Prenylated C17orf37 Induces Filopodia Formation to Promote Cell Migration and Metastasis

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Post-translational modification by covalent attachment of isoprenoid lipids (prenylation) regulates the functions and biological activities of several proteins implicated in the oncogenic transformation and metastatic progression of cancer. The largest group of prenylated proteins contains a CAAX motif at the C-terminal that serves as a substrate for a series of post-translational modifications that convert these otherwise hydrophilic proteins to lipidated proteins, thus facilitating membrane association. C17orf37 (chromosome 17 open reading frame 37), also known as C35/Rdx12/MGC14832, located in the 17q12 ampli-
con, is overexpressed in human cancer, and its expression correl-
ates with the migratory and invasive phenotype of cancer cells. Here we show that C17orf37 contains a functional CAAX motif and is post-translational-ly modified by protein gera-
nylgeranylation with a 20-carbon chain by GGTase-I (8). The

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methyltransferase (Icmt) (isoprenylcysteine-O-carboxyl methyltransferase) enzyme. These final modifications are also thought to be important for correct subcellular localization and biological activities of these proteins (9, 10). The addition of the isoprenyl tail facilitates binding to the cellular membranes and protein-protein interaction and increases the stability of the protein, thereby regulating the overall functional property of these proteins (7). Several oncoproteins that undergo prenylation processing are known to promote cancer progression by activating proliferation, migration, and invasion of cancer cells, and inhibiting prenylation of these proteins reduces their functional activity (10). Hence, enzymes involved in the prenylation processing are attractive targets for cancer therapy (10, 11).

We investigated the importance of C-terminal isoprenylation of C17orf37 protein with respect to its function. We show for the first time that C17orf37 is geranylgeranylated by GGTase-1 and also undergoes postprenylation processing, and these modifications are important for correct localization of the C17orf37 protein. We also show that membrane-associated C17orf37 increases cellular migration by inducing increased filopodia formation at the leading edge of the migrating cell, whereas prenylation-deficient mutant C17orf37 failed to do so. We further confirm that ectopic expression of C17orf37 modulates the dissemination of cancer cells and facilitates formation of metastatic nodules in a mouse model of metastasis. Our data clearly indicate that C17orf37 prenylation is sufficient to drive cellular migration and colonization of injected nontransformed fibroblast cells to distant organs compared with the inactive mutant C17orf37 protein. These findings directly establish role for C17orf37 in the migration and metastatic dissemination of cancer cells and further elucidates the importance of isoprenyl modification in the function of C17orf37.

### EXPERIMENTAL PROCEDURES

**Cloning, Expression, and Purification of Recombinant C17orf37**—Full-length wild type human C17orf37 cDNA of 347 bp was cloned into pGEX-4T-1 vector (GST-C17orf37-WT) and pEGFP-C1 vector (GFP-C17orf37) as described previously (4). The Cys-112 residue in the CVIL motif of C17orf37 (cloned in pGEX-4T1 vector and pEGFP-C1 vector) was mutated to serine, or the entire CVIL motif of C17orf37 (cloned in pEGFP-C1 vector) was deleted by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene). The primer pairs used are as follows: C17orf37 WT, 5’-CAG CCG TCC TCC CAG CGT CAT CCT GTG-3’/5’-GTC GGC AGG AGG GTC GCA GTA GGA CAC-3’; C17orf37 WT, 5’-GCC GTG GGC GTG GAA CTC TCT CCT GAG TAA TCA TTC TGA GAC 3’/5’- CGG CAG GAG GGA CTC AGT AGG ACA CTG-3’. GST-C17orf37-WT (C17orf37) and GST-C17orf37-C112S (C17orf37 Δ112) fused protein was expressed in Escherichia coli BI-21 strain and purified using glutathione-Sepharose 4B column (GE Healthcare) according to the manufacturer’s instructions.

