CLPTM1L Promotes Growth and Enhances Aneuploidy in Pancreatic Cancer Cells

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Abstract

Genome-wide association studies (GWAS) of 10 different cancers have identified pleiotropic cancer predisposition loci across a region of chromosome 5p15.33 that includes the TERT and CLPTM1L genes. Of these, susceptibility alleles for pancreatic cancer have mapped to the CLPTM1L gene, thus prompting an investigation of the function of CLPTM1L in the pancreas. Immunofluorescence analysis indicated that CLPTM1L localized to the endoplasmic reticulum where it is likely embedded in the membrane, in accord with multiple predicted transmembrane domains. Overexpression of CLPTM1L enhanced growth of pancreatic cancer cells in vitro (1.3–1.5-fold; \( P_{\text{DAYS}} < 0.003 \)) and in vivo (3.46-fold; \( P_{\text{DAYS}} = 0.039 \)), suggesting a role in tumor growth; this effect was abrogated by deletion of two hydrophilic domains. Affinity purification followed by mass spectrometry identified an interaction between CLPTM1L and non-muscle myosin II (NMM-II), a protein involved in maintaining cell shape, migration, and cytokinesis. The two proteins colocализed in the cytoplasm and, after treatment with a DNA-damaging agent, at the centrosomes. Overexpression of CLPTM1L and depletion of NMM-II induced aneuploidy, indicating that CLPTM1L may interfere with normal NMM-II function in regulating cytokinesis. Immunohistochemical analysis revealed enhanced staining of CLPTM1L in human pancreatic ductal adenocarcinoma (\( n = 378 \)) as compared with normal pancreatic tissue samples (\( n = 17; P = 1.7 \times 10^{-5} \)). Our results suggest that CLPTM1L functions as a growth-promoting gene in the pancreas and that overexpression may lead to an abrogation of normal cytokinesis, indicating that it should be considered as a plausible candidate gene that could explain the effect of pancreatic cancer susceptibility alleles on chr5p15.33.

Introduction

Risk variants in the TERT-CLPTM1L gene region on chromosome 5p15.33 have been reported in genome-wide association studies (GWAS) for 10 cancer types, including bladder, breast, glioma, lung, melanoma, non-melanoma skin cancer, ovarian, pancreas, prostate, and testicular germ cell cancer (1–13). The TERT gene encodes the catalytic subunit of the telomerase reverse transcriptase complex known for its role in maintaining telomere ends and the increased telomerase activity often seen in human cancers (14). The CLPTM1L gene encodes the cleft lip and palate-associated transmembrane 1-like protein (CLPTM1L), and was originally identified in a screen for genes conferring resistance to cisplatin in ovarian cancer cells (15). When overexpressed in ovarian cancer cells, CLPTM1L induced apoptosis in cisplatin-sensitive cells, giving rise to its original name: cisplatin resistance–related protein (CRR9; ref. 15). CLPTM1L was later shown to protect lung cancer cells from apoptosis after treatment with DNA-damaging agents via Bcl-xL (16).

Gain of chromosome 5p is one of the most recurrent chromosomal abnormalities in human cancers (17). Although most commonly seen in thyroid, lung, and cervical cancer, 5p gain is also frequent in other cancers, including gastric, ovarian, colorectal, hepatocellular, esophageal, bladder, and pancreatic adenocarcinoma (17–19). The most common event in early stages of non–small-cell lung cancer is gain at 5p15.33 involving both TERT (78%) and CLPTM1L (53%; ref. 20). However, a recent study of cervical cancer noted that CLPTM1L, but...
not TERT, was among the multiple genes on 5p (33%) that were both amplified and overexpressed (21, 22).

The most significant GWAS risk variants on 5p15.33 for pancreatic cancer lie in intron 13 of the CLPTM1L gene and are located approximately 27 kb from the transcriptional start of TERT (11). Although this does not exclude TERT as a plausible candidate gene explaining this pancreatic cancer risk allele, CLPTM1L should be considered a potential target gene. Thus, to explore a possible function for CLPTM1L in pancreatic cancer, we examined its role in growth control in vitro and in vivo, and searched for interacting proteins that could provide clues to its function.

Materials and Methods

Cell lines and antibodies

The human embryonic kidney cell line HEK293T, human pancreatic cancer cell line PANC-1, and mouse kidney cell line IMCD3 (all from the American Type Culture Collection) were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Mediatech Inc) supplemented with 10% FBS (Life Technologies).

Commercial antibodies used included those for endogenous CLPTM1L (HPA014791; Sigma), FLAG-tagged CLPTM1L (M2 F1804; Sigma), the endoplasmic reticulum (ER) marker Calnexin, (C4731; Sigma), a mitochondrial marker (MTC02, ab3298; Abcam), the centrosome marker γ-tubulin (T5192; Sigma), the Golgi marker GM130 (G7295; Sigma), α-tubulin (ab7291; Abcam), and MYH9/MYH10 (sc-33729; Santa Cruz Biotechnology). Secondary antibodies were Alexa Fluor594 or Alexa Fluor488 donkey anti-mouse or anti-rabbit IgG (H + L; A21202, A21203, and A21206; Life Technologies).

