcloned from pCAG:CRE-GFP plasmid # 13776 (Addgene). Virus was packaged in 293T cells and functionally titered by infecting 3T3 cells (LSL-LacZ), which express LacZ upon Cre recombinase activation. Mice were anesthetized with 200 μL Avertin (40 μg/mL). About 10⁵ or 10⁶ active virus particles in 50 μL of PBS was delivered via intratracheal intubation using a 22-gauge i.v. catheter under general anesthesia to 10 transgenic mice per group [LSL-K-RasG12D (Mouse Models of Human Cancers Consortium (MMHCC) Strain 01XJ6, Jackson Laboratory #008179 (B6), #008180 (129)]. After 24 weeks, mice were anesthetized and euthanized by cervical dislocation. The thoracic cavity was surgically opened to expose the lungs. The trachea was cannulated with a 22-gauge catheter and lungs were inflated with Tellyesniczky’s solution (70% ethanol, 2% formaldehyde, and 5% glacial acetic acid) at 25 cm of pressure by gravity. Lungs were fixed overnight and the solution was exchanged to 70% ethanol the next day. Lungs were photographed, lobes were separated and cleaned. Tumors were counted and measured with digital calipers. A cutoff for visible tumors of 0.2 mm diameter was used. Tumor volume was determined by the following formula: 

\[ V = \frac{4}{3} \pi r^3 \]

Values were determined using a 2-tailed Student t test.

Transformation assays

NIH3T3 cells acquired within the last 6 months from ATCC were cultured in DMEM 10% FBS to 80% confluence before cotransfecting with the indicated expression and shRNA vectors using Lipopectamine LTX (Invitrogen). Cells were split, allowed to attach, and placed on puromycin selection for 3 days or until mock-transfected cells were dead. Cells were plated at the indicated densities and fed as needed. For whole-plate staining, cells were fixed in cold methanol and stained with crystal violet. For anchorage-independent growth, cells were suspended in 0.4% agarose in complete growth media and plated over 0.8% bottom agar at 10,000 cells per well (H-Ras) or 20,000 cells per well (K-Ras) of a 6-well tissue culture dish in triplicate. Cells were fed twice a week over 4 weeks in culture, and colonies were stained using cell staining reagent and protocol from Millipore’s Cell Transformation Detection Assay. Images of wells were captured and analyzed by ImageJ software to count colonies. Values were determined using a 2-tailed Student t test.

Anoikis assay

A total of 2 × 10⁵ cells were plated on either conventional treated-6-well tissue culture plates (TPP) or on poly-HEMA-coated, nonadherent 6-well tissue culture plates (Sciencell) with 1 μmol/L CellPlayer green fluorescent caspase-3-substrate (Essen Bioscience) and analyzed on an Incucyte FLR live cell imager (Essen Bioscience) over 44 hours in culture for caspase-positive cells.

Nude mouse xenograft assay

Tumor cells stably expressing shRNA vectors as described above were cultured, counted, and resuspended in sterile PBS at a concentration of 2.5 × 10⁶ cells/mL. A volume of 200 μL (5 × 10⁵ cells, respectively) was injected subcutaneously into the right (vector) or left (shRNA) flank of athymic nude mice at an age of 8 weeks. The health of these mice was monitored 3 times weekly and tumor sizes were measured periodically until sacrifice at 4 weeks postinjection. Tumors were removed and weighed. Protein was collected from approximately 30 mg of tumor tissue by homogenization with a TissueLyser LT and 5 mm steel beads (Qiagen) in radioimmunoprecipitation assay (RIPA) buffer (Sigma) with protease and phosphatase inhibitors. P values were determined by 2-tailed Student t test.