**Cell Lines, Culture Conditions, Treatment, and Transfection Procedures**—DU-145 and SKBR-3 cells were obtained from ATCC and maintained in RPMI1640 supplemented with 10% FBS and 1% penicillin-streptomycin. NIH3T3 mouse fibroblast cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Wild type mouse embryonic fibroblasts (MEFs), Icmt−/−, and Rce1−/− were grown in DMEM supplemented with 15% calf serum, 1% nonessential amino acid, 1% penicillin-streptomycin, and 3.6 μl of β-mercaptoethanol (12). The cells were transfected using Lipofectamine 2000 (Invitrogen) with plasmid DNA for a period of 6 h in OPTI-MEM (Invitrogen). After transfections, the cells were grown in complete medium overnight before mounting on slides using Vectashield (Vector Laboratories, Burlingame, CA) for confocal microscopy.

For generation of stable cells, NIH3T3 cells were transfected using Lipofectamine 2000, with GFP (empty vector), GFP-C17orf37-WT (C17orf37 WT), GFP-C17orf37-C112S (C17orf37 WT), or GFP-C17orf37-D112–115 (C17orf37 Δ112–115) plasmid DNA for 24 h. Stable transfected cell populations were challenged in complete medium supplemented with 250 μg/ml G418 (Invitrogen) for about 3 weeks. For subsequent experiments, polyclonal pooled clones obtained from the transfected cells were used. DU-145 stable cells expressing C17orf37 or GFP were generated as mentioned previously (4). GGTI-DU-40 and FTI-2148 (Calbiochem) inhibitors were dissolved in Me2SO and mixed with complete medium at the indicated dose prior to treatment for a period of 24 h. The cells were either fixed and stained with C17orf37 antibody for confocal microscopy or subjected to Western immunoblot.

**Antibodies and Reagents**—Antibodies used for the study are as follows: mouse polyclonal C17orf37 (Abnova, Taiwan; 1:500), rabbit polyclonal anti-PGK, mouse monoclonal anti-NA-K-ATPase (Developmental studies hybridoma bank, Colorado), rabbit polyclonal anti-GFP (Cell Signaling and Roche Applied Science), mouse monoclonal anti-transferrin receptor (Zymed Laboratories Inc.), mouse monoclonal anti-Ki-67 (DAKO), GAPDH (Santa Cruz), Tom20 (13), Lamin A/C (Cell Signaling), and rabbit polyclonal anti-Goα2 (Santa Cruz). Geranylgeranyl dipiphosphate (GGPP) was purchased from Biomol, Inc; [3H]GGPP and [3H]FFPP from PerkinElmer Life Sciences; [3H]GGOH (American Radiolabeled Chemicals); medastatin (Sigma); Enhancer (GE Healthcare), and FTI-2148 was purchased from Calbiochem.

**Confocal and TIRF Microscopy**—GFP-tagged cells were grown on coverslips and mounted on glass slides with Prolong Gold mounting medium (Invitrogen). Confocal images were obtained using Zeiss confocal microscope LSM 510 under 40×, 1.2-numerical aperture water immersion objective at a 0.6-μm Z-section as previously described (12). Lung sections were immunostained with GFP and Ki-67 antibody, and images were obtained under 20× objective. For TIRF microscopy, DU-145 cells were grown on coverslips and then treated with Me2SO or 5, 10, and 20 μM of GGTI for 24 h. The cells were then fixed by 2% paraformaldehyde. Unpermeabilized cells were then washed with PBS and treated with C17orf37 followed by Alexa-568 conjugated secondary antibody. The coverslips were then mounted on specialized cover glass 1 (22 × 50 mm size; Corning, Lowell, MA). For TIRF images, the cells were visualized on an Olympus IX71 microscope with commercial TIRF attachment as described previously (4) by 60× oil immersion objective.
In Vitro Prenylation Analysis—Prenylation of C17orf37 and Rac1 were measured by incorporation of radiolabeled isoprenoid as described previously (14). Briefly, purified mammalian GGTase-I or FTase (10 ng, expressed in Sf9 cells) (15) were used to initiate reactions containing 10 μM GGPP or FPP incubated with either 2.5 μM of purified GST-tagged C17orf37 substrates (wild type or C1125-mutant) or 5 μM GST-tagged Rac1 or Ras. The reagents were incubated for 60 min at 30 °C before precipitation, product termination, and measurement of [3H]GGPP or [3H]FPP incorporation.

