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# DHHC2 Affects Palmitoylation, Stability, and Functions of Tetraspanins CD9 and CD151

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**Although palmitoylation markedly affects tetraspanin protein biochemistry and functions, relevant palmitoylating enzymes were not known. There are 23 mammalian "DHHC" (Asp-His-His-Cys) proteins, which presumably palmitoylate different sets of protein substrates. Among DHHC proteins tested, DHHC2 best stimulated palmitoylation of tetraspanins CD9 and CD151, whereas inactive DHHC2 (containing DH→AA or C→S mutations within the DHHC motif) failed to promote palmitoylation. Furthermore, DHHC2 associated with CD9 and CD151, but not other cell surface proteins, and DHHC2 knockdown diminished CD9 and CD151 palmitoylation. Knockdown of six other Golgi-resident DHHC proteins (DHHC3, -4, -8, -17, -18, and -21) had no effect on CD9 or CD151. DHHC2 selectively affected tetraspanin palmitoylation, but not the palmitoylations of integrin β4 subunit and bulk proteins visible in [$^3$H]palmitate-labeled whole cell lysates. DHHC2-dependent palmitoylation also had multiple functional effects. First, it promoted physical associations between CD9 and CD151, and between α3 integrin and other proteins. Second, it protected CD151 and CD9 from lysosomal degradation. Third, the presence of DHHC2, but not other DHHC proteins, shifted cells away from a dispersed state and toward increased cell–cell contacts.**

## INTRODUCTION

Transmembrane proteins in the tetraspanin family are widely distributed in nearly all cell types from fungi to mammals (Gourgues *et al*., 2002; Fradkin *et al*., 2002; Moribe *et al*., 2004; Huang *et al*., 2005). In brain, in the immune system, on oocytes, and on metastasizing tumor cells, tetraspanins regulate cell motility, morphology, fusion, signaling, proliferation, and apoptosis (Boucheix *et al*., 2001; Geisert *et al*., 2002; Hemler, 2003; Wright *et al*., 2004; Stein *et al*., 2004; Levy and Shoham, 2005). Tetraspanins assemble together with integrins, signaling molecules and other proteins, into multiprotein cell surface complexes known as tetraspanin-enriched microdomains (TEMs) (Hemler, 2003; Hemler, 2005; Nydegger *et al*., 2006; Odintsova *et al*., 2006). Many proteins within TEMs are extensively and irreversibly palmitoylated by a mechanism that requires an intact Golgi (Yang *et al*., 2002; Yang *et al*., 2004). Palmitoylation plays a key role in the assembly and/or maintenance of TEMs, while also affecting tetraspanin subcellular distribution, stability during biosynthesis, and cell signaling, motility, and morphology (Berditchevski *et al*., 2002; Charrin *et al*., 2002; Yang *et al*., 2002; Zhou *et al*., 2004; Cherukuri *et al*., 2004; Yang *et al*., 2006).

Palmitic acid is a 16-carbon fatty acid that can be added to intracellular cysteines of various cytoplasmic and transmembrane proteins, through the action of thiol-directed *p*rotein *a*cyl*t*ransferases (PATs) (Mitchell *et al*., 2006). The recently characterized "DHHC" family of PATs is composed of transmembrane proteins, typically containing four transmembrane domains and two extracellular/luminal loops, flanking a signature cytoplasmic Asp-His-His-Cys (DHHC) motif (Politis *et al*., 2005; Mitchell *et al*., 2006; Ohno *et al*., 2006). The cysteine within the DHHC motif may play a central role in the transfer of palmitate to substrate (Mitchell *et al*., 2006). There are at least 23 distinct mammalian DHHC proteins and eight yeast DHHC proteins, residing in diverse tissues and subcellular locations (Ohno *et al*., 2006). A few of these DHHC proteins, possessing PAT activity, have been shown to target specific cytoplasmic substrates (Fukata *et al*., 2006; Mitchell *et al*., 2006).

Many transmembrane proteins undergo palmitoylation. These include not only tetraspanins but also integrins (α6, α3, and β4), claudins, G protein-coupled receptors, glutamate receptors, amino acid permeases, and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins (Percherancier *et al*., 2001; Yang *et al*., 2004; Keller *et al*., 2004; Hayashi *et al*., 2005; Valdez-Taubas and Pelham, 2005; Roth *et al*., 2006; Kovalenko *et al*., 2007). In general, palmitoylation of transmembrane proteins serves to regulate subcellular trafficking and/or protein degradation (Percherancier *et al*., 2001; Hayashi *et al*., 2005; Valdez-Taubas and Pelham, 2005). Despite the functional importance of transmembrane protein palmitoylation, in only a few cases has the involvement of specific DHHC proteins been ascertained. For example, palmitoylations of yeast transmembrane proteins Tlg1 and Chs3 are mediated by DHHC proteins Swf1 (Valdez-Taubas and Pelham, 2005) and Pfa4 (Lam *et al*., 2006), respectively, whereas mammalian DHHC3/GODZ targets glutamate receptor subunits (Keller *et al*., 2004; Hayashi *et al*., 2005).

Given the considerable biological relevance of TEMs and their component tetraspanin proteins, and the importance of tetraspanin palmitoylation, we sought to identify specific mammalian DHHC proteins that might be involved. Using gene expression and small interfering RNA (siRNA) approaches, we implicate DHHC2 in the functionally relevant palmitoylation of multiple tetraspanin proteins.

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Abbreviations used: DHHC, Asp-His-His-Cys motif that defines a family of enzymes involved in protein palmitoylation; PAT, protein acyl transferase; TEM, tetraspanin enriched microdomain.

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## MATERIALS AND METHODS

### Antibodies, Reagents, and Cells

Antibodies to CD151 (5C11 and 1A5), CD9 (MM2/57), and integrin $\alpha 3$ (A3X8 and D23) have been described previously (Stipp *et al*., 2003; Yang *et al*., 2006). Antibodies to $\beta$-catenin, c-Raf, and green fluorescent protein (GFP) (monoclonal antibody [mAb] and polyclonal antibody [pAb]) were from BD Biosciences (San Jose, CA); actin and FLAG (M2) were from Sigma-Aldrich (St. Louis, MO); c-Src (pAb) and CD44 (mAb) were from Santa Cruz Biotechnology (Santa Cruz, CA); and $\beta 4$ integrin (mAb and pAb) were from Millipore Bioscience Research Reagents (Temecula, CA). G418 sulfate was obtained from Cellgro (Herndon, VA). Cerulenin (an agent that inhibits fatty acid synthesis and diminishes levels of endogenous palmitate) and bafilomycin A1 were purchased from Sigma-Aldrich and dissolved in ethanol and dimethyl sulfoxide, respectively. [$^3$H]Palmitic acid and [$^{35}$S]methionine were from Perkin Elmer-Cetus (Boston, MA). Lipofectamine 2000 and Lipofectamine RNAiMAX reagent were from Invitrogen (Carlsbad, CA). RNA isolation kit and reverse transcription-polymerase chain reaction (RT-PCR) reaction reagents were from QIAGEN (Valencia, CA). Human embryonic kidney (HEK) epithelial 293 cells and human epidermoid carcinomal cells A431 and MDA 231 were grown in DMEM with 10% fetal bovine serum, 10 mM HEPES, and antibiotics. For transient transfection, cells were transfected with Lipofectamine 2000. Stable transfections were also performed with Lipofectamine 2000, and later A431 cells were selected on 1.5 mg/ml G418 sulfate.

### Plasmid Construction and Mutagenesis

Cloned cDNA for DHHC2, 5, 7, 9, and 11 were from American Type Culture Collection (Manassas, VA) and DHHC15 was from Invitrogen. All (except DHHC5) were cloned into pEGFP N1 vector (Clontech, Mountain View, CA) to make C-terminal GFP fusion proteins. DHHC5 was cloned into pcDNA 3.1 vector (Invitrogen), and a FLAG tag was inserted at its C-terminal end. Inactivating point mutations in DHHC2 and -15 were generated by PCR, and cloned into pEGFP N1 vector to make C-terminal GFP fusion proteins, and then verified by DNA sequencing.

