Systematic siRNA Screen Unmasks NSCLC Growth Dependence by Palmitoyltransferase DHHC5

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Abstract

Protein S-palmitoylation is a widespread and dynamic post-translational modification that regulates protein–membrane interactions, protein–protein interactions, and protein stability. A large family of palmitoyl acyl transferases, termed the DHHC family due to the presence of a common catalytic motif, catalyzes S-palmitoylation; the role of these enzymes in cancer is largely unexplored. In this study, an RNAi-based screen targeting all 23 members of the DHHC family was conducted to examine the effects on the growth in non–small cell lung cancer (NSCLC). Interestingly, siRNAs directed against DHHC5 broadly inhibited the growth of multiple NSCLC lines but not normal human bronchial epithelial cell (HBEC) lines. Silencing of DHHC5 by lentivirus-mediated expression of DHHC5 shRNAs dramatically reduced in vitro cell proliferation, colony formation, and cell invasion in a subset of cell lines that were examined in further detail. The phenotypes were restored by transfection of a wild-type DHHC5 plasmid but not by a plasmid expressing a catalytically inactive DHHC5. Tumor xenograft formation was severely inhibited by DHHC5 knockdown and rescued by DHHC5 expression, using both a conventional and tetracycline-inducible shRNA. These data indicate that DHHC5 has oncogenic capacity and contributes to tumor formation in NSCLC, thus representing a potential novel therapeutic target.

Implications: Inhibitors of DHHC5 enzyme activity may inhibit non–small cell lung cancer growth. Mol Cancer Res; 13(4); 784–94. ©2015 AACR

Introduction

Protein palmitoylation is increasingly understood as a common posttranslational modification of widespread regulatory significance (1–3). Dynamic cycles of palmitoylation and de-palmitoylation control protein–protein interactions, protein–membrane interactions, and protein stability (4–7), and complex reciprocal palmitoylation and phosphorylation events within proteins has been recognized (8, 9). Palmitoylation of intracellular proteins on cysteine residues (or more generally, protein S-fatty acylation) is carried out by members of a family of protein S-acyltransferases (PAT) that share a common motif, aspartic acid–histidine–histidine–cysteine (DHHC), with 23 family members encoded in the human genome (10, 11). The founding member of the mammalian PATs, DHHC9, palmitoylates N- and H-Ras, a modification that is required for membrane association of these oncogenic proteins (12–15). Several other DHHC family members are transforming and have recently been implicated in cancers (16–18).

NSCLC is a complex and remarkably heterogeneous disease marked by a unique natural history and molecular evolution in each patient, providing opportunities for development of targeted therapies (19–21). Mutations in signaling receptors that act at the plasma membrane as receptor tyrosine kinases include members of the ErbB family (including the EGF receptor) and other tyrosine kinases such as ALK, LTK, ROS, RET, FLT3, Eph, IGF-1R, DDR, and MET (22, 23). These mutations activate signaling pathways, largely the Ras/Raf-MEK/ERK or PI-3K/AKT/mTOR, Src, or JAK/STAT3 pathways. As a number of signaling proteins in these pathways are palmitoylated in addition to being phosphorylated, we wondered whether DHHC family members might contribute to the growth of NSCLC. Published data from an initial genome-wide siRNA screen (24) led us to perform a secondary screen using pooled siRNAs corresponding to 23 mammalian PATs for a larger number of NSCLC and HBEC lines. In the current article, we demonstrate that a subset of NSCLC cell lines is highly dependent on DHHC5 for growth and tumor xenograft formation and that the catalytic activity of DHHC5 is needed for this effect.

Materials and Methods

Cell culture

All human NSCLC cell lines and immortalized HBECs used in this study were established in the laboratories of Dr. Adi Gazdar and Dr. John Minna at the Hamon Center for Therapeutic Oncology Research (Dallas, TX; refs. 25, 26). The NSCLC cell lines were maintained in RPMI1640 (Sigma-Aldrich) supplemented with 5% heat-inactivated FBS and cultured at 37 °C in a humidified
atmosphere containing 5% CO2. HBECs were cultured with keratinocyte serum-free media (Life Technologies) containing 5 ng/mL of EGF and 50 μg/mL of bovine pituitary extract. All cell lines have been DNA fingerprinted using PowerPlex 1.2 kit (Promega) and confirmed to be mycoplasma-free using e-Mycokit (Boca Scientific). Histology and mutation status of NSCLCs used in the study is provided in Supplementary Table S1.

