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DHHC5 REGULATES BETA-ADRENERGIC SIGNALING IN THE HEART THROUGH PALMITOYLATION OF G ALPHA PROTEINS

Jie Chen

DHHC5 REGULATES BETA-ADRENERGIC SIGNALING IN THE HEART

THROUGH PALMITOYLATION OF G ALPHA PROTEINS

by

(Jessica) Jie Chen, B.A.

APPROVED:

_____
Darren Boehning, Ph.D.
Advisory Professor

_____
Vasanthi Jayaraman, Ph.D.

_____
Ilya Levental, Ph.D.

_____
Kartik Venkatachalam, Ph.D.

_____
Ambro van Hoof, Ph.D.

APPROVED:

_____
Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

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A

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DOCTOR OF PHILOSOPHY

by

(Jessica) Jie Chen, B.A.
Houston, Texas

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Dedication

This dissertation is dedicated to my parents:

Wei Chen

and

Dr. Jin Huang

Thank you for being the most supportive and understanding family one can ask for. You encourage me to pursue my goals and always believe in me.

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DHHC5 REGULATES BETA-ADRENERGIC SIGNALING IN THE HEART THROUGH PALMITOYLATION OF G ALPHA PROTEINS

(Jessica) Jie Chen, B.A.

Advisory Professor: Darren Boehning, Ph.D.

S-palmitoylation is a reversible posttranslational modification that plays an important role in regulating protein localization, trafficking, and stability. Recent studies have shown that some proteins undergo extremely rapid palmitoylation/depalmitoylation cycles after cellular stimulation supporting a direct signaling role for this posttranslational modification. The work in this dissertation investigated whether β-adrenergic stimulation led to stimulus-dependent palmitoylation of downstream signaling proteins in immortalized and primary cell models. I found that β-adrenergic stimulation led to rapidly increased Gαs and Gαi palmitoylation only in primary cardiomyocytes. The kinetics of palmitoylation was temporally consistent with the downstream production of cAMP and contractile responses. I identified the plasma membrane-localized palmitoyl acyltransferase DHHC5 as an important mediator of the stimulus-dependent palmitoylation in cardiomyocytes. Knockdown of DHHC5 showed that this enzyme is necessary for palmitoylation of Gαs, Gαi, and functional responses downstream of β-adrenergic stimulation. A palmitoylation assay with purified components revealed that Gαs and Gαi are direct substrates of DHHC5. Finally,

we provided evidence that the C-terminal tail of DHHC5 can be palmitoylated in response to stimulation and such modification is important for its dynamic localization in the plasma membrane. My results reveal that DHHC5 is a central regulator of signaling downstream of β-adrenergic receptors in cardiomyocytes.

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Abbreviations

ABE	Acyl biotin exchange
ABHD	α/β-hydrolase fold
AC	Adenylyl cyclase
Acyl-RAC	Acyl-resin-assisted capture
APT	Acyl protein thioesterase
β-AR	Beta-adrenergic receptor
2-BP	2-bromopalmitate
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CoA	Coenzyme A
cTnI	Cardiac troponin I
ER	Endoplasmic Reticulum
FTase	Farnesyltransferase
$GABA_B$	γ-aminobutyric acid
GGTase	Geranylgeranyltransferase
GPCR	G protein coupled receptor
GPI	Glycosylphosphatidylinositol
GRK	G protein coupled receptor kinase
HA	Hydroxylamine
HPLC	High performance liquid chromatography
IMM	Inner mitochondria membrane
ISO	Isoproterenol

LTCC	L-type calcium channel
MEND	Massive endocytosis
MMTS	Methyl methanethiosulphonate
NEM	N-ethylmaleimide
NMT	N-myristoyltransferase
NRVM	Neonatal rat ventricular cardiomyocyte
OMM	Outer mitochondria membrane
PalmB	Palmostatin B
PAT	Palmitoyl acyltransferase
PKA	Protein kinase A
PLB	Phospholamban
PLM	Phospholemman
PTP	Permeability transition pore
RGS	Regulators of the G protein signaling
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SERCA	Sarcoplasmic reticulum calcium ATPase
SR	Sarcoplasmic reticulum
TCR	T cell receptor

Chapter I: Introduction

This chapter is partially based upon a review article originally published in

Chen, J. J., & Boehning, D. (2017). Protein lipidation as a regulator of apoptotic calcium release: Relevance to cancer. *Frontiers in oncology*, *7*, 138.

Lipidations and their enzymatic regulators

Protein lipidation is the cotranslational or posttranslational covalent addition of a variety of lipids, including fatty acids, isoprenoids and cholesterol, to target proteins (**Table 1**). Lipidation can be categorized into two types based on the location of the modified proteins: those that are modified in the endoplasmic reticulum (ER) lumen and secreted, and those that are modified in the cytoplasm or on the cytoplasmic face of membrane [1]. The former type includes glycosylphosphatidylinositol (GPI) anchor and cholesterylation, and the latter type includes N-myristoylation, acylation, and prenylation.

GPI anchor was first discovered in the parasite *Trypanosoma brucei* where the highly expressed variant surface glycoprotein (VSG) is anchored to the cell surface *via* a glycolipid containing phosphatidylinositol [2-4]. Since then, many proteins in mammals and lower eukaryotes such as protozoa have been shown to contain GPI anchors with enormous structural variety, most of which include an ethanolamine attached to the carboxyl terminus of the protein, a

glycan core, inositol, and lipid moieties [5-7]. GPI-anchored peptides often include a cleavable N-terminal signal sequence, which directs the peptide to the ER lumen, and a hydrophobic C terminal sequence that is cleaved at the time of GPI anchor addition [6, 8, 9]. GPI anchors facilitate tethering of proteins to the extracellular face of the plasma membrane and are important for many cellular functions, including adhesion, membrane trafficking and immune system signaling [10-12].

The GPI precursor, formed in the ER lumen, is transferred to target proteins by GPI transamidase, a membrane-bound multi-subunit enzyme [13-16]. GPI transamidase cleaves the C-terminal signal peptide of the target proteins, and forms an enzyme-substrate intermediate, allowing the nucleophilic attack by the terminal amino group of the preformed GPI [17].

Cholesterylation is a characteristic of the mammalian Hedgehog family proteins, which are secreted signaling proteins that regulate embryonic patterning of many tissues and structures [18, 19]. The Hedgehog protein undergoes an autocatalytic processing that internally cleaves between the conserved Gly257 and Cys258 at the GCF motif, and yields a ~20kD N-terminal signaling domain and a ~25kD C-terminal catalytic domain [20, 21]. The N-terminal domain receives a cholesterol moiety and is active in signaling [19, 21, 22]. Interestingly, multiple studies have detected other potentially cholesterylated proteins [19, 23]. However, the identification of these potential cholesterylation targets remains to be elucidated. Cholesterylation of the N-terminal signaling

domain of Hedgehog seems to be only dependent on the presence of the C-terminal catalytic domain, suggesting that this process is autocatalytic [19].

N-myristoylation is the attachment of the 14-carbon myristic acid to a N-terminal Gly residue *via* an amide bond [24]. It was first identified as a blocking group that prevents Edman degradation on the N-terminus of the catalytic subunit of cyclic AMP-dependent protein kinase and the calcium-binding β-subunit of calcineurin [25, 26]. Many other proteins regulating key signaling pathways, including the Src family non-receptor protein tyrosine kinases [27, 28] and Gα proteins [29, 30] were shown to be myristoylated. These proteins contain the N-terminal sequence Met-Gly- and often have a Ser/Thr/Cys at position 6 [24, 31]. Myristoylation can happen cotranslationally following the removal of the initiator methionine residue [32]. Although myristoylation is required for membrane targeting of many proteins, it is not sufficient for stable membrane anchoring due to its weak hydrophobic nature and often needs subsequent lipid modifications [33-35]. Additionally, myristoylation can also happen posttranslationally during apoptosis following the caspase cleavage of substrate proteins that exposes an internal glycine [36-39]. Many apoptotic proteins, including Bid, gelsolin and p21-activated kinase 2, require posttranslational myristoylation following caspase cleavage for proper subcellular localization and subsequent functions [36-38].

N-myristoylation is catalyzed by N-myristoyltransferases (NMTs) [40-42]. NMTs bind first to myristoyl-CoA and then to the peptide, followed by a direct nucleophilic addition-elimination reaction and subsequent release of CoA and the

myristoylated peptide [43]. Studies in various tissues and cell types have shown that the enzymatic activity of NMTs is predominantly distributed in the cytosolic fraction [44-47]. Some studies have shown that low levels of myristoyl-CoA may be rate limiting for NMT activity [48, 49]. However, the transcriptional up-regulation of NMTs under pathological conditions suggests that this might not always be the case [50].

Acylation is the addition of various fatty acids, such as palmitic acid, oleic acid and stearic acid, on different amino acid residues [51-53]. One of the best-studied types of acylation is S-palmitoylation, which is characterized by the reversible addition of the 16-carbon saturated palmitic acid to Cys residues *via* labile thioester bonds [53, 54]. Despite the presence of multiple algorithms to predict palmitoylation sites, there is no validated consensus sequence for palmitoylation [55-57]. One key aspect of palmitoylation is that its reversibility allows for a palmitoylation and depalmitoylation cycle that regulates the posttranslational trafficking and functions of target proteins, such as H- and N-Ras [58]. Palmitoylation can also occur on large transmembrane proteins, including ion channels and G protein coupled receptors [59-63]. The roles of transmembrane protein palmitoylation include regulation of channel maturation/quality control and association with lipid rafts [64-67].

Protein S-acylation is regulated by palmitoyl acyltransferases (PATs) and acyl-protein thioesterases, which add and remove the lipid group, respectively. There are 23 PATs in mammals, all of which share a common DHHC (Asp-His-His-Cys) motif within a cysteine rich domain [68, 69]. PATs are polytopic

membrane proteins that are localized to distinct subcellular compartments, primarily the Golgi apparatus and the plasma membrane [70]. Some DHHC enzymes show preference for certain types of proteins (i.e. transmembrane proteins), and in some cases the same substrates can be palmitoylated by multiple DHHC enzymes [68, 71-73]. Compared to the large amount of studies on PATs, thioesterases are relatively poorly characterized. Two protein palmitoyl thioesterases (PPT1/2) and two acyl protein thioesterases (APT1/2) have been identified [74-77]. PPTs predominantly localize to the lysosomal lumen and are involved in depalmitoylation during protein degradation, whereas APTs have cytosolic localization and are shown to depalmitoylate and recycle signaling proteins such as Ras and growth associate protein (GAP-43) from the plasma membrane back to Golgi [75-78]. Very recently, two independent groups found that the α/β-hydrolase domain (ABHD) family of serine hydrolases are potent depalmitoylating enzymes for select substrates, including PSD-95 and N-Ras [79, 80].

Prenylation is the addition of the 15-carbon farnesyl or the 20-carbon geranylgeranyl isoprenoid lipid on cysteine residues *via* stable thioether bonds [81, 82]. It requires a C-terminal CAAX motif, where C is a cysteine, A is aliphatic amino acids and X can be any amino acid. Prenylation at the CAAX motif is found in many proteins, including mammalian Ras proteins [83, 84]. In addition to its role in membrane association, prenylation can also regulate protein-protein interactions and subcellular distribution of the modified targets [85, 86].

Prenylation is catalyzed by the enzymes farnesyltransferase (FTase), geranylgeranyltransferase I (GGTase 1), and Rab geranylgeranyltransferase (GGTase 2) [87-89]. The prenylating enzymes localize to the cytosol and conjugate isoprenoids generated from the mevalonate/HMG-CoA reductase pathway to target proteins. Specifically, the isoprenoids farnesyl and geranylgeranyl are transferred to a C-terminal CAAX motif on target proteins. Unlike FTase and GGTase 1, geranylgeranyl transfer by GGTase 2 requires the co-factor REP (Rab escort protein) [90]. GGTase 1 and FTase generally have high specificity for the protein targets, depending on the X residue [91-93]. However, they can act on each other's substrates. One example is that K-Ras and N-Ras, usually targets of FTase, can be geranylgeranylated in Ras-mutant human cancer cells treated with FTase inhibitors [94, 95]. Removal of the –AAX tripeptide and methylation of the prenyl-cysteine, catalyzed by the ER membrane proteins RCE1 and ICMT, respectively, are two post-prenylation steps required for maturation of prenylated proteins [96-98].

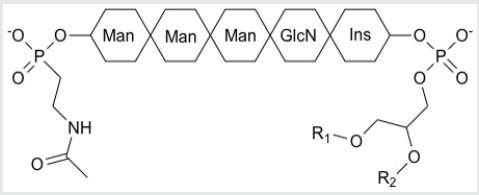
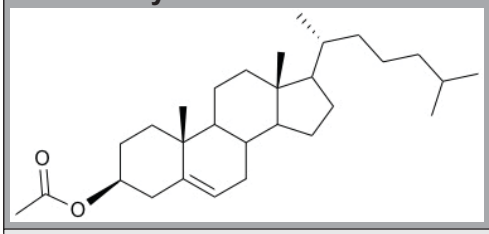
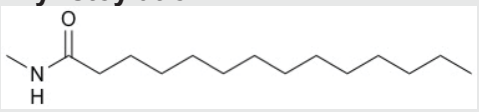
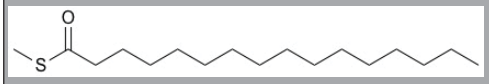
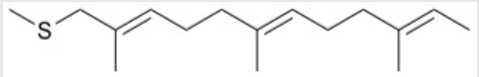
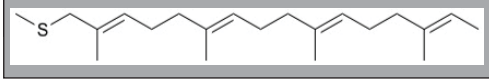
Lipid Structure	Effects on proteins	Enzymatic regulators
GPI anchor 	Plasma membrane tethering, Incorporation into specific membrane domains, Protein-protein interaction	GPI transamidase
Cholesterylation 	Hedgehog signaling activation	Autocatalytic
Myristoylation 	Membrane localization, Autoinhibition	N-myristoyltransferases
Palmitoylation 	Plasma membrane localization, Partitioning into lipid rafts, Protein maturation/quality control	Palmitoyl acyltransferases, Acyl protein thioesterases, α/β-hydrolase fold family proteins
Farnesylation 	Membrane localization Conformational change Protein-protein interaction	Farnesyltransferase
Geranylgeranylation 	Membrane localization Protein-protein interaction	Geranylgeranyl -transferase

Table 1. Summary of Different Types of Lipidation.

The lipid moiety structures, effects on target proteins, and enzymatic regulators of various lipidation. This table is from *Chen, J. J., & Boehning, D. (2017). Protein lipidation as a regulator of apoptotic calcium release: Relevance to cancer. Frontiers in oncology, 7, 138.* Copyrights remain with the authors.

Palmitoylation and signaling transduction

S-Acylation, more commonly known as S-palmitoylation, is the only reversible lipidation and therefore its roles in signaling transduction have been studied extensively. Recent advances in detecting lipidated proteins by proteomic and targeted approaches have revealed that lipidation of signaling proteins is essential for regulating a wide variety of signaling pathways.

One of the earliest studies reporting S-palmitoylation in mammalian cells relies on the incorporation of radiolabeled fatty acid [^{3}H]palmitate into live cells, followed by fluorography with long exposure (multiple days) [99]. To detect palmitoylation of specific proteins of interest, either purified proteins are needed for cell-free labeling with [^{3}H]palmitate, or exposing cells to [^{3}H]palmitate for extended period of time is needed [100-102]. While these methods have been used to identify various palmitoylation targets, they have many drawbacks. Other than the potential health hazard and the time-consuming autoradiograph exposure step, the sensitivity and the efficiency of radiolabeling are extremely low for some proteins [103].

Click chemistry based chemical probes are developed as an alternative to better detect protein palmitoylation (**Figure 1A**) [104]. Synthetic fatty acid analogues with alkyne groups at the terminal end furthest away from the carboxyl group (ω-position) are incorporated into live cells [105]. Cells are then fixed (for fluorescence imaging) or lysed (for biochemical assays), followed by a copper(I)-catalyzed reaction with azide-conjugated fluorescent groups or biotin for detection [106]. Notably, synthetic fatty acids with different chain lengths are

shown to incorporate onto proteins *via* thioester linkage with different efficiency, suggesting that while palmitoylation is the most commonly studied S-acylation, other lipid groups can be added [104]. Click chemistry based probes are especially useful in studies that distinguish the selectivity of different fatty acid groups [107].

While click chemistry has led to many important discoveries in protein palmitoylation (reviewed in [108]), the efficiency of incorporating alkyne conjugated probes onto substrates vary, and thus is not suitable for unbiased proteomic studies in native tissues. Recently acyl-biotin exchange (ABE, **Figure 1B**) has been developed to detect S-acylated proteins in various tissues and organisms [71, 109, 110]. The first step of ABE is blocking the free cysteine sites on proteins using the thioreactive compound N-ethylmaleimide (NEM) or methyl methanethiosulphonate (MMTS). Then the labile thioester bonds at the palmitoylated cysteines are cleaved using hydroxylamine (HA, neutral PH), exposing the free thiol groups that can be subsequently biotinylated. After biotinylation, proteins are pulled down using streptavidin beads and eluted. Standard Western blotting can be used to detect the palmitoylation levels of proteins of interest [103], or mass spectrometry can be used for global proteomic analysis [71].

