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14. ABSTRACT Palmitoylation is an important post-translational modification that plays a critical role in regulating protein localization, activity, and stability, as well as multiprotein complex formation. Our preliminary data suggested that DHHC3, a palmitoylating enzyme, plays a critical role in breast cancer growth, invasion, and/or metastasis, at least partly by palmitoylating certain proteins resident in tetrapanin-enriched microdomains. However, very few proteins have been identified as DHHC3 substrates. Thus, we proposed to comprehensively identify DHHC3 substrates in breast cancer by integrating our palmitoyl protein identification and site characterization method with triplex SILAC. We established this multiplexed quantitative palmitoyl-proteomics method in year 1. Here, we applied this multiplexed quantitative palmitoyl-proteomics method to identify DHHC3 substrates in breast cancer MDA-MB 231 cells. We identified over 680 candidate palmitoyl proteins, among 70 candidate palmitoyl proteins, among which 70 are candidate DHHC3 substrates.					
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Introduction

Tetraspanin-enriched microdomains (TEMs) are critical signaling platforms, playing a key role in cancer progression [1, 2]. Many TEM-resident proteins, including laminin-binding integrins, tetraspanins, and their partner proteins, are regulated by post-translational palmitoylation. Protein palmitoylation, the only reversible lipid modification, is critical for the regulation of protein localization, activity, and stability as well as multiprotein complex formation [3]. Palmitoylation in human is carried out by 23 DHHC enzymes [4]. Dr. Martin Hemler's lab showed that the ablation of DHHC3 not only inhibits the palmitoylation of integrin $\alpha 6$ and $\beta 4$ subunits [5], but also disrupts TEMs and markedly alters cell morphology, invasion and signaling through focal adhesion kinase (FAK) in breast cancer cell lines. The results strongly suggested that DHHC3 plays a critical role in breast cancer progression. Because the 23 human DHHC proteins catalyze the palmitoylation of hundreds of cellular proteins, it is likely that at least dozens of proteins are palmitoylated by DHHC3. However, little is known about which proteins, except integrin $\alpha 6$ and $\beta 4$ subunits, are DHHC3 substrates in breast cancer cells.

We developed a novel palmitoyl-proteomics method termed Palmitoyl Protein Identification and Site Characterization (PalmPISC) [6]. In this project, we proposed to comprehensively analyze DHHC3 substrates in breast cancer cells (Aim 3). We planned to integrate our PalmPISC method with stable isotope labeling by amino acids in cell culture (SILAC) [7], a very accurate and robust quantitative proteomics method, to identify known and novel DHHC3 substrates. In year 1, we successfully integrated our PalmPISC method with triplex SILAC labeling. Here, we applied the quantitative palmitoylproteomics method to comprehensively identify DHHC3 substrates in breast cancer MDA-MB-231 cells.

Body

As shown in Figure 1, three populations of MDA-MB-231 cells were metabolically labeled with isotopically different SILAC amino acids in parallel. One group of control cells were cultured in "light" medium containing natural lysine (Lys0) and arginine (Arg0), DHHC3-knockdown cells were cultured in "heavy" medium containing $^{13}\text{C}_6, ^{15}\text{N}_2$ -lysine (Lys8) and $^{13}\text{C}_6, ^{15}\text{N}_4$ -arginine (Arg10), and the other group of control cells were cultured in "medium" medium containing 4,4,5,5-D₄-lysine (Lys4) and $^{13}\text{C}_6$ -arginine (Arg6). After six doublings, when cellular proteins were at least 98% labeled with SILAC amino acids, control cells labeled with Lys0 and Arg0 and DHHC3-knockdown cells labeled with Lys8 and Arg10 were mixed at 1:1 ratio and palmitoyl proteins were isolated using our PalmPISC method. During the development of our PalmPISC method, we noticed that a small population of nonpalmitoylated proteins is always co-purified with palmitoyl proteins. Thus, to distinguish palmitoyl proteins from these contaminating proteins, we omitted hydroxylamine—a chemical provides selectivity for palmitoyl proteins—from our PalmPISC condition and isolated the contaminating proteins from control cells labeled with Lys4 and Arg6. Finally, we mixed the purified proteins together and performed in-depth quantitative proteomics analysis using GeLC-MS/MS and analyzed the SILAC dataset with MaxQuant (v1.0.13.13), a free software suite for SILAC data analysis [8].