Tumor microarray
A NSCLC microarray produced from preexisting samples maintained at MD Anderson Cancer Center (Houston, TX; ref. 27) was stained using the anti-DHHC5 antibody (Sigma, HPA014670) at a 1:100 dilution under conditions as optimized in the Human Protein Atlas: http://www.proteinatlas.org/ENSG00000156599/antibody. The slides were stained concurrently with a positive control tissue (fallopian tube) using an automated HIC stainer (Dako Autostainer Plus). Antigen retrieval was performed in a pressure cooker with pH 6.0 citrate. The slides were read by two pathologists (H. Liu and J. Rodriguez-Canales) and scored as described under Supplementary Table S2.

shRNA stable expression in lung cancer lines and HBECs
pGPZIP lentiviral vectors containing a GFP reporter gene and shRNA sequences specific for DHHC5 were purchased from Open Biosystems (V2LHS_211792 and V3LHS_403115). A non-silencing shRNA (control) was also used (RHS4346). These lentiviral vectors are referred to as pGPZIP-shDHHC5-1, pGPZIP-shDHHC5-2, and pGPZIP-shCON, respectively. Two different shRNAs targeting DHHC5 were used, designated shDHHC5-1 (AGATCATTTT-TACAGCA) and shDHHC5-2 (TAAACCAGA); shDHHC5-1 (AGATCATTTT-TACAGCA). HEK293T cells were used for lentivirus preparation by transiently transfecting with packaging, envelope, and shRNA transfer vectors using FuGENE6 (Roche; ref. 28). At 48 to 72 hours after transfection, the medium containing the lentivirus was collected and used for the transduction of lung cancer cells with the addition of 8 μg/mL of polybrene (Sigma-Aldrich). Stable shRNA-expressing NSCLC cell lines were generated after 10 to 14 days in culture in puromycin (Sigma-Aldrich). After puromycin selection, DHHC5 expression level was then assessed by lentivirus expression in H1299 with siRNAs targeting DHHC5. Further selection was performed.

For the production of DHHC5 knockdown HBECs, cells were transduced with the pGPZIP-shDHHC5-1 and pGPZIP-shCON and selected on the basis of GFP fluorescence, as the HBEC cell lines used in the current study are puromycin resistant owing to the presence of vector sequences used to immortalize the cells. GFP sorting was performed on a Cytomation MoFlo flow cytometer (Beckman Coulter), and carried out with a 488 nm argon ion laser. The GFP fluorescence was measured using a band pass filter at 530/20 nm. We collected the top 25% GFP-positive cells for further experiments. As >90% knockdown was achieved, no further selection was performed.

Tetraacycline-inducible ("Tet-On") pTRIPZ lentiviral vectors (Open Biosystems) were used to establish inducible stable cell lines in H1299. The pTRIPZ vector contains a tetracycline response element (TRE), which drives the expression of a TurboRFP reporter in addition to the shRNA construct. The negative control pTRIPZ nonsilencing vector was purchased from Open Biosystems (RHS4743), whereas the shRNA construct of DHHC5 shDHHC5-1 was moved from pGPZIP vector to pTRIPZ on a 345 bp Xhol/MluI DNA fragment to generate the DHHC5 targeting vector. This vector is designated pTRIPZ-shDHHC5-1. The lentiviruses were prepared similarly as for nontetracycline inducible vectors as above, and stable inducible shRNA-expressing H1299 cell lines were created after 2 weeks of puromycin selection (1.25 μg/mL). Doxycycline (Sigma-Aldrich, 1 μg/mL) was used to induce TurboRFP/shRNA expression, which reached full intensity by 72 hours, whereas TurboRFP/shRNA expression could also be turned off by withdrawal of doxycycline for approximately 72 hours.