Acyl-resin-assisted capture (Acyl-RAC, **Figure 1B**) is a recently developed approach similar to ABE. Instead of using biotin conjugation and streptavidin pull-down, thioreactive Sepharose resin were used after the HA cleavage step [111]. This method reduces the length of the procedure and is shown to have improved

sensitivity for detecting some palmitoylated proteins while missing some other proteins detected by ABE [112].

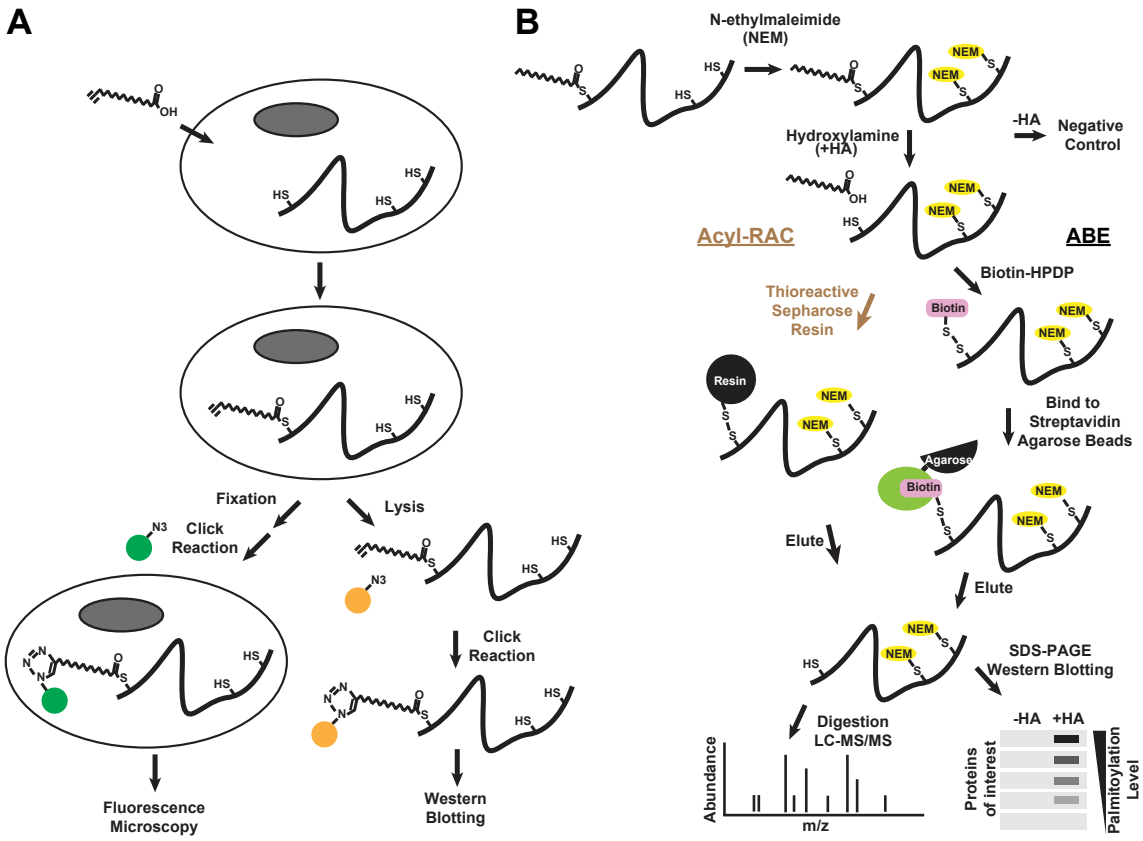


Figure 1 Schematics of methods to detect protein palmitoylation.

(A) ω-alkynyl fatty acid is incorporated into live cells, followed by click chemistry to detect palmitoylation using azide-conjugated fluorophore (green) or biotin/streptavidin-HRP (yellow). **(B)** Procedures of using acyl-biotin exchange (ABE) and acyl-resin-assisted capture (Acyl-RAC) to study the palmitoyl proteome or proteins of interest.

Due to the proliferation of targeted and proteomic studies using click chemistry based probes, ABE, or Acyl-RAC to identify fatty acylated proteins, protein palmitoylation is shown to play central roles in various signaling pathways [105]. Our group [113] and others [114-117] have investigated the role of palmitoylation in regulating death receptor signaling. We found that components of the T cell receptor (TCR) complex such at Lck, Zap-70, PLC-γ1, and other TCR components were required for apoptotic calcium release in T cells after engagement of the Fas receptor with Fas ligand [118]. The Src kinase Lck is myristoylated and doubly palmitoylated on the N-terminus, and these modifications regulate its plasma membrane localization and partitioning into lipid rafts. It is known that the Fas macromolecular complex assembles and signals in lipid rafts [119], so we asked whether Fas stimulation resulted in rapid palmitoylation of Lck. Fas stimulation resulted in a rapid increase in *de novo* palmitoylation of Lck detectable within minutes of Fas receptor engagement [113]. Surprisingly, after longer period of stimulation (>10 min) of the Fas receptor, Lck palmitoylation levels rapidly decreased to even lower than the levels seen in non-stimulated cells. Importantly, the rapid but transient increase in palmitoylation of Lck closely matched the activation kinetics of signaling proteins involved in the initiation of Fas receptor signaling [113]. We therefore proposed that the enzymatic mechanisms controlling stimulus-dependent protein palmitoylation and depalmitoylation likely are directly activated by components of the Fas signaling pathway. In the case of Fas signaling, the plasma membrane localized DHHC21 protein is essential [113]. Presumably Fas stimulation rapidly

activates DHHC21 and a yet unidentified acyl-protein thioesterase to regulate Lck lipidation levels. How the activation of these enzymes occurs is unknown, but could possibly be regulated either directly or indirectly by calcium ions (Ca^{2+}).

In order to better understand the role of stimulus-dependent rapid palmitoylation and their roles in signaling transduction, it is worthwhile to consider the kinetics of the enzymatic machinery. Over 15 years ago it was shown that β-AR stimulation resulted in rapid turnover of the palmitoylation [120, 121] and depalmitoylation [122] cycle of Gαs. The model was based upon availability of free Gαs: dissociation from the βγ subunits allowed putative palmitoylating and depalmitoylating enzymes access to the protein. However, a later study found that stimulation of beta-adrenergic receptors (β-ARs) in kidney fibroblast-like COS cells and S49 lymphoma cells did not lead to detectable changes in Gα protein palmitoylation levels using high performance liquid chromatography (HPLC) [123]. Since then, other proteins such as PSD-95, eNOS, and Ras have been shown to have increased turnover of palmitoyl groups in response to various stimuli, suggesting rapid cycling of lipids in various signaling cascades [124]. However, further studies on the rapid palmitoylation of endogenous proteins and their downstream functional roles are still needed, especially in specific cell types including cardiac cells.

Beta-adrenergic signaling in cardiomyocytes

One of the most well studied pathways in the heart is the β-AR signaling pathway. Drugs that block the activation of this pathway, termed beta-blockers,

are among the most commonly used medications for pathological conditions such as heart failure [125, 126]. Heart failure is the leading cause of death in many developed countries such as the United States [127]. The hallmarks of heart failure include reduction in response to sympathetic stimulation and thus inability to increase cardiac output [128]. One of the key signaling pathways that regulate these processes in cardiomyocytes is the β-AR signaling pathway.

β-ARs are transmembrane G-protein coupled receptors that transmit extracellular signals to mediate contractility in the heart. Under conditions where organs and tissues have increased demand for oxygen delivery, the sympathetic nervous system is activated and neurotransmitters norepinephrine and epinephrine are produced [129]. When ligands such as norepinephrine and epinephrine bind to the β-ARs, they interact with distinct classes of the heterotrimeric G-proteins, consisting of α, β, and γ subunits, and allow for the exchange of GDP for GTP [130, 131]. Active Gα and Gβγ subunits then dissociate and trigger different downstream signaling events.

There are two major sub-types of β-ARs in mammalian hearts—β_1-AR and β_2-AR. β_1-AR is the predominant type and accounts for 75-80% of cardiac β-ARs, whereas β_2-AR accounts for 20-25% [132]. Different subtypes of β-ARs can couple with distinct subfamilies of G-proteins (**Figure 2A**). Both β_1-AR and β_2-AR associate with Gαs, which results in activation of downstream adenylyl cyclase (AC) and production of cAMP. The primary target of cAMP is protein kinase A (PKA), which phosphorylates substrates such as L-type calcium channel (LTCC), phospholamban (PLB), ryanodine receptor (RyR), and cardiac troponin I (cTnI)

[133]. PKA-mediated phosphorylation of the plasma membrane localized LTCC leads to increased Ca^{2+} uptake into the cell [134]; Phosphorylation of PLB reduces its inhibitory effects on sarcoplasmic reticulum calcium ATPase (SERCA), which results in increased Ca^{2+} storage in the sarcoplasmic reticulum (SR); RyR phosphorylation leads to increased Ca^{2+} release from the SR [135]; and cTnI phosphorylation leads to altered Ca^{2+} affinity for the myofilaments and cross-bridge kinetics [136]. All of these events result in increased contractility of cardiomyocytes, also known as positive inotropy. Positive inotropic effect is defined by an increase in muscular contractile forces [137]. The force production in cardiomyocytes is dependent on the actin-myosin cross-bridge, which is regulated by intracellular Ca^{2+} levels [138]. The majority of the Ca^{2+} needed for cardiac contraction is released from the SR through a process called calcium-induced calcium release [139]. Depolarization of the membrane results in the opening of LTCC and a small amount of Ca^{2+} influx, which leads to the opening of RyR and large amount of Ca^{2+} release from the SR (reviewed in [140]). Therefore, positive inotropic effects induced by activation of the β-AR signaling pathway result in increased intracellular Ca^{2+} levels.

In addition to Gαs, β_2-ARs also bind to Gαi, which inhibits the AC-cAMP-PKA pathway [141]. Given the relative low numbers of Gαi-coupled receptors compared to Gαs-coupled receptors under normal physiological conditions, the overall effect of stimulation by agonists such as isoproterenol (ISO, agonist for both β_1AR and β_2AR) leads to positive inotropic effects [142]. However, β_2-ARs shift from Gαs coupling to Gαi coupling when phosphorylated by PKA after

prolonged stimulation, contributing to desensitization of the pathway [143, 144]. Studies in multiple heart failure models observed increased expression and receptor coupling of Gαi [145-147]. Additionally, β_2-AR stimulation can also activate the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3'-kinase (PI3K) pathways in a Gαi-dependent fashion and has protective effect in cardiomyocytes against apoptosis [148]. Spatial and temporal regulations of signaling proteins are important to mediate subtype-specific responses to β-AR stimulations. In cardiomyocytes, β_2-AR, ACs and certain PKA subunits are enriched in caveolae, lipid-raft-like structures that form invaginations [149]. Caveolae exist in many tissues and their structure is dependent on the scaffolding protein caveolin [150, 151]. There are three members in the human caveolin gene family and caveolin-3 is specific to muscle cell types [152, 153]. Domain mapping studies identified caveolin-binding motifs that are present on various signaling proteins downstream of β-ARs, including Gα proteins [154, 155]. Caveolae provide the spatial proximity of these upstream proteins in the β-AR signaling cascade that favors the efficient and rapid recruitment of effectors following receptor activation [149]. Recently, proteomic studies revealed that palmitoylated proteins are enriched in caveolae, suggesting that palmitoylation is a better predictor of caveolae localization than caveolin binding motifs in cardiomyocytes [32]. Therefore, studying stimulus-dependent palmitoylation of endogenous proteins is crucial for understanding β-AR signaling in cardiomyocytes.

Another key aspect of β-AR signaling in the heart is its negative feedback *via* receptor desensitization, which is mediated by the serine/threonine kinases G-protein coupled receptor kinases (GRKs, **Figure 2B**). GRK2 and GRK5 are the most abundantly expressed GRK family members in the heart [156, 157]. Both GRK2 and GRK5 are crucial players in cardiac pathology. In failing heart, increased expression of GRK2 and GRK5 contribute to decreased β-AR signaling efficiency and thus disable the contractile function of the heart to meet its demand [158, 159]. GRKs phosphorylate the ligand-bound receptors, leading to recruitment of β-arrestin and internalization of the receptor [160]. Plasma membrane localization of GRKs is crucial for receptor desensitization. Interestingly, GRK2 is known to go through isoprenylation, a non-reversible lipid modification at the C-terminal CAAX domain, but isoprenylation alone is not sufficient for its plasma membrane targeting [161]. Multiple studies have demonstrated that GRK2 directly binds the dissociated Gβγ subunits, and the formation of the complex is important for its plasma membrane localization and functions [162, 163]. Further studies deciphering whether GRK proteins are subject to other forms of lipidation in cardiomyocytes are necessary.

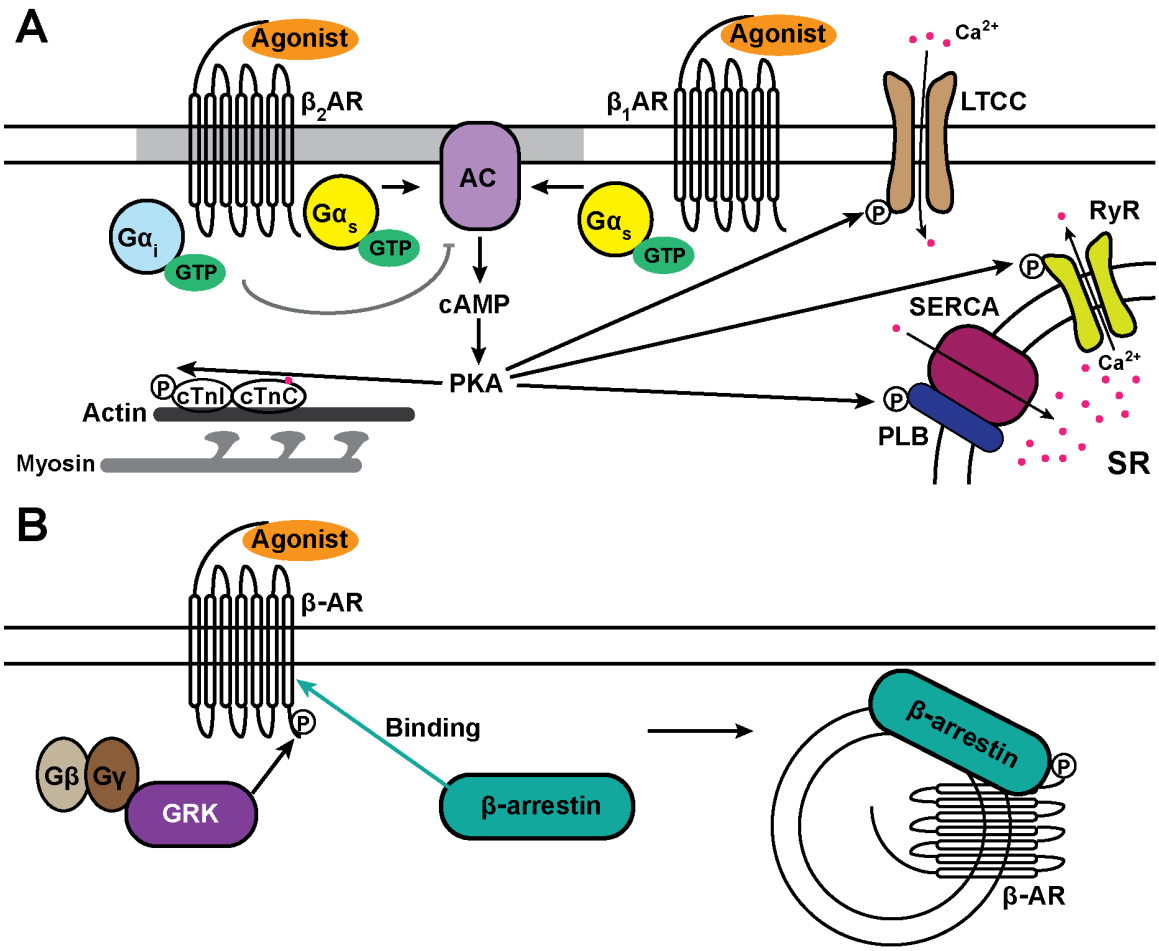


Figure 2 Beta-adrenergic signaling pathways in cardiomyocytes

(A) Agonist stimulation of beta-adrenergic receptors (β-ARs) leads to activation of adenylyl cyclase (AC) and production of cAMP. Protein Kinase A (PKA) is subsequently activated and phosphorylates L-type calcium channel (LTCC), phospholamban (PLB), ryanodine receptor (RyR), and cardiac troponin I (cTnI). Lipid subdomain in the plasma membrane is shown in grey. **(B)** G protein coupled receptor kinase (GRK) phosphorylates β-AR, which results in β-arrestin binding and receptor internalization.

Beta-adrenergic signaling and palmitoylation

Many proteins in the β-AR signaling pathway are palmitoylation targets. In HEK293 cells overexpressing β_1-AR, mutation studies using [^{3}H]palmitate showed that β_1AR is palmitoylated at Cys 392, 393, and 414 [164]. Loss of palmitoylation at these sites did not affect protein stability or plasma membrane localization, but only the Cys 414 mutant showed reduced internalization following stimulation. Interestingly, click-chemistry based pulse-chase labeling showed stable palmitoylation at position 392/393 and fast turnover rate (15 min) at position 414, suggesting differential regulation and potentially distinct functions of palmitoylation at these sites.