Generation of CLPTM1L expression plasmids and creation of stable cell lines

Full-length human CLPTM1L cDNA (Invitrogen, Ultimate Mammalian Expression System) was cloned into pCAGGS (a Gateway system adapted version of Sigma’s p3xFLAG-CMV10) using the Gateway system (Invitrogen). Three constructs were generated from the full-length human CLPTM1L cDNA (RefSeq NM_030782.3) and verified by Sanger sequencing WT CLPTM1L (full-length CLPTM1L), CLPTM1L-DLoop [missing amino acids 36–280 between transmembrane (TM) domains 1 and 2] and CLPTM1L-DActerm (lacking amino acids 455–538). The designed constructs include three tandem FLAG tags at the amino terminus of CLPTM1L. A fourth construct contained a FLAG tag at the C-terminus of CLPTM1L, to compare the effect of FLAG tags on the N- or C-termini on growth rates in vitro.

Stable cell lines were generated by transfecting PANC-1 cells (Lipofectamine 2000, 11668-019; Life Technologies) with selecting transfectants PANC-1 cells (Lipofectamine 2000, 11668-019; Life Technologies) with selection in 400 μg/mL G418 (Mediatech; 30-234-CI). The constructs above, expressing wild-type (WT) or mutant CLPTM1L from the cytomegalovirus (CMV) promoter, were used to generate the following stable lines: PANC1-vo (empty vector), PANC1-CLPTM1L (full-length CLPTM1L), PANC1-CLPTM1L-DLoop (loop deletion), or PANC1-CLPTM1L-DActerm (C-terminal deletion). Transient transfections for HEK293T and mIMCD3 cells were performed with the same constructs.

Prediction of the topology of WT CLPTM1L was assessed using: TMHMM v.2.0, TMPred, and TopPred2 (http://www.cbs.dtu.dk/services/TMHMM-2.0/; http://www.ch.embnet.org/software/TMPRED_form.html; http://www.sbc.su.se/~erikw/toppred2/; refs. 23–26).

In vitro and in vivo growth assays

Cell proliferation was measured in vitro by seeding PANC-1 stably expressing CLPTM1L (full-length or deletion mutants) at 3 × 10^3 cells per well in 96-well plates. Time points were taken every 2 days (days 1, 3, 5, and 7) and cell growth was assessed using the WST-1 reagent (Roche Applied Science) for 30 minutes. The optical density change created by the metabolizing of the reagent was evaluated in a spectrophotometer (Tecan) at 450 nm. Absorbance at the reference wavelength of 600 nm was subtracted from the A590 values.

CLPTM1L knockdown was performed using the Dharmacon DharmaFECT siRNA transfection reagent (Thermo Scientific Dharmacon; #T-2001-01) according to the manufacturer’s instructions. Dharmacon ON-TARGET plus SMARTpool siRNA specifically targeting CLPTM1L (L-015661-02-0005) or a control nontarget siRNA (D-001810-02-05) was purchased from Thermo Scientific Dharmacon. Cell proliferation experiments were performed 48 hours after transfection with 100 nmol/L siRNA. The efficiency of CLPTM1L knockdown was assessed by isolating RNA from PANC-1 cells, using the mirVana RNA kit (ABI). Briefly, 1 μg RNA (RIN scores > 9.0) was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed on a 7900HT system (ABI) using TaqMan gene expression assays for CLPTM1L (Hs00363947_m1) and B2M (Hs00187842_m1) from Life Technologies. Each reaction was run in quadruplicate and analyzed according to the ΔΔCt method using B2M as the housekeeping gene.

Tumor growth was measured in vivo by using a xenograft mouse model. Female nude mice (8–10 weeks old) were purchased from the Animal Production Area, NCI, Frederick, MD, and housed in a pathogen-free environment. Briefly, 10^6 PANC-1 cells, stably transfected with different CLPTM1L constructs or the vector control, were injected subcutaneously into the flank of each mouse. Tumor size was measured by a caliper three times a week for up to 77 days using the formula of length × width × width/2 to estimate tumor volumes in mm^3, or when protocol experimental end points were reached (tumor diameter reached 2 cm). For each group, 5 mice were injected per stable cell line per experiment. After seeing similar results for two independent constructs expressing WT CLPTM1L with FLAG tags at either end, the CLPTM1L constructs tagged on the N-terminus were chosen for further work. Final results were pooled from three independent experiments: two that were performed with PANC1-vo (empty vector) and PANC1-CLPTM1L cells, and a third experiment that used PANC1-vo, PANC1-CLPTM1L, PANC1-CLPTM1L-DLoop, and PANC1-CLPTM1L-DActerm cells. The difference in growth rates was analyzed by comparing tumor volumes at day 68 using the Mann–Whitney U test. Animal care and experimental procedures were approved by the NIH Animal Care and User Committees (PB-047 M1 to Dr. Javed Khan, Pediatric Oncology Branch, National Cancer Institute, NIH, Bethesda, MD).
Affinity purification of protein complexes, tryptic digestion, and fractionation