Cell proliferation assays (MTS assays)
Cell proliferation was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the manufacturer's instructions. Cells were plated onto a 96-well plate at a density of 1 × 10^3 cells per well. At 24, 48, 72, and 96 hours, the medium was replaced with fresh medium containing 20 μL of methanethiosulfonate/phenazine methosulfate (MTS/PHS) solution, and incubated at 37°C for an additional hour. Optical density at 490 nm was measured using a SpectraMax 190 absorbance microplate reader. Each individual experiment was performed at least three times independently using 8 wells each.

Colonies formation assays
To evaluate anchorage-dependent colony formation, 500 cells were suspended in 2 to 3 mL of RPMI1640 medium with 5% FBS and plated in a 6-well plate to grow for 1 to 3 weeks. The cells then were fixed and stained with 1 mL of 0.05% crystal violet (Sigma-Aldrich) to count the colonies using scanned images with ChemiDoc MP System (Bio-Rad). The experiments were performed in triplicate wells and repeated three times.

Cell invasion assays
In vitro cell invasion assays were performed using a BD BioCoat Matrigel Invasion Chamber (BD Biosciences) containing inserts with an 8 μm pore size PET membrane, which is precoated with a thin layer of growth factor–reduced Matrigel. The top chamber was seeded with 2.5 × 10^4 cells suspended in 0.5 mL of serum-free RPMI1640 medium, and the bottom chamber contained 0.75 mL of RPMI1640 medium with 5% FBS. After 18 to 24 hours of incubation at 37°C in a humidified atmosphere with 5% CO2, noninvasive cells on the top surface of the membrane were wiped off and membranes were fixed with 30% methanol and stained with 1× Giemsa staining solution (Sigma-Aldrich) at room temperature for 1 hour. The membranes were then photographed and the total migrating cells were counted.

Reverse transfection in H1299 with siRNAs targeting DHHC5
Two siRNAs with the same targeting sequences as the shDHHC5-1 and shDHHC5-2 were synthesized by Sigma Aldrich, designated siDHHC5-1 and siDHHC5-2. Accordingly, two siRNAs with mismatches (bases 9 through 11 of the siRNA are replaced with their complement, referred to us the "c911 control") were used as controls for off-target effects and were
used. A full-length mouse DHHC5 cDNA and its mutant were subcloned into pCI-neo mammalian expression vector (Promega) from pEF-BOS-HA-DHHC5 (30) and pEF-BOS-HA-
SD. Animals were sacrificed for each experiment and the data were presented as mean ± SD. Animals were sacrificed when the tumor reached 1,000 to 1,500 mm³, after which tumors were dissected and frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for further analysis.

For the study of xenograft tumor formation of the tetracycline-inducible DHHC5 knockdown cell line, NOD/SCID mice were injected with 1 × 10⁶ of H1299-TRIPZ-shDHHC5-1 cells. Mice were randomized to receive vehicle (1% sucrose) or vehicle plus 2 mg/mL doxycycline in the drinking water, and fresh doxycycline (or vehicle) was provided every 3 days. The vehicle-only mice were further randomized to receive vehicle or doxycycline when tumors reached a volume of approximately 350 mm³. The doxycycline-treated mice were subsequently randomized to receive either continued doxycycline or changed to vehicle at day 59 (mean volume of around 100 mm³). Five mice were used for each treatment group. All tumor measurements were performed in a blinded fashion without knowledge of treatment assignment.

**Results**

Pooled siRNAs directed against DHHC5 inhibit growth over a panel of NSCLC lines

A panel of 53 NSCLC lines and 5 HBECs were reverse transfected with pools of 4 siRNAs corresponding to all 23 human DHHC family members (Supplementary Fig. S1). Pooled siRNAs directed only against DHHC5 were broadly suppressive over the NSCLC lines and therefore DHHC5 was chosen for validation and further study.