β_2-AR is one of the first G protein coupled receptors (GPCRs, following the light-activated GPCR rhodopsin [59]) to be identified as a palmitoylation target. The first reported palmitoylation site on β_2-AR is Cys 341, and mutation at this site led to reduced downstream cAMP production in the Chinese hamster fibroblast cell line CHW-1102 [61]. Later studies showed that loss of Cys 341 palmitoylation increased receptor phosphorylation level at Ser 345/346 and uncoupling from Gαs, a process mediated by PKA [165, 166]. In cardiomyocytes, palmitoylation of β_2-AR at Cys 341 is crucial for its association with phosphodiesterase (PDE) 4D and subsequent β-arrestin mediated internalization [167]. Recently, a second palmitoylation site Cys 265 was identified within β_2-AR by using acyl-RAC [168]. Palmitoylation at Cys 265 is evident after 20 min of ISO stimulation and peaks after 120 min. Additionally, this study by Adachi *et al.* also revealed that Golgi-localized DHHC9/14/18 redundantly palmitoylate Cys 265,

but not Cys 341. Adachi *et al.* proposed that palmitoylation at Cys 265 contributes to preserving β_2-ARs at the plasma membrane after sustained adrenergic stimulation. However, this study used 2-bromopalmitate (2-BP) and palmostatin B (PalmB) to inhibit and enhance palmitoylation of β_2-ARs, respectively. These methods are not designed for analyzing the functions of palmitoylation under physiological conditions. Additionally, 2-BP also inhibit other enzymes including APT1, which catalyzes depalmitoylation [169], and therefore cannot be used as a reliable way to inhibit palmitoylation.

Gα subunits, including the s, i, o, z, and q, are all shown to incorporate [^{3}H]palmitate at cysteines near the N-terminus in COS cells or insect Sf9 cells [170]. Mutation studies showed that palmitoylation of Gαs, Gαq, and Gαo are essential for their membrane association [171, 172]. In addition to plasma membrane localization, Gαi and Gαz palmitoylation alters their interaction with various regulators of the G protein signaling (RGS) proteins in brain [173]. Mutation studies at Cys 3 reported various effects on downstream signaling events, including activation of AC [174] and MAPK [175]. As mentioned briefly before, stimulation of β-ARs in cells lines led to increased turnover of the palmitoylation/depalmitoylation cycle on Gαs [122]. Interestingly, dynamic palmitoylation of Gα proteins is not specific to the β-AR signaling pathway. Gαi, Gαo, and Gαq had increased palmitoylation turnover upon serotonin receptor activation in brain [176].

Additionally, GRK proteins are also shown to be palmitoylation targets. There are six members in the human GRK gene family [177]. In kidney fibroblast

COS-7 cell line, GRK6 was shown to be palmitoylated at three cysteines (561, 562, and 565) in the C-terminal domain and the palmitoylation targets the protein to membrane [178]. Subsequently, all of the four GRK4 isoforms were shown to be palmitoylated [179]. Mutation studies in COS cells showed that loss of GRK6 palmitoylation did not affect its kinase activity, but greatly reduced its ability to sequester β_2AR following stimulation [180].

GRK2 and GRK5 are the most abundantly expressed GRK family members in the heart [156, 157]. However, neither of them was shown to incorporate [^{3}H]palmitate [178]. It is proposed that the basic amino acids at position 552-562 near the C-terminal domain of GRK5 are important for its association with the membrane [181]. The positive charges from these basic amino acids can form electrostatic interaction with the negatively charged lipid head groups and stabilize GRK5 at the membrane [179, 182]. Multiple studies have demonstrated that GRK2 directly binds the dissociated Gβγ subunits, which are isoprenylated, and the formation of the complex is important for its plasma membrane localization and functions [162, 163]. Given the recent development in methods to detect endogenous protein palmitoylation levels, we are interested in identifying the dynamic palmitoylation levels of signaling proteins and their functional outcomes in cardiomyocytes.

Palmitoyl acyltransferases and their functions in cardiomyocytes

To better understand the role of palmitoylation on signaling transduction in cardiomyocytes, it is worth considering the enzymatic regulators that catalyze the

attachment of palmitic acid moiety onto substrates. As mentioned briefly in previous sections, 23 DHHC (referring to the conserved amino acids at the catalytic core) enzymes exist in the human genome [69]. This class of enzymes was first discovered in yeast for palmitoylation of Ras [183]. *In vitro* assays using [^{3}H]palmitoyl-CoA revealed that DHHC enzymes are sufficient for the transfer of palmitic acid from CoA to target cysteines, and they go through autoacylation, presumably at the DHHC site [184]. A later study using DHHC2 and DHHC3 showed that DHHC autoacylation at the catalytic Cys site serves as an acyl-enzyme intermediate in a two-step ping-pong mechanism [185]. Recently, the crystal structures of zebrafish DHHC15 and human DHHC20 were solved [186]. This study revealed that the binding of Zn^{2+} ions is crucial for positioning the catalytic Cys, which is positioned at the protein-lipid interphase, and that the transmembrane domains form a hydrophobic pocket where acyl-CoA chains of specific lengths can fit [186]. Despite a highly conserved catalytic domain, the human DHHC family members display considerable amino acid sequence divergence, especially in the C-terminal tail region (reviewed in [187]). The roles of DHHC enzymes and their targets have been extensively studied in the context of neurological diseases and cancers (reviewed in [188]). However, fewer studies were dedicated to their functions in cardiac cells.

One of the DHHC enzymes that have been implicated to play a crucial role in the heart is DHHC16. DHHC16 deficient mice showed severe birth defects including partial embryonic lethality, eye malformation, and cardiomyopathy [189]. Zhou *et al.* identified phospholamban (PLB) as one of the DHHC16

substrates and palmitoylation of PLB is crucial for obtaining its quaternary structure. Additionally, Zhou *et al.* found that introducing PLB deficiency into the DHHC16 deficiency mice rescued some of the cardiac phenotypes [189]. The study by Zhou *et al.* provided the first evidence that DHHC16 is crucial for heart development. However, many questions remain such as what other substrates are palmitoylated by DHHC16 in cardiomyocytes? Is there dynamic regulation of PLB palmitoylation after activation of the β-AR/AC/PKA pathway? One recent study in the HAP1 cell line reported a palmitoylation cascade in which DHHC16 palmitoylates DHHC6 at three cysteines in the C-terminal SH3 domain [190]. This also raises the question of whether the cardiac phenotypes observed in DHHC16 mouse model are due to direct effects of DHHC16 or through another DHHC enzyme.

DHHC5 is one of the best characterized PATs in the heart and palmitoylation of its substrate phospholemman (PLM) is crucial for mediating a process called massive endocytosis (MEND). PLM is a regulatory subunit of the cardiac Na/K pump [191]. PLM can be phosphorylated by PKA and PKC, and subsequently activates the Na/K pump [192]. On the other hand, PLM can also be palmitoylated to inhibit Na/K pump activity [193]. Co-immunoprecipitation experiments showed that DHHC5, along with DHHC 4, 6, and 7, binds PLM. In adult rat ventricular myocytes (ARVMs), DHHC2, 4, 5, and 7 are most abundantly expressed at the mRNA level [194]. DHHC5 is one of the few PATs that are shown to localize at the plasma membrane [70], where its target PLM is located. Over-expression and knockdown of DHHC5 enhanced and suppressed,

respectively, PLM palmitoylation levels in cell lines. Additionally, the study by Howie *et al.* also identified the PLM palmitoylation site to be at a single Cys in ARVM [194].

To further elucidate how DHHC5 potentially affects cellular activities in cardiomyocytes, a DHHC5 knockout mouse model was used to study MEND, a cellular process that happens during reperfusion of anoxic heart [195] (**Figure 3**). MEND is characterized by internalization of more than 70% of the plasma membrane [196]. MEND can be triggered by the release of Coenzyme A (CoA) from the mitochondria upon calcium overload and oxidative stress. The presence of CoA in the cytoplasm allows the synthesis of Acyl-CoA, which can then be used to palmitoylate surface membrane proteins [196]. Interestingly, MEND can be promoted by overexpression of palmitoylated PLM [193], providing a potential mechanism by which DHHC5-mediated palmitoylation of PLM plays an essential role in mediating MEND. Indeed, myocytes isolated from DHHC5 deficient mice showed increased Na/K pump activity as well as more surface localized PLM [197]. Additionally, MEND is inhibited in post-anoxia reperfusion models in the DHHC5 deficient heart [197]. These data suggest that the availability of Acyl-CoA can be one potential mechanism by which DHHC5-mediated PLM palmitoylation is regulated in cardiomyocytes. Interestingly, both PLM and DHHC5 are shown to be enriched in caveolae [194], suggesting that the physical proximity of the substrate may be another way to regulate DHHC5-mediated palmitoylation.

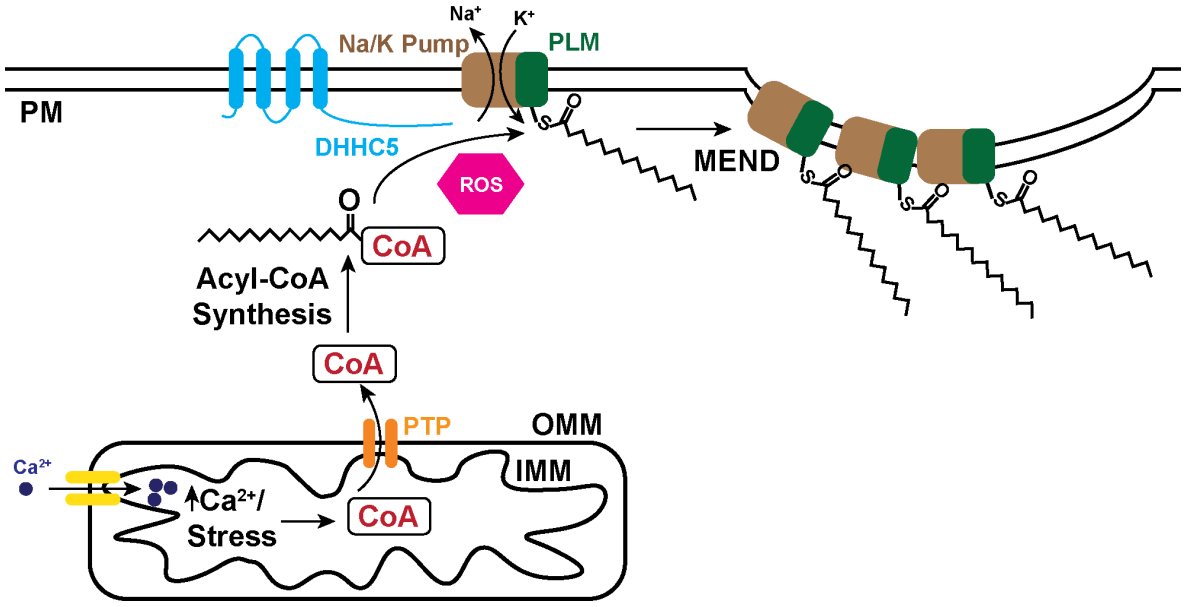


Figure 3 DHHC5 mediates massive endocytosis in cardiomyocytes

Increased calcium storage in mitochondria or oxidative stress leads to export of Coenzyme A (CoA) through permeability transition pore (PTP) that spans the inner and outer mitochondria membranes (IMM and OMM). Palmitoyl-CoA is then synthesized and used by DHHC5 to palmitoylate phospholemman (PLM) in the presence of reactive oxygen species (ROS). PLM palmitoylation leads to massive endocytosis (MEND) and internalization of Na/K pumps.

It is worth noting that truncation studies have shown that the C-terminal domain between N218 and T334 is crucial for DHHC5 substrate recognition [194]. Additionally, emerging evidence has shown that DHHC5 can be posttranslationally modified in the C-terminal tail. In neurons, the tyrosine kinase Fyn phosphorylates DHHC5 at Y533 and forms a DHHC5/Fyn/PSD-95 protein complex to keep them at the synaptic membrane [198]. Proteomic approaches in adult mouse forebrain extracts revealed that DHHC5 was found to be palmitoylated at three potential cysteines in the C-terminus tail (Cys 236, 237, and 245), some of which are conserved in other DHHC enzymes [199]. Therefore, further studies are needed to identify post-translational modifications of DHHC5 and how they regulate its activities in cardiomyocytes.

Chapter II: Material and Methods

This chapter is partially based upon a preprint article:

> Chen, J. J., Marsden, A. N., Akimzhanov, A. M., & Boehning, D. (2018) The palmitoyl acyltransferases DHHC5 mediates beta-adrenergic signaling in the heart by targeting Gα proteins and G protein-coupled receptor kinase 2. *bioRxiv* preprint.

Antibodies, constructs, and reagents

Mouse monoclonal anti-Gαs was purchased from Millipore (MABN543). Rabbit monoclonal anti-Gαi/o and rabbit polyclonal anti-calnexin was purchased from Abcam (ab140125, ab22595). Rabbit polyclonal anti-GRK2, anti-PKA C-α, anti-PKA RI-α/β were purchased from Cell Signaling (#3982, 4782, 3927). Rabbit polyclonal anti-AC5/6 was purchased from Millipore (ABS573). Rabbit polyclonal anti-DHHC5 was purchased from Proteintech (21324-1-AP). Secondary antibodies conjugated to Alexa-488, Alexa-555 (Molecular Probes), peroxidase (Jackson ImmunoResaerch), IRDye 680LT, and 800CW (LI-COR) were used. The 3xFLAG-DHHC5 construct was a gift of Dr. Yusuke Ohno (Sapporo, Japan). DHHS5 mutant was built using q5 mutagenesis kit (NEB) with primers 5'-TGATCATCACAGCCCCTGGGTG-3' and 5'-AATTCCTCCACACAGTTGTCAC-3'. DHHC5 Cys 236 to Cys 245 deletion (DHHC5Δ236-245) mutant was built with primers 5'-AGTTCTCCAGCACCCAGG-3' and 5'- GCCATTGGTGAAGGGATTC-3'. DHHC5 Cys 236 to Ser (DHHC5C236S) mutant was built with primers 5'-

CACCAATGGCAGCTGTAACAACG-3' and 5'-AAGGGATTCACACCTCCC-3'. DHHC5 Cys 245 to Ser (DHHC5C245S) mutant was built with primers 5'-CCGTGTCCTCAGCAGTTCTCCAG-3' and 5'-CTAACGTTGTTACAGCAGCC-3'. DHHC5C236S_C245S double mutant was built using the same primers as DHHC5C245S in the DHHC5C236S background. DHHC5 tail-less (DHHC5TL) mutant was built with primers 5'-ATTTCATGTGTAACTGGTGGCTAGGGGAC-3' and 5'- CCTGTGAGGCCAGCTACA-3' so that Val 209 was changed to a stop codon. DHHC5 truncation mutant that changes Ser 376 to a stop codon (DHHC5S376STOP) was built with primers 5'-CGCCAAGTTGTGACGTGGAGACA-3' and 5'-CTGGAGGAATGAGGCATG-3'. DHHC5 truncation mutant that changes Ser 584 to a stop codon (DHHC5S584STOP) was built with primers 5'-CCCCCGTACTTGATCCTCCTCAGATG-3' and 5'- GCATGGCCTGAGCCTGGT-3'. DHHC5 was cloned into the pmCherry-C1 Vector from Clontech (632524) using the NEBuilder High-Fidelity DNA Assembly Cloning (NEB, E5520) with primers 5'-CGACGGTACCGCGGGCCCGGGATCCATGCCCGCAGAGTCTGGAAAG-3' and 5'-TCAGTTATCTAGATCCGGTGGATCCTCACACCGAAATCTCATAGGTGG-3' following the manufacturer's protocol. Isoproterenol was purchased from Tocris (#1747) and was prepared fresh before use. Silencer Select DHHC5 siRNAs (s168613, s168614) and negative control medium GC duplex siRNA (12935112)

were purchased from ThermoFisher Scientific. All other reagents were purchased at the highest possible purity from Sigma-Aldrich.

Cell culture

The rat myoblast H9c2 and HEK293 cell lines were obtained from the American Type Culture Collection (ATCC) and maintained according to ATCC guidelines. Neonatal rat ventricular cardiomyocytes (NRVMs) were prepared from 1 to 2 day-old Sprague-Dawley rat pups, as previously described [200]. Animals were purchased from Texas Animal Specialties (Humble, TX) and processed on the same day. Cardiomyocytes were plated on Primaria polystyrene plates (Corning) or fibronectin-coated glass coverslips. Cells were initially maintained in a mixture of 40% DMEM, 40% Ham's F10 culture medium, and 20% FBS. Forty-eight hours after plating, the media was replaced by a mixture of 50% DMEM and 50% Ham's F10 culture medium, supplied with human recombinant insulin (Gibco), transferrin, and thyroxine (Sigma). Cytosine-B-D-arabino-furanoside hydrochloride (10μM, Sigma) was present in all culture medium to prevent proliferation of fibroblasts or endothelial cells. Cardiomyocytes were transfected with Lipofectamine 3000 following manufacturer's instruction. All siRNA knock-down experiments were carried out 48 hours following transfection and within one week of initial plating. The vertebrate animal procedures were approved by the Animal Welfare Committee (AWC) at UTHealth. For each assay, n represents number of biological replicates from separate NRVM preps.