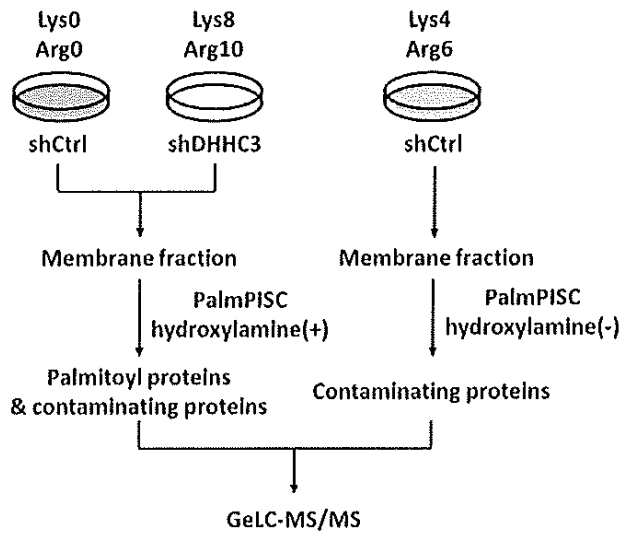


Figure 1. Workflow for the identification of DHHC3 substrate in breast cancer.

Theoretically, proteins that are palmitoylated by DHHC3 (*i.e.*, DHHC3 substrates) will have a pattern of SILAC spectra shown in Fig. 2A, because the knockdown of DHHC3 reduces the palmitoylation level of its substrates while the omission of hydroxylamine prevents the purification of the substrates. In contrast, DHHC3 knockdown will not affect other palmitoylated proteins, thus these non-DHHC3-substrates will have a pattern shown in Fig. 2B. In addition, contaminating proteins will have a ratio of 1:1:1 (Fig. 2C), because DHHC3 knockdown or the presence/absence of hydroxylamine will not affect their purification.

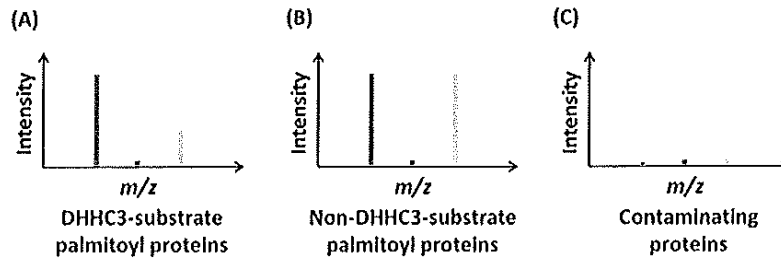


Figure 2. Theoretic patterns of SILAC spectra for (A) palmitoylated proteins that are DHHC3 substrates, (B) palmitoylated proteins that are not DHHC3 substrates, and (C) contaminating proteins.

Our quantitative palmitoyl-proteomics analysis led to the identification of 1097 proteins with a false-discovery rate of 1%; among these proteins about 930 were quantitated (see Table S1). Using a cutoff value of 0.606 ($p < 0.05$) for the “medium”/ “light” (M/L) SILAC ratio, we identified 687 candidate palmitoyl proteins. Moreover, using a cutoff value of 0.606 ($p < 0.05$) for the “heavy”/ “light” (H/L) SILAC ratio, we identified 70 candidate palmitoyl proteins as candidate DHHC3 substrates (Table 1). Figure 3A showed a representative SILAC spectrum of a peptide derived from cytoskeleton-associated protein 4 (CKAP4), a known palmitoyl protein [3]. DHHC3 knockdown led to the decrease of the palmitoylation level of CKAP4, suggesting that

CKAP4 is a candidate substrate of DHHC3. In contrast, as shown in Figure 3B, the palmitoylation level of flotillin-1 (FLOT1), also a known palmitoyl protein [4], was not affected by DHHC3 knockdown, indicating that flotillin-1 is unlikely a DHHC3 substrate.