**Immunoblotting**

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing protease inhibitor cocktail (Roche). Immunoblot analysis was conducted with 25 to 30 μg of total cell protein. The protein concentration of cell lysates was determined with the Bradford reagent (Bio-Rad). Equal amounts of total protein were subjected to SDS-PAGE on a 6% or 8% gel, and then transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% milk, and then incubated in PBST buffer with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5,000, Thermo Fisher Scientific) for 1 hour at room temperature. Membranes were exposed to HyBlot CL film at room temperature. Primary antibodies used were anti-DHHC5 (HPA014670, Sigma-Aldrich), anti-GAPDH (GT239, GeneTex), anti-HSP90 (SC-13119, Santa Cruz Biotechnology).

**Statistical analyses**

Student t test (two-tailed) was used to determine the significance of the in vitro cell proliferation, liquid colony formation, and cell invasion data between different groups. The significance of tumor size in vivo was determined using the two-way ANOVA test for overall significance among different treatment groups. The Gehan–Breslow–Wilcoxon test was used to analyze human survival data.

**Study approval**

All animal experiments were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory Committee.

**DHHC5 is overexpressed in NSCLC cell lines and in HBECs transformed to malignancy**

Eleven NSCLC cell lines representing major subtypes (adenocarcinoma, large cell neuroendocrine, and squamous cell carcinoma) were chosen; data concerning the mutational status of these cell lines are presented in Supplementary Table S1. Immunoblotting of cell lysates using an anti-DHHC5 antibody (Fig. 1A) revealed low DHHC5 protein expression in immortalized normal human lung bronchial epithelial cell lines (HBEC) and overexpression at the protein level in 9 of 11 of the tested NSCLC cell lines. Quantitation of these immunoblots (Fig. 1B) suggested a 5- to 8-fold increase in protein expression.

Sato M. and colleagues (32) has developed a model of lung cancer tumorigenesis in which immortalized HBECs are transformed stepwise to malignancy by introducing genetic manipulations commonly found in lung cancer, through p53 inactivation, transfection of a K-Ras–mutant allele, and c-myc overexpression. Interestingly, immunoblotting over this preexisting panel revealed overexpression of DHHC5 only in the most tumorigenic cell line (Fig. 1C).

**DHHC5 protein expression in human lung cancers and lack of correlation with survival**

A lung tumor tissue microarray representing 218 lung cancer specimens was subjected to immunostaining using the anti-DHHC5 antibody. Data for 194 evaluable cases are presented in Fig. 1D and E and in Supplementary Table S2. As DHHC5 has been reported to be primarily localized to the plasma membrane
(33), and owing to variably observed high background cytoplasmic staining, only membrane staining was scored. Multiple tumor cores from each patient scored for high, moderate, or low staining (3 = high, 2 = moderate, 1 = low, 0 = none; average values expressed on a scale of 0–300) by a pathologist (Supplementary Table S2). A continuum of DHHC5 staining was observed (Fig. 1E). About 10% of adenocarcinomas were negative for DHHC5, with the remainder and all squamous carcinomas displaying variable staining.

When the top and bottom quartiles of DHHC5 expression were compared with respect to survival of patients from time of diagnosis, no significant difference in overall survival was noted (Fig. 1F).

DHHC5 depletion suppresses cell proliferation and colony formation of some NSCLC cell lines but not HBECs

To investigate whether endogenous DHHC5 plays a role in the growth of NSCLC cells, we employed a lentivirus-mediated shRNA (shDHHC5-1) to stably knock down the expression of DHHC5 in 11 NSCLC cell lines as well as 3 HBEC lines. DHHC5 depletion resulted in varying degrees of reduction in anchorage-dependent liquid colony formation in the 11 tested NSCLC cell lines, whereas there was no effect in 3 HBEC lines (Fig. 2A). Similarly, suppression of DHHC5 caused reduction in cellular proliferation as measured by MTS assay in NSCLC cell lines, whereas there was almost no change observed in the HBECs (Fig. 2B). The degree of silencing of DHHC5 expression was assessed by
immunoblotting in each cell line and was judged to be greater than 90% (Fig. 2C and data not shown).