### Metabolic Labeling and Immunoprecipitation

For [$^3$H]palmitate labeling, cells (30 h after transfection) were serum starved (1.5 h) and then pulsed (2 h) with [$^3$H]palmitic acid (0.2 mCi/ml) in a medium containing 5% dialyzed serum. After washing in phosphate-buffered saline (PBS), cells were lysed in 25 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, and protease inhibitor cocktail, with either 1% Brij 96 or NP-40 (1 h). After preclearing with protein G-Sepharose beads (GE Healthcare, Uppsala, Sweden), lysates containing equal amounts of protein were used for immunoprecipitation (Yang *et al*., 2006). Proteins were visualized by immunoblotting, and $^3$H-labeled proteins were detected using BioMax MS film and Biomas TRANSCREEN LE intensifying screen (Eastman Kodak, Rochester, NY) for 1–2 wk at −80°C, as described previously (Yang *et al*., 2004). Quantitation was done using Image Quant, version 5.2 software (GE Healthcare) or Gene Tools, version 3.00.22 (Syngene, Frederick, MD). For $^{35}$S-labeling, cells were incubated (1.5 h) in cysteine/methionine-free media, pulsed with [$^{35}$S]methionine/cysteine (0.2 mCi/ml; 1.5 h) in 5% dialyzed serum, and then chased for variable times in media containing 25× unlabeled L-methionine/cysteine.

### siRNA Transfection and RT-PCR

All duplex siRNAs were obtained from Dharmacon RNA Technologies (Lafayette, CO) and Ambion (Austin, TX). Sense strand codes were CCAAGGAUCUUCCCAUCUAUU (DHHC2 #6), GGAUCUUCCCAUCUAUACCtt (DHHC2 #10), GCAGGUCUUUGGCAUGA (CD151 #4), and GACAGAUGCCAACUUAUAAUU (control siRNA). Mixtures of siRNAs were used to target all other DHHC proteins. RNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen). Cells were then used after 72 h, and the extent of knockdown was quantified by RT-PCR. Primer sequences for all DHHCs were indicated previously (Ohno *et al*., 2006).

### Flow Cytometry

Cells (3 d after siRNA transfection) were stained with specific mAbs (1–2 $\mu$g/well) in 96-well plates, for 30 min at 4°C. After washing with PBS, fluorescein isothiocyanate-conjugated secondary antibody was added (30 min; 4°C), and cells were analyzed by FACSCalibur (BD Biosciences). To

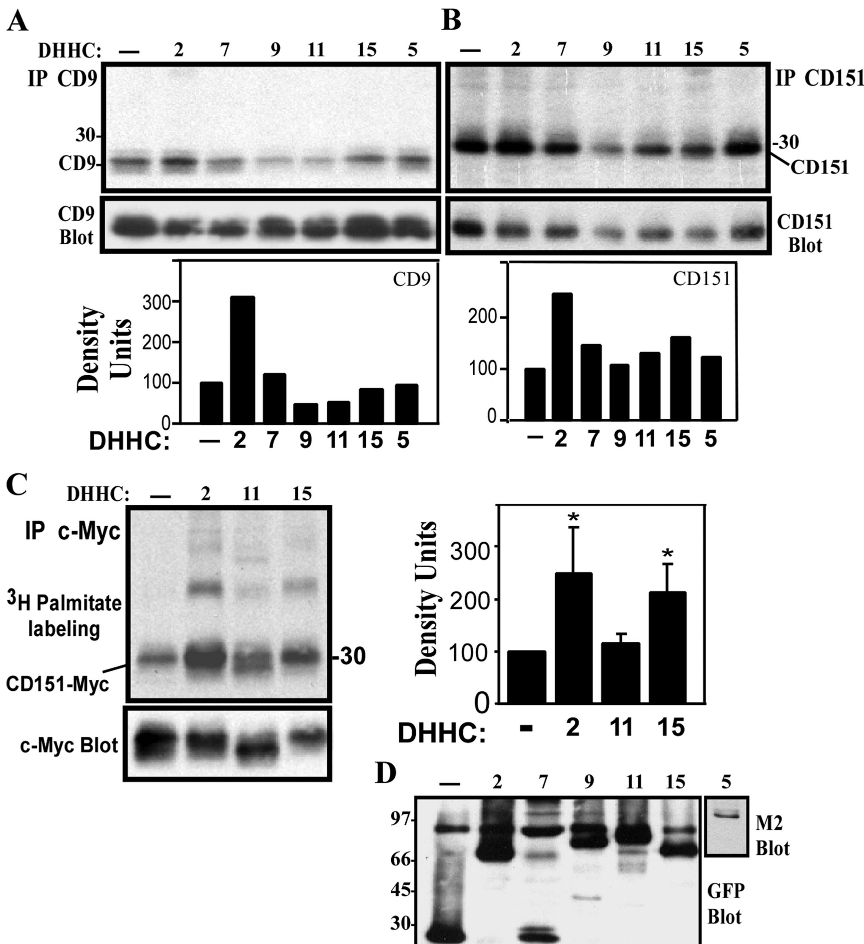


**Figure 1.** Screening of DHHC proteins for palmitoylation of CD9 and CD151. HEK293 cells were transiently transfected with the indicated DHHC proteins. After 36 h, cells were starved in a media containing 5 $\mu$g/ml cerulenin for 1 h, and then they were metabolically labeled with [$^3$H]palmitic acid for 2 h before lysing in a buffer containing 1% Brij 96. CD9 (A) and CD151 (B) were immunoprecipitated by using MM2/57 and 5C11 mAbs, respectively, to show $^3$H-labeled proteins (top) and total immunoprecipitated CD9 and CD151 protein (middle). Also shown is density of $^3$H-labeled protein normalized for total immunoprecipitated protein (bottom). (C) HEK293 cells were cotransfected with CD151-Myc and DHHC-GFP proteins. After 36 h, cells were starved for 1 h and metabolically labeled with [$^3$H]palmitic acid for 2 h. Cells were lysed in a buffer containing 1% NP-40. CD151 was immunoprecipitated from the lysates by Myc mAb. The membrane was then probed for CD151 expression by Myc mAb. The palmitoylation signal, normalized relative to protein expression, is shown as density units (right; *$p < 0.05$). (D) Lysates from each transfection were analyzed for expression of DHHC proteins by blotting with anti-GFP and -FLAG antibodies.

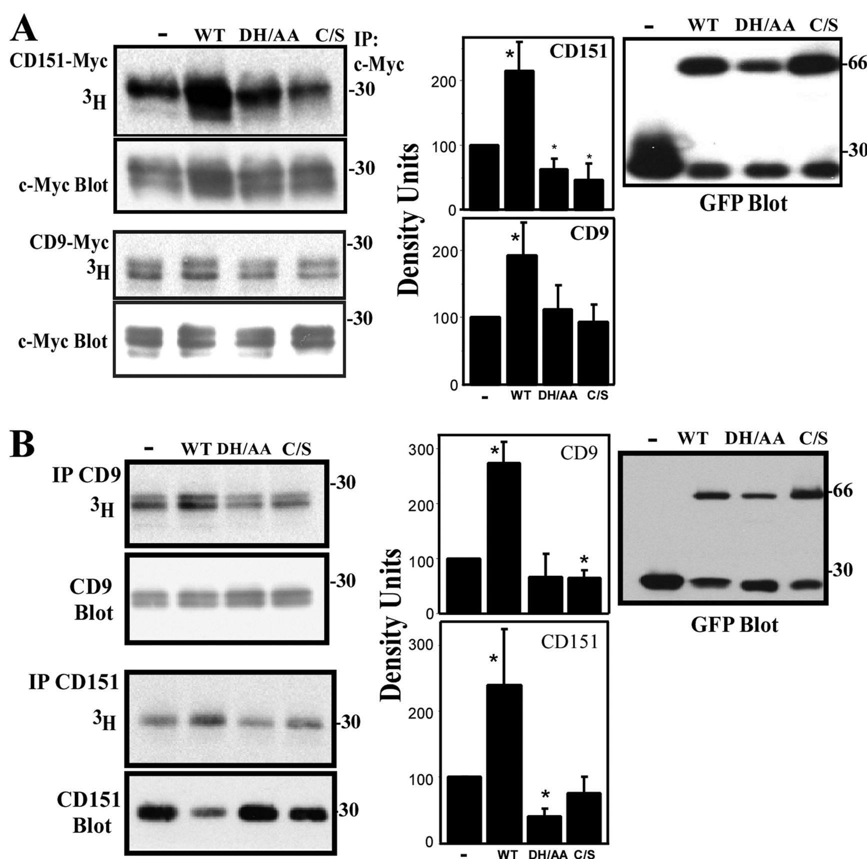


**Figure 2.** Effects of wild-type and mutant DHHC2 on CD9 and CD151 palmitoylation. (A) HEK293 cells were cotransfected with DHHC2 or its mutants along with either CD151-myc or CD9-myc. After 36 h, cells were metabolically labeled with [$^3$H]palmitic acid, CD151, and CD9 were immunoprecipitated using anti-myc antibody, and proteins were then detected based on [$^3$H]palmitate emission, or c-Myc blotting. Density units, normalized for protein expression, are shown as bar graphs (*$p < 0.05$). (B) A431 cells stably expressing DHHC2 and its mutants were metabolically labeled with [$^3$H]palmitate. Then, endogenous CD9 and CD151 were immunoprecipitated and visualized based on [$^3$H]palmitate emission, and immunoblotted using anti-CD9 and -CD151 antibodies. Right, density unit quantitation (*$p < 0.05$). In A and B, expression of GFP-tagged wild-type and mutant DHHC2 proteins is verified by blotting for GFP (right).