Incorporation of [3H]GGOH into DU-145 Cells and Immunoprecipitation of Prenylated Proteins—In vivo labeling of C17orf37 protein with [3H]GGOH in DU-145 cells was performed as described in Ref. 16. Briefly, 2 × 10^6 DU-145 cells were seeded in 6-well plates and after 12 h in culture, cells were treated with 5 μM mevastatin for 4 h. After the cells were then transfected with GFP, C17WT, or C17C1125, and after 6 h were incubated in complete medium containing 5 μM mevastatin and 30 μCi/ml of [3H]GGOH. After 40 h, the cells were washed and resuspended in lysis buffer to obtain the whole cell lysate. One half of the lysate was immunoprecipitated using anti-GFP antibody (Roche Applied Science) followed by capture of the protein-IgG complex using protein A/G-agarose beads (Santa Cruz). The beads were washed, and eluted fractions were subjected to SDS-PAGE. The gel was washed, fixed, stained with Ponceau S, destained, and scanned. The immunoblots were quantified using Kodak’s digital imaging analyzer (Kodak, Germany). To locate prenylated C17orf37 in DU-145 cells, C17orf37 protein was immunoprecipitated with an anti-FLAG antibody (M2, Sigma), and the beads were washed, and eluted fractions were subjected to SDS-PAGE. After electrophoresis, the gel was washed, fixed, stained, destained, and scanned. The immunoblots were quantified using Kodak’s digital imaging analyzer (Kodak, Germany).

Isoprenylation Regulates C17orf37 Localization and Function

RESULTS

The C17orf37 Protein Is Prenylated by GGTase-I Enzyme—C17orf37 localization in cells has been reported to be both cytosolic and associated with membranes (4). In a survey of several computational algorithms, including SOSUI, TMAP, TMHMM, TMpred, and TopPred, we could not identify a prenylation motif, CVIL, at the C terminus of the protein, a motif predicting the protein to be prenylated. In addition to the CAA motif, certain upstream clusters of polybasic amino acids are also known to enhance efficient membrane association of the prenylated proteins (12, 20). Comparison of the 30 amino acids of C17orf37 preceding the CAA motif with the known geranylgeranylated proteins RhoA, RhoB, RhoC, Rac1, and Cdc42 revealed the presence of multiple basic amino acids in C17orf37 protein as well (Fig. 1A). Because the last amino acid of CAAX box is leucine, C17orf37 is predicted to be pre-
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nylated by GGTase-I at Cys-112 compared with FTase, whereas GGTase-II, which requires two cysteine residues in the CAX box for isoprenyl group addition, did not show any match. To directly determine whether GGTase-I could catalyze the addition of a geranylgeranyl isoprenoid to Cys-112 of the C17orf37 CAX motif, we performed an in vitro prenylation assay using GST-fused recombinant protein C17orf37 wild type (C17WT) and C17orf37-C112S (C17C112S) was used for in vitro prenylation assay. Direct geranylgeranylation or farnesylation of C17WT (2.5 μM) was achieved in the presence of recombinant GGTase-I enzyme containing [3H]geranylgeranyl pyrophosphate or FTase enzyme containing [3H]FPP. Rac1 (5 μM) and Ras were used as positive controls, and GST protein was used as negative control. C incorporation of [3H]GGOH into geranylgeranylated proteins was achieved by labeling DU-145 cells transfected with GFP, C17WT, and C17C112S constructs. Left panel, SDS-PAGE followed by autoradiography showing total protein that incorporated [3H]GGOH. Right panel, immunoprecipitation (IP) of the whole cell lysate using GFP antibody showing the incorporation of [3H]GGOH only in C17WT.