Preparation of adult left ventricular lysates

Frozen left ventricle tissue from adult rats was a gift of Dr. Karmouty-Quintana (UTHealth) and Drs. Youker and Amione-Guerra (Houston Methodist Hospital). Frozen tissue was homogenized and lysed in 1% β-D-maltoside in PBS, supplemented with a protease inhibitor mixture (Roche 11873580001) and the acyl protein thioesterases inhibitor ML211 (10μM, Cayman Chemicals), followed by centrifugation (8000 xg, 5min) to remove insoluble material. The cleared supernatant was stored at -80°C.

Acyl biotin exchange

NRVMs were plated at a density of 1,000 cells per mm^2 on 6-well plates. For control and DHHC5 knockdown experiments, the total amount of siRNA transfected was 30 pmol per 900,000 cells. NRVMs were stimulated with 10 μM ISO at room temperature for indicated times, followed by scraping on ice in ice-cold lysis buffer. Cells were lysed in 1% β-D-maltoside or 0.5% SDS (for DHHC5 tail palmitoylation experiments) in PBS, supplemented with a protease inhibitor mixture (Roche 11873580001) and acyl protein thioesterases inhibitor ML211 (10 μM, Cayman Chemicals). Acyl biotin exchange was performed as described previously with slight modifications [71]. Briefly, proteins were precipitated by choloroform-methanol (CM) and incubated with 10 mM N-ethylmaleimide (NEM) overnight at 4°C with gentle mixing. Following three rounds of CM precipitation, samples were incubated with 400 mM hydroxylamine (HA, pH=7, freshly prepared) and 1 mM HPDP-biotin for 50 min at 37°C with gentle mixing. When a

minus-HA treated sample was included, the sample was divided into two equal parts after the third precipitation and sodium chloride was used instead of HA. After three rounds of CM precipitation, samples were incubated with Streptavidin-agarose (Pierce) beads for at least 90 min up to 8 hrs at room temperature with gentle mixing. Following four rounds of washes, bound proteins were eluted for 15 min at 80°C with shaking in 30 µl of elution buffer containing 1% β-mercaptoethanol and 2 mM DTT. The supernatants were transferred to new tubes and 20 µl of the samples were loaded onto a 4-20% gradient SDS-PAGE gel (BIO-RAD), followed by transfer to nitrocellulose membrane and immunoblotting.

cAMP ELISA

For quantification of intracellular cAMP levels, NRVMs were plated at a density of 175 cells per mm^2 on 24-well plates. For siRNA knockdown experiments, cells were transfected with 5 pmol of siRNA for 48 hr. For knockdown and rescue experiments, cells were transfected with 5 pmol of siRNA for a total of 48 hr and 2 µg of DNA were transfected 24 hr after the siRNA transfection. Cells were stimulated with 1 µM ISO and assayed using the direct cAMP ELISA kit (Enzo) per manufacturer's instructions. Each well was lysed in 250 µl provided lysis buffer by scraping the wells. Final results were analyzed using a Tecan Spark Multimode Microplate Reader. Each experiment was performed with at least three biological replicates.

Calcium imaging

NRVMs were plated at a density of 300 cells per mm^2 on fibronectin-coated glass coverslips. Cardiomyocytes were loaded with Fura-2 AM as described previously [200]. Briefly, cardiomyocytes were incubated with Fura-2 AM in imaging solution (1% BSA, 107mM NaCl, 20 mM HEPES, 2.5 mM $MgCl_2$, 7.5 mMKCl, 11.5mM glucose, and 1 mM $CaCl_2$) for 30 min at room temperature. Then cardiomyocytes were incubated in imaging solution without Fura-2 AM for 20 min at room temperature before mounting onto the microscope. Images were taken on a Nikon TiS inverted microscope with a 40X oil immersion objective, and images were taken every second with a Photometrics Evolve EMCCD camera. ISO (10 μM) was added after 2 min of baseline recording. An oscillation was counted when the Fura-2 ratio rose 10% above the baseline ratio. Oscillation frequencies before and after ISO were calculated from five coverslips for each condition and mean ± SEM were plotted as described [200].

TIRF microscopy and analysis

NRVMs were plated at a density of 200-250 cells per mm^2 on fibronectin-coated glass coverslips and transfected with mCherry-DHHC5 or mCherry-DHHC5Δ236-245 for 24 hrs using Lipofectamine 3000. Images were taken on a Nikon Eclipse TiS TIRF microscope with a 60X Apo TIRF oil immersion objective every 500 ms with an Andor Zyla sCMOS camera. Beating cardiomyocytes were located and imaged for three minutes before treating with ISO (10 μM). Cells were continuously imaged for 10 min after stimulation. Images were analyzed using the TrackMate v3.8.0 Plugin for Fiji [201]. We used the LoG detector for particles,

and the thresholds were determined for each cell based on the fluorescence intensity. We used the LAP tracker (max distance 15 pixel, max frame gap 4) for particle tracking. All output files from TrackMate were used for data analysis. “Links in track statistics” data from each cell was exported from TrackMate as comma-separated values (csv) files and analyzed using custom Python scripts utilizing the pandas and NumPy packages for data analysis. Tracking files from individual cells were merged together into wildtype and mutant data frames. Time bins of 30 s were created for the 13-minute experiment duration and each row of the data frames, representing 0.5 s, were assigned to these time bins. Downstream analyses were carried out using these 30 s time bins. Particle lifetime analysis was performed by extracting all particles which existed during a 30 s bin at the start of the experiment (time 0 min) and during one bin at the end of the experiment (time 10 min). The time that a particle existed (in frames) within this window was calculated for each particle and the normalized distribution was plotted along with the kernel-density-estimate (KDE) of the distribution [202]. Plotting was performed in Python using the seaborn and matplotlib packages. The mean squared displacement of every particle was calculated in Python using the following formula at each frame (n) (0.5s) for a total of 60 frames (N) where x and y represent the coordinate position of the particle as determined by TrackMate:

$$MSD(n) = \frac{1}{N-n}\sum_{i=1}^{N-n}[(x_{n+i} - x_i)^2 + (y_{n+i} - y_i)^2]$$

The MSD at each frame was plotted for a 30 s bin immediately prior to treatment (frames 300-360) and for a 30 s window 10 minutes post-treatment (frames 1560-1620).

Immunofluorescence staining

NRVMs were plated at a density of 250 cells per mm^2 on fibronectin-coated glass coverslips. Immunofluorescence staining was performed as described previously [200]. Briefly, H9c2 or NRVMs were fixed using 100% ice-cold methanol at -20°C for 20 min, and permeabilized using 0.1% saponin in PBS at room temperature for 10 min. After washing with PBS, samples were blocked in 5% FBS and 1% BSA in PBS. Primary antibodies were diluted in the same buffer as blocking. Anti-DHHC5 was used at a concentration of 1:100. Secondary antibodies were used at a concentration of 1:500. Samples were mounted in VECTASHIELD antifade mounting medium with DAPI, sealed, and kept at 4°C. Images were taken on a ZEISS Cell Observer SD laser scanning confocal system with a 63x oil objective. For the quantification of plasma membrane localization of DHHC5, the percentage of cells displaying near plasma membrane localization (example shown in **Figure 7**) from five separate fields was scored in a blinded manner.

***In vitro* DHHC5 activity assay**

HEK293T cells were not transfected (control) or transfected with FLAG- or mCherry- tagged DHHS5 and DHHC5 using Lipofectamine 3000 for 18-24 hrs. Cells were lysed in lysis buffer containing 0.5% SDS, 150mM NaCl, 50mM Tris,

1% Triton and 1mM EDTA, in the presence of protease inhibitor (Roche). Lysates are incubated at 4°C with rotation for 15 min, sonicated until no longer viscous, and diluted using lysis buffer to a final concentration of 0.01% SDS. Rabbit monoclonal anti-FLAG (CST147935) or RFP-Trap (ChromoTek) were used to immunoprecipitate DHHS/C5. The reaction mix contains 500 nM recombinant human Gαs (Origene TP314318), non-myristoylated rat Gαi proteins (Gift of Dr. Carmen Dessauer and Dr. Tanya Baldwin) or recombinant human GAPDH (abcam ab77109), 200 nM DTT, 300 μM DDM, and 5 μM palmitoyl CoA{N-[(7-nitro-2-1,3- benzoxadiazol-4-yl)-methyl]amino} (Avanti lipids). For samples mixed with hydroxylamine (HA), final concentration of 400 mM neutral HA is present in the reaction mix. Reactions were started by adding palm-CoA-NBD and kept shaking at 37°C. At the indicated time points, a portion of the reaction was taken out and stopped by adding lysis buffer and heating at 80°C for 15 min. Beads were boiled in the presence of 2% BME to check for DHHS/C5 proteins at the end of the reactions. All samples were run on 4-20% gradient gels, transferred, and imaged using a ChemiDoc XRS+ system (BIO-RAD) with a Blue Epi illumination and a 530/25 filter for the NBD signals. Standard Western Blotting was used to check for the level of total substrate proteins.

Acyl-PEG-Exchange

H9c2 cells were lysed in 1% β-D-maltoside in PBS, supplemented with a protease inhibitor mixture and APT inhibitor ML211 (10 μM). Acyl polyethylene glycol (PEG) exchange was performed as described previously with slight

modifications [203]. Briefly, proteins were precipitated by chloroform-methanol (CM) and incubated with 0.2% MMTS at 42°C for 15 min. Following four rounds of CM precipitation, protein pellet was dissolved in buffer containing 2% SDS, 5mM EDTA, 100mM HEPES, PH 7.4 (refer to as 2SB). Samples were divided into two equal parts, and one part was incubated with 2M Hydroxylamine (HA, PH=7) while the other part with 2M NaCl (-HA, negative control) for 1 hr at room temperature with end-to-end rotation. Samples were washed with three rounds of CM precipitation and dissolved in 2SB. Equal volume of 2mM PEG maleimide (mPEG) was added to samples and incubated at room temperature for 2 hr with gentle mixing. Excess mPEG was removed using three rounds of CM precipitation and the protein pellet was dissolved in 2SB. Loading buffer containing 2% BME and 2mM DTT were added to the samples and heated at 80°C for 15 min. Samples were ran on 4-20% gradient gels, followed by standard western blotting.

Chapter III: DHHC5 Mediates Beta-adrenergic Signaling Though Palmitoylation of G Alpha Proteins in Cardiomyocytes

This chapter is partially based upon a preprint article:

> Chen, J. J., Marsden, A. N., Akimzhanov, A. M., & Boehning, D. (2018) The palmitoyl acyltransferases DHHC5 mediates beta-adrenergic signaling in the heart by targeting Gα proteins and G protein-coupled receptor kinase 2. *bioRxiv* preprint.

Introduction

Beta-adrenergic receptor (β-AR) signaling is one of the central regulators of contractile function in the heart. Like other G protein-coupled receptors (GPCRs), β-ARs associate with heterotrimeric G proteins, composed of α, β, and γ subunits [204, 205]. Upon activation, β-ARs act as guanine nucleotide exchange factors and replace the GDP with GTP in the G protein α subunits [206]. Cardiac β-ARs associate with both Gαs and Gαi, which can stimulate or inhibit adenylyl cyclase, respectively [207, 208]. Adenylyl cyclase produces the secondary messenger cAMP, which activates multiple downstream effectors, including protein kinase A (PKA) [209, 210]. PKA phosphorylates many substrates that alter contractile functions, including ryanodine receptors, phospholamban, and cardiac troponin I [211-213]. Given the crucial roles of β-AR signaling in regulating cardiac function, it is not surprising that this pathway is tightly controlled.

Multiple members of the β-AR signaling pathway undergo protein palmitoylation, a posttranslational modification that adds a 16-carbon palmitic acid to cysteine residues *via* a labile thioester bond. Protein palmitoylation is catalyzed by a class of enzymes termed palmitoyl acyltransferases (PATs). There are at least 23 mammalian PATs that share a conserved Asp-His-His-Cys (DHHC) domain that is essential for PAT activity [68]. Most of the DHHC PATs are localized to the Golgi apparatus and play crucial roles in the cycling of palmitoylated substrates between the Golgi apparatus and the plasma membrane. Recently our lab showed that the plasma membrane-localized DHHC21 is responsible for rapid, agonist-induced palmitoylation of substrates downstream of the Fas death receptor in T-cells [113].

Small G proteins such as Gα are classic palmitoylated proteins, and this modification is crucial for their plasma membrane targeting and subsequent functions [170, 171]. Studies in HeLa cells and primary hippocampal neurons suggested that Gα proteins can be palmitoylated by Golgi-localized DHHC3 and -7 [214]. The enzymatic activity regulating Gα protein palmitoylation in cardiac tissue is incompletely understood. Recently, the β_2-AR was reported to be palmitoylated at two cysteine residues in cardiomyocytes in a PKA-dependent manner resulting in internalization after 20 minutes of isoproterenol (ISO) stimulation [168]. In this study it was found that the Golgi-associated DHHC9, 14, and 18 may mediate β_2-AR palmitoylation. In order for palmitoylation to regulate GPCR signaling with kinetics consistent with a signaling role, presumably a plasma membrane localized DHHC enzyme should be activated. In

cardiomyocytes, the plasma membrane localized DHHC5 enzyme has been shown to be concentrated in caveolae and regulate the dynamic palmitoylation of phospholemman, a regulatory subunit of the Na pump [193, 194]. Importantly, phospholemman palmitoylation is dependent upon PKA phosphorylation, indicating it is likely palmitoylated in a stimulus-dependent manner [193]. Whether DHHC5 regulates the dynamic palmitoylation of other substrates after β-AR stimulation in cardiomyocytes is not known.

Here I show that Gαs and Gαi are palmitoylated within minutes of β-AR activation in ventricular cardiomyocytes. The palmitoylation kinetics are temporally consistent with the downstream cAMP production and changes in contractility. Knockdown of DHHC5 significantly inhibits agonist-induced palmitoylation and downstream responses indicating that this enzyme is required for β-AR signaling in cardiomyocytes. Additionally, I show that Gαs and Gαi are direct substrates of DHHC5 *in vitro*. These results uncover a novel and essential palmitoylation-dependent signaling cascade downstream of β-ARs in cardiomyocytes.

Results

Acyl-biotin exchange reveals known and novel palmitoylation proteins in the cardiac β-AR signaling pathway

Multiple methods have been developed to detect protein palmitoylation. The traditional method of metabolically labeling target proteins with [^{3}H]-palmitate is powerful, but not ideal for unbiased identification of multiple palmitoylation

substrates. The efficiency of labeling is dependent on the ability of cells to incorporate labeled palmitate and the palmitate turnover rate of each individual protein [103]. In addition, exogenous addition of palmitic acid to cells may drive non-physiological processes including altering the acylation state of signaling proteins. More recently, acyl-biotin exchange (ABE, **Figure 1B** in **Chapter I**) has been exploited as an efficient way of detecting the endogenous palmitoyl proteome in various tissues and organisms [71, 109, 110]. The first step of ABE is blocking the free cysteine sites on proteins in cell lysates by using the thioreactive compound N-ethylmaleimide (NEM). Subsequently the labile thioester bond at the palmitoylation site is cleaved using neutral hydroxylamine (HA), exposing the free thiol group that can be subsequently biotinylated. After biotinylation, proteins are pulled down using streptavidin beads and eluted, followed by Western blotting to detect palmitoylated proteins. I used multiple rat cardiac cell types, including the myoblast cell line H9c2, neonatal rat ventricular myocytes (NRVM), and adult murine left ventricular tissue for detection of palmitoylated proteins downstream of β-AR (**Figure 4A-C**). I used calnexin as a positive control for protein palmitoylation in all experiments [215, 216]. As expected, I found that Gαs and Gαi are palmitoylated in both cell types and in cardiac tissue. Additionally, I also identified G protein-coupled receptor kinase 2 (GRK2), a negative regulator of β-AR signaling, as a novel palmitoylation target in H9c2 cells. Though GRK2 has never been shown as a palmitoylated protein, another member of the GRK family, GRK6, has been shown to be palmitoylated at the carboxyl-terminal domain in COS-7 cells [178]. The PKA regulatory

subunit showed low levels of palmitoylation in H9c2 cells consistent with a previous proteomic study [217]. However, I cannot conclude that PKA regulatory subunit is indeed palmitoylated in H9c2 cells because the signal was only marginally stronger than the background signal in the -HA lane. Other methods, for example acyl-RAC, may be more suitable for confirming the palmitoylation state of PKA regulatory subunits. No palmitoylation was detected for other proteins downstream of β-ARs, including adenylyl cyclase 5/6 or PKA catalytic subunits (**Figure 4A-C**). Consistent with our ABE results in adult rat ventricular tissue (**Figure 4C**), a palmitoyl proteomic study in adult rat ventricular cardiomyocytes found that Gαs and Gαi are palmitoylated proteins [218]. Interestingly, Wypijewski *et al.* identified adenylyl cyclase 6, but not any other proteins listed in my study, as a potential palmitoylated protein.