obtain mean fluorescence intensity (MFI) values, background staining (from negative control antibody) was subtracted from specific mAb staining.

## RESULTS

### *DHHC2 Promotes Tetraspanin Palmitoylation*

Preliminary studies, involving mass spectrometry, suggested that DHHC2, DHHC5, DHHC7, and DHHC11 might be present within tetraspanin-enriched microdomains (data not shown). These four proteins, together with DHHC9 (negative control) and DHHC15 (which shows sequence similarity to DHHC2) were then tagged and overexpressed in HEK293 cells. From [$^3$H]palmitate-labeled cell lysate, endogenous tetraspanins CD9 and CD151 were immunoprecipitated, and the extent of palmitoylation of CD9 (Figure 1A) and CD151 (Figure 1B) was determined. DHHC2 expression yielded a substantial increase in palmitoylation for both CD9 and CD151, as indicated directly (top), and from densitometric quantitation (bottom, bar graphs) after normalization for variations in CD9 and CD151 recovery (middle). By contrast, palmitoylation was increased to a lesser extent, or not at all, upon expression of the other DHHC proteins, which were synthesized at levels shown in D. In a separate experiment, GFP-tagged DHHC proteins were tested for effects on palmitoylation of Myc-tagged CD151 in HEK293 cells. Increased palmitoylation was again seen with DHHC2, and to a lesser extent with DHHC15, but not with DHHC11 (C), DHHC7, DHHC9, or DHHC5 (data not shown).

### *DHHC2 and Tetraspanin Palmitoylation: Further Evidence*

To understand further the role of DHHC2, inactive forms were generated. As seen for other DHHC proteins, mutations within the DHHC motif (especially those involving cysteine) can reduce or eliminate PAT activity (Mitchell *et al.*, 2006). Hence, DH→AA and C→S mutants of DHHC2 were prepared. Neither of these mutant forms of DHHC2 stimulated palmitoylation of Myc-tagged CD151 or CD9 in HEK293 cells (Figure 2A, left). In fact, after normalization for Myc-CD151 and Myc-CD9 recovery, the extent of palmitoylation was variably diminished compared with that yielded by wild-type DHHC2 (bar graphs). In a separate experiment, we analyzed endogenous CD9 and CD151 in a different cellular environment (A431 instead of HEK293 cells). On stable expression of DHHC2, palmitoylation of CD9 and CD151 was again increased, compared with vector control cells, whereas mutant forms of DHHC2 (DH/AA and C/S) again failed to stimulate palmitoylation (Figure 2B). Compared with wild-type DHHC2, there is 40–50% less DH/AA mutant, as seen in B and A, respectively. Nonetheless, the DH/AA mutant still inhibits palmitoylation by 30–70% in three of the four examples shown in Figure 2. Presumably, if wild-type and DH/AA proteins were present at the same level, even more inhibition would be seen. Costaining with Golgi matrix protein GM130 (Supplemental Figure 1) confirmed that DHHC2-GFP was almost entirely localized to the Golgi.

### *DHHC2 Selectivity*

Next, we assessed the selectivity of DHHC2 effects on protein palmitoylation. Whereas palmitoylation of CD151 in A431 cells was again stimulated by expression of wild-type DHHC2, and partly inhibited by mutant DHHC2, palmitoylation of the integrin β4 subunit (a partner for CD151) was not affected in the same experiment (Figure 3A). Analysis of total palmitate labeling from whole HEK293 cell lysates revealed minimal positive or negative effects of DHHC2, its

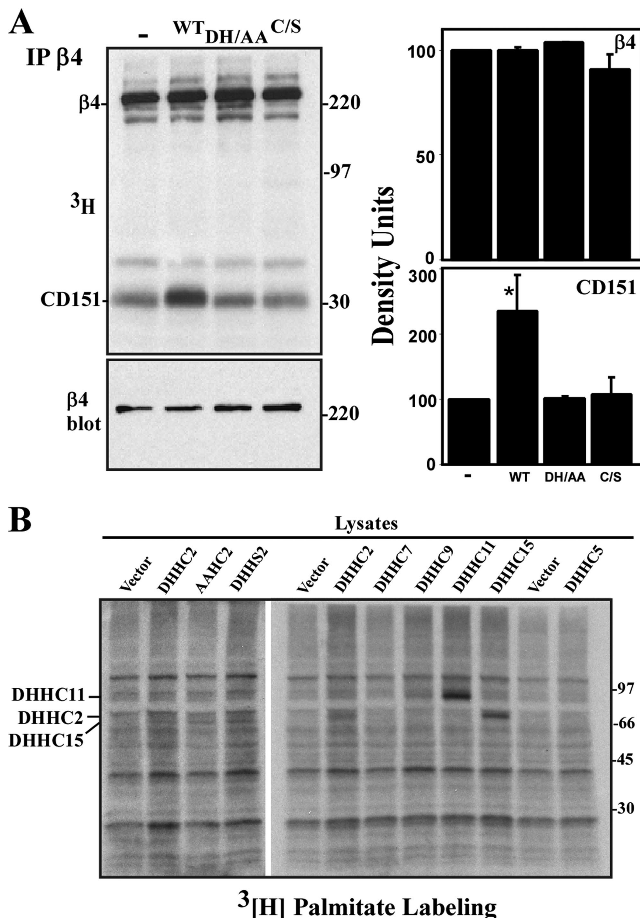


**Figure 3.** DHHC2-mediated palmitoylation specificity. (A) A431 cells stably expressing wild-type and mutant DHHC2 were metabolically labeled with [$^3$H]palmitate, lysed in 1% Brij 96, and then β4 integrin was immunoprecipitated. [$^3$H]Palmitate labeling (top), β4 protein (bottom) and CD151 protein (data not shown) were detected. Density units (labeling/protein) are shown in the bar graphs (*$p < 0.05$). (B) HEK293 cells were transiently transfected with wild-type and mutant DHHC2 and other DHHC proteins. After [$^3$H]palmitate labeling, labeled proteins were visualized in whole cell lysates.

mutant forms, or other DHHC proteins on the various abundantly palmitoylated proteins shown (Figure 3B). The only palmitoylated proteins that stand out are autopalmitoylated forms of DHHC2, DHHC11, and DHHC15, in the 65- to 75-kDa region (Figure 3B). Because it is not yet clear the extent to which autopalmitoylation is proportional to enzyme activity, we hesitate to make inferences regarding DHHC protein activity from these data.