FIGURE 1. C17orf37 is geranylgeranylated by GGTase-I at Cys 112. A, sequence alignment of last 30 amino acids of representative geranylgeranylated proteins. Basic amino acids are shown in red, and the CAX motif is in bold and underlined. B, schematic diagram showing that the CAX box located at the C-terminal end of C17orf37 was mutated to serine (C112S), and GST-fused recombinant protein C17orf37 wild type (C17WT) and C17orf37-C112S (C17C112S) was used for in vitro prenylation assay. Direct geranylgeranylation or farnesylation of C17WT (2.5 μM) was achieved in the presence of recombinant GGTase-I enzyme containing [3H]geranylgeranyl pyrophosphate or FTase enzyme containing [3H]FPP. Rac1 (5 μM) and Ras were used as positive controls, and GST protein was used as negative control. C, incorporation of [3H]GGOH into geranylgeranylated proteins was achieved by labeling DU-145 cells transfected with GFP, C17WT, and C17C112S constructs. Left panel, SDS-PAGE followed by autoradiography showing total protein that incorporated [3H]GGOH. Right panel, immunoprecipitation (IP) of the whole cell lysate using GFP antibody showing the incorporation of [3H]GGOH only in C17WT.
plasma membrane (24). TIRF microscopy was performed in DU-145 cells treated with either vehicle control Me2SO or an increasing dose of GGTI. DU145 cells treated with vehicle (Me2SO) showed numerous dense spots of C17orf37 protein, whereas increasing doses of GGTI treatment particularly at 10 and 20 μM concentration reduced membrane association of C17orf37 (supplemental Fig. S2). These results demonstrate that C17orf37 is indeed a substrate of GGTase-I and that modification by GGTase-I is required for localization of C17orf37 to the plasma membrane.

Postprenylation Processing Is Required for Proper Subcellular Localization of C17orf37—For CAA Proteins, prenylation is followed by two additional important biochemical processes (25). Proteolytic cleavage of the last three amino acids (AAX) by Rce1 and carboxyl methylation of the prenylated cysteine by Icmt are important postprenylation events required for the correct localization and biological functions of many CAA proteins (12, 26). Gene disruption studies in mice have confirmed that Icmt and Rce1 enzymes are essential for mouse development (27–29). To determine whether geranylgeranylation of C17orf37 is followed by Rce1-mediated cleavage and carboxymethylation, we evaluated the subcellular localization of C17orf37 in MEF deficient in Rce1 and Icmt enzymes. Wild type MEF cells transiently transfected with GFP-tagged C17orf37 showed evenly distributed C17orf37 in the membrane and cytosol. However, in Rce1<sup>−/−</sup> cells, C17orf37 localization was abrogated and displayed increased nuclear and cytoplasmic accumulation (Fig. 3A). Surprisingly, in Icmt<sup>−/−</sup> cells, C17orf37 was found to be localized as dense punctuated spots, presumably some form of vesicles, and also in the cytosol and nucleus (Fig. 3B). It seems likely that the absence of carboxymethylation results in a tendency for C17orf37 protein to associate with vesicles. We also generated a GFP-tagged C17orf37 mutant in which Cys-112 was mutated to Ser (C17C112S). Expression of this form of the protein in wild type, Rce1<sup>−/−</sup>, and Icmt<sup>−/−</sup> MEFs showed significant nuclear accumulation (Fig. 3, A and B, bottom panels), demonstrating mis-targeting of the mutant protein. Taken together, these findings provide evidence that efficient membrane association of geranylgeranylated C17orf37 is also dependent on postprenylation processing mediated by Rce1 and Icmt enzymes.