To investigate the possibility of off-target effects in the phenotypes observed, a second shRNA targeting DHHC5 (shDHHC5-2) was used to stably knockdown DHHC5 in H1299 cells. The stable cell lines generated from shDHHC5-2 showed virtually the same results as shDHHC5-1 (Fig. 3A). Loss of DHHC5 led to significantly reduced cellular proliferation, liquid colony formation, and cell invasion. As a further control, two "C911" control siRNAs corresponding to the sequences in shDHHC5-1 and -2 were used in transient knockdowns of DHHC5 in H1299 (Fig. 3B). C911 control siRNA bear mutations in bases 9 through 11 of the siRNA in which nucleotides are replaced with their complement. C911 control siRNAs show greatly improved performance in distinguishing on-target from off-target effects (29). Immunoblots confirmed the efficient knockdown of DHHC5 by siDHHC5-1 and siDHHC5-2, but not the C911 controls (Fig. 3B). Three days after the reverse transfection, cell proliferation was reduced by about 50% in cells transfected with wild-type siRNAs, whereas those transfected with C911-mutant siRNAs gave similar results as the negative controls (Fig. 3B). These results suggest that the cell growth suppression phenotype is likely related to an on-target effect directed at DHHC5.

Cell growth, colony formation, and cell invasion is rescued by wild-type DHHC5 but not the DHHS active site mutant

As shDHHC5-1 targets sequences in the 3'-untranslated region of the DHHC5 gene, DHHC5-containing cDNA plasmids are insensitive to this shRNA, and transfection in the knockeddown cells recovers DHHC5 expression. In the next series of experiments, both wild-type DHHC5 and a plasmid in which the putative active site (31, 34) of DHHC5 (DHHS) was mutated were overexpressed in H1299, H2009, and H358 stable DHHC5 knockdown cells. Suppression and reexpression of DHHC5 and DHHS in these three cell lines were confirmed by immunoblotting.

Figure 2.

DHHC5 depletion leads to decreased colony formation and cell proliferation of NSCLC cell lines, but not HBECs, in vitro. A, cell lines shown were transduced with a lentiviral shRNA directed against DHHC5 or a control shRNA (pGIPZ-shDHHC5-1 or pGIPZ-shCON) and their ability to form colonies in liquid medium was assessed. Number of colonies is expressed as a percentage of the nontargeted control. B, cell lines described in A were seeded and grown for 96 hours and cell proliferation assessed by MTS assay. Growth rate is reported as a percentage of the nontargeted control. C, representative data for two cell lines sensitive (left) and resistant (right) to DHHC5 knockdown. Immunoblotting for DHHC5, cell proliferation (MTS assays) and liquid colony formation are shown.
The effect of DHHC5 knockdown on anchorage-independent colony formation and growth

H1299 and H358 DHHC5 knockdown and control cells were injected subcutaneously into the flanks of NOD/SCID mice and tumor volume was measured over a 7- to 8-week period. Significantly, suppression of DHHC5 led to virtual absence (H1299) or severe suppression (H358) of tumor xenograft formation (Fig. 6). Consistent with the data showing rescue of liquid colony, cell proliferation and soft agar formation by DHHC5, tumor xenograft formation was also rescued by DHHC5 in H1299 cells. (H358 was not tested). Tumors were examined at the time of sacrifice and confirmed to have pathology typical of lung carcinomas. Immunostaining for DHHC5 consistently showed expected membrane staining in both control and DHHC5 “rescued” tumors (Supplementary Fig. S4) and lack of DHHC5 staining was confirmed in the small tumors arising from DHHC5 knockdown H358 xenografts (Supplementary Fig. S5).