Results

High expression of CLPTM1L in lung tissue correlates with disease-associated genotype

In 30 lung adenocarcinomas, CLPTM1L expression averaged 2.8-fold greater in tumor tissue than in matched normal lung tissue (P < 0.005; Fig. 1A). Of these patients, 25 (83%) overexpressed CLPTM1L over adjacent normal tissue [19 (63%) by >1.5-fold], with a maximum of 8.7-fold. Publicly available data analyzed and visualized using Oncomine from Compendia Bioscience in Ann Arbor similarly show highly significant upregulation of expression of CLPTM1L in lung squamous cell carcinoma (P < 5 × 10⁻³³) and lung adenocarcinoma (P < 5 × 10⁻¹⁴) compared with normal lung tissue (Supplementary Fig. S1), as well as in many other cancer types (data not shown). With the knowledge that CLPTM1L is commonly overexpressed in lung adenocarcinoma (Fig. 1A; ref. 2) and that genetic polymorphisms within CLPTM1L are associated with risk of developing lung cancer (8, 9, 11, 15), we investigated whether expression in tumor-adjacent normal lung tissue correlated with the disease associated polymorphisms within the gene. We therefore genotyped the rs31489 lung cancer variant and evaluated expression of CLPTM1L transcripts in tumor adjacent normal lung tissues of 32 patients with adenocarcinoma. This variant is one of the most significant risk variants in multiple GWAS studies (9, 17) and is in strong linkage disequilibrium (LD) with rs402710 and rs401681 lung cancer variants in multiple GWAS studies (9, 17) and is in strong linkage disequilibrium (LD) with rs402710 and rs401681 lung cancer variants. High expression of CLPTM1L in tumor adjacent normal lung tissues of 32 patients with adenocarcinoma strongly correlated with the risk genotype at rs31489 (C) (P < 0.0005; Fig. 1B and Supplementary Fig. S2). In agreement with our findings, a 2012 study by Grundberg and colleagues (27) showed an association of CLPTM1L expression with the same CLPTM1L SNPs in adipose tissue of 856 healthy female twins (Supplementary Fig. S3). The SNPs identified in this study are in perfect concordance with lung cancer–associated single-nucleotide polymorphisms (SNP).
CLPTM1L interacts with PI3K and is required for Ras-induced AKT activation and Bcl-xL accumulation

Given evidence that CLPTM1L may interact with catalytic subunits of PI3K (PIK3C3 and PIK3Calpha; ref. 5) and regulates survival of tumor cells (2), we investigated the effect of CLPTM1L on AKT phosphorylation and its interaction with PI3K. Coimmunoprecipitation was conducted on NIH3T3 cell lysates using PI3K, CLPTM1L, or β-actin control antibody immobilized covalently on a resin column. Immunoprecipitates and control lysate were immunoblotted for PI3K or CLPTM1L. CLPTM1L coprecipitated with PI3K class III and class I alpha catalytic subunits, but not with actin control antibody, both when used as bait and prey (Fig. 2A).

Survival signaling by PI3K in tumor cells is often mediated by phosphorylation of AKT (6). To investigate the effect of CLPTM1L on AKT phosphorylation and its interaction with PI3K, Coimmunoprecipitation was conducted on NIH3T3 cell lysates using PI3K, CLPTM1L, or β-actin control antibody immobilized covalently on a resin column. Immunoprecipitates and control lysate were immunoblotted for PI3K or CLPTM1L. CLPTM1L coprecipitated with PI3K class III and class I alpha catalytic subunits, but not with actin control antibody, both when used as bait and prey (Fig. 2A).

CLPTM1L is required for Ras-induced oncogenic transformation and anchorage-independent growth