HEK293T cells were grown to approximately 60% confluence and transfected with 3xFLAG-tagged WT CLPTM1L (experimental analysis) or vector only (control) in a complex of polymer PEI (polyethyleneimine; Polysciences Inc) and DNA at a ratio of 5:2. After 48 hours, cells were harvested on ice in RIPA buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1.0% NP-40) with 1× protease inhibitor cocktail (Roche). Lysates were incubated with 25 μL of anti-FLAG M2 agarose (Sigma) for 2 hours followed by washing and elution via a 3×-FLAG peptide as previously described (Das PMID: 20968308). The eluates were subjected to overnight digestion with trypsin (1 μg) at 37°C followed by lyophilization, reconstitution, and fractionation using strong cation exchange liquid chromatography (LC) and mass spectrometry analysis as described (27). Proteomics data analysis was performed as previously described (27).

Validation of CLPTM1L-MYH9 interaction by co-immunoprecipitation

HEK293T cells were transfected with 3xFLAG-tagged WT CLPTM1L expression plasmids or the vector control only, and collected 48 hours after transfection. PANC-1 cells stably expressing WT CLPTM1L, CLPTM1L-Δloop, or CLPTM1L-Δterm were cultured to a confluence of approximately 80%. Cells were washed with ice-cold PBS and harvested in lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 1.0% NP-40, with 1× protease inhibitor cocktail). Immunoprecipitation of FLAG-tagged CLPTM1L was performed by incubating the supernatant with anti-FLAG M2 agarose for 2 hours at 4°C. Affinity complexes were washed and interacting complexes released from FLAG beads by boiling in 2× LDS sample buffer (Invitrogen) and resolved on a 3% to 8% Tris Acetate gel (Invitrogen). Samples were subjected to Western blot analysis using antibodies to FLAG M2, Myosin 9/10 and β-actin followed by appropriate secondary mouse or rabbit antibodies (Thermo Scientific) before detection of the signal with ECL (Thermo Scientific).

Cell cycle and DNA content analysis

The effect of CLPTM1L and MYH9 on cell-cycle progression was studied by flow cytometric analysis of DNA content and BrdUrd incorporation using the FITC BrdU Flow Kit (557891; BD Pharmingen BioSciences) according to the manufacturer’s recommendation. Briefly, logarithmically growing cells transiently transfected HEK293T or stably transfected PANC-1 cells were labeled with 10 μmol/L BrdUrd for 30 minutes at 37°C, fixed, permeabilized, and treated with DNase. A fluorescein isothiocyanate (FITC)–conjugated anti-BrdUrd antibody was used for staining DNA with incorporated BrdUrd, and 7-amino-actinomycin D (7-AAD) for total DNA staining. Fluorescence of 30,000 cells was acquired on a FACSCalibur instrument (Becton Dickinson). The resulting DNA histograms were quantified by using the CellQuest Pro software (Becton Dickinson) and the percentage of G0–G1, S, G2–M and aneuploid (>4N) cells determined.

For knockdown experiments, cells were plated in six-well plates 24 hours before transfection with siRNA, so they reached 80% confluence at the time of transfection. The Dharmacon DharmaFECT siRNA transfection reagent (Thermo Scientific Dharmacon; #TF-2001-01) was used for transfection according to the manufacturer’s instructions. Dharmacon ON-TARGET plus SMARTpool siRNA specifically targeting MYH9 (L-007668-00-0005) or a control nontarget siRNA (D-001810-02-05) was purchased from Thermo Scientific Dharmacon. Twenty-four hours after transfection with 100 nmol/L siRNA, the media were replaced with fresh complete media. Assays were performed 48 hours after transfection.

Efficiency of MYH9 knockdown was assessed by isolating RNA from PANC-1 cells, using the mirVana RNA kit (ABI). Briefly, 1 μg RNA (RIN scores > 9.0) from cell lines was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). qRT-PCR was performed on a 7900HT system (ABI) using TaqMan gene expression assays for MYH9 (Hs00159522_ml) and B2M (Hs00187842_ml) from Life Technologies. Each reaction was run in quadruplicate and analyzed according to the ΔΔCt method using B2M as the housekeeping gene.