Preprenylated C17orf37 Is Associated with Plasma Membrane—To demonstrate that the Cys-112 residue is required for C17orf37 prenylation and subsequent association with the plasma membrane, we generated stable polyclonal pooled populations of C17orf37-null NIH3T3 cells, expressing GFP vector, GFP-C17orf37, or the C17orf37 mutants C17C112S and C17orf37<sup>Δ112−115</sup>, in which the entire CAAX box was deleted (supplemental Fig. S3). Although C17orf37<sup>Δ112−115</sup> mutant behaved as expected, the abundance of the protein recovered...
from NIH3T3 cells was low, suggesting that deletion of entire CAAX box affected protein stability (supplemental Fig. S3). Subcellular fractionation of cells by differential centrifugation revealed distribution of C17\textsuperscript{WT} protein exclusively in the cytosolic and plasma membrane fractions, whereas C17\textsuperscript{C112S} is mostly cytosolic (Fig. 4A) and very less in the membrane fractions. Surprisingly, C17\textsuperscript{C112S} mutant was not found to be in the nuclear fraction probably because of increased molecular weight caused by fusion with GFP. GAPDH, TOM20, Na-K/ATPase, and Lamin A/C were used to check the purity of the cytosolic, mitochondrial, plasma membrane, and nuclear fractions.

The plasma membrane is a dynamic structure composed of extremely complex set of 500 different lipid species with proteins embedded within (30). Most prenylated proteins have the ability to interact with cellular membranes, although many maintain a substantial cytoplasmic soluble pool (31). Studies have shown that farnesylated Ras protein has the ability to aggregate in cholesterol-enriched membrane microdomains known as “lipid rafts,” and there is a dynamic relation between raft and nonraft associated proteins (32). We determined the spatial and temporal distribution of prenylated C17orf37 in the membrane microdomains. Total protein recovered from NIH3T3 cells stably expressing either C17\textsuperscript{WT} or C17\textsuperscript{C112S} were subjected to differential centrifugation on a discontinuous sucrose gradient, and fractions collected based on their density on sucrose gradients were analyzed. The cholesterol-rich low density membrane fractions (fractions 2–4) were distributed at the interface between 5 and 45% sucrose and contain an enrichment of the raft marker Ga\textsubscript{12}, the heavier membrane fractions (fractions 5–8) show a predominant expression of transferrin receptor, whereas most cellular protein were localized to the bottom of the gradient in fractions (fractions 9–12) (Fig. 4, B and C). C17\textsuperscript{WT} was distributed predominantly in two different pools: as soluble fraction in the cytosol (fractions 9–12) and in the membrane fractions enriched in transferrin receptor (fractions 5–8) and less in the raft fractions (Fig. 4B). As expected, C17\textsuperscript{C112S} was mostly localized in the soluble fractions comprised of cytosolic proteins (fractions 9–12) and less in the membrane (Fig. 4C). Our studies provide evidence that prenylated C17orf37 localizes outside the cholesterol-enriched raft region at the plasma membrane, consistent with previous reports that geranylgeranylated proteins cluster in microdomains outside the lipid rafts (33, 34).

C17orf37 Prenylation Regulates Functional Response of the Protein—We next investigated whether the function of C17orf37 shows a dependence on the post-translational modifications at the CAAX motif. Ectopic expression of C17orf37 increases the migratory ability of cancer cells (4). Hence, in the first set of studies to examine the role of C17orf37 prenylation, we assessed migration of NIH3T3 cells stably expressing GFP vector, C17\textsuperscript{WT}, or C17\textsuperscript{C112S} (supplemental Fig. S4) in scratch wound assays. Scratch assays are commonly used to study the ability of cells to polarize and migrate into the wound with time (35, 36). Cells expressing the C17orf37\textsuperscript{WT} were able to migrate efficiently and cover nearly all of the wounded area within 18 h (2-fold increase in cell migration), whereas cells expressing the GFP vector or the C17\textsuperscript{C112S} were much less efficient in this process (Fig. 5, A and B). Further, in a Transwell migration assay, expression of C17orf37\textsuperscript{WT} led to a ~2.5-fold increase in migration compared with GFP vector or C17\textsuperscript{C112S} in NIH3T3 cells (Fig. 5, C and D), indicating that the ability of C17orf37 to enhance cell migration is dependent on its prenylation.