To determine whether CLPTM1L is required for oncogenic transformation by Ras, we cotransfected NIH3T3 mouse fibroblasts along with shRNA targeting CLPTM1L (shCLP). Phenotypic results with multiple shRNA constructs targeting CLPTM1L minimize the possibility of any off-target effects. The sh2 construct demonstrated the best knockdown efficiency and was subsequently used for knockdown studies in mouse cells (hereafter referred to as shCLP). Western blotting of lysates from NIH3T3 cells with K-RasV12 and/or shCLP demonstrated a decrease in Bcl-xL expression with loss of CLPTM1L (Fig. 2B). These data are consistent with our previous observations in mouse and human lung tumor cells, in which both a decrease in Bcl-xL expression and sensitivity to genotoxic apoptosis accompanied CLPTM1L depletion (Supplementary Fig. S4A). Phenotypic results with multiple shRNA constructs targeting CLPTM1L minimize the possibility of any off-target effects. The sh2 construct demonstrated the best knockdown efficiency and was subsequently used for knockdown studies in mouse cells (hereafter referred to as shCLP). Western blotting of lysates from NIH3T3 cells with K-RasV12 and/or shCLP demonstrated a decrease in Bcl-xL expression with loss of CLPTM1L (Fig. 2B). These data are consistent with our previous observations in mouse and human lung tumor cells, in which both a decrease in Bcl-xL expression and sensitivity to genotoxic apoptosis accompanied CLPTM1L depletion (2) and (Supplementary Fig. S4). Expression of K-RasV12 in NIH3T3 cells increased levels of both Bcl-xL and phosphorylated (T308) AKT (Fig. 2B). However, when CLPTM1L was stably depleted with shRNA in K-RasV12-expressing cells, the elevation of both Bcl-xL and phospho-AKT was ablated.

CLPTM1L is required for Ras-induced oncogenic transformation and anchorage-independent growth

To determine whether CLPTM1L is required for oncogenic transformation by Ras, we cotransfected NIH3T3 mouse fibroblasts with expression vectors for H-RasV12 or K-RasV12 and shRNA targeting CLPTM1L (shCLP). Stable transfection of...
either H-RasV12 or K-RasV12 induced a transformed morphology (Fig. 3A). The transformed cells grew in crossing spindle patterns with foci growing into dense spheroids that sometimes detached and became free-floating, as has been previously described in oncogenic K-Ras–expressing NIH3T3 cells (28). However, upon stable cotransfection of shCLP with either H-RasV12 or K-RasV12, a nearly complete reversion of the phenotype occurred, and no spheroids were observed. Cells with RasV12/shCLP displayed altered morphology compared with vector controls, but they did not form dense foci or grow in anchorage-independent spheroids. Cells with shCLP alone did not demonstrate morphologic changes compared with vector controls. Exogenous overexpression of RasV12 and knockdown of CLPTM1L was confirmed by Western blotting. To further determine the effect of CLPTM1L depletion on the ability of Ras-transformed NIH3T3 cells to grow in an anchorage-independent manner, soft agar tissue culture was used. Expression of H-RasV12 induced anchorage independence, forming an average of 18 colonies per well in soft agar (Fig. 3B), whereas H-RasV12 expressing cells with CLPTM1L depletion formed an average of only one colony per well in soft agar, representing a 94% inhibition of colony formation (P < 0.05). Similarly, K-RasV12 transfection transformed NIH3T3 cells. With twice as many cells plated as were plated for HRasV12 soft agar assays, K-RasV12 transformed NIH3T3 cells formed 56 colonies per well, which was inhibited by 47% upon CLPTM1L depletion (P < 0.05), demonstrating a requirement for CLPTM1L for oncogenic Ras-induced anchorage-independent growth.

AKT activity and Bcl-xL expression are independently sufficient to reconstitute Ras oncogenic transformation in CLPTM1L-depleted cells

To investigate the role of AKT phosphorylation and Bcl-xL expression in the CLPTM1L-mediated effects on Ras transformation, we expressed constitutively active myristoylated AKT (myrAKT), Bcl-xL, or the corresponding empty vectors in our stable NIH3T3 cell lines expressing K-RasV12 and shCLP. Expression of myrAKT rescued the transformed phenotype in NIH3T3 cells with K-RasV12 expression and CLPTM1L depletion (Fig. 4A). Reconstitution of either AKT signaling or Bcl-xL resulted in formation of spheroids and macroscopically visible foci. Similarly, re-expression of exogenous Bcl-xL rescued Ras transformation, indicating that either maintenance of AKT phosphorylation or Bcl-xL regulation is sufficient for the effect of CLPTM1L on Ras transformation. Expression of phosphorylated myrAKT and Bcl-xL was confirmed by Western blotting (Fig. 4B). Expression of myrAKT did not alter Bcl-xL expression or its regulation by CLPTM1L, indicating that Bcl-xL is regulated by a separate mechanism and that each is independently sufficient for Ras transformation in CLPTM1L-depleted cells.