DHHC5 knockdown suppresses tumor xenograft formation and growth

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Tetracycline-inducible suppression of DHHC5 inhibits growth of cell lines and xenografts

To begin to address questions concerning the temporal nature of the requirement for DHHC5 in tumor formation, we developed an inducible shRNA to deplete the expression of DHHC5 in H1299 cells. A tet-on-based regulated expression system was used, which expresses both tet repressor (tet-R) and the shDHHC5-1 upon induction by doxycycline. As a first step, inducible stable cell lines that expressed either shDHHC5-1 or a control shRNA were generated in H1299 cells. The DHHC5 knockdown was reversible and time dependent, with the maximal protein depletion detected 3 days postinduction. DHHC5 protein was restored 3 days after doxycycline withdrawal (Fig. 7A). On doxycycline addition, shDHHC5-infected cells showed reduced clonogenicity as well as cell proliferation, whereas cells bearing a control shRNA showed no effect (Fig. 7B and C). With doxycycline withdrawal, the colony formation of the knockdown cells recovered to baseline (Fig. 7B).

Doxycycline-inducible DHHC5 suppression in H1299 cells also eliminated their ability to form tumors in vivo similar to the noninducible model as described above (Fig. 7D).

In the next set of experiments, all mice were injected with H1299 cells bearing the tet-inducible shDHHC5-1. Mice were randomized to receive either vehicle or doxycycline in the drinking water, either early (from the time of cell injection) or after tumors were established. Fully blinded assessments were carried out. As expected, sizeable tumors developed by day 56 in vehicle-treated mice in which DHHC5 was not suppressed (Fig. 7E, left) and minimal or no tumors developed in the doxycycline-treated mice. To test the effect of DHHC5 suppression on already established tumors, on day 56 (when the tumors reached around 350 mm$^2$) mice were randomized to receive continued vehicle or added doxycycline to suppress DHHC5. In this case, untreated tumors continued to grow (Fig. 7E, center), whereas doxycycline-treated tumors (DHHC5 suppressed) stopped growing but did not regress. The effect of release from doxycycline was also tested (Fig. 7E, right). For mice receiving doxycycline from day 0, mice were randomized on day 59 to receive either continued doxycycline or vehicle (to release from DHHC5 suppression). In this case, a slight increase in tumor growth was seen, but the rate of growth was much less as compared with previously untreated cells. These results suggest that the effects of DHHC5 suppression may persist for some time after release from suppression and that the effects of DHHC5 may be directed toward a longer lived cell population.

Figure 4.
Rescue of the growth-suppressive phenotype of DHHC5 stable knockdown by plasmid-mediated expression of DHHC5, but not the active site mutant, DHHS, in H1299, H358, and H2009 cells. Immunoblots for DHHC5 expression of control, knockdown, wild-type and mutant DHHC5 rescue are shown as well as colony formation and cell proliferation assessments in H1299 (A), H358 (B), and H2009 (C) cell lines. All graphs show the mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student t test.
Discussion

The current study has shown that the protein palmitoyltransferase DHHC5 contributes to the proliferation, anchorage-dependent and -independent colony formation of NSCLC lines and is required for the establishment of tumor xenografts in mice for a subset of NSCLC cell lines. The enzyme is not required for growth of HBECs, which express low levels of the enzyme. The catalytic activity of DHHC5 is implicated, because anchorage-dependent NSCLC growth suppressed by DHHC5 knockdown was rescued by DHHC5 but not by a catalytically inactive mutant. These results suggest that inhibiting DHHC5 may be a strategy for treating a subset of NSCLCs.

Agents that target another protein lipid modification, the farnesytransferase inhibitors (FTI), have had limited success in

![Image](image-url)
cancer clinical trials (reviewed in ref. 35). FTIs were developed to block Ras farnesylation and membrane association. However, N-Ras and K-Ras are resistant to FTIs as a consequence of alternative geranylgeranylation. Our experiments showing that a knockdown of a single DHHC palmitoyltransferase suggests that, at least in some cell lines, DHHC5 is not subject to the same sort of redundancy. Furthermore, as there are a larger number of PATs (23 PATs vs. 4 prenyltransferases) the opportunity for development of more specific and therefore less toxic inhibitors may be greater.

It was previously shown (31, 36) that genetically engineered mice bearing a hypomorphic allele of DHHC5 (5%–7% of wild-type level) are phenotypically normal except for a defect in learning and memory (36). Therefore, it could be argued that specific inhibitors of DHHC5 may not display serious toxicity outside of the central nervous system. It should be noted, however, that embryonic lethality was noted in the DHHC5 knockdown mice, which were born at only 50% of the expected rate, so reproductive toxicity of targeted inhibitors may be anticipated.