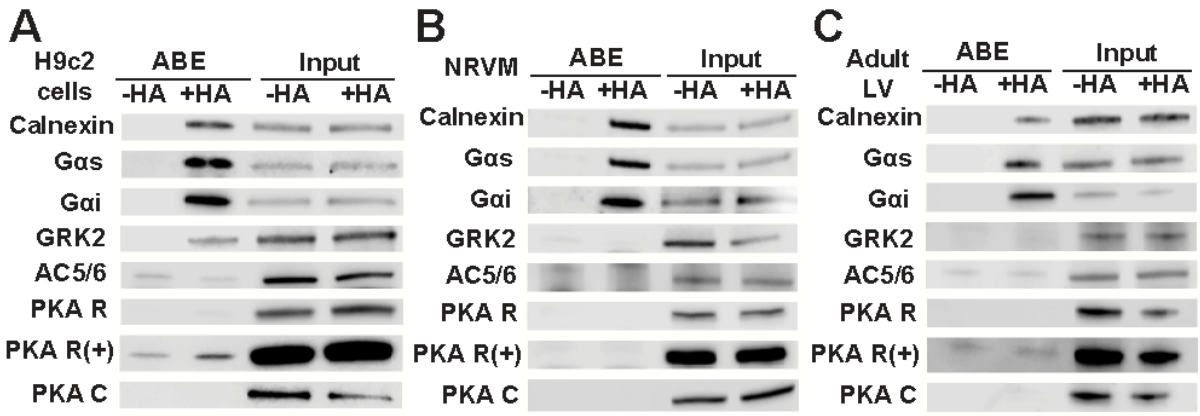


Figure 4 Palmitoylation of cardiac proteins as determined by acyl-biotin exchange (ABE)

ABE was performed on H9c2 cells (A), neonatal rat ventricular myocytes (NRVMs, B), and adult rat left ventricular (LV) tissue (C). Calnexin was used as a positive control. The absence of hydroxylamine (-HA) was used as a negative control for non-specific binding to the streptavidin beads. Abbreviations are as follows: GRK2, G protein-coupled receptor kinase 2; AC, adenylyl cyclase; PKA R, protein kinase A regulatory subunit; PKA C, PKA catalytic subunit. Overexposure of the PKA R blot is indicated as a (+). This figure is modified from Chen, J. J., Marsden, A. N., Akimzhanov, A. M., & Boehning, D. (2018) The palmitoyl acyltransferases DHHC5 mediates beta-adrenergic signaling in the heart by targeting Gα proteins and G protein-coupled receptor kinase 2. *bioRxiv* preprint. Copyrights for this article remain with the authors.

Activation of β-ARs induces rapid protein palmitoylation in cardiomyocytes

Previous studies indicate that stimulation of β-ARs increases the rate of turnover of palmitate groups on Gα [122, 171, 219]. To examine whether Gαs and Gαi proteins are palmitoylated upon β-AR activation in cardiomyocytes, I stimulated primary NRVMs with the β-AR agonist isoproterenol (ISO) and collected lysates from 0 to 30 minutes. I then performed ABE to quantify palmitoylation levels of proteins of interest. I found that both Gαs and Gαi had significantly increased palmitoylation levels within a minute of ISO stimulation (**Figure 5A,B**). Calnexin is a well-characterized and stably palmitoylated protein [215]. I found that palmitoylation levels of calnexin were marginally elevated after ISO stimulation. It is known that calnexin is localized to the ER [220], a separate membrane system from the plasma membrane where many of the β-AR signaling proteins are localized. Therefore, due to the modest increases in calnexin palmitoylation levels and localization to a separate membrane system, I used calnexin to normalize the changes in palmitoylation levels of Gα proteins (**Figure 5B**). By 30 min the palmitoylation levels of Gαs and Gαi were significantly reduced suggesting a temporally distinct activation of acyl protein thioesterases. Interestingly, I also found that GRK2 can be palmitoylated after ISO stimulation (**Figure 6**). The kinetics of GRK2 palmitoylation were characterized by increased levels at later time points compared to Gα proteins and were maintained throughout the 30 min time course (**Figure 6B**). These delayed kinetics may be functionally relevant to the negative regulatory role of GRK2 in the β-AR pathway. I next examined whether the agonist-induced

palmitoylation is temporally consistent to downstream events mediating contractile functions. To test whether the kinetics of Gα palmitoylation correspond to downstream cAMP production, I measured intracellular cAMP levels in NRVMs after ISO stimulation. We found that cAMP levels increased within a minute of ISO stimulation and then decreased within 30 min (**Figure 5C**), which was temporally consistent with the kinetics of Gα palmitoylation (**Figure 5A,B**). ISO is known to induce positive inotropy in NRVMs [221]. I used Fura-2 AM, a ratiometric calcium indicator, to monitor the intracellular calcium levels in spontaneously beating NRVMs. I observed an increase in calcium oscillation frequency (corresponding to beating frequency) within a minute of ISO stimulation, which like cAMP production, was temporally consistent with the kinetics of Gα palmitoylation (**Figure 5D**). These results suggest that rapid palmitoylation of signaling proteins after β-AR stimulation may play a role in the assembly of the macromolecular β-AR signaling complex and efficient downstream modulation of cardiomyocyte contractility. To directly address this possibility, I targeted the enzymatic machinery potentially responsible for rapid palmitoylation of signaling proteins in cardiomyocytes.

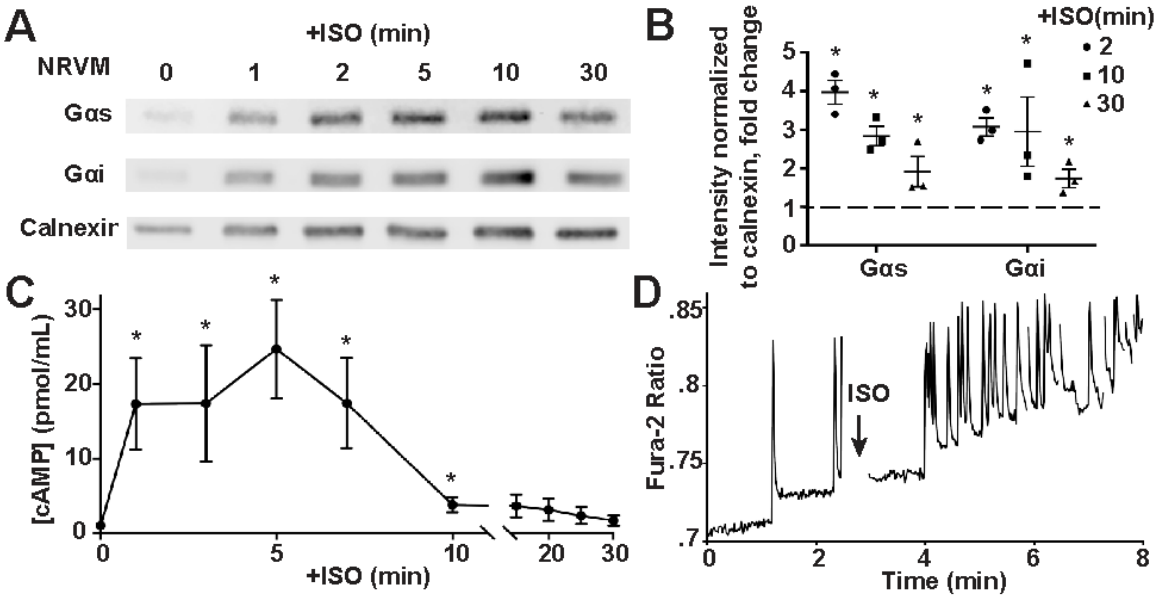


Figure 5 Isoproterenol induces rapid and reversible palmitoylation of Gαs and Gαi in cardiomyocytes

(A) Neonatal rat ventricular myocytes (NRVMs) were treated with 10 μM isoproterenol (ISO) for the indicated times. ABE was used to detect palmitoylation levels. Calnexin, which has a relatively slow turnover of palmitoylation groups, was used to normalize turnover rates assessed by ABE. (B) Fold changes of palmitoylation levels of Gαs and Gαi normalized to calnexin at the indicated time points compared to 0 min (n=3). (C) NRVMs were treated with 10 μM ISO for indicated time points and intracellular cAMP concentration was measured using ELISA (n=5). (D) Representative single cell trace of the Fura-2 ratio in a NRVM cell treated with 10 μM ISO at the indicated time point. *p<0.05 (paired t test). This figure is modified from Chen, J. J., Marsden, A. N., Akimzhanov, A. M., & Boehning, D. (2018) The palmitoyl acyltransferases

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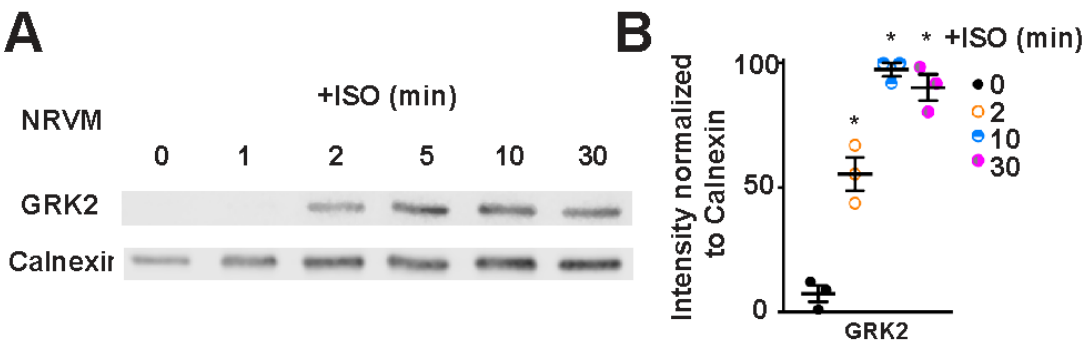

Figure 6 Isoproterenol induces rapid palmitoylation of GRK2 in primary cardiomyocytes

(A) Neonatal rat ventricular myocytes (NRVMs) are treated with 10 μM isoproterenol (ISO) for the indicated times. ABE was used to detect palmitoylation levels of GRK2 in response to ISO stimulation. This blot was generated from the same ABE reaction as shown in Figure 5. (B) Quantification of palmitoylation levels normalized to calnexin at the indicated time points. *p<0.05 (paired t test, compared to time 0). This figure is modified from Chen, J. J., Marsden, A. N., Akimzhanov, A. M., & Boehning, D. (2018) The palmitoyl acyltransferases DHHC5 mediates beta-adrenergic signaling in the heart by targeting Gα proteins and G protein-coupled receptor kinase 2. *bioRxiv* preprint. Copyrights for this article remain with the authors.

The palmitoyl acyltransferase DHHC5 mediates β-AR signaling in cardiomyocytes

I have previously shown that the plasma membrane-localized DHHC enzyme DHHC21 is responsible for rapid agonist-induced palmitoylation of components of the Fas receptor pathway in T cells [113]. Therefore, I hypothesized that the DHHC enzyme that catalyzes the ISO-induced palmitoylation in cardiomyocytes must similarly be localized to the plasma membrane. In addition to DHHC21, DHHC5 is one of the few DHHC enzymes that has been shown to be at least partially plasma membrane localized [70]. At the transcript level, DHHC5 is abundantly expressed in rat cardiomyocytes [194]. I analyzed the localization of endogenous DHHC5 in NRVMs. Immunofluorescence staining showed that DHHC5 has Golgi/ER and plasma membrane localization (**Figure 7A**). To determine whether DHHC5 is responsible for ISO-induced palmitoylation in NRVMs, I used siRNA to knockdown DHHC5 (**Figure 7B,C**). I also confirmed that siRNA knockdown of DHHC5 did not affect the total proteins level of Calnexin, Gαs, or Gαi (**Figure 7D,E**). I then performed ABE and found that agonist-induced palmitoylation of Gαs and Gαi is undetectable in DHHC5 knockdown cells (**Figure 8A-B**). Next I determined whether DHHC5 activity is required for signaling downstream of ISO stimulation in NRVMs. I found that DHHC5 knockdown cells have diminished levels of cAMP production following ISO stimulation (**Figure 8C**). Additionally, I found that DHHC5 knockdown cells no longer have an increased calcium oscillation frequency in response to ISO stimulation (**Figure 8D**). These data indicate that

DHHC5 is essential for signaling through the β-AR pathway in cardiomyocytes, and this is likely mediated at least in part by regulating the palmitoylation state of Gα proteins. To address whether my observation in cardiomyocytes are due to Gαs and Gαi being direct substrates of DHHC5, I utilized an *in vitro* assay to test DHHC5 enzymatic activity in the presence of recombinant protein substrates.

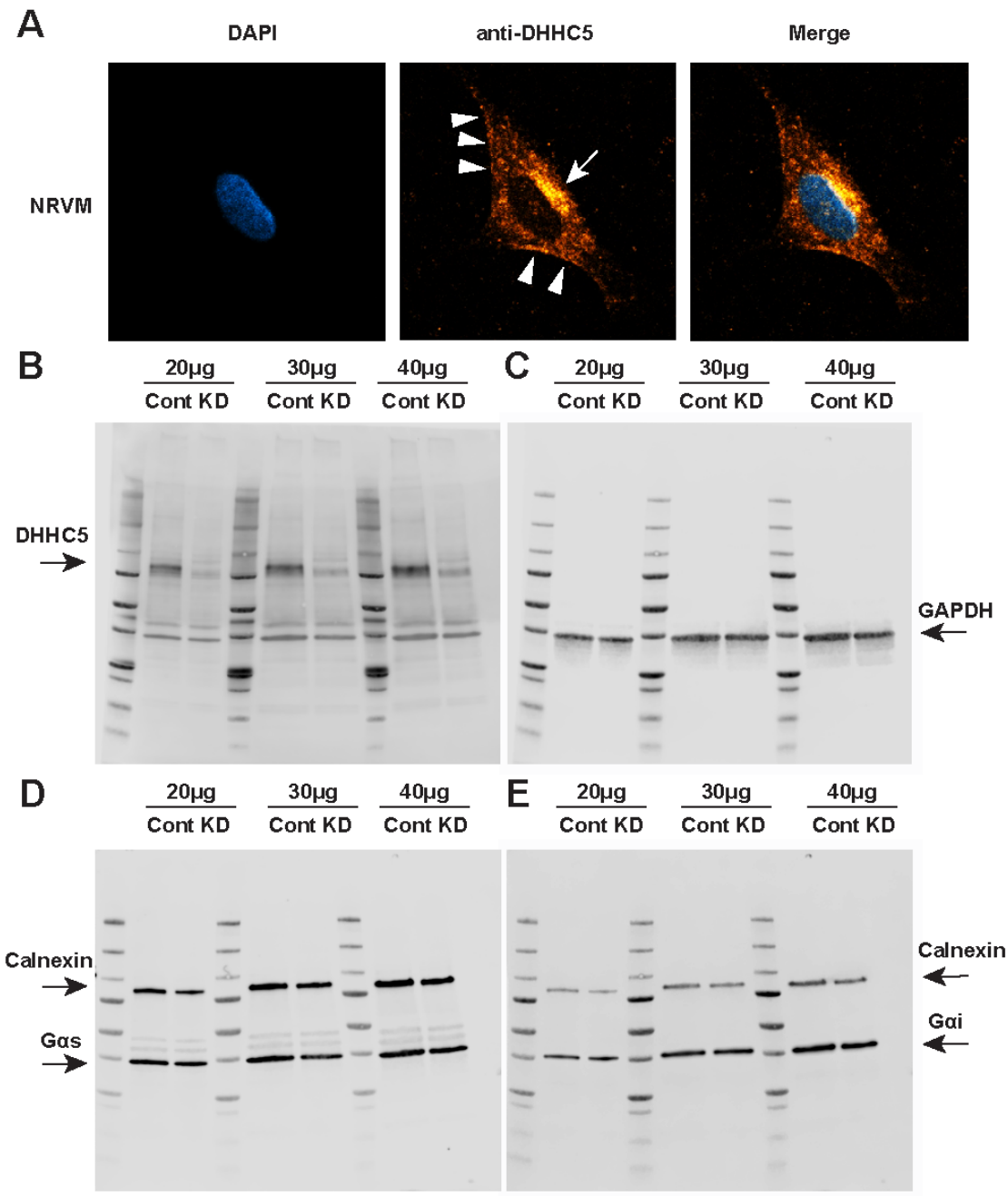

Figure 7 Localization of DHHC5 in primary cardiomyocytes

(A) Immunofluorescence staining of neonatal rat ventricular myocytes (NRVM) with anti-DHHC5. Golgi apparatus localization is indicated by arrows, and plasma membrane localization is indicated by arrowheads. 71% of all NRVM cells (n=24 from 5 samples) showed plasma membrane localization as determined by

blinded scoring. (B-E) Western blot analysis of NRVMs transfected with control (Cont) and a mixture of two siRNAs targeting DHHC5 (KD). GAPDH is used as a loading control. Same blot was stripped and re-probed with Calnexin, Gαs, and Gαi to make sure there was no off-target effect on the proteins of interest. 20-40 μg of total proteins were loaded for the indicated lanes. The same siRNAs are used to generate the data in Figure 8 of this chapter.