CLPTM1L protects human lung tumor cells from anoikis and is required for tumorigenesis

To determine whether CLPTM1L is similarly necessary in lung tumor cells for survival of anchorage detachment, assays for anoikis were used. A549 cells with stable CLPTM1L depletion using 2 independent shRNA vectors (shCLP or shCLP*) or scrambled shRNA control were plated in triplicate on polyHEMA–coated plates to prevent attachment, as well as conventional coated tissue culture plates. Nonadherent cells transfected with control vector grew in clusters of refractile cells (Fig. 5A). Nonadherent cells with CLPTM1L depletion grew in clusters that generally appeared flat and dull. Apoptotic cell numbers were monitored in real-time using a fluorescent caspase-3 substrate and live cell imaging system over 44 hours in culture. Apoptosis was induced to a significant degree on a nonadherent surface only when CLPTM1L was depleted using either shRNA vector, demonstrating that CLPTM1L is critical for protection of lung tumor cells from anoikis (P < 0.05; Fig. 5B). Depletion of CLPTM1L in A549 cells with shCLP and shCLP* was confirmed by Western blotting (Fig. 5C). Similar robust induction of anoikis with CLPTM1L depletion

![Diagram](image-url)

**Figure 2.** CLPTM1L regulates AKT pathway and Bcl-xL accumulation. A, coimmunoprecipitation of PI3KCA, PIK3C3, and CLPTM1L from NIH3T3 cell lysates using each as bait or prey. Actin represents a nonspecific control IP antibody. The control column represents lysate passed through an actin antibody–loaded column (lysat with no relevant immunoprecipitation). B, representative Western blot analyses for phosphorylated and total AKT, Bcl-xL, K-Ras, and CLPTM1L in NIH3T3 cells with K-RasV12 expression and/or shRNA CLPTM1L depletion or scrambled shRNA control.
using shCLP\textsuperscript{*} was observed in H838 human lung adenocarcinoma cells (K-H- and N-Ras wild-type/p53E62\textsuperscript{+/+}), demonstrating that the critical survival role of CLPTM1L is not limited to Ras-mutant or p53-competent tumor cells ($P < 5 \times 10^{-5}$; Fig. 5D and E). H838 cells with scrambled vector grew as mostly separated cells in poly-HEMA–coated wells, whereas CLPTM1L-depleted H838 cells grew in aggregates (Fig. 5D). Depletion of CLPTM1L was confirmed by Western blotting (Fig. 5F). We conducted xenograft experiments in athymic nude mice to determine whether CLPTM1L depletion affected the ability of A549 human lung tumor cells to form tumors. A total of $5 \times 10^5$ A549 cells with either CLPTM1L depletion or with scrambled shRNA control were injected subcutaneously into the left or right flank, respectively. Over 4 weeks, A549 cells with CLPTM1L depletion formed significantly smaller tumors (average weight = 72 mg), compared with control tumors (average weight = 254 mg; $P < 0.00005$; Fig. 6A). Depletion of CLPTM1L protein in tumors was confirmed by Western blotting (Fig. 6B).