Membrane-bound Prenylated C17orf37 Induces Cell Migration by Increased Filopodia Formation—Cell migration depends on coordinated polymerization of actin filaments result-
ing in protrusive structures at the leading edge of motile cell called lamellipodia (37). From the lamellipodia arise thin finger-like projections filled with parallel bundles of F-actin, known as filopodia (38). Surprisingly, the ectopic expression of C17WT in NIH3T3 cells dramatically increased filopodia formation in more than 70% of the cells counted (Fig. 6, open bars). However, in cells expressing GFP vector or C17C112S, only 15–20% counted cells showed filopodia formation (Fig. 6, open bars), and there was a ~4-fold decrease in the number of filopodia/cell when compared with C17WT (Fig. 5D, open bars). Filopodia are often referred to as “tentacles” used by the migrating cells to probe their microenvironment and known to facilitate directional movement (34).

Inducing migration by wounding scratch in NIH3T3 cells, we observed increased actin polymerization and stress fiber formation in both wild type and mutant C17orf37 (Fig. 6B). Filopodia originating from the migratory cells were found to protrude toward to the wound, supporting a directional migration (Fig. 6B); however, the number of filopodia radiating from C17WT cells was significantly higher than that of C17C112S cells (Fig. 6, B and D, gray bars), suggesting the involvement of prenylated C17orf37 in filopodium formation. We also observed a global increase in the formation of filopodia, particularly in cells surrounding the wound (Fig. 6D, gray bars), and the number of cells with filopodium increased upon induction of migration (Fig. 6B, gray bars), supporting the notion that filopodia formation is a well regulated process by the dynamic balance of actin polymerization (39). Nevertheless, our findings strongly suggest a mechanism by which prenylated C17orf37 induces increased migratory behavior in cells.

C17orf37 Prenylation Enhances Metastatic Dissemination and Colonization of Cells—Migration and invasion of cancer cells are the hallmark of metastasis, and ectopic expression of C17orf37 increases the migratory and invasive behavior of cancer cells. In clinical specimens, increased expression of C17orf37 protein has been detected in patients with metastatic cancer (3). To determine whether C17orf37 prenylation could drive oncogenesis and metastatic progression of the disease in...
vivo, we used the mouse model of experimental lung metastasis. NIH3T3 cells stably expressing GFP vector, C17WT, or C17C112S were injected into the tail veins of athymic nude mice. NIH3T3 is a nontransformed cell line and does not form tumors or lung metastasis when injected into nude mice (40). However, stable overexpression of different oncogenes has been known to induce oncogenic transformation of NIH3T3 cells (40). Eleven weeks after the injection, we did not observe any change in animal behavior, and the animals looked healthy. The isolated lungs were free of any visible external metastatic nodules (Fig. 7B, top panels). However, quite unexpectedly, we observed increased colonization of C17WT-expressing cells in the lung parenchyma when compared with mice injected with cells expressing the GFP vec-

![Image](https://example.com/image.png)