Immunofluorescence and in situ proximity ligation assay

To visualize the subcellular localization of the endogenous or FLAG-tagged CLPTM1L proteins, cells were grown on glass coverslips, fixed with 4% paraformaldehyde or 100% methanol and stained with antibodies against endogenous CLPTM1L (1:500), FLAG-tagged CLPTM1L (1:500), calnexin (1:500), mitochondrial marker (1:500), GM130 (1:500), α-tubulin (1:1000), and γ-tubulin (1:1000) followed by visualization by confocal microscopy (Zeiss LSM 510 Meta). Assessment of colocalization with MYH9 was performed in the same manner using antibodies against MYH9 (1:500). Secondary antibodies used were Alexa Fluor594 or 488 donkey anti-mouse or anti-rabbit IgG (H+L; Invitrogen). Cells were counterstained with 4′-diamidino-2-phenylindole (DAPI).

The Duolink in situ Proximity Ligation Assay (DPLA; in situ red starter kit; #92101 from Olink Bioscience) was used according to the manufacturer’s protocol to evaluate interactions between CLPTM1L and other proteins. Briefly, cells were grown on coverslips and fixed with 4% paraformaldehyde or 100% methanol for 10 minutes at room temperature and air-dried, followed by blocking and labeling with antibodies to endogenous CLPTM1L (Sigma; HPA014791) or FLAG-tagged CLPTM1L (Sigma; F1804), MYH9/10 (Santa Cruz Biotechnology; SC-33729), at a dilution of 1:500 in a preheated humidity chamber at 37°C for 30 minutes. After washing, slides were incubated with anti-rabbit and anti-mouse secondary antibodies with attached DPLA probes (dilution 1:5) for 30 minutes in a preheated humidity chamber at 37°C. Finally, cells were counterstained by DAPI and signals were observed by confocal microscopy (Zeiss LSM 510 Meta); a red fluorescence signal indicates that two proteins are separated by less than 40 nm (28).

Cells were treated with 50 μmol/L of the DNA-damaging agent cisplatin (ALX-400-040-M250, Enzo Life Sciences) for 0, 24, or 48 hours for immunofluorescence and Duolink experiments as indicated.
Immunohistochemistry

Tissue microarrays (TMA) with normal- and tumor-derived pancreatic tissue samples were obtained from the Mayo Clinic and from the Surveillance, Epidemiology, and End Results (SEER) registries (29, 30). The TMAs from the Mayo Clinic (n = 111), PDAC (pancreatic ductal adenocarcinoma) TMA1 (n = 111), PDAC GEM (gemcitabine; n = 156), PanIN (pancreatic intraepithelial neoplasia) TMA (n = 13), and pancreatic islet cell tumors (islet cell TMA; n = 41) along with three cores of tissues from normal pancreas that could be evaluated for CLPTM1L expression. The SEER TMA contained 14 cores from normal pancreas (adjacent to tumor) in addition to PDAC (n = 111) and islet cell tumors (n = 7).

Immunohistochemistry for CLPTM1L was performed with a rabbit polyclonal antibody (HPA014791; Sigma) at 1:1,600 dilution. Briefly, slides were deparaffinized in xylene and graded alcohols, and subject to antigen retrieval in a pressure cooker with citrate buffer (pH 6.0) for 20 minutes. Endogenous enzyme activity was blocked with 3% hydrogen peroxide in methanol with an additional block in 2% nonfat milk used to reduce nonspecific reactions. Subsequently, slides were incubated with primary antibody for 60 minutes at room temperature. Staining extent of cells was also scored on a scale of 0–4 as follows: 0, negative; 1, background; 2, weak; 3, positive; and 4, strong. Combined scores for intensity and extent (individual range, 0–4) were calculated by multiplying scores for intensity and extent (individual range, 0–4; combined range, 0–16). For statistical analysis, CLPTM1L staining was classified as negative/weak (combined scores, 0–4; intermediate/weak, 4–6); A 2 × 2 χ² test was used to assess the difference in staining between normal- and tumor-derived samples (df = 1). A Pearson uncorrected P value was reported.

Results

Subcellular localization of CLPTM1L

The protein product of the CLPTM1L gene is predicted to contain six transmembrane domains and two large hydrophilic domains: a loop between the first and second transmembrane domains, and a C-terminal tail. It is highly conserved in primates and relatively well conserved in flies and nematodes (31). We assessed the subcellular localization of endogenous CLPTM1L in pancreatic cancer cell lines by immunofluorescence analysis and demonstrated perinuclear cytoplasmic staining and a punctate pattern of CLPTM1L over the nucleus, indicating possible nuclear or nuclear membrane staining (Fig. 1A). Co-localization with markers for cytoplasmic organelles showed that CLPTM1L localized mainly to the ER (Fig. 1B) in accord with its six predicted transmembrane domains. No staining was detected in the Golgi apparatus (Fig. 1D). To confirm that the ER localization of overexpressed WT FLAG-tagged CLPTM1L observed was not due to defects in polarized sorting, we transiently expressed CLPTM1L in mouse kidney IMCD3 epithelial cells and observed colocalization with the ER marker calnexin (Supplementary Fig. S1). Two of the three programs used to predict the topology of CLPTM1L indicated that the N- and C-termini of the protein protrude into the lumen of the ER and other organelles it resides in (TMpred and TMHMM), but one (TopPred) predicted an opposite topology with both ends on the cytoplasmic face of the ER (23). The hydrophilic loop structure between TM1 and TM2 is expected to be on the opposite side of the membrane as compared with the N- and C-termini.