**CLPTM1L is required for lung tumorigenesis in a shRNA/K-Ras\textsuperscript{LSL-G12D/+} mouse model**

A recently developed model of lung cancer in K-Ras\textsuperscript{LSL-G12D/+} transgenic mice uses intratracheal delivery of lentivirus expressing CRE recombinase to activate oncogenic K-Ras expression. This model also permits simultaneous expression of shRNAs targeting a gene of interest, via the same viral vector (26) and is thus particularly well suited to the investigation of potential modifiers of lung tumorigenesis. We used this model to induce oncogenic K-Ras–driven lung tumors in a cellular environment that is depleted of CLPTM1L (Fig. 7A). We also evaluated depletion of TERT in this model to agnostically approach 5p susceptibility candidates. Several lentiviral shRNA vectors targeting TERT or CLPTM1L were designed and evaluated for knockdown of their target transcripts in the Spon8 mouse lung tumor cell cells. Vectors shCLP2 and shTERT1 accomplished knockdown at the transcript level of 80% and 50% for CLPTM1L and TERT, respectively (Fig. 7B and Supplementary Fig. S5), and were subsequently used in the mouse model studies. Virus particles were packaged and tittered by infection of 3TZ cells for quantification of the number of CRE-dependent LacZ-inducing particles per milliliter. Mice were subsequently infected with 10\textsuperscript{4} functional particles (high-dose group) or 10\textsuperscript{3} particles (low-dose group) by intratracheal intubation. After 24 weeks, lungs were harvested and lung tumors were counted and sized for the high-dose group. Most tumors were $\leq 1$ mm in diameter with a few larger tumors. Mice in the high-dose group with a nonspecific scrambled shRNA had an average of 115 tumors per mouse with an average tumor load of 13.2 mm\textsuperscript{3} (Fig. 7C and E), whereas mice with CLPTM1L depletion had an average of 46 tumors per mouse with an average tumor load of 3.4 mm\textsuperscript{3}. This equates to a 60% inhibition of tumor number ($P < 0.05$) and a 74% inhibition of tumor load ($P = 0.05$) in mice with CLPTM1L depletion. Mice with TERT depletion had an average of 122
tumors per mouse with an average tumor load of 10.2 mm³ and were statistically similar to the control group (P = 0.58). The low-dose group was counted at 28 weeks postinfection. Scrambled control and shTERT mice had an average of 6 and 5 tumors, with average tumor loads of 0.50 and 0.41 mm³, respectively (Fig. 7D and E). Only one mouse with CLPTM1L shRNA (n = 7) had a single tumor for an average tumor number of 0.14 and load of 0.01 mm³. This represents 98% inhibition of both tumor number and load (P < 0.005 and P < 0.005, respectively). There was no significant difference or trend in tumor number or load between scrambled shRNA mice and mice with TERT depletion.

Discussion

The association of high CLPTM1L expression with disease SNPs at the 5p locus is highly suggestive that cis-regulation of CLPTM1L expression contributes to lung cancer risk. It is expressed in normal lung tissue, rather than in tumor tissue that is relevant to cancer susceptibility. Therefore, our SNP association analysis focused on normal tissue. The majority of tumors may acquire increased CLPTM1L expression regardless of genotype or the effect of genotype on basal expression. In accordance with this notion, the correlation of genotype with expression in tumor tissues trended higher with the risk genotype but was not significant (data not shown). Our results agree with the study by Grundberg and colleagues (27), which showed cis-regulation of CLPTM1L expression in adipose tissue of 856 healthy female twins (Supplementary Fig. S3). This twin’s study was able to separate heritable expression differences from those influenced by environment. By regulatory trait concordance (RTC) methodology, the authors showed perfect concordance of CLPTM1L regulatory SNPs with lung cancer risk SNPs, suggesting that cis-regulation of this gene is likely involved in heritable risk. We intend to conduct eQTL studies on a larger number of normal lung tissues to thoroughly investigate the relationship of CLPTM1L cis-regulation with lung cancer risk.