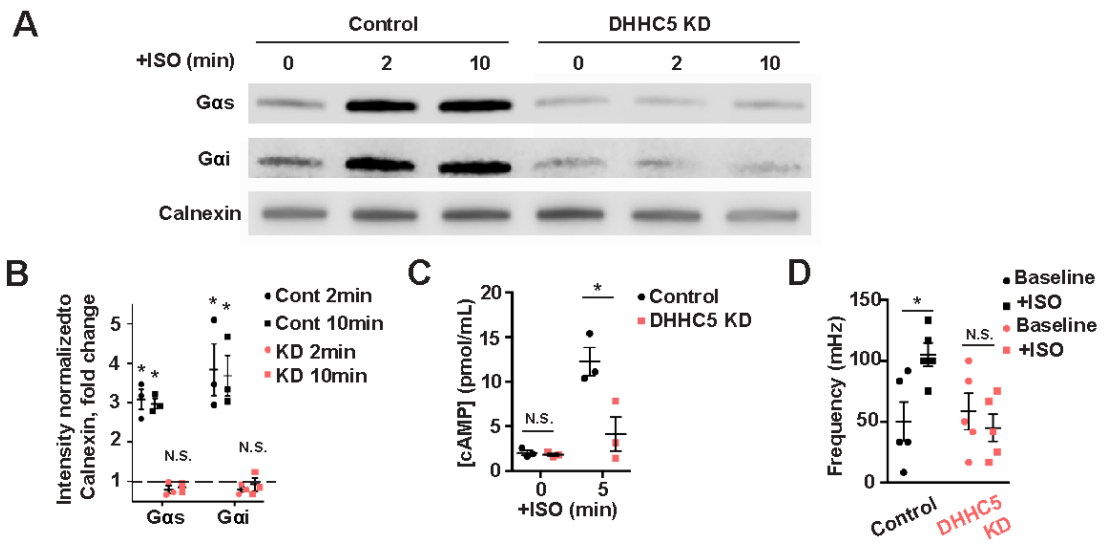

Figure 8 DHHC5 is required for signaling downstream of β-adrenergic receptor stimulation in cardiomyocytes

(A) Palmitoylation of Gαs, Gαi, and calnexin in control and DHHC5 knockdown (KD) NRVMs treated with 10 μM isoproterenol (ISO) for indicated time points. (B) Fold changes of palmitoylation levels of Gαs and Gαi normalized to calnexin at the indicated time points compared to 0 min (n=3). (C) Quantification of cellular cAMP levels before and after ISO (5 min) in control (black) and DHHC5 knockdown (red) NRVMs (n=3). (D) Quantification of beating frequency monitored by Fura-2 before (baseline) and after ISO (+ISO) in control (black) and knockdown (red) cells (n=5). *p<0.05; n.s. = not significant (paired t test). This figure is modified from Chen, J. J., Marsden, A. N., Akimzhanov, A. M., & Boehning, D. (2018) The palmitoyl acyltransferases DHHC5 mediates beta-adrenergic signaling in the heart by targeting Gα proteins and G protein-coupled receptor kinase 2. *bioRxiv* preprint. Copyrights for this article remain with the authors.

DHHC5 palmitoylates Gαs and Gαi *in vitro*

In collaboration with Dr. Marsden, I adopted a previously described *in vitro* palmitoylation assay using a fluorescently labeled palmitoyl-CoA analog, palmitoyl CoA{N-[(7-nitro-2-1,3- benzoxadiazol-4-yl)-methyl]amino} (NBD). It was used to demonstrate that purified human DHHC20 and zebrafish DHHC15 can robustly palmitoylate *Legionella* ubiquitin ligase GobX and human SNAP25B, respectively [186]. We overexpressed FLAG-tagged human DHHC5 or the catalytically inactive form DHHS5 in HEK293 cells, immunoprecipitated DHHC/S5, and performed an on-bead enzymatic assay. To establish that the NBD fluorescent signal is attached to the substrates *via* thioester bonds, we added hydroxylamine to the reaction mix as a negative control (**Figure 9**). For both no enzyme and catalytically inactive DHHS5 controls, we observed some level of autoacylation of Gα proteins, which has been reported in previous studies using [^{3}H]Palmitoyl-CoA *in vitro* (**Figure 10A,B**; [222]). In the presence of DHHC5, the rate of Gαs and Gαi palmitoylation was accelerated. In contrast, the known palmitoylated protein GAPDH [223] showed no increased palmitoylation in the presence of DHHC5 indicating substrate specificity (**Figure 10C**). Additionally, an N-terminal mCherry-fused variant of DHHC5 performed similarly to the FLAG-tagged variant validating that N-terminal tags do not appreciably impact enzymatic activity or substrate recognition of this enzyme (**Figure 11**).

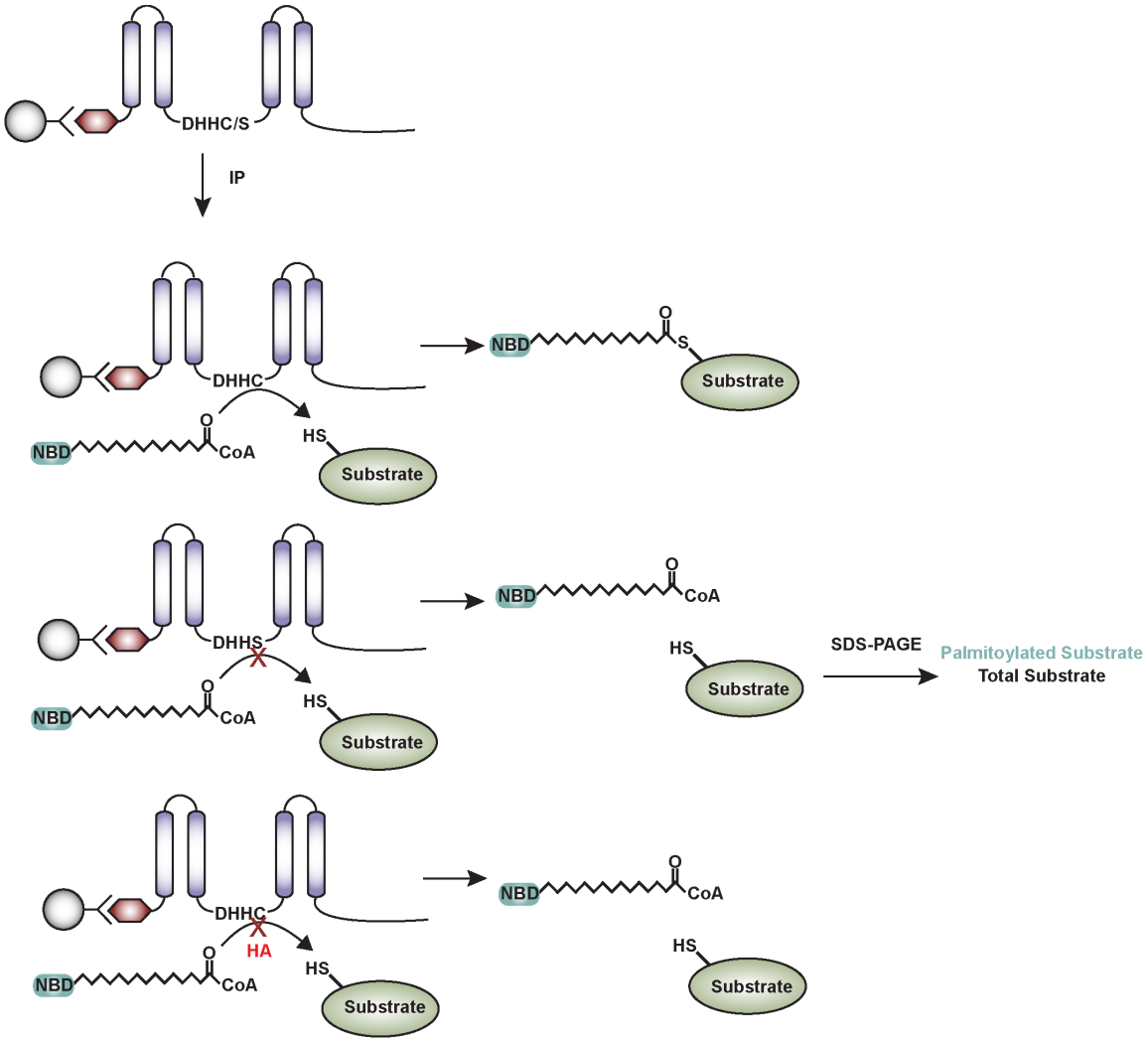

Figure 9 Procedures of in vitro assay to identify direct substrates of DHHC5

First, flag-tagged DHHC5 or catalytically inactive DHHS5 were immunoprecipitated (IP). The reaction was carried out on-bead with recombinant substrate proteins and the Palm-CoA{N-[(7-nitro-2-1,3- benzoxadiazol-4-yl)-methyl]amino} (NBD). Hydroxylamine (HA, red) was added as a negative control to ensure the NBD signal detected is link to substrates *via* thioester bonds.

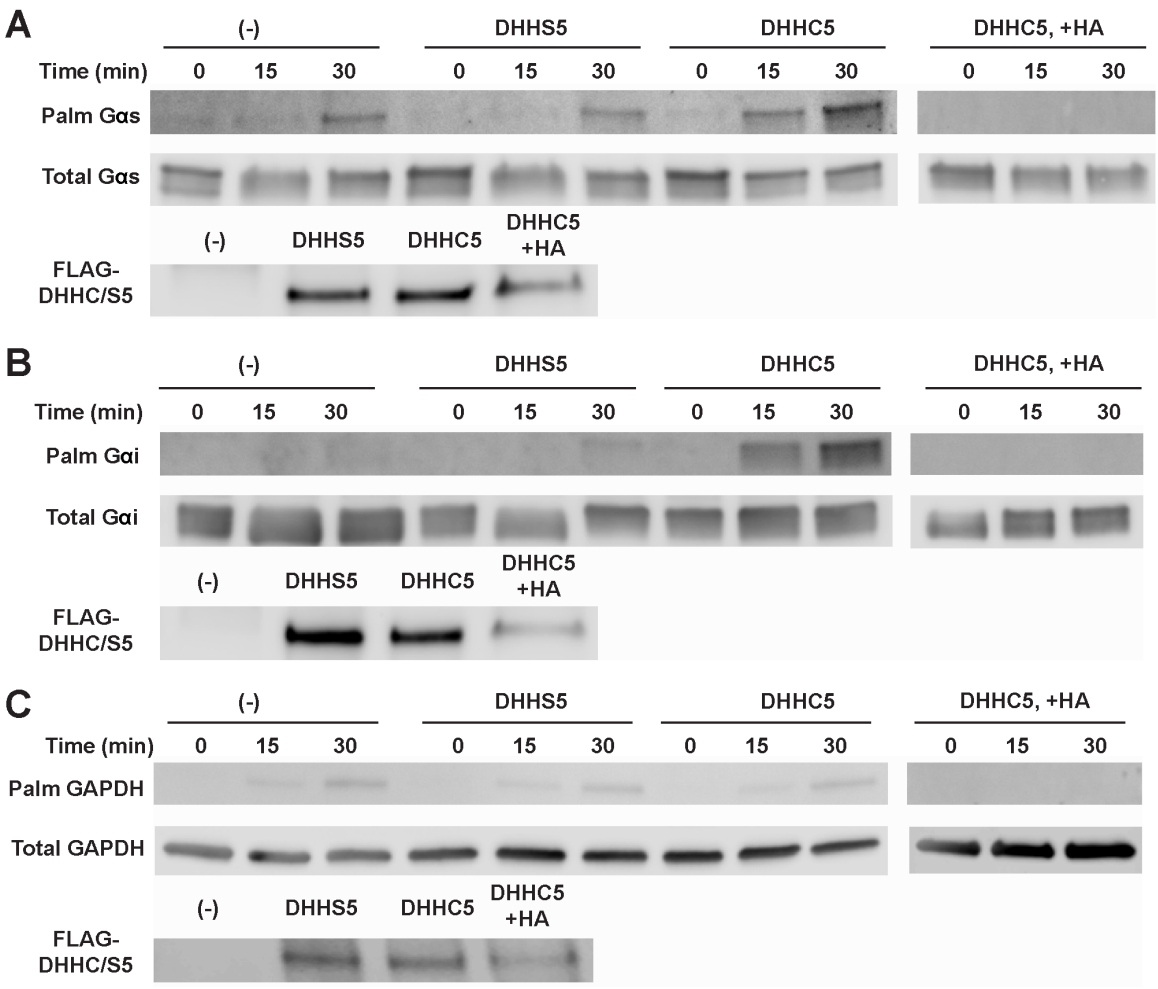

Figure 10 Gαs and Gαi are direct substrates of DHHC5.

Palmitoylation of recombinant Gαs (A), Gαi (B), and GAPDH (C) *in vitro* with mock IP (-), catalytically inactive form of DHHC5 (DHHS5), and DHHC5. As a negative control, hydroxylamine (HA) is added to cleave palmitoylation. DHHC total input as determined by Western blotting is shown below the palmitoylation panels.

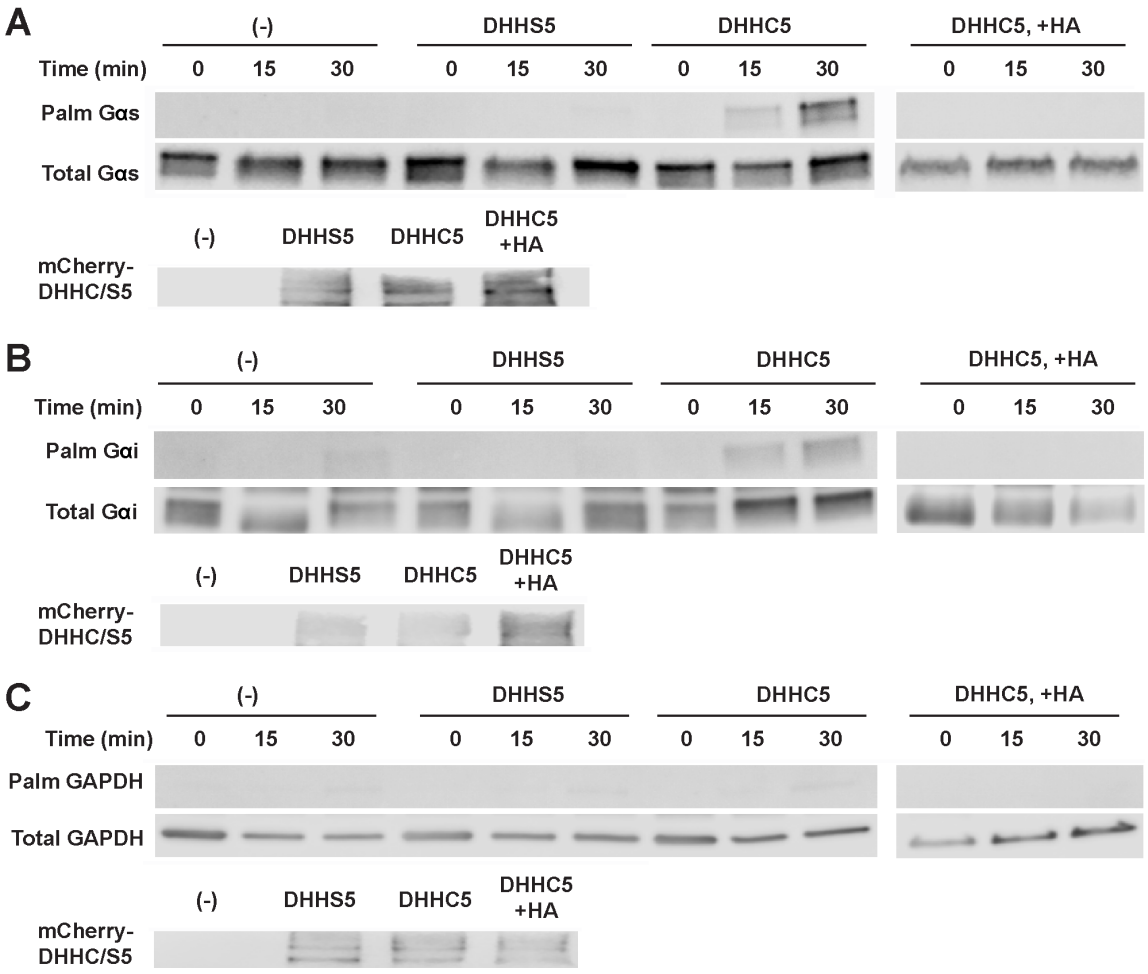

Figure 11 mCherry-DHHC5 is catalytically active

(A-C) *In vitro* DHHC assay as in Figure 4 of the main text using mCherry-DHHC5 instead of FLAG-DHHC5. Palmitoylation of recombinant Gαs (A), Gαi (B), and GAPDH (C) *in vitro* with mock IP (-), catalytically inactive form mCherry-DHHS5, and mCherry-DHHC5. As a negative control, hydroxylamine (HA) is added to cleave all thioester bonds.

Discussion

Rapid, agonist-induce palmitoylation in signaling transduction

Palmitoylation is the only reversible lipid modification, and a growing list of signaling proteins are known to undergo cycles of palmitoylation and depalmitoylation [224, 225]. Recently, our group showed that the tyrosine kinase Lck can be palmitoylated within a minute of Fas ligand stimulation in T cells, suggesting that palmitoylation plays key role in rapid signal transduction analogous to other post-translational modifications such as phosphorylation [113]. Multiple members of the Gα protein family, including Gαs and Gαi, have been shown to be palmitoylated in the N-terminus [170, 219]. In cell lines, overexpression of Gαs and Gαi palmitoylation mutants showed defects in membrane association [171, 226]. Stimulation of β-ARs in kidney fibroblast-like COS cells and S49 lymphoma cells did not lead to detectable changes in Gα protein palmitoylation levels using high performance liquid chromatography (HPLC) [123], though an earlier study found that [^{3}H]palmitate had increased turnover after ISO stimulation [122]. One possible explanation is that [^{3}H]palmitate loading only labels a small fraction of the total Gα pool, which cannot be detected using HPLC. Recent advances in palmitoylation detection methods allow us to better study dynamic palmitoylation of endogenous Gα proteins with endogenous lipids and their functional outcomes in specific cell types. My data demonstrate extremely rapid palmitoylation kinetics of Gα proteins following activation of the β-ARs and its temporal correlation with downstream events in cardiomyocytes. The requirement of palmitoylation for β-

AR signaling were functionally demonstrated by transient knockdown of DHHC5, which reduced or eliminated the majority of downstream effects of ISO stimulation in cardiomyocytes.