CLPTM1L overexpression enhances cell proliferation in vitro and in vivo

To investigate whether CLPTM1L, influenced growth, we overexpressed full-length or mutant CLPTM1L in HEK293T (transient transfection) and PANC-1 (stable transfection) cells. These included four FLAG epitope–tagged cDNA expression constructs: WT full-length CLPTM1L (tagged on either C- or N-termini), and two deletion mutants tagged at the N-termini: CLPTM1L ΔCterm (hydrophilic C-terminal domain after the last transmembrane domain deleted) and CLPTM1L Δloop (hydrophilic loop between the first and second transmembrane domains deleted). Figure 2 shows a schematic figure of the resulting proteins (top) as well as protein expression of the three different forms of CLPTM1L (WT and two mutant) in transiently transfected HEK293T cells (by Western blotting, bottom left) and in PANC-1 cells stably expressing the proteins (by FLAG immunoprecipitation and Western blotting, bottom right).

We assessed cell proliferation in vitro and observed that cells overexpressing full-length WT CLPTM1L grew faster than cells containing empty vector (Fig. 3A). Two sets of PANC-1 cells stably transfected with CLPTM1L (with N- or C-terminal FLAG epitope tags) showed similar results (1.3–1.5-fold increase at day 7; P = 0.0019 and P = 0.0027, respectively, indicating that the FLAG tag does not influence the growth-promoting function of the protein. This effect was abolished by the two mutants, CLPTM1L Δloop and CLPTM1L ΔCterm (Fig. 3B), and inhibited by siRNA targeting CLPTM1L (Supplementary Fig. S2). These results prompted an investigation of whether the same growth effect was mediated by CLPTM1L in vivo. PANC-1 cells stably overexpressing WT CLPTM1L generated larger tumors in nude mice in vivo than those containing empty vector (average tumor volume is shown for one representative experiment in Fig. 3C; median tumor volume is shown for all three experiments combined in Fig. 3D). A significantly larger tumor size was observed in vivo for cells overexpressing CLPTM1L as compared with the empty vector (3.46-fold at day 68; P = 0.039). The two deletion mutants did not induce growth as compared with the empty vector, indicating that these two domains are critical for the growth-promoting function of the CLPTM1L protein. In fact, PANC-1 cells expressing CLPTM1L Δloop and CLPTM1L ΔCterm grew slower than controls, indicating a possible dominant negative effect (Fig. 3D). This effect was significant for the CLPTM1L construct lacking...
the Loop structure ($P = 0.012$) but not for the construct lacking the C terminus ($P = 0.69$). To rule out the possibility that the lack of growth promotion by the mutant CLPTM1L proteins is caused by abrogated subcellular localization, we evaluated the localization of the two mutants in PANC-1 cells. Both mutants localized to the ER (Supplementary Fig. S3), indicating that mislocalization does not explain the reduced biologic effect. However, a defect in folding of the mutants within the membranes cannot be ruled out.

WT CLPTM1L interacts with non-muscle myosin II in pancreatic cancer cells

To investigate further the molecular function of the CLPTM1L protein, we searched for interacting proteins using affinity purification, followed by liquid chromatography and mass spectrometry analysis. Because CLPTM1L is predicted to be highly hydrophobic, we performed the screen in RIPA buffer to facilitate solubilization of membrane-associated proteins. The most frequently observed peptides (Supplementary Table S1) belonged to Myosin-9 (MYH9) and Myosin-10 (MYH10), two heavy-chain non-muscle myosin type II (NMM-II) proteins involved in maintaining cell shape, migration, secretion, and cytokinesis. PANC-1 cells express approximately 25-fold higher amounts of MYH9 compared with MYH10 at the RNA level (Supplementary Fig. S4), suggesting that this interaction may be primarily between CLPTM1L and MYH9. Interestingly, the ER marker calnexin was identified as a potential CLPTM1L-interacting protein (Supplementary Table S1), consistent with the observed ER localization shown above using immunofluorescence analysis.