### *DHHC2 and Tetraspanin-enriched Microdomains*

Consistent with its role in tetraspanin palmitoylation, GFP-labeled DHHC2 physically associated with tetraspanin CD9, and more weakly with CD151, and α3 integrin, which is a major partner for CD151 (Figure 4A). Immunoprecipitation of control proteins such as CD147 (a cell surface IgSF protein; Tang and Hemler, 2004) and c-Raf1 did not yield DHHC2 (Figure 4A). Because a key consequence of tetraspanin palmitoylation is to promote heterologous associations among different tetraspanins (Berditchevski *et al*., 2002; Charrin *et al*., 2002; Yang *et al*., 2002; Zhou *et al*., 2004), we predicted that DHHC2 overexpression might enhance CD9–CD151 association. Indeed, DHHC2 expression enhanced the recovery of CD151 upon immunoprecipitation of CD9 (Figure 4B, top) and enhanced recovery of CD9 upon immunoprecipitation of CD151 (Figure 4B, second panel) from 1% Brij 96 lysates of A431 cells. There was also a slight enhancement of CD9-CD151 association upon DHHC15 overexpression, but not nearly to the same extent as DHHC2. Mutant forms of DHHC2 and DHHC15 seemed to diminish, rather than stimulate CD9–CD151 association. Although there were small variations in the recoveries of immunoprecipitated CD151 (Figure 4B, third panel), association of CD151 with α3 integrin was relatively unaffected by DHHC protein expression (Figure 4B, fourth panel). Control experiments indicated that comparable amounts of CD9, CD151, and GFP-tagged DHHC proteins were present in total cell lysates (Figure 4B, bottom 3 panels).

In another experiment, the presence of DHHC2 in HEK293 cells enhanced the association of α3 integrin with several unknown proteins, as seen upon immunoprecipitation of α3 from [$^3$H]palmitate-labeled 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate lysate (Figure 4C). Notably, association of these palmitoylated proteins was markedly diminished upon expression of the inactive DHHC2 mutants (Figure 4C). Expression of other DHHC proteins (DHHC7, -9, -11, and -5) did not enhance α3 association with these other proteins (data not shown).

### *Knockdown of DHHC2 Affects Tetraspanin Palmitoylation and Expression*

To evaluate further the functional consequences of DHHC2-dependent palmitoylation, DHHC2 siRNA knockdown experiments were carried out. As indicated in Supplemental Figure 2A, DHHC2 was appreciably diminished (by >70%) upon treatment of HEK293 cells with two different siRNAs (#6 and #10). In parallel, steady state amounts of CD9 (Figure 5, A and B) and CD151 (Figure 5B) were also diminished (~50%), upon treatment of two different cell lines with DHHC2 siRNA, but not control siRNA. Other cellular proteins (β-catenin, c-Src, integrin α3 subunit, and actin) were unaffected (Figure 5, A and B). In a control experiment, CD151 siRNA almost eliminated CD151, without affecting CD9 or the other proteins (Figure 5B). Cell surface CD9 and CD151 were also significantly decreased (by 35–40%) in DHHC2 siRNA-treated samples, as seen by flow cytometry (Figure 5C). By contrast, levels of other cell surface proteins (integrin α3, major histocompatibility complex class I, integrin β1, EWI-F, CD44, and CD147) were relatively unchanged (Figure 5C). In another control experiment, $^3$H-labeled proteins in a whole cell lysate from MDA-231 cells showed minimal changes in response to DHHC2 knockdown (Figure 5D).

Besides DHHC2, at least 11 additional DHHC proteins reside in the Golgi (Ohno *et al*., 2006; Fernandez-Hernando *et al*., 2006) and thus could promote tetraspanin palmitoylation. Among those, DHHC12, DHHC15, and DHHC22 were only minimally expressed on the majority of cell lines analyzed by semiquantitative RT-PCR (Supplemental Figure 3), and DHHC7 and DHHC9 were already analyzed in Figure 1. The remaining 6 DHHC proteins (DHHC3, -4, -8, -17, -18, and -21) were knocked down by ≥75% in HEK293 cells (Supplemental Figure 2B). However, in contrast to DHHC2 knockdown, none of these other knockdowns significantly diminished CD9 palmitoylation or protein levels (Figure 6, A and C) or CD151 protein levels (Figure 6, B and C). Control experiments show that protein levels for α3 integrin and β-catenin are not affected, even by DHHC2 (Figure 6B).

Results in Figures 5 and 6 suggested that loss of palmitoylation diminished CD9 and CD151 protein expression.

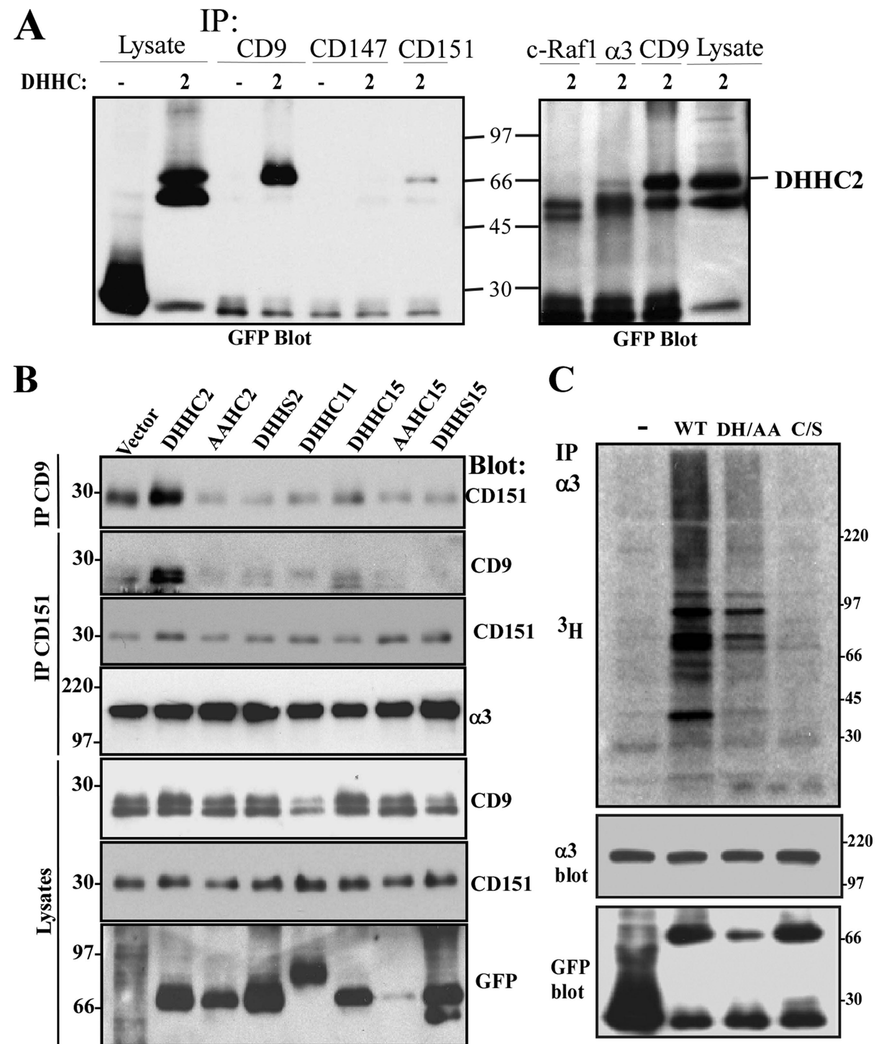


**Figure 4.** DHHC2 and tetraspanin association. (A) A431 cells stably expressing DHHC2 (GFP tag) and vector (GFP) were lysed in 1% Brij 96; endogenous CD9, CD147, CD151, integrin α3, and C-Raf1 were immunoprecipitated; and then associated DHHC2 was detected by blotting for GFP. (B) A431 cells stably expressing DHHC2 and its mutants, DHHC15 and its mutants, and DHHC11 were lysed in a buffer containing 1% Brij 96. Endogenous CD9 and CD151 were immunoprecipitated and blotted for CD151 and CD9, respectively. CD151 immunoprecipitates were also blotted for α3 integrin. In the bottom three panels, lysates from each A431 transfectant were probed for CD9, CD151, and GFP to assess total protein levels. (C) HEK293 cells were transiently transfected with wild-type and mutant DHHC2, labeled with [$^3$H]palmitate, and then α3 integrin was immunoprecipitated using mAb A3X8. The same membrane was blotted for α3 expression. $^3$H-labeled proteins (top), α3 integrin (middle), and GFP-tagged DHHC proteins (bottom) were detected.

Consequently, we predicted that a palmitoylation-deficient mutant of CD9 should be insensitive to DHHC2 knockdown. Indeed, whereas DHHC2 knockdown decreased wild-type CD9 expression by ~50%, mutant CD9 (in which 6 membrane proximal cysteines were converted to alanine) was not affected (Figure 7A, top and bar graph). In control experiments, endogenous CD151 showed 50% loss of expression, regardless of whether wild-type or mutant CD9 was present in the same cell (Figure 7A, second panel), and integrin α3 protein was unaffected (Figure 7A, third panel).