DHHC5 is a novel regulator of cardiac functions

Few studies have examined the cellular function of DHHC5 and its link to human diseases. One of the known substrates of DHHC5 is the neuronal scaffolding protein PSD-95, which is enriched at the postsynaptic sites in neurons [227]. Mice homozygous for a hypomorphic allele of *Zdhhc5* gene showed defects in learning and memory [227]. Curiously, PSD-95 palmitoylation levels are not decreased in the hypomorphic mice, likely because PSD-95 can also be palmitoylated by other DHHC enzymes in neurons [228]. Some of the homozygous DHHC5 hypomorphic mice show lethality, suggesting its role in early development [227]. Cardiomyocytes isolated from these mice showed reduced massive endocytosis, a process that happens during re-oxygenation of anoxic hearts [197]. Phospholemman and the cardiac Na/Ca exchanger were identified as potential substrates of DHHC5 in regulating massive endocytosis [197]. Several other studies on cardiac functions of DHHC5 also focus on its substrate phospholemman in regulating sodium pumps in adult ventricular myocytes [193, 194]. My data identify β-AR signaling, a pathway that is closely associated with heart failure and is a major therapeutic target in cardiovascular diseases, as a key target for the enzymatic action of DHHC5.

Efficient detection of DHHC enzyme substrates *in vitro*

Several methods have been used to study palmitoylation of a specific substrate by a DHHC enzyme of interest. For example, in the first study that identified yeast transmembrane protein Akr1p to be a PAT, an *in vitro* assay was used to showed that Akr1p can palmitoylate the type I casein kinase Yck2p [184]. This method utilized [^{3}H]palmitoyl-CoA, purified Yck2p and Akr1p, in the presence of ATP, MES, and bovine liver lipids [184]. Recently, a radiolabeling-free method was used to show that purified human DHHC20 and zebrafish DHHC15 palmitoylate ubiquitin ligase GobX and SNAP25B, respectively [187]. A dye conjugated to palmitoyl-CoA was used instead of [^{3}H]palmitoyl-CoA. My study modified this method further by performing an on-bead assay after immunoprecipitating DHHC5, thus eliminating the necessity of having purified DHHC enzymes. Additionally, my method showed substrate specificity because DHHC5 did not palmitoylate GAPDH, a known palmitoylated protein.

Chapter IV: The H9c2 cell line as an alternative to study protein palmitoylation in β-AR pathways

Introduction

The H9c2 cell line is derived from BDIX rat ventricular heart tissue after selective serial passages [229]. The original study described that H9c2 cells are multinucleated, non-beating, spindle-shaped cells that show morphological, biochemical, and electrophysiological features of muscle cells. Specifically, electron microscopy revealed that the T-tubule structures of H9c2 cells were more similar to those of skeletal muscle cells but not cardiac muscle cells [229]. One later study found that the majority of H9c2 cells are mononucleated, and did not have gap junctions, caveolae, or T-tubules [230]. Further examination of the electrophysiological properties revealed that after couple of weeks in culture, H9c2 cells show inward voltage-dependent calcium currents similar to that of cardiac muscle cells but not skeletal muscle cells. They also showed that H9c2 cells responded to isoproterenol stimulation and increased intracellular cAMP [230]. Therefore, many studies focusing on cardiac signaling pathways have since used H9c2 cells as a model. For example, one study found that H9c2 cells, similar to NRVMs, display hypertrophic responses to both angiotensin II and endothelin-1 [231].

Interestingly, H9c2 cells have 30% β_1-AR and 70% β_2-AR, a ratio that is the opposite of that in rat heart tissue, but similar to that in human heart [232]. In response to prolonged β-AR stimulation, β_2-AR was down regulated both at the

protein level and at the mRNA level [232], similar to the observation in NRVMs that β_2-ARs were reduced at the cell surface [233]. Despite the lack of caveolae in H9c2 cells [230], one recent study showed that β_1-AR and β_2-AR are confined to certain regions of the plasma membrane, and their interactions with anchoring proteins are crucial for these localization patterns [234]. Given the similarities of H9c2 cells with other cardiac cell models, I am interested in using it as an alternative model to study DHHC5-mediated palmitoylation of Gα proteins. One study showed that distinct classes of proteins can be palmitoylated in H9c2 cells using [^{3}H]palmitate labeling [235]. However, the identities of those proteins remain uncharacterized. Additionally, the localizations and functions of DHHC enzymes in H9c2 cells remain unknown.

Results

Activation of β-ARs does not induce rapid protein palmitoylation in H9c2 cells

Previous studies have shown that H9c2 cells are prone to further differentiation when maintained in culture [236]. To verify that my culture of the H9c2 cells still possess cardiac muscle properties, I stimulated the H9c2 cells with endothelin-1 (ET-1), a peptide released by endothelial cells that stimulates the ET_A and the ET_B receptors [237]. Previous study showed that H9c2 cells expressed high level of ET_A receptors and showed calcium release from the SR storage in response to ET-1 stimulation, a process mediated by the RyRs [238]. I used Fura-2 AM to monitor intracellular calcium level in live H9c2 cells. After

stimulation with ET-1, in over 70% of the cells imaged we observed a calcium transient (**Figure 12A**). Next, I want to study if there is stimulus-dependent rapid palmitoylation of Gα proteins after ISO stimulation in H9c2 cells, like I observed in NRVMs. I stimulated the cells with 10 μM ISO and performed ABE at the indicated time points. I observed no change in palmitoylation levels of either Gαs or Gαi proteins (**Figure 12B**). Downstream of the β-AR signaling pathway, I found no significant change in cAMP levels after ISO stimulation (**Figure 12C,D**). Additionally, there is no increase in intracellular calcium levels after ISO stimulation (**Figure 12E**). These data suggested that despite the presence of the β-AR signaling pathway, there is no rapid palmitoylation or inotropic responses to ISO stimulation in H9c2 cells like those observed in NRVMs.

Next, I investigated if DHHC5 has similar localization patterns in H9c2 cells. Immunofluorescence staining showed that unlike in NRVMs, DHHC5 has only Golgi/ER but no plasma membrane localization in H9c2 cells (**Figure 13A**). These data combined suggested that H9c2 is not an ideal model for studying rapid palmitoylation or more generally, contractile responses following β-AR stimulation.

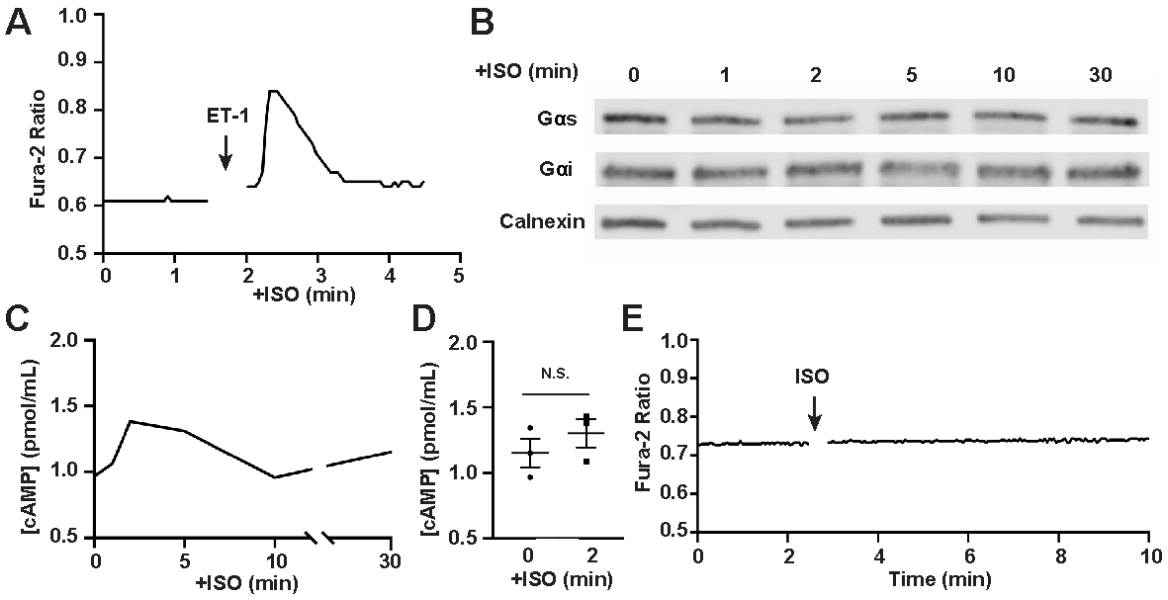

Figure 12 Isoproterenol induces no increased palmitoylation of Gα proteins in H9c2 cells

(A) Representative single cell trace of the Fura-2 ratio in a H9c2 cell treated with 1 μM endothelin-1 (ET-1) at the indicated time point. (B) H9c2 cells were treated with 10 μM isoproterenol (ISO) for the indicated times. ABE was used to detect palmitoylation levels. (C) Representative trace of H9c2 cells treated with 10 μM ISO for indicated time points and intracellular cAMP concentration was measured using ELISA. (D) Quantification of cellular cAMP levels before and after ISO (2min) in H9c2 cells (n=3). (E) Representative single cell trace of the Fura-2 ratio in a H9c2 cell treated with 10 μM ISO at the indicated time point.

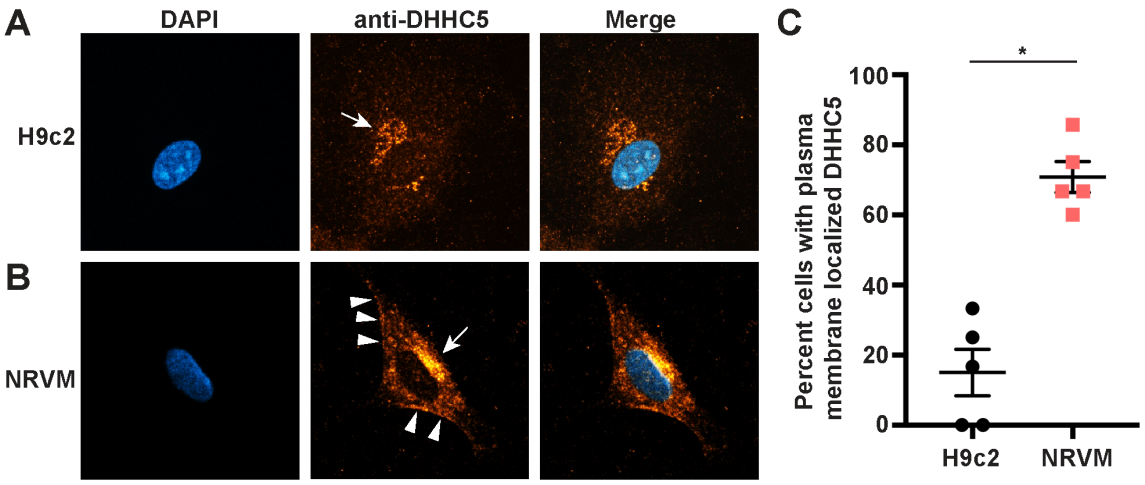

Figure 13 Localization of DHHC5 in H9c2 cells and NRVMs

Immunofluorescence staining of H9c2 cells (A) and neonatal rat ventricular myocytes (B) with anti-DHHC5. Golgi apparatus localization is indicated by arrows, and plasma membrane localization is indicated by arrowheads. (C) Quantification of the percentage of cells with plasma membrane localize DHHC5 (n=5, * $p<0.05$, unpaired T test).

Identifying potential cardiac protein palmitoylation sites in H9c2 cells

My ABE data showed that in h9C2 cells, GRK2 was palmitoylated with no ISO stimulation (**Figure 4A**). Since there was no previous report on GRK2 being a palmitoylated substrate, we used Acyl-PEG exchange (APE, **Figure 14A**) assay [203] to find the number of potential palmitoylation sites on GRK2. APE shares similar steps with ABE—free cysteine sites were first blocked using NEM, followed by cleaving the palmitoylation site. Then a 5 kDa PEG was added to the palmitoylation site and analyzed after Western blotting. Calnexin has two palmitoylation sites [215] and was used as a positive control. I found that Gαs and Gαi showed one single shift in molecular weight, consistent with previous findings that Gαs and Gαi are palmitoylated at one single cysteine at the N-terminus (**Figure 14B**; [170]). I found that there was a single shift of GRK2 protein indicating that there is only one palmitoylation site (**Figure 14B**). However, further mutagenesis study is still needed to identify the palmitoylation site.

Discussion

In **Chapter III** we showed that in NRVMs, ISO stimulation leads to rapid palmitoylation of Gα proteins by DHHC5. Despite previous studies reporting that the β-ARs and their downstream signaling proteins are present in H9c2 cells, I do not find similar rapid palmitoylation or downstream inotropic effects in my study. Since I proposed that rapid palmitoylation of Gα proteins recruits them

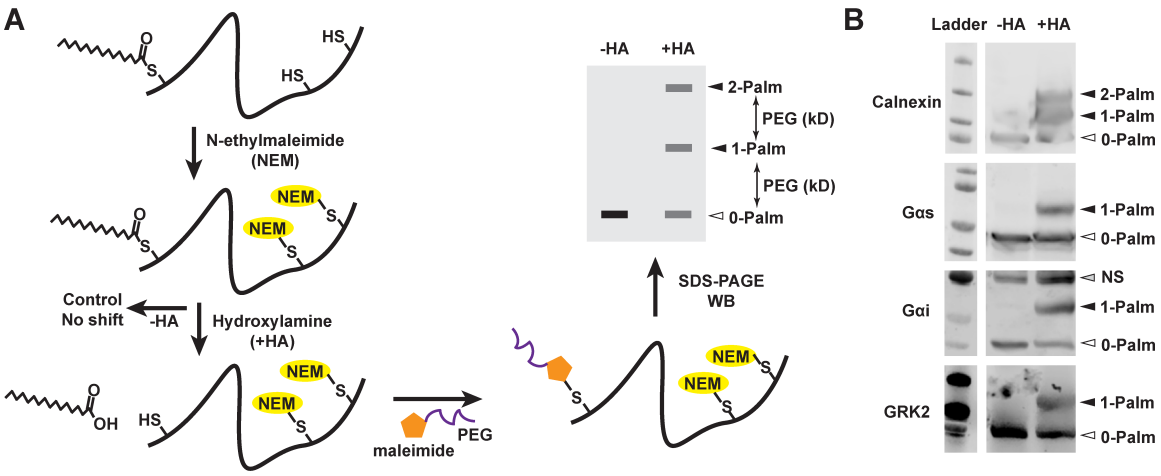

Figure 14 Identifying number of palmitoylation sites using Acyl-PEG exchange assay

(A) Schematics of acyl-PEG exchange (APE) assay. The free cysteine sites are blocked, followed by cleaving the thioester bond at the palmitoylation site, and addition of a mass-tag of certain size. (B) APE assay using 5kD PEG on H9c2 cells. Calnexin was used as positive control. White arrowheads indicate non-palmitoylated proteins. Black arrowheads indicate palmitoylated proteins. Grey arrowheads indicate non-specific background signals that are equal in -HA and +HA lanes (NS).

into membrane subdomain caveolae for efficient signaling transduction, it is possible that the lack of caveolae in H9c2 cells [230] lead to the absence of such mechanisms. Additionally, I also found that endogenous DHHC5 is almost exclusively localized to the Golgi/ER membrane, but not on the plasma membrane in H9c2 cells. Though I cannot exclude the possibility that other plasma membrane localized DHHC enzymes (DHHC20 or 21) may be able to mediate rapid palmitoylation near the plasma membrane, it is clear that the mechanisms of ISO-induced rapid signaling events are absent in H9c2 cells, and therefore it is not a suitable model for studying stimulus-dependent palmitoylation downstream of β-AR signaling.

Since GRK2 is palmitoylated without stimulation in H9c2 cells, I was able to identify a single palmitoylation site on the protein. Previous studies have shown that two other GRK family proteins, GRK4 and 6, are palmitoylated [178, 179]. GRK6 is palmitoylated at a cluster of cysteines near the C-terminal end at position 561, 562, and 565 [178]. Based on an analogous alignment, GRK4 is predicted to be palmitoylated at position 561 and 578 [179]. However, these sites are not conserved in GRK2 and therefore further studies are required for identifying the palmitoylation site(s).

Chapter V DHHC5 is regulated by palmitoylation and phosphorylation of the C-terminal tail

Introduction

DHHC5 has a ~500 amino acid (more than 70% total length of the protein) C-terminal tail that is proposed to contain regulatory components that are crucial for substrate recognition, localization, activation, and other potential functions [194, 198, 199]. Truncation mutations of DHHC5 showed that the region between amino acids 218 and 334 is necessary for recognizing substrates including phospholemman and flotillin 2 [194]. In neurons, the tyrosine kinase Fyn phosphorylates DHHC5 at Y533 and stabilizes it at the synapse by forming a DHHC5/Fyn/PSD-95 protein complex [198]. In adult mouse forebrain extracts, DHHC5 was found to be palmitoylated at three potential cysteines in the C-terminus tail, some of which are conserved in other DHHC enzymes [199]. One recent study showed that DHHC6 is palmitoylated in the C-terminal tail by DHHC16, revealing a novel palmitoylation cascade to fine-tune DHHC activities [190]. However, the functional consequences of DHHC5 C-terminal tail palmitoylation remain largely unknown.