Results of our in vivo tumorigenesis experiments demonstrate for the first time that CLPTM1L likely plays a protumorigenic role in lung cancer. Proliferative rate in Beas-2B immortalized bronchial epithelial cells was not affected by CLPTM1L depletion, demonstrating that CLPTM1L is not required for normal proliferative function (Supplementary Fig. S6). Although CLPTM1L was necessary for anchorage-independent survival of both A549 and H838 lung tumor cells, proliferation on an adherent surface was only affected in H838 cells with CLPTM1L loss in association with increased cell death, not in A549 cells. However, A549 cell xenograft tumorigenesis was robustly and significantly inhibited upon depletion of CLPTM1L. This is presumably due to the demonstrated dependence on CLPTM1L for anchorage-independent survival.
The effect of CLPTM1L depletion on Ras-driven lung tumorigenesis was quite striking, significantly inhibiting both tumor incidence and load by up to 98%. This approach to validate modifiers of tumorigenesis allows for the inhibition of expression of a gene of interest with shRNA concurrently and exclusively in cells that are K-RasG12D induced (26). Our study further demonstrates the use of this model to validate modifiers of lung tumorigenesis, without necessitating the need to generate and/or cross the corresponding knockouts. This is especially useful in the investigation of candidate genes identified by lung cancer GWAS.

We did not see an effect on tumorigenesis with knockdown of TERT expression. This may indicate one or a combination of the following: (i) TERT expression does not immediately and directly affect lung tumorigenesis, (ii) this model may miss indirect, long-term or trans-generational affects, potentially related to maintenance of telomere length, (iii) longer telomere length in the mouse may mask the effect of TERT knockdown, and (iv) inefficient knockdown of TERT in vivo. Mice with shTERT also served as an additional nonspecific control for CLPTM1L-knockdown mice. TERT is well-studied for its role in telomere maintenance, bypass of replicative senescence and...
Overexpression of TERT allows cellular immortalization through telomere maintenance, which is the most likely mechanism by which TERT may contribute to the 5p association with cancer risk. A recent study suggests that 5p risk variants may be associated with hypermethylation in the TERT promoter (30); however, existing data regarding association of 5p variants with TERT expression and telomere maintenance are conflicting at best (31–33). In an analysis of SNPs in the 5p region, we have found that lung cancer–associated SNPs are not associated with telomere length (Supplementary Fig. S7), which is in agreement with two other studies (31, 32). In contrast, a study by Rafnar and colleagues showed an association between 5p variants (rs401681 and rs2736098) and telomere length (\(P = 0.017\) and \(0.027\), respectively), although this effect was only seen in women older than 75 years with homozygous genotypes (34).

Our demonstration that stable depletion of CLPTM1L ablates morphologic transformation and anchorage-independent growth of NIH3T3 cells by oncogenic H-Ras or K-Ras establishes CLPTM1L as possessing an important protumorigenic function relevant to not only cancer of the lung, but other cancers as well. We did not compare inhibition of colony formation by CLPTM1L depletion between Ras isoforms as equal numbers of cells were not used in K-Ras and H-Ras experiments. Given some heterogeneity in knockdown in a pooled population of cells and in the assay system, 100% inhibition would not be expected. The results obtained were quite robust for assays of this type. It is known that H-Ras and K-Ras can prevent downregulation of Bcl-xL upon detachment from the extracellular matrix, thereby avoiding anoikis or apoptosis due to detachment (35). Here, we present evidence that CLPTM1L is necessary for the sustained Ras-induced accumulation of Bcl-xL. Depletion of CLPTM1L and the resultant depletion of survival signals are associated with a nearly complete reversion of spheroid formation and anchorage-independent growth induced by Ras. Interestingly, depletion of CLPTM1L ablated Ras-induced AKT phosphorylation. The addiction of cancers to Ras can be reduced to PI3K/AKT signaling (36). NF-\(\kappa\)B signaling, which is downstream of AKT, has recently been shown to be required for lung tumorigenesis in a K-Ras–driven model very similar to that used in this study (37), although we did not see strong evidence that NF-\(\kappa\)B–dependent transcription was significantly affected by CLPTM1L (data not shown). AKT signaling is known to confer resistance to anoikis (38–40) and apoptosis induced by TRAIL (38), etoposide chemotherapy (41), and the p53 pathway (41, 42). Together, these data suggest that CLPTM1L may be an important regulator of the AKT survival signaling pathway. In fact, constitutively active AKT restores the transforming phenotype of Ras in CLPTM1L-depleted cells. The interaction of CLPTM1L with PI3K is likely to be necessary for its function, providing a potential target for therapy if the nature of the interaction is delineated. Investigation of this interaction and how it may be targeted will be the subject of future studies. However, Bcl-xL expression, modulation of which is required for the effect of CLPTM1L on genotoxic apoptosis in human lung tumor cells (2), was unaffected by constitutively active AKT, suggesting that CLPTM1L may promote survival of tumor cells by mechanisms in addition to AKT signaling. The effect of CLPTM1L appears to be upstream of both AKT signaling and Bcl-xL stabilization, both of which may independently play a role in the protumorigenic effect of CLPTM1L. Abrogation of the effect of CLPTM1L depletion on K-Ras transformation by
either myrAKT or Bcl-xL expression mechanistically implicates these pathways in CLPTM1L-dependent oncogenic transformation. The fact that both AKT signaling and Bcl-xL expression must be concomitantly inhibited to reverse Ras transformation is of great clinical importance. It has recently been shown that inhibition of Bcl-xL inhibition is synergistic with PI3K/AKT inhibition in human cancer (43).