**FIGURE 5. Expression of prenylation-deficient C17orf37 mutant inhibits cell migration.** A, scratch wound assays were performed in NIH3T3 cells stably expressing vector (GFP), GFP-fused wild type C17orf37 (C17WT), or GFP-fused C17orf37-C112S mutant (C17C112S). Confluent monolayers of cells were wounded, and healing of the wound by cell migration was monitored for 18 h. Images were taken at different time points: 0 h (left column), 9 h (middle column), and 18 h (right column). B, migrated cells in the wound area at 9 h (open bar) and 18 h (gray bar) were counted from five different fields and expressed as the means ± S.E. of five independent experiments. C and D, NIH3T3 cells expressing different constructs as mentioned for A were prelabeled with calcein AM fluorescent dye and Transwell® migration assay performed with 10% serum as a chemoattractant in the lower chamber. C, representative images of migrated cells from two different fields were obtained after 24 h, and the fold change of migration was calculated normalized to parental NIH3T3 cells and expressed as the means ± S.E. of three independent experiments as shown in D.
tor alone or C17<sup>C112S</sup> (Fig. 7A). Most of the C17<sup>WT</sup> cells densely populated the lung parenchyma, predominantly infiltrating from the blood vessels (Fig. 7, A, bottom panels, and B, middle panels), whereas minimal GFP stain was observed in GFP vector or C17<sup>C112S</sup> lung sections (Fig. 7B, middle panels). Ki-67 staining of the lung sections was basal (Fig. 7B, bottom panels), and no significant differences were found between the groups (supplemental Fig. S5B). This clearly suggests that C17orf37 prenylation markedly increases the migratory ability of cells, an aggressive trait required for dissemination and metastatic colonization. On the other hand, preventing prenylation of C17orf37 by genetically altering the CAA box completely suppressed this effect.

When DU-145 prostate cancer cells stably expressing GFP-fused C17orf37 (C17<sup>WT</sup>) (supplemental Fig. S3) were injected in athymic nude mice via tail vein, increased lung metastasis was observed 56 days postinjection compared with the DU-145 cells expressing GFP vector alone (Fig. 7C). Animals injected with C17<sup>WT</sup> showed enhanced lung colonization of tumor cells (Fig. 7C), increased number of metastatic lesions, and a ~4-fold increase in lung metastasis (Fig. 7, D and E). Two mice injected with DU-145 expressing C17<sup>WT</sup> cells died 50 days postinjection because of increased lung metastasis (animals 3 and 7) (supplemental Fig. S5A). Microscopic examination of the lung sections from animals injected with C17<sup>WT</sup>-expressing cells confirmed abundant expression of GFP-positive cells in the metastatic lesions, compared with few GFP-expressing cells in lung tissues of animal injected with vector alone (Fig. 7D, middle panels). Although C17orf37 expression does not significantly alter cancer cell proliferation and growth in DU-145 cells in vitro,<sup>4</sup> we observed a ~4-fold increase in Ki-67 staining (Fig. 7F) in metastatic lesions of the lungs expressing C17<sup>WT</sup>, compared with minimal stain in the vector alone (Fig. 7, D, bottom panel, and F). This increased proliferation may be attributed to the tumorigenic ability of parental DU-145 cells, suggesting that ectopic expression of C17orf37 enhanced the migratory and metastatic ability of the cells to colonize to the lungs, whereas proliferation and increased Ki-67 staining are due to the inherent malignant phenotype of DU-145 cells. Taken together, our findings strongly indicate that overexpression of C17orf37 functionally predisposes cells to increased migratory and metastatic phenotype

<sup>4</sup>S. Dasgupta and J. K. Vishwanatha, unpublished observations.
and that the prenylation at CVIL motif is critical for the C17orf37-mediated effect.

**DISCUSSION**

Information concerning the importance of the novel gene C17orf37 in cancer biology is still emerging. Prompted by the finding that the gene is located in an important amplicon of chromosome 17q12 known as the “hot spot locus of cancer,” previous studies identified C17orf37 to be overexpressed in breast, colon, and prostate cancer (1, 2, 4) and postulated that it could play an important role in cancer. In an attempt to understand the post-translational modifications of C17orf37 that may regulate its function, we identified a consensus prenylation motif CVIL at the C-terminal end. Both in vitro and in vivo prenylation assays confirmed C17orf37 is a substrate for the GGTase-I enzyme that catalyzes addition of 20-carbon isoprenyl group to the cysteine residue of CVIL motif (Fig. 1). Pharmacologic inhibition of GGTase-I and genetic inhibition of Icmt and Rce1 enzymes revealed that post-translational modifications are required for membrane association of C17orf37 protein (Figs. 2 and 3).