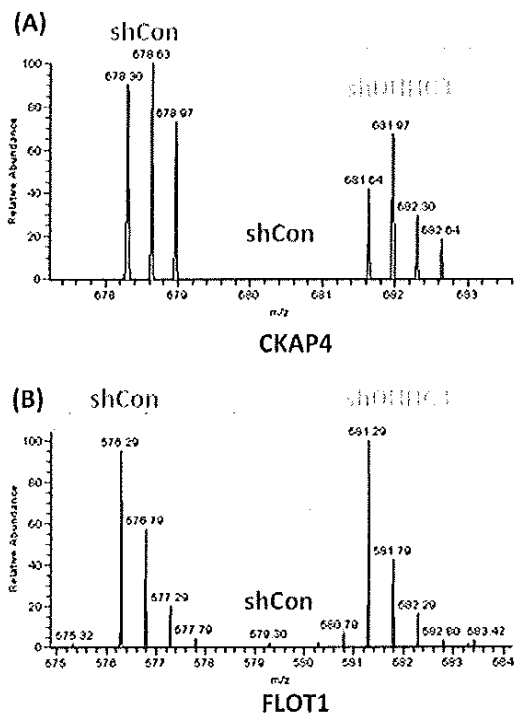


Figure 3: Representative SILAC spectra of a peptide derived from (A) a candidate DHHC3 substrate cytoskeleton-associated protein 4 (CKAP4) and (B) an unlikely DHHC3 substrate flotillin-1 (FLOT1).

Table 1. List of candidate DHHC3 substrates

Gene Name	Protein Name	Median/Light Ratio	Heavy/Light Ratio
S100A9	Protein S100-A9	0.160	0.008
SCAMP3	Secretory carrier-associated membrane protei	0.009	0.034
SUMF2	Sulfatase-modifying factor 2	0.074	0.075
PGRMC1	Membrane-associated progesterone receptor component 1	0.470	0.280
CTSD	Cathepsin D	0.297	0.294
NOL6	Nucleolar protein 6	0.382	0.305
LAMP4A	Lysosomal-associated transmembrane protein 4A	0.099	0.340
CMTM3	CKLF-like MARVEL transmembrane domain-containing protein 3	0.136	0.345
HSPB1	Heat shock protein beta-1	0.520	0.369
ITGA6	Integrin alpha-6	0.163	0.372
TMEM192	Transmembrane protein 192	0.090	0.380
MREG	Melanoregulin	0.008	0.380
FAM108A1	Abhydrolase domain-containing protein	0.021	0.396
TMEM97	Transmembrane protein 97;Protein MAC30	0.155	0.407
NPC1	Niemann-Pick C1 protein	0.122	0.409
HIST1H1C	Histone H1.2	0.553	0.416
CMTM6	CKLF-like MARVEL transmembrane domain-containing protein 6	0.222	0.474