The interaction between CLPTM1L and NMM-II was confirmed by coimmunoprecipitation in both PANC-1 (Fig. 4A) and 293T cells (Fig. 4B). An interaction between the two CLPTM1L deletion mutants and NMM-II was absent or very low (Fig. 4A), indicating that the loop and C-terminal domains are required for the interaction between the two proteins. To assess where in the cells CLPTM1L and NMM-II interact, we performed colocalization experiments by DPLA (Fig. 4C) and immunofluorescence (Fig. 4D) and confirmed the interaction. Because CLPTM1L has been shown to protect cells from cisplatin-induced apoptosis (16, 32), we treated PANC-1 stably transfected cells with 50 μmol/L cisplatin for 0, 24, or 48 hours and observed a shift in the extent of colocalization after 48 hours of cisplatin treatment (Fig. 4C). Instead of widespread

Figure 1. Localization of endogenous CLPTM1L in pancreatic cells and colocalization of CLPTM1L with cytoplasmic organelles. A, localization of endogenous CLPTM1L in PANC-1 pancreatic cancer cells. Cytoplasmic and punctate nuclear, or nuclear membrane, staining is seen (red). Counter staining was performed for α-tubulin (green). B, colocalization of WT CLPTM1L (red) with the ER marker calnexin (green) in PANC-1 cells stably transfected with WT CLPTM1L. Colocalization of WT CLPTM1L (red) with a mitochondrial marker (green; C), or a Golgi marker (GM130 in green; D), was not seen in PANC-1 cells stably transfected with WT CLPTM1L. Cells were counterstained with DAPI (blue).
cytoplasmic colocalization for CLPTM1L and NMM-II, fewer areas of colocalization were seen after cisplatin treatment, especially after 48 hours (Fig. 4C, bottom). These were often as few as one or two prominent dots per cell, located close to the nucleus (Fig. 4C, bottom right). This pattern was confirmed using immunofluorescence, showing prominent colocalization of CLPTM1L and NMM-II after cisplatin treatment in dot-like structures with tubular projections located at the nuclear periphery (Fig. 4D, right). We hypothesized that these structures were centrosomes, and confirmed that both CLPTM1L and NMM-II colocalized with the centrosome marker γ-tubulin, as shown in Fig. 5A for the former and in Fig. 5B for the latter, indicating that the two proteins probably interact specifically at centrosomes after DNA damage induction by cisplatin. Colocalization of CLPTM1L and γ-tubulin was confirmed using immunofluorescence (Fig. 5C), indicating colocalization in dot-like structures close to the nucleus consistent with a single centrosome per cell.

**Overexpression of WT CLPTM1L results in increased aneuploidy**

On the basis of the interaction with NMM-II and colocalization with γ-tubulin at the centrosome, we assessed DNA content and cell-cycle progression by fluorescence-activated cell sorting (FACS) analysis after transiently or stably overexpressing WT CLPTM1L in HEK293T cells and PANC-1 cells, respectively. This analysis did not reveal a significant difference in the fraction of cells in G0–G1, S, or G2–M phase (data not shown) but indicated that the percentage of aneuploid cells with DNA content double than that of the majority of cells was enhanced in cells overexpressing CLPTM1L: a 1.8-fold increase was seen in PANC-1 cells (left) and a 2.3-fold increase was seen in HEK293T cells (right) as compared with empty vector (Fig. 6A). It has previously been shown that inhibiting the function of NMM-II with a specific chemical inhibitor (blebbistatin), or with specific siRNA, results in abnormal cytokinesis and increased rates of aneuploidy (33). To address this, we assessed aneuploidy in PANC-1 cells stably expressing WT and mutant CLPTM1L. After knockdown of MYH9 expression with a specific siRNA (knockdown efficiency, 77.8% to 86.0%; Supplementary Fig. S5), we noted an increase in the fraction of aneuploid cells in PANC-1 cells containing empty vector (1.54-fold) and in cells that overexpressed WT CLPTM1L. (1.36-fold) as compared with negative siRNA (Fig. 6B, bars 1–4). Simultaneous overexpression of WT CLPTM1L and knockdown of MYH9 gave the highest fraction of aneuploid cells (2.01-fold), suggesting an additive effect. On the other hand, PANC-1 cells expressing the two deletion mutants showed lower levels of aneuploid cells than cells containing empty vector or WT-CLPTM1L. Knockdown of MYH9 in these cells increased the fraction of aneuploid cells, but not to the levels seen in cells containing empty vector or expressing WT-CLPTM1L (Fig. 6B, bars 5–8).