Bafilomycin A1 inhibits vacuolar proton-translocating ATPases, thereby raising lysosomal pH and causing degradation of lysosomal proteinases (Ishidoh *et al*., 1999). The extent of disappearance of CD9 and CD151, caused by DHHC2 knockdown, was diminished when HEK293 cells were treated with bafilomycin A1 for 36 or 24 h (Figure 7B, first and second panels). Levels of control proteins (integrin α3, third panel) and actin (data not shown) were relatively unaffected. Another inhibitor of lysosomes ($NH_4Cl$) and inhibitors of lysosomal proteases (leupeptin + pepstatin A) also prevented loss of CD9 and CD151 protein upon DHHC2 knockdown (Table 1), yielding recoveries (86–100%) comparable with those seen upon bafilomycin A1 treatment (59–71%). By contrast, inhibition of the ubiquitin–proteosome degradation pathway (by MG132 or ALLM), or inhibition of matrix metalloproteinases (by GM6001) mostly did not prevent loss of CD9 and CD151 that is caused by knockdown of DHHC2 (Table 1). Together, these results suggest that knockdown of DHHC2 causes a loss of CD9 and CD151 palmitoylation, leading to enhanced lysosomal proteolysis.

After DHHC2 knockdown, rapid degradation of non-palmitoylated CD9 and CD151 prevented us from confirming that they were indeed less palmitoylated. To circumvent this problem in DHHC2 siRNA-treated cells, palmitoylation was analyzed in the presence of bafilomycin A1. Under those conditions, upon DHHC2 knockdown, more total unlabeled CD151 protein was recovered. Consequently, the amount of $^3$H-labeling/protein was diminished for CD9 (data not shown) and for endogenous CD151, as seen in two different cell lines (Figure 7C).

### *DHHC2 Knockdown Accelerates Lysosomal Degradation and Triggers Cell Dispersion*

To further assess protein degradation, [$^{35}$S]cysteine/methionine pulse-chase labeling experiments were performed. After treatment with siRNA, HEK293 cells were pulsed with $^{35}$S-label, chased for various time periods, and then CD9 and α3 integrin were immunoprecipitated from 1% Triton X-100 lysates. As indicated, degradation of CD9 was markedly enhanced in cells in which DHHC2 was knocked down (half-life, 10 h), compared with control siRNA-treated cells (half-life,

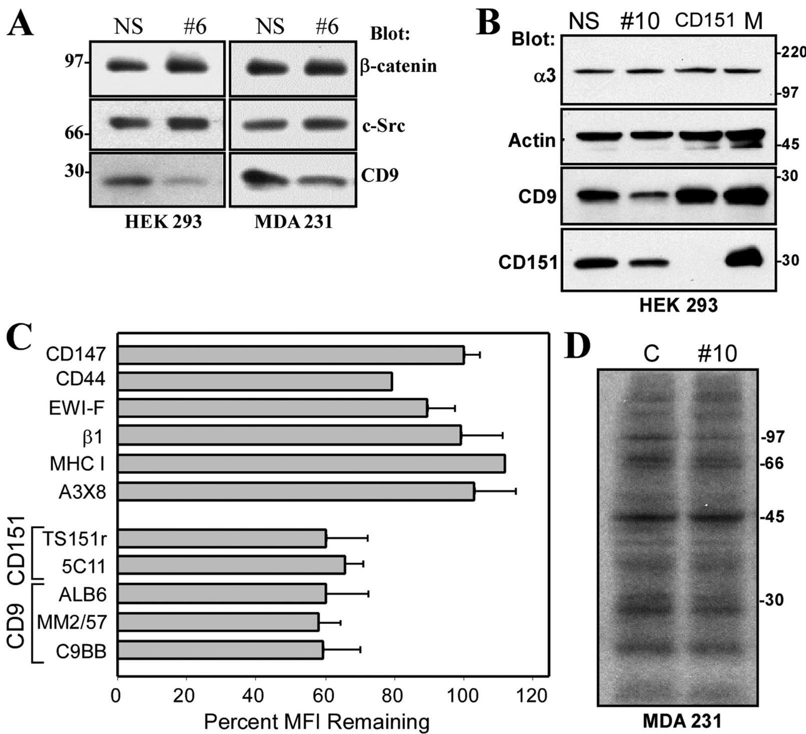


**Figure 5.** siRNA knockdown of DHHC2. (A) HEK293 and MDA-231 cells were transfected with siRNAs and after 72 h, Brij 96 cell lysates were blotted for β-catenin, c-Src, and CD9 proteins. (B) HEK293 cells were transfected with siRNAs, and then after 72 h, cells were lysed in 1% Brij 96, and blotted for integrin α3, actin, CD9, and CD151 proteins. (C) HEK293 cells treated with control and DHHC2 siRNAs were analyzed by flow cytometry, which counted ~10,000 cells/experiment. Using MFI values, the percentage of each cell surface protein remaining after DHHC2 knockdown was determined. n = 3 in all cases, except when n = 2 (α3 and β1 integrins and CD44) or n = 1 (MHC I and CD147). Two different anti-CD151 and three different anti-CD9 mAbs yielded similar results. Control MFI values ranged from 17 to 25 (for α3 and CD44) and from 50 to 200 for all other cell surface proteins. (D) MDA-231 cells were transfected with control or DHHC2 #10 siRNAs, labeled with [$^3$H]palmitate (2 h) and then labeled proteins were visualized in total cell lysates.

22 h; Figure 8A). By contrast, degradation of α3 integrin was minimally affected (Figure 8B). Diminished cell surface expression of CD151, due to knockdown of DHHC2, also could be detected by antibody staining and immunofluorescence microscopy (Figure 9). Not only was the intensity of immunofluorescent staining diminished but also cell organization was altered. In particular, most of the cells treated with DHHC2 siRNA (oligo #10) showed dispersed, single cell morphology, whereas only <10% of control cells occurred as single cells (Figure 9). Similar results were obtained using a second DHHC2 siRNA (oligo #6; data not shown). Cotransfection with a fluorescent siRNA confirmed that transfection efficiency was nearly 100% (Supplemental Figure 4).

## DISCUSSION

### *Tetraspanin Palmitoylation by DHHC2*

Here, we show that DHHC2, a member of the DHHC family of PATs, regulates palmitoylation of tetraspanins CD9 and CD151. This conclusion is based on four key results. First, overexpression of DHHC2 promoted palmitoylation of both

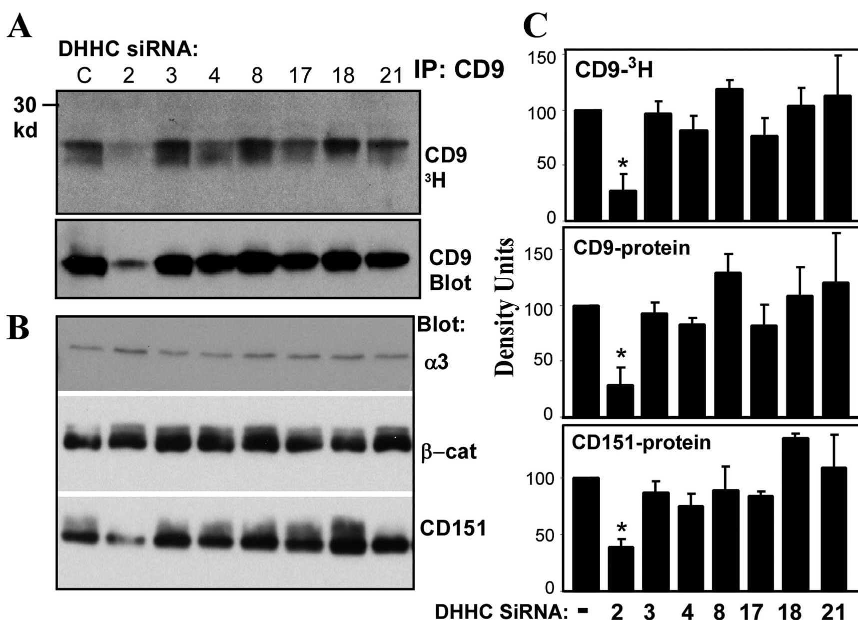


**Figure 6.** Knockdown of multiple DHHC proteins. (A) HEK 293 cells were transfected with different DHHC and control siRNAs, metabolically labeled with [$^3$H]palmitate (2 h), and then cells were lysed in 1% Brij 96 and endogenous CD9 was immunoprecipitated. Proteins were visualized by [$^3$H]palmitate radioactivity (top) and by blotting (bottom). (B) HEK293 cell lysates were also blotted for α3 integrin, β-catenin, and CD151 proteins. (C) The amount of CD9 was quantitated from [$^3$H]palmitate labeling and from immunoblotting. The amount of CD151 was quantitated from CD151 protein immunoblotting, normalized relative to control β-catenin protein (*$p < 0.01$).