TMEM179B	Transmembrane protein 179B	0.0139	0.480
OSTC	Oligosaccharyltransferase complex subunit OSTC	0.293	0.492
BRI3BP	BRI3-binding protein	0.353	0.494
TMEM55A	Transmembrane protein 55A	0.100	0.501
KIAA0090	KIAA0090	0.449	0.505
RFFL	E3 ubiquitin-protein ligase rififylin	0.014	0.509
NFXL1	NF-X1-type zinc finger protein NFXL1	0.370	0.514
TMED1	Transmembrane emp24 domain-containing protein 1	0.138	0.517
STARD3NL	MLN64 N-terminal domain homolog	0.010	0.519
IMP3	U3 small nucleolar ribonucleoprotein protein IMP3	0.450	0.519
GPX8	Probable glutathione peroxidase 8	0.161	0.522
M6PR	Cation-dependent mannose-6-phosphate receptor	0.006	0.525
PGRMC2	Membrane-associated progesterone receptor component 2	0.501	0.529
AGPAT1	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	0.030	0.529
VAPB	Vesicle-associated membrane protein-associated protein B/C	0.522	0.532
METTL7B	Methyltransferase-like protein 7B	0.045	0.534
ROMO1	Reactive oxygen species modulator 1	0.059	0.541
HSP90B1	Endoplasmic	0.446	0.545
SLC4A7	Solute carrier family 4 sodium bicarbonate cotransporter member 7	0.223	0.548
MFSD1	Major facilitator superfamily domain-containing protein 1	0.129	0.550
BET1	BET1 homolog	0.117	0.551
VKORC1	Vitamin K epoxide reductase complex subunit 1	0.389	0.556
SELI	Ethanolaminephosphotransferase 1	0.595	0.559
DERL2	Derlin-2	0.352	0.561
KIAA0754	Uncharacterized protein KIAA0754	0.493	0.562
PTRH2	cDNA FLJ32471 fis, clone SKNMC2000322, highly similar to Peptidyl-tRNA hydrolase 2, mitochondrial (EC 3.1.1.29)	0.144	0.562
FAM36A	Protein FAM36A	0.416	0.564
SPCS1	Signal peptidase complex subunit 1	0.221	0.566
BANF1	Barrier-to-autointegration factor	0.259	0.566
CDKAL1	CDK5 regulatory subunit-associated protein 1-like 1	0.284	0.566
MRPL43	Mitochondrial ribosomal protein L43	0.456	0.566
SPRY2	Protein sprouty homolog 2	0.169	0.568
AUP1	Ancient ubiquitous protein 1	0.046	0.568
CKAP4	Cytoskeleton-associated protein 4	0.025	0.571
MAN1B1	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	0.021	0.572
SARIA	GTP-binding protein SAR1a	0.546	0.573
PRDX4	Peroxiredoxin-4	0.572	0.575
SOAT1	Sterol O-acyltransferase 1	0.301	0.582
SCARB1	Scavenger receptor class B member 1	0.079	0.582
SPINT2	Kunitz-type protease inhibitor 2	0.509	0.583
DNAJA1	DnaJ homolog subfamily A member 1	0.448	0.589
LMF2	Lipase maturation factor 2	0.125	0.589
RHOT2	Mitochondrial Rho GTPase 2	0.453	0.592
PCBP2	Poly(RC)-binding protein 2 isoform b variant	0.496	0.592
TXN	Thioredoxin	0.178	0.596
SCAP	Sterol regulatory element-binding protein cleavage-activating protein	0.138	0.597
ZDHHC6	Probable palmitoyltransferase ZDHHC6	0.0154	0.597
STX7	Syntaxin-7	0.109	0.599
TPI1	Triosephosphate isomerase	0.549	0.601
RAB27B	Ras-related protein Rab-27B	0.539	0.602

DNAJC11	DnaJ homolog subfamily C member 11	0.332	0.602
ERGIC3	Endoplasmic reticulum-Golgi intermediate compartment protein 3	0.050	0.603

Key Research Accomplishments

1. Identification of about 700 candidate palmitoyl proteins from breast cancer MDA-MB-231 cells.
2. Identification of 70 candidate DHHC3 substrates.

Reportable Outcome

Conclusion

In summary, by integrating RNAi, SILAC, and PalmPISC, we developed a powerful tool for rapid identification of substrates for an individual palmitoyl acyltransferase. By using this quantitative palmitoyl-proteomics method, we identified integrin $\alpha 6$, a known DHHC3 substrate, and 69 novel candidate DHHC3 substrates.

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