When this paper was under revision, Katz (41) identified another motif at the N terminus of C17orf37 protein called immunoreceptor tyrosine-based activation motif, which is cross-phosphorylated at two tyrosine residues, and they show that phosphorylation is important for C17orf37 function. Therefore, it is quite evident that post-translational modification codes regulate the activity and stability of C17orf37 protein.

Functionally, C17orf37 protein enhances the migratory and invasive ability of prostate cancer cells. Mutation of the Cys residue that is the site of prenylation (C112S) results in a protein that fails to be modified by GGTase-I and is functionally inactive (Fig. 5). Through both in vitro and in vivo studies, we find that prenylated C17orf37 enhances the migratory and metastic potential of prostate cancer cells. This suggests that prenylation at CVIL motif is critical for the C17orf37-mediated effect.
astatic ability of tumor cells to increase their colonization to distant organs, whereas a prenylation-defective mutant of C17orf37 fails to do so (Fig. 7). Because prenylated C17orf37 promotes migratory ability not proliferation of cells, its role may be restricted to the intravasation and extravasation steps of tumor metastasis.

In a migrating cancer cell, C17orf37 preferentially localizes to the leading edge of the cell (4), which is comprised of protrusive structures formed by polymerization of actin cytoskeleton. Mechanistic studies reveal that prenylated C17orf37 modulates filopodia formation and facilitates directional migration of cancer cells (Fig. 6). Filopodia formation is a tightly regulated process mediated by cortical actin polymerization in response to stimuli. Several proteins have been identified to mediate the assembly and disassembly of actin, which includes small GTPases like Rho family of proteins, Rac1, and Arp2/3 (actin-related protein) (38). Interestingly, most of these proteins are post-translationally modified by isoprenyl group addition important for protein-protein interaction and GTP loading. Although C17orf37 protein does not have a canonical GTP-binding site, it will be important to identify how C17orf37 protein induces filopodia formation. Because it is known that C17orf37 can act as a signaling molecule through the PI3K/Akt axis and activate NF-κB downstream genes MMP-9, uPA, and VEGF, it will be interesting to find whether C17orf37-induced filopodium formation is a direct effect mediated by the Akt pathway or by other regulatory processes engaged by C17orf37.

Membrane is a vast dynamic structure composed of different classes of lipids. Our studies indicate that a majority of the prenylated C17orf37 protein localize to the membrane low in cholesterol composition (30), whereas the mutant C17orf37 accumulates in the cytosol. Recently, there has been a great debate in the field to better understand the raft and nonraft lipid compositions and re-evaluate our understanding of the membrane microdomains (42). Although it is clear that rafts are enriched in cholesterol and glycosphatidylidyinositol (34), the lipid composition of non-cholesterol-rich membrane fraction is poorly understood (42). Studies in prostate cancer cells have that revealed protein kinase B/Akt located in different compartments of plasma membrane (5) regulates distinctive downstream signaling pathways. Our studies indicate that prenylated C17orf37 localizes outside the raft region (Fig. 4), and further studies are required to identify interactors of C17orf37 and lipid composition that favor its binding to the plasma membrane.

Collectively, the findings of the present study broaden our understanding regarding the importance of C17orf37 in metastatic progression of cancer and the implications of post-translational isoprenylation on the functional activity of the protein. The present data also indicate that membrane association of C17orf37 is dependent on its post-translational modification at the C-terminal prenylation domain, which facilitates cellular migration by inducing filopodia formation and subsequent dissemination of cancer cells to distant organs. Because metastasis of cancer cells increases the mortality rate of human carcinomas, it is plausible that therapeutic strategies targeting C17orf37 may prove to be clinically useful to impede metastasis.

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