**CLPTM1L protein levels were increased in human pancreatic adenocarcinoma**

To assess CLPTM1L protein expression in normal- and tumor-derived pancreatic samples, we performed immunohistochemical staining of formalin-fixed paraffin-embedded tumor- and normal-derived pancreatic samples. Initially, we stained TMAs obtained from the Mayo Clinic, containing...
normal (n = 3) and pancreatic adenocarcinoma (PDAC TMA1, n = 111; and PDAC GEM, n = 156) pancreatic samples. To increase the sample size for normal pancreatic samples, we stained a TMA from the SEER registries with normal (n = 14) and tumor (SEER PDAC; n = 111) tissue samples. All normal samples scored as negative or weak (combined “intensity/extent” or histoscores, 0–2), whereas PDAC-derived samples scored in all four categories (histoscores, 0–16). In fact, the normal-derived samples on both TMAs never scored above a histoscore of 2. The distribution of average histoscores across all PDAC samples combined (PDAC TMA1, PDAC GEM, and PDAC SEER) were 24.9% negative, 28.8% weak, 22.2% intermediate, and 24.1% strong (see scores and percentages for individual TMAs in Table 1 and representative staining in Supplementary Fig. S6). When score categories were collapsed into two groups with lower (negative and weak; histoscore, 0–4) versus higher (intermediate and strong; histoscores, 6–16) scores, a statistically significant difference was seen for CLPTM1L staining between the normal- and tumor-derived samples (P = 1.7 x 10^{-3}), indicating elevated expression levels of CLPTM1L in pancreatic tumors of PDAC histology. Significant correlations between CLPTM1L protein levels (histoscore) with survival, gender, age, stage, and grade were not seen (data not shown). PanINs (n = 13) stained mostly in the negative (46.2%) and weak (46.2%) categories but with a small fraction (7.7%) of samples falling within the intermediate group. The average percentages of staining scores for islet cell tumors (n = 48) on both TMA sets were 6.3% negative, 2.1% weak, 8.3% intermediate, and 83.3% strong. Negative or weak staining was seen in islets in the normal samples, indicating that CLPTM1L levels may also be increased in pancreatic endocrine tumors.

Discussion

Chromosome 5p15.33 contains a pleiotropic cancer predisposition locus identified in GWAS of at least 10 cancer types (34). Multiple independent loci, in the TERT gene or the neighboring CLPTM1L gene, have been identified in this region (1–13, 35, 36). Some, but not all, risk loci have been associated with TERT expression or with telomere length (35, 36). To further define the underlying biology of the
susceptibility alleles on 5p15.33, we investigated the function of a plausible candidate gene, CLPTM1L, and its encoded protein, CLPTM1L. We observed a positive effect on tumor cell growth both in vitro and in vivo, after overexpression of full-length CLPTM1L. Deletion mutants of CLPTM1L lacking either one of the two large hydrophilic domains of this predominantly hydrophobic and presumably membrane-bound protein did not induce growth in vitro or in vivo, indicating that these domains may be crucial for the growth-promoting function of the protein.

A search for interacting proteins identified NMM heavy chain, types IIA and IIB (NMM-II) as protein partners. The two hydrophilic domains of CLPTM1L were required for this interaction. NMY9 and NMY10 are heavy chains of Myosin, a hexameric protein containing two heavy chains, two light chains, and two regulatory light chains. Myosin is involved in various cellular processes such as muscle contraction, cell motility, and intracellular transport. The interaction between CLPTM1L and NMM-II was confirmed by in situ PLA with or without treatment with 50 μmol/L cisplatin for 24 or 48 hours. The interaction is seen as red dots where the two proteins are in close proximity. Two representative figures are shown for the 48 hours post-treatment point. A red arrow points to a prominent area of colocalization located close to the nucleus. D, immunofluorescence analysis showing colocalization of WT N-FLAG CLPTM1L and NMM-II as orange to yellow staining after treatment with cisplatin for 48 hours.
chains, and two regulatory chain subunits, and are important for maintaining cell shape, migration, and secretion (37–39). In addition, NMM-II plays a role in cytokinesis in which it, in concert with actin and furrow components, drives the contraction needed for cytokinesis, the final stage of cell division (33).

A well-known effect of blocking the expression or function of NMM-II in model organisms is a defect in cytokinesis that leads to the formation of multinucleate cells (40–42). In HeLa cells, treatment with blebbistatin, a chemical inhibitor of NMM-II, inhibits contraction of the cell cleavage furrow, resulting in an increased number of binucleate cells (33). NMM-II is also important for the migration of centrosomes to the opposite sides of chromosomes during assembly of the mitotic spindle, indicating an important role during multiple steps of cell division (43).

In our study, under normal growth conditions, CLPTM1L and NMM-II colocalized to the ER. After treatment with a DNA-damaging agent, both CLPTM1L and NMM-II localized to centrosomes, where they appear to interact. As seen in other cell types (33), knocking down the levels of NMM-II resulted in an increase in aneuploidy. The opposite was true for CLPTM1L, as increased levels resulted in enhanced rates of aneuploidy. This suggests that the two proteins could have antagonizing effects on cells proceeding through cell division, which may involve a disruption of normal NMM-II function by CLPTM1L.

The interaction between MYH9 and CLPTM1L seems to be mediated through the loop domain and C-terminal tail of CLPTM1L. Because the ER membrane is continuous with the nuclear membrane, which is absorbed into the ER during mitosis (44), CLPTM1L may reside in the outer nuclear membrane (as per punctate staining pattern in Fig. 1A) and interact with centrosome proteins during cell division. It is also possible that the interaction between the two proteins in the ER and other subcellular organelles has additional functional consequences that may influence cell growth, apoptosis, or other cellular activities. CLPTM1L has recently been shown to be overexpressed in lung cancer in which it protects cells from apoptosis after treatment with DNA-damaging agents via Bcl-xL, indicating a negative role on mitochondrial apoptotic pathways (16, 32).