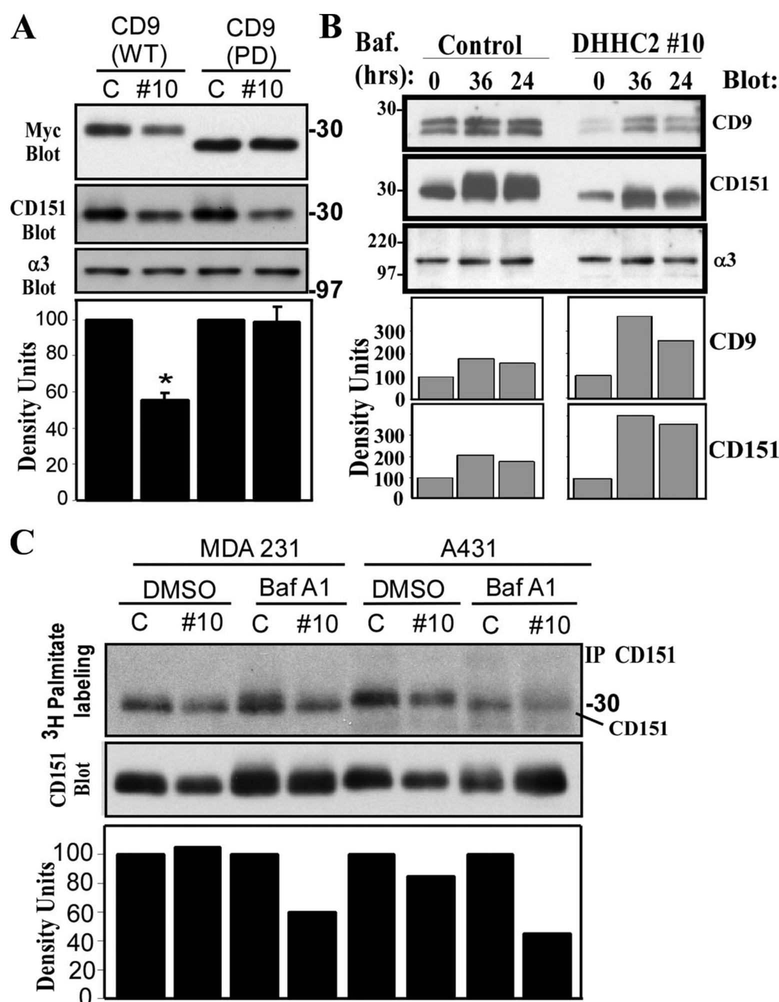


**Figure 7.** Loss of palmitoylation is accompanied by loss of expression. (A) HEK293 cells were transiently transfected with control, or DHHC2 #10 siRNA. After 24 h, they were transiently transfected with either unmutated CD9-myc, or palmitoylation-deficient CD9-myc (lacking membrane-proximal cysteines). After another 48 h, cells were lysed in 1% Brij 96, and then they were blotted for Myc, CD151, and α3 integrin. Density units are from densitometric scans of Myc blots (e.g., as in lanes 2 and 4), normalized relative to controls (e.g., as in lanes 1 and 3, respectively) (*$p < 0.05$). (B) HEK293 cells were transfected with control and DHHC2 #10 siRNA. Bafilomycin A1 (100 nM), an acidic protease inhibitor, was added to DHHC2 siRNA-treated samples at 36 and 48 h after transfection. Cells were then lysed after 72 h and steady-state expression of CD9, CD151, and α3 was determined by immunoblotting. Two representative experiments are shown. Combined results from four experiments show that knockdown of DHHC2 significantly enhanced the 36-h stimulation effects of bafilomycin. Bafilomycin stimulation, 1.27 ± 0.3 with control siRNA; 2.40 ± 0.74 with DHHC2 siRNA ($p < 0.03$). (C) MDA-231 and A431 cells were transfected with control, or DHHC2 #10 siRNA. Bafilomycin A1 (100 nM) was added 36 h after transfection, and at 72 h, cells were lysed in 1% Brij 96, and CD151 was immunoprecipitated. Proteins were visualized by detection of [$^3$H]palmitate (top) and by blotting for CD151 (middle), which enabled calculation of normalized density units (bottom). Two representative experiments are shown. Combined results from three experiments show that DHHC2 siRNA causes palmitoylation to be significantly decreased, when bafilomycin is also present. Palmitoylation, 95 ± 17% (DHHC2 siRNA, no bafilomycin); 45 ± 10% (DHHC2 siRNA, plus bafilomycin) ($p < 0.02$).

overexpressed and endogenous CD9 and CD151 in multiple cell types. Second, inactive forms of DHHC2 (DH/AA and C/S mutations within the DHHC motif) not only failed to enhance DHHC2-induced palmitoylation but also variably suppressed basal palmitoylation, consistent with a possible dominant-negative effect on endogenous DHHC2-dependent activity. A precedent was established when a C/S mutation in the DHHC motif of DHHC15 seemed to have a dominant-negative effect on palmitoylation of PSD-95, a neuronal scaffold protein (Fukata *et al*., 2004). Third, knockdown of endogenous DHHC2, but not other DHHC proteins, caused a marked decrease in the palmitoylation of

**Table 1.** Effects of protease inhibitors on loss of CD9 and CD151 caused by DHHC2 knockdown

| Inhibitor[a] | Tetraspanin | Decrease (−inhibitor) (%) | Decrease (+inhibitor) (%) | Recovery (%) |
|---|---|---|---|---|
| Bafilomycin A1 | CD9 | 75 | 22 | 53/75 = 71 |
| | CD151 | 56 | 23 | 33/56 = 59 |
| Leupeptin/pepstatin | CD9 | 58 | 8 | 50/58 = 86 |
| | CD151 | 37 | 0 | 37/37 = 100 |
| $NH_4Cl$ | CD9 | 60 | 0 | 60/60 = 100 |
| | CD151 | 38 | −15 | 53/38 = 139 |
| GM6001 | CD9 | 58 | 52 | 6/58 = 10 |
| | CD151 | 80 | 67 | 13/80 = 16 |
| ALLM | CD9 | 70 | 68 | 2/70 = 3 |
| MG132 | CD9 | 40 | 60 | −20/40 = −50 |

[a] Inhibitors used were bafilomycin A1 (100 nM), combined leupeptin (10 μM) and pepstatin A (100 μ M), $NH_4Cl$ (10 mM), GM6001 (10 μ M), ALLM (50 μ M), and MG132 (10 μ M). HEK293 cells were transfected with control and DHHC2 #10 siRNA. Inhibitors were added (at 36 h), cells were lysed (at 72 h), and CD9 and CD151 were detected by immunoblotting. CD9 and CD151 band densities were determined by densitometry (after knockdown by DHHC2 or control siRNA), and percentage of decrease (due to DHHC2 knockdown) was calculated.