As the effect of CLPTM1L is apparently through regulation of mitochondrial apoptosis and as the CLPTM1L locus is associated with all lung cancer histologies (21), we hypothesize that its effect may not be exclusive to Ras-mutant–driven tumors but may rather influence any tumors with aberrant growth signaling. In agreement with this premise, a majority of lung tumors we tested overexpressed CLPTM1L compared with adjacent normal tissue, a much higher number than would be expected to harbor K-Ras mutations. DNA was unavailable for mutational analysis in these patients and all available RNA was used. In support of the hypothesis that the effect of CLPTM1L is not exclusive to Ras-driven tumors, we did not observe higher CLPTM1L expression in lung tumor cell lines harboring oncogenic K-Ras mutations than those with wild-type K-Ras (data not shown). Nevertheless, conclusive determination of CLPTM1L levels in relation to K-Ras mutation is warranted. Likewise, investigation of the effect of CLPTM1L on transformation by mutant forms of other common oncogenes such as EGFR is justified.

It has been shown that depletion of Bcl-xL with siRNA can sensitize cisplatin-resistant human lung adenocarcinoma cells (44). Our previous studies have shown that Bcl-xL, but not other apoptotic regulators Bcl-2 and Bax, is regulated by CLPTM1L and that this regulation is required for the effect on cisplatin sensitivity (2). Similar in vitro results in regard to regulation of survival proteins and resistance to apoptosis were obtained in Spon8 mouse lung tumor cells (Supplementary Fig. S4) and in H838 human lung tumor cells (2), which are p53-mutant (184G>T; p.E62”), suggesting that the effect is not exclusive to p53 wild-type lung cancers and that the phenotype is repeatable in multiple cell lines. H838 cells are wild-type for
all Ras isoforms, again suggesting that the effects are not specific to Ras-transformed cells. In fact, induction of anoikis upon CLPTM1L depletion was more significant in H838 cells than in A549 cells. Protection from apoptosis upon DNA damage may lead to an accumulation of such damage and subsequent accumulation of mutations leading to cancer. In fact, the lung cancer–associated SNP rs402710 within CLPTM1L has recently been found to be associated with high levels of bulky aromatic and hydrophobic DNA adducts (45).

Together these results strongly implicate CLPTM1L–dependent protection from apoptosis through regulation of survival signaling as a mechanism necessary for Ras transformation and lung tumorigenesis. The current study demonstrates a clear tumorigenic role for CLPTM1L, which until now has only been functionally related to cisplatin resistance in ovarian tumor cells (1). High CLPTM1L expression may represent an important biomarker for chemoresistance and tumor progression, and there is high potential for its use as a therapeutic target influencing anoikis and anchorage-independent growth. These findings justify further investigation of the influence of CLPTM1L on cancer risk, its function and its use as a target for cancer prevention, treatment, and sensitization to genotoxic therapy.

References