In a recent proteomics screen of the human autophagy network, CLPTM1L was shown to bind PIK3C3 (phosphatidylinositol 3-kinase catalytic subunit type 3), indicating a possible role in autophagy (45). Although PIK3C3 plays a central role in

![Figure 5. CLPTM1L and non-muscle Myosin II localize to centrosomes.](image)

![Figure 6. CLPTM1L overexpression increases the fraction of aneuploid cells.](image)
Table 1. CLPTM1L protein levels are enhanced in pancreatic cancer

<table>
<thead>
<tr>
<th>Score</th>
<th>Normal TMA1</th>
<th>PanIN TMA1</th>
<th>PDAC TMA1</th>
<th>PDAC GEM</th>
<th>Islet cell TMA</th>
<th>SEER TMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Negative</td>
<td>2 (66.7)</td>
<td>6 (46.2)</td>
<td>25 (22.5)</td>
<td>10 (6.4)</td>
<td>0 (0.0)</td>
<td>8 (57.1)</td>
</tr>
<tr>
<td>Weary</td>
<td>2–4</td>
<td>1 (33.3)</td>
<td>6 (46.2)</td>
<td>42 (37.8)</td>
<td>20 (12.8)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>5–8</td>
<td>0 (0)</td>
<td>1 (7.7)</td>
<td>31 (27.9)</td>
<td>49 (31.4)</td>
<td>2 (4.9)</td>
</tr>
<tr>
<td>Strong</td>
<td>9–16</td>
<td>0 (0)</td>
<td>0 (0.0)</td>
<td>13 (11.7)</td>
<td>77 (49.4)</td>
<td>39 (55.1)</td>
</tr>
<tr>
<td>Total</td>
<td>3 (100)</td>
<td>13 (100)</td>
<td>111 (100)</td>
<td>156 (100)</td>
<td>41 (100)</td>
<td>14 (100)</td>
</tr>
</tbody>
</table>

NOTE: Combined staining intensity and extent for CLPTM1L by immunohistochemistry in normal-derived pancreas, PanIN, PDAC, and pancreatic islet cell tumors. CLPTM1L immunohistochemical staining extent (area) and intensity was scored from 0 to 4 and the combined extent intensity was calculated and shown above (histoscore).

Abbreiation: Islet cell, islet cell tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Dr. Javed Khan, Pediatric Oncology Branch, National Cancer Institute, NIH, for guidance with mouse experiments.

Grant Support
This study was supported in part by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health (NIH) under Contract No. HHSN26120080001E. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 6, 2013; revised February 3, 2014; accepted February 25, 2014; published OnlineFirst March 19, 2014.

autophagy, it is also important for vesicle trafficking, receptor signaling, and cytokinesis. Depletion of PIK3C3 in mammalian cells leads to an arrest of cytokinesis and an increased number of multinucleate cells (46). Interestingly, in the autophagy screen, both CLPTM1L and MYH9 were also listed as interacting proteins for CLN3 (45), a transmembrane vesicle trafficking protein that regulates cytokinesis in yeast (47) and is mutated in Batten disease (48).

Immunohistochemical analysis showed that a substantial number of pancreatic cancers have higher levels of the CLPTM1L protein than normal pancreatic tissues, approximately 46% (range, 5%–81%) of pancreatic adenocarcinomas, and 92% (range 43%–100%) of islet cell tumors, indicating a possible role in carcinogenesis in both the exocrine and endocrine pancreas. A possible limitation of our study is that we have a substantially larger number of pancreatic cancers (PDAC; n = 378) compared with normal samples (n = 17). Thus, our findings would benefit from additional validation studies.

In summary, our results show that CLPTM1L promotes growth of pancreatic cancer cells in vitro and in vivo and that its function, at least in part, may be through abrogation of cell division. We have shown that CLPTM1L interacts with NMM-II, and that the two proteins have opposing effects on aneuploidy. Because NMM-II is required for cytokinesis, it is possible that CLPTM1L blocks this process, perhaps by interfering with processing, localization, or function of NMM-II. Furthermore, we show that CLPTM1L is overexpressed in a substantial fraction of pancreatic tumors. Although further studies are needed to characterize CLPTM1L function in greater detail, our results indicate that CLPTM1L influences processes that are important for cancer growth and thus represents a plausible candidate gene for investigating the biologic basis of cancer susceptibility alleles on chr5p15.33, particularly for pancreatic cancer.

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CLPTM1L Promotes Growth and Enhances Aneuploidy in Pancreatic Cancer Cells


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