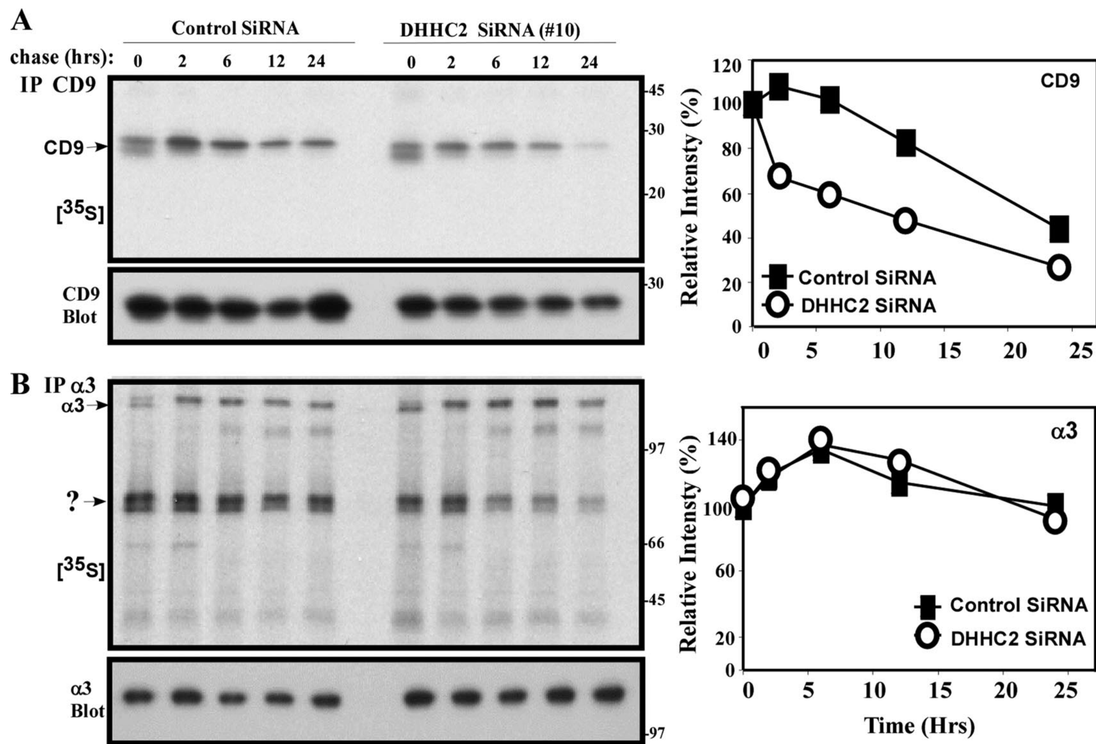


**Figure 8.** DHHC2 knockdown enhances CD9 degradation. HEK293 cells were transfected with either control or DHHC2 siRNA. After 48 h, cells were pulsed with 0.2 mCi/ml [$^{35}$S]methionine for 1.5 h, and then either lysed (chase = 0 h) or chased with a medium containing excess cold methionine for different times. The cells were then lysed in 1% Triton X-100 and endogenous CD9 (A) and $\alpha$3 integrin (B) were immunoprecipitated. Membranes were then probed for total CD9 and $\alpha$3 integrin, by blotting with mAb MM2/57 and pAb D23, respectively. Graphs show relative protein levels normalized to the 0-h time point.

CD9 and CD151. This is a critical complement to our other results, which are based on DHHC2 overexpression. Fourth, we demonstrated that DHHC2 can indeed associate specifically with tetraspanin substrates (CD9 and CD151), just as another DHHC protein (DHHC3/GODZ) was found previously to physically associate with its polytopic transmembrane protein substrates (Mittelbrunn *et al.*, 2002). DHHC2-substrate association supports the idea that DHHC2 is having a direct, rather than indirect effect on palmitoylation. Finally, DHHC2 is present in the Golgi (Fukata *et al.*, 2006) where tetraspanin palmitoylation occurs (Yang *et al.*, 2002, 2004).

### *Palmitoylation Specificity*

Among the 12 different DHHC proteins tested either by overexpression (DHHC2, DHHC5, DHHC7, DHHC9, DHHC11, and DHHC15) or by siRNA knockdown (DHHC2, DHHC3, DHHC4, DHHC8, DHHC17, DHHC18, and DHHC21) only DHHC2 promoted tetraspanin palmitoylation. The closest homologues of DHHC2 are DHHC15 and DHHC20 (Ohno *et al.*, 2006). However, DHHC15 was generally much less effective than DHHC2, and it was not detectable in four of five cell lines in which CD9 and CD151 palmitoylation occurs. DHHC20 was not considered further because expression is

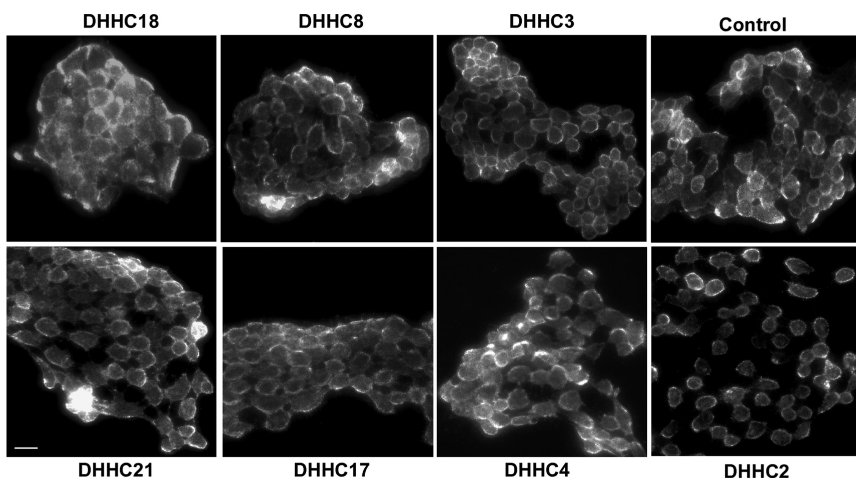


**Figure 9.** Knockdown of DHHC2 affects A431 cell dispersion. A431 cells were transfected with control, DHHC2 #10, or other DHHC siRNAs. After 72 h, cells were stained with anti-CD151 mAb 1A5 and then Alexa 594-conjugated second antibody. Images were acquired using a Nikon Eclipse TE300 inverted fluorescence microscope, using Spot software (Diagnostics Instruments, Sterling Heights, MI). Bar, 20 $\mu$m.

limited to the plasma membrane (Ohno *et al.*, 2006). DHHC2 has also been suggested to promote palmitoylation of PSD-95, a neural synapse protein (Fukata *et al.*, 2006). However, 1) those results were obtained when both DHHC2 and PSD-95 substrate were highly overexpressed, and 2) DHHC2 was not as effective as DHHC15 for that substrate.

DHHC2 did not have a global effect on cellular palmitoylation. It physically associated with CD9, CD151, and to a small extent with $\alpha$3 integrin, but not with control proteins (CD147 and c-Raf1). Neither overexpression nor knockdown of DHHC2 had much effect on the many proteins detectable in [$^3$H]palmitate-labeled whole cell lysates.

Furthermore, a quantitative proteomics approach aimed at identifying DHHC2 substrates revealed 50 palmitoylated proteins that are unaffected by DHHC2, whereas one protein (called CKAP4/p63) showed only a marginal ~30% decrease in palmitoylation upon DHHC2 knockdown (Zhang *et al.*, 2008). Also, DHHC2 overexpression did not alter integrin $\beta$4 palmitoylation, even though $\beta$4-associated CD151 was markedly affected in the same experiment. Hence, $\beta$4, a type I transmembrane protein, is palmitoylated by a different mechanism than tetraspanin proteins CD9 and CD151. Indeed, as summarized elsewhere, type I transmembrane proteins and polytopic transmembrane proteins may be palmitoylated by different DHHC PATs (Mitchell *et al.*, 2006; Linder and Deschenes, 2007).

Tetraspanins CD9 and CD151 each contain six membrane-proximal cysteines (proximal to each of the four transmembrane domains), and all of these most likely undergo palmitoylation (Berditchevski *et al.*, 2002; Charrin *et al.*, 2002; Yang *et al.*, 2002; Kovalenko *et al.*, 2004). At present, we do not know whether DHHC2 targets all of these cysteines, which are each surrounded by different amino acids. There is not yet evidence for DHHC proteins recognizing specific palmitoylation motifs (Mitchell *et al.*, 2006). Hence, we suspect that within TEMs, DHHC2 may gain proximity to tetraspanin membrane cysteines, thereby enabling palmitoylation, regardless of flanking amino acids. Because CD9 and/or CD151 can readily associate with many other tetraspanins (e.g., CD37, CD53, CD63, CD81, CD82, and TSPAN4) (Tachibana *et al.*, 1997; Levy *et al.*, 1998), we predict that their membrane-proximal cysteines will also be palmitoylated by DHHC2. In this regard, multiple proteins containing membrane-proximal cysteines, but lacking a conserved consensus motif, were palmitoylated by Swf1, a DHHC PAT protein in yeast (Roth *et al.*, 2006).