How DHHC5 contributes to the formation of tumors is probably complex. While we did not see a strong correlation between DHHC5 protein and sensitivity to DHHC5 knockdown among the 11 cancer cell lines examined, a larger sample set may be required to conclusively examine this relationship. With more data we may also be able to correlate sensitivity of DHHC5 knockdown to molecular signatures of lung cancer subtypes (24). This may be important for developing predictive biomarkers of DHHC5 response. DHHC5 is localized to the plasma membrane in association with detergent-resistant microdomains (31, 37, 38), and as the catalytic activity is implicated, the mechanism for tumor dependence on DHHC5 for growth is likely to depend on palmitoylation of one or more substrates in these domains. Only a few substrates of DHHC5 are known. Flotillin-2 is a substrate of DHHC5 in neuronal stem cells (31) and...
cardiomyocytes (39), and cardiomyocytes derived from DHHC5 knockdown mice are defective in a form of floxillin-dependent endocytosis. Floxillins have been implicated in receptor tyrosine kinase signaling and epithelial cancers, including non-small cell lung and breast cancers (40). However, we did not detect changes in flotillin-2 palmitoylation in DHHC5 knockdown lung cancer cell lines (not shown). DHHC5 also palmitoylates ankyrin-G (ANK3) in polarized MDCK epithelial cells, and the palmitoylation is required for establishment of lateral epithelial membrane assembly (37). DHHC5 has been most studied in the context of neuronal function because DHHC5 is very highly expressed in the brain and neuronal tissues. DHHC5 is localized to dendrites, where it palmitoylates GRIP1B, a regulator of AMPA receptor trafficking (41) and β-catenin (CTNN2D1), a component of synaptic cadherin adhesion complexes that are key regulators of synaptic plasticity involved in memory formation (42). Interestingly, β-catenin is upregulated in NSCLC, and increased expression is associated with poor prognosis (43). Of note, DHHC5 and a homolog of β-catenin (CTNN1D1) are located in close proximity in an amplicon of approximately 0.4 Mb at 11q12 that is amplified in about 3% of lung cancers (44). Unfortunately, our sample did not include a lung cancer with amplification in this region.

Other DHHC family members have been reported to contribute to oncogenesis in certain cell types and could also represent therapeutic targets, either alone or in combination of inhibitors of DHHC5. DHHC8, a PAT that shares approximately 50% homology at the amino acid level to DHHC5, was recently identified as a radiation susceptibility gene. DHHC8 depletion enhanced the therapeutic efficacy of X-irradiation in a mesothelioma model (45). DHHC8 is present in the Golgi apparatus and/or endoplasmic reticulum (ER; ref. 33) and shares some common substrates with DHHC5 (37, 41). Interestingly, we saw that a number of NSCLC lines were sensitive to DHHC8 siRNAs (Supplementary Fig. S1), although these results remain to be validated. Other DHHC family members implicated in cancer include DHHC7 and DHHC21, which are PATs for sex steroid receptors (estrogen, progesterone, and androgen), relevant to breast and prostate cancer (46). In addition, DHHC17 (also known as HIP14; ref. 18) and DHHC20 (17) transform mammalian cells, and DHHC14 is activated via a translocation in acute biphenotypic leukemia (16).

In the future, profiling of palmitoylated proteins using some of the cell lines generated in the current study will provide a list of candidate substrates and define pathway(s) responsible for dependence on DHHC5 for cell growth. This will be a major undertaking, as a previous study suggests the number of substrates is potentially quite large (31). However, it may not be necessary to identify the relevant substrates of DHHC5 to develop inhibitors. For example, protein kinase inhibitors were initially developed using assays that track inhibition of phosphorylation of artificial substrates or autophosphorylation of the kinase even before relevant substrates were known (see refs. 47–49 and references therein). Autopalmitoylation is currently being used in a screening assay for DHHC enzyme inhibitor development (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Tian, J.-Y. Lu, J.D. Minna


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