### *Functional Consequences*

DHHC2-dependent tetraspanin palmitoylation has several functional consequences. First, DHHC2 promotes TEM interactions, as evidenced by DHHC2 overexpression causing a marked increase in CD9–CD151 association. Other DHHC proteins and inactive DHHC2 mutants notably lacked this effect, indicating that enhanced palmitoylation was responsible for the increased association. Although not tested here, we suspect that homo- and hetero-clustering of many additional tetraspanins will also be promoted by DHHC2 overexpression. In this regard, prior studies involving mutation of membrane-proximal cysteines showed that palmitoylation promotes hetero- and homo-clustering among tetraspanins, including CD9, CD151, CD81, CD63, and CD53 (Berditchevski *et al.*, 2002; Charrin *et al.*, 2002; Yang *et al.*, 2002).

DHHC2 overexpression did not stimulate CD151 association with integrin $\alpha3\beta1$ or $\alpha6\beta4$. This is not surprising considering that the interaction of CD151 with integrins $\alpha3\beta1$ and $\alpha6\beta4$ involves a direct protein–protein interaction between extracellular domains, and it is not diminished upon mutation of CD151 palmitoylation sites (Yauch *et al.*, 2000; Yang *et al.*, 2002). Although DHHC2 did not affect $\beta$4 or $\alpha$3 palmitoylation, it did promote increased association with five to seven unknown proteins associated with $\alpha$3 integrin. At least one of these is likely to be autopalmitoylated DHHC2 itself. The others possibly represent additional proteins in $\alpha$3–CD151–CD9 complexes that undergo enhanced palmitoylation. Their identification awaits further study.

A second functional consequence of DHHC2-dependent palmitoylation is stabilization of target protein expression. When DHHC2 was present (i.e., not siRNA depleted), detection of CD9 and CD151 was enhanced, both in total cell lysates and on the cell surface. By contrast, levels of nine other proteins were unaffected by DHHC2 depletion. Pulse-chase experiments confirmed that DHHC2 knockdown enhanced the degradation of CD9, without altering initial biosynthesis. In some cases, loss of transmembrane protein palmitoylation leads to proteolytic degradation by the ubiquitin–proteosome pathway (Valdez-Taubas and Pelham, 2005). However, degradation of CD9 and CD151 was not affected by proteosome inhibitors MG132 and ALLM. Instead, disappearance of CD9 and CD151 was partially reversed upon treatment of cells with agents that disrupt lysosomes (bafilomycin A1 and $NH_4Cl$) or inhibit lysosomal proteases (leupeptin + pepstatin A). In this regard, CD9 and CD151 resemble chemokine receptor CCR5. In the absence of palmitoylation, that polytopic cell surface receptor also showed enhanced degradation in lysosomes, but not proteosomes (Percherancier *et al.*, 2001).

Why is the palmitoylation-deficient CD9 mutant highly expressed in Figure 7A, when it should be rapidly degraded? Because palmitoylation-deficient CD151 is degraded at a faster rate (Yang *et al.*, 2002), we suspect that mutant CD9 may also show accelerated degradation (although we have not yet measured it). However, despite faster degradation, mutant CD151 is readily expressed on cells at a high steady-state level (Berditchevski *et al.*, 2002; Yang *et al.*, 2002), and mutant CD9 also can be expressed at high levels (Charrin *et al.*, 2002; Kovalenko *et al.*, 2004). Hence, we cannot infer much about degradation of palmitoylation-deficient CD151 or CD9 mutants from steady-state levels. Furthermore, palmitoylation-deficient CD9 and CD151 not only lose palmitoylation but also they lack membrane proximal cysteines. This could help to explain why loss of CD9 and CD151 expression is not that dramatic for palmitoylation-deficient mutants (Berditchevski *et al.*, 2002; Charrin *et al.*, 2002; Yang *et al.*, 2002), compared with DHHC2 knockdown conditions.

Due to rapid protein degradation, we were initially unable to capture and study CD9 and CD151 in DHHC2-depleted cells, to confirm that they were indeed deficient in palmitoylation. However, in subsequent experiments, treatment of intact cells with bafilomycin A1 inhibited protein degradation, thus enabling demonstration that palmitoylation was diminished in CD9 and CD151 proteins upon DHHC2 knockdown. Hence, tetraspanin degradation is closely related to loss of palmitoylation. Consistent with this, DHHC2 knockdown accelerated CD9 degradation to an extent similar to that seen when CD151 palmitoylation sites were mutated (Yang *et al.*, 2002). Also, there is precedent for protein degradation being accelerated upon loss of palmitoylation (Linder and Deschenes, 2007). We suggest that CD151, which normally traffics through endosomal/lysosomal-type vesicles (Sincock *et al.*, 1999), is exposed to resident proteases when palmitoylation is impaired.

A third functional role for DHHC2 is to promote cell–cell association, as indicated by DHHC2 knockdown causing cell dispersion. Because complete substrate profiles are not known for DHHC2 or any other DHHC protein, we cannot be certain which DHHC2 substrates might be involved in the cell dispersion phenotype. However, all evidence available so far points toward tetraspanins as being major substrates (see discussion above). Tetraspanin proteins, in the context of TEMs, are associated with a variety of cellular functions (e.g., motility, morphology, signaling, and fusion) (Berditchevski *et al*., 2002; Charrin *et al*., 2002; Yang *et al*., 2002; Zhou *et al*., 2004; Cherukuri *et al*., 2004; Yang *et al*., 2006) that could play key roles indirectly promoting cell–cell contacts instead of cell dispersion. Notably, mutation of four CD151 palmitoylation sites previously caused a related shift in cell morphology, except that cells containing palmitoylation-deficient CD151 became more epithelial-like and less fibroblastic (Yang *et al*., 2002). At present, we cannot explain why the functional consequences of diminishing palmitoylation of multiple tetraspanins (via DHHC2 knockdown) are somewhat opposite to the effects of overexpressing a single palmitoylation-deficient tetraspanin mutant. One possibility is that DHHC2 depletion effects on multiple other tetraspanins (e.g., CD9, CD82, and CD63) override its effects on CD151.

### *Novelty and Broader Implications*

Despite the functional importance of tetraspanin palmitoylation, the responsible PAT had not been identified. Now our results strongly implicate DHHC2 as playing a major role.

The gene coding for human DHHC2 is ubiquitously expressed (Oyama *et al*., 2000). Hence, DHHC2 should be widely available to promote tetraspanin palmitoylation, which has been observed in all primary cells and tumor cell lines so far examined.

In previous studies, tetraspanin palmitoylation was studied using the inhibitor 2-bromopalmitate, or by mutation of relevant cysteines. However, 2-bromopalmitate partially and nonspecifically inhibits nearly all palmitoylation, and replacement of cysteines may alter a protein in a manner that goes beyond simply preventing palmitoylation. Because we can now manipulate DHHC2, we are no longer so reliant on 2-bromopalmitate or cysteine mutagenesis to study tetraspanin palmitoylation. Indeed, our results now provide new insight into how tetraspanin palmitoylation may be selectively enhanced or inhibited, via DHHC2 overexpression, mutation, and/or knockdown. We suspect that manipulation of DHHC2 will prove to be of considerable utility in future studies aimed at understanding the functions of TEMs, because they contribute to events such as cell signaling, motility, morphology, fusion, and human immunodeficiency virus assembly.

It remains to be determined whether reduced DHHC2 expression in colorectal cancers (Oyama *et al*., 2000) is associated with diminished tetraspanin palmitoylation. Also, it is intriguing to consider that a suggested tumor suppressor function for DHHC2 in colon cancer (Oyama *et al*., 2000) may be related to its role in palmitoylating CD9, which itself has been suggested to be a tumor suppressor (Ikeyama *et al*., 1993; Miyake *et al*., 2000). Although we do not yet know which DHHC2 substrate(s) is(are) most critical, we have gained new insight into DHHC2 function. The shift of DHHC2-knockdown A431 cells from a state of cell–cell association toward one of almost complete cell dispersion resembles an epithelial-mesenchymal transition (EMT). EMT is the process in which epithelial cells lose epithelial morphology and markers and gain a fibroblastic morphology during tumor progression (Zavadil *et al*., 2001; Janda *et al*., 2002; Thiery, 2002). A role for DHHC2 in maintaining an epithelial phenotype would be consistent with its putative tumor suppressor role (Oyama *et al*., 2000).

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