Palmitoylation of various transmembrane proteins has been shown to affect their localizations in membrane subdomains or their interaction with other transmembrane proteins [64, 239-241]. To elucidate the potential functional outcomes of DHHC5 palmitoylation specifically at the plasma membrane, we need to exclude the population of DHHC5 proteins localized at the Golgi apparatus membrane. Therefore, we utilized total internal reflection fluorescence

(TIRF) microscopy to monitor fluorescently tagged DHHC5 proteins in live cells. This technique is based on the theory of total internal reflection at the interface between the coverslip (glass) and the sample (cells in media) when the light source hits at a specific angle (critical angle) [242]. The reflection results in an electromagnetic field generating the evanescent wave that excites only the fluorophores close to the coverslip [243]. The thickness of the evanescent field is dependent on the wavelength of the laser, illumination angle, and other factors [242]. Some of the advantages of TIRF microscopy compared to traditional epifluorescence microscopy include less photobleaching, little out-of-focus fluorescence signals, and higher signal-to-noise ratio. TIRF microscopy, sometimes combined with other techniques such as fluorescence resonance energy transfer, has been used to monitor membrane protein properties including their lateral mobility, clustering, and endocytosis in live cells [244-247]. In live neonatal and adult ventricular myocytes, TIRF microscopy has been used to monitor dynamic SR formation and rearrangements, ryanodine receptor localization and clustering, and L-type calcium channel localization [248, 249]. Therefore, we believe that TIRF microscopy is useful in assessing the dynamic localization of DHHC5 in NRVMs.

Results

The C-terminal tail of DHHC5 is palmitoylated upon stimulation of β-ARs to modulate downstream signaling in cardiomyocytes

DHHC5 contains a C-terminal tail that can be a target for post-translational modifications to modulate enzyme structure/function (**Figure 15**) [250]. Three potential palmitoylation sites in the C-terminal tail of DHHC5 have previously been identified in mouse forebrain extracts (highlighted in **Figure 15**) [199]. In order to determine if these sites are palmitoylated in NRVMs, I created single mutants that changes Cys 236 or 245 to Ser, double mutant that changes both Cys 236 and 245 to Ser, and removed all three potential palmitoylation sites by deleting amino acids 236-245 (refer to as Δ236-245). DHHC enzymes are autocatalytically modified at the active site with palmitate [185], and thus I performed all ABE experiments using the catalytically dead mCherry-DHHS constructs expressed in NRVM to only examine palmitoylation in the C-terminal tail. I observed that the single mutants, the double mutant, and the deletion mutant all showed no palmitoylation in the C-terminal tail (**Figure 16A)**. These data suggest that all three sites are palmitoylated and the absence of any one site interferes with the palmitoylation at other sites. Next, Ie examined whether the palmitoylation of the three Cys in the C-terminal tail changes after ISO stimulation. I performed an ABE time course on the catalytically dead mCherry-DHHS5 and mCherry-DHHSΔ236-245. I found that DHHS5 is palmitoylated within one minute upon ISO stimulation, while this is lost in the DHHSΔ236-245 mutant (**Figure 16B**). This indicates that the C-terminal tail of DHHC5 is very

rapidly palmitoylated in this cluster of Cys residues upon β-AR stimulation. To address the functional outcomes of DHHC5 tail palmitoylation upon stimulation, I knocked down DHHC5 in NRVMs and rescued downstream cAMP production with both wild-type mCherry-DHHC5 and mCherry-DHHC5Δ236-245 (**Figure 17A**). I found that wild-type mCherry-DHHC5 restored cAMP production following ISO stimulation, while mCherry-DHHC5Δ236-245 was incapable of rescuing cAMP production (**Figure 17B**). Therefore, I conclude that palmitoylation of the DHHC5 C-terminal tail is crucial for β-AR signaling. In collaboration with Dr. Marsden and Dr. Scott, I next tested the hypothesis that DHHC5 palmitoylation in the C-terminal tail leads to sequestration in membrane microdomains where it can assemble with other components of the β-AR signaling pathway to facilitate downstream signaling [149, 251, 252].

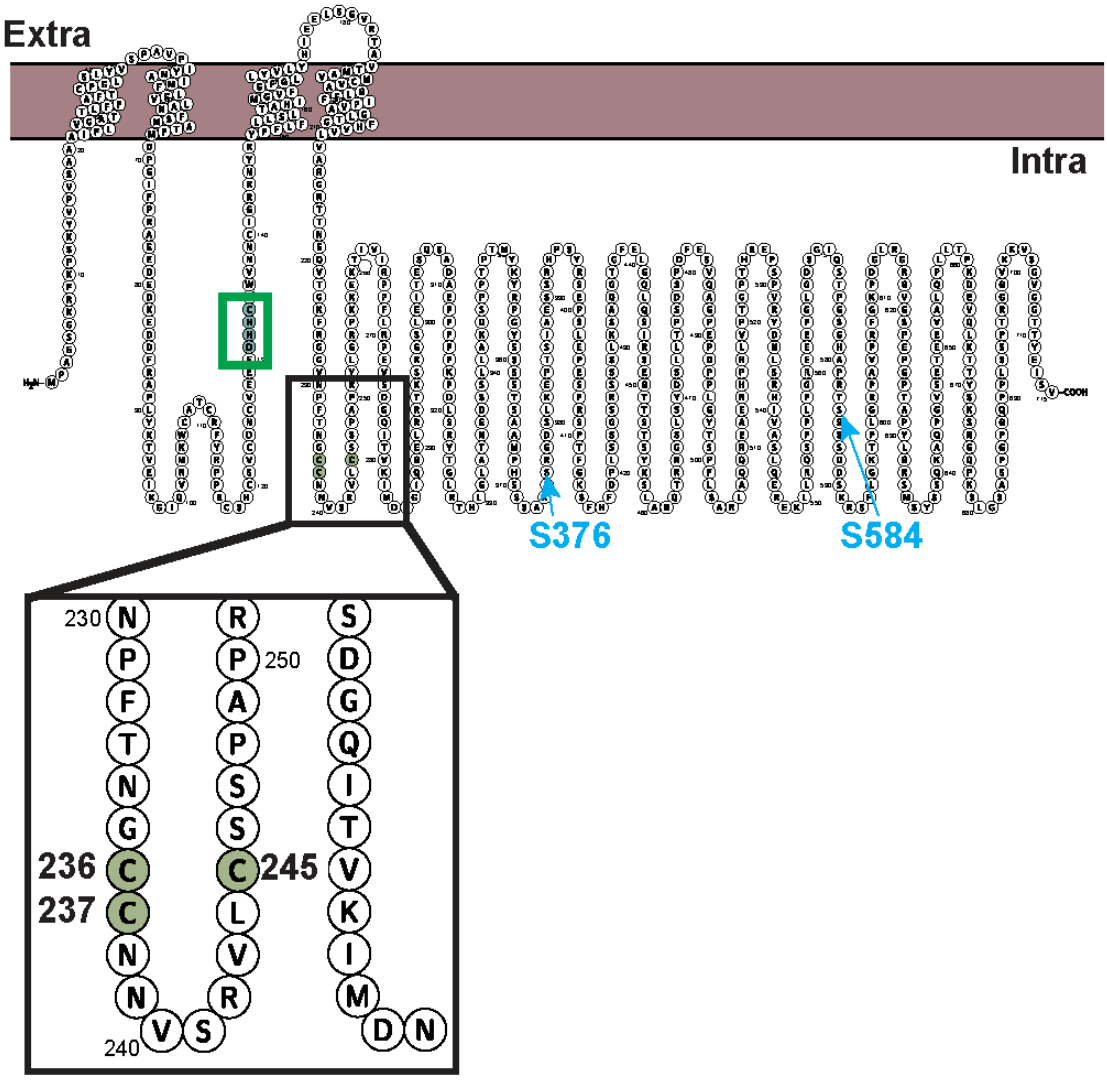

Figure 15 Schematic of DHHC5 protein and the potential posttranslational modifications in the C-terminal tail

Schematic of DHHC5 created by Protter [250]. The catalytic site DHHC is highlighted in the green box. Three potential palmitoylation sites (236, 237, and 245) within the tail are enlarged and highlighted. Serine 376 and Serine 584, at which truncation mutants have been made, are heighted (blue arrows).

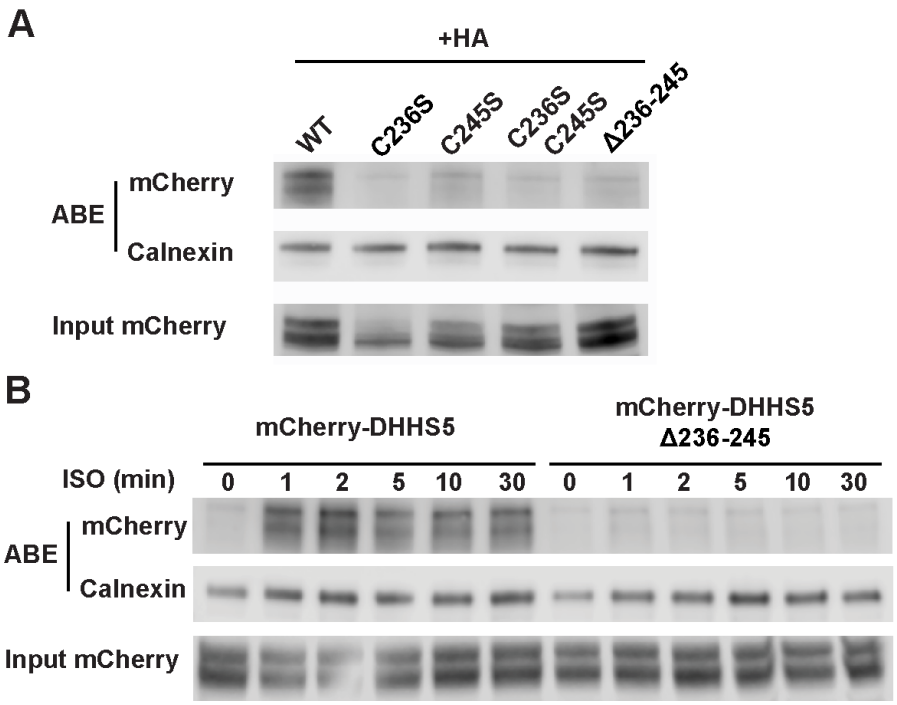

Figure 16 DHHC5 is palmitoylated at cysteines position 236, 237, and 245 after ISO stimulation

(A) ABE was used to detect palmitoylation of mCherry-DHHS5 wild-type (WT), Cys 236 to Ser (C236S), Cys 245 to Ser (C245S) single mutants, Cys 236 & 245 to Ser double mutant (C236S C245S), and Cys 236 to Cys 245 deletion mutant (Δ236-245) in NRVMs. (B) Palmitoylation of mCherry-DHHS5 and mCherry-DHHS5Δ236-245 in NRVMs treated with 10 μM isoproterenol (ISO) for indicated time points. Calnexin is used as positive control.

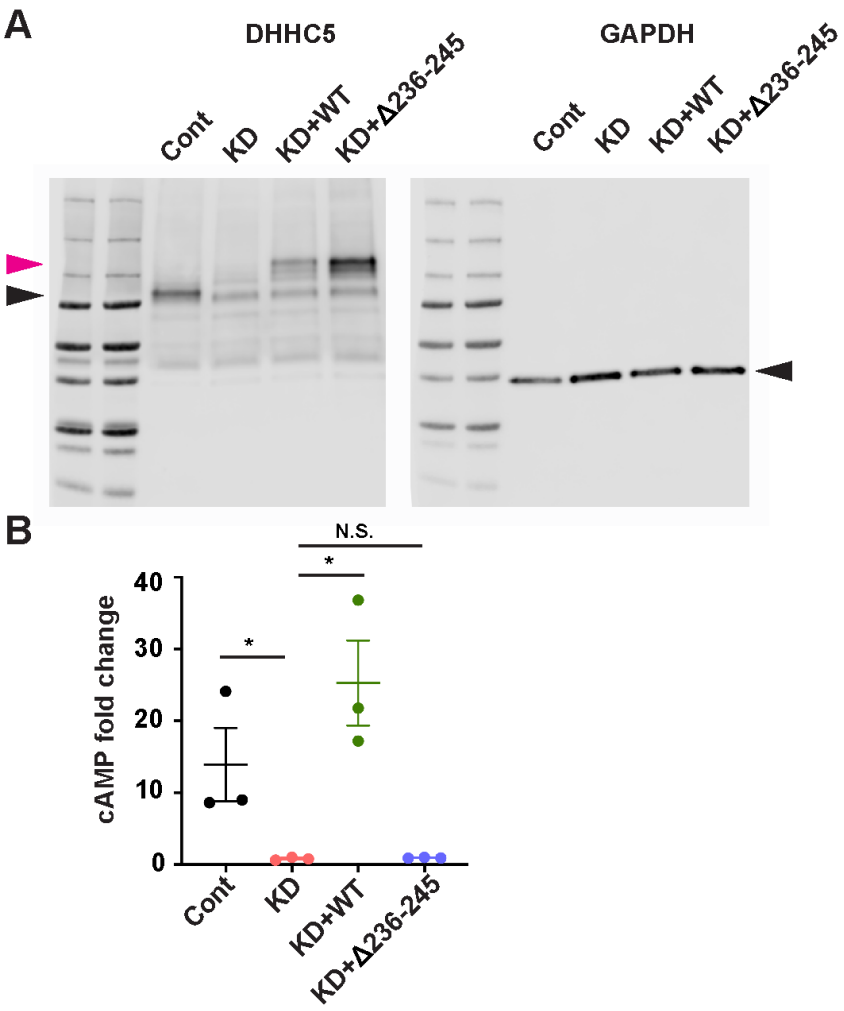

Figure 17 Palmitoylation of the C-terminal tail of DHHC5 regulates signaling in NRVM

(A) Western blot analysis of NRVMs transfected with control siRNA (Cont), siRNA targeting DHHC5 (KD), siRNA targeting DHHC5 and wild-type mCherry-DHHC5 (KD+WT), siRNA targeting DHHC5 and mCherry-DHHC5Δ236-245 (KD+Δ236-245). Endogenous DHHC5 and overexpressed mCherry-DHHC5 are indicated by black and red arrowheads (left), respectively. GAPDH (black arrowhead, right) is used as a loading control. (B) Quantification of cellular cAMP

levels in control (black), knockdown (red), and knockdown and rescue with either wild-type mCherry-DHHC5 (green) or mCherry-DHHC5Δ236-245 (blue) in NRVMs. *$p<0.05$; N.S. = not significant (paired t test, n=3).

Palmitoylation of the C-terminal tail of DHHC5 regulates stimulation-dependent plasma membrane motility

To determine the effect of palmitoylation on DHHC5 membrane motility, Dr. Marsden, Dr. Scott and I utilized total internal reflection fluorescence (TIRF) microscopy [243]. We imaged live, beating NRVMs transfected with mCherry-DHHC5 and mCherry-DHHC5Δ236-245. Both proteins were expressed in the TIRF plane as laterally mobile puncta of various fluorescent intensities presumably representing either single molecules or oligomers (**Figure 18A-B**) [72, 253]. First, we confirmed that mCherry-DHHC5Δ236-245 can localize to the plasma membrane appropriately like the wild-type proteins, with similar number of observed particles in the TIRF plane (**Figure 18C**). We then examined the lifetime of mCherry-DHHC5 particles in the TIRF plane to examine whether there is stimulus-dependent removal or insertion of DHHC5 into the plasma membrane. To accomplish this we used kernel density estimate analysis [254, 255] to calculate the lifetime of particles before and after 10 min of ISO stimulation (**Figure 18D-E**). When comparing the particle distributions before and after ISO stimulation, we observed a shift to longer lived particles after β-ARs were activated (**Figure 18D**). In contrast, the mCherry-DHHC5Δ236-245 showed the opposite result (**Figure 18E**). Thus, ISO stimulation led to the stabilization of DHHC5 particles at the membrane, an effect that is lost in the palmitoylation deficient DHHC5 mutant.

TIRF imaging has been widely used to examine lateral mobility of proteins in response to receptor stimulation in multiple systems [256-258]. We next

examined the lateral mobility of DHHC5 in the plasma membrane before and after ISO stimulation. To quantify this, we calculated the mean squared displacement (MSD) of all particles present before and after 10 min of ISO stimulation. MSD calculates the summation of particle movement over time [259]. Stalling particles will show a straight horizontal line as a function of time, while those moving quickly will show a steep slope. We found that the MSD of mCherry-DHHC5 particles remains essentially unchanged before and after ISO stimulation indicating no change in lateral mobility (**Figure 18F**). In contrast, the mCherry-DHHC5Δ236-245 particles have an increase in the MSD after ISO stimulation indicative of increased mobility (**Figure 18G**). In total, these data indicate that DHHC5 is palmitoylated in the C-terminal tail after ISO stimulation (**Figure 16B**), leading to stabilization in the plasma membrane (**Figure 18D,F**). The DHHC5Δ236-245 mutant cannot be palmitoylated (**Figure 16B**), leading to decreased lifetime at the membrane and increased motility (**Figure 18E,G**), presumably reflecting increased ISO-stimulated removal from the TIRF plane. Thus, palmitoylation of the DHHC5 C-terminal tail is critical for maintaining the enzyme at the membrane after β-AR stimulation with potentially significant consequences for palmitoylation-dependent signaling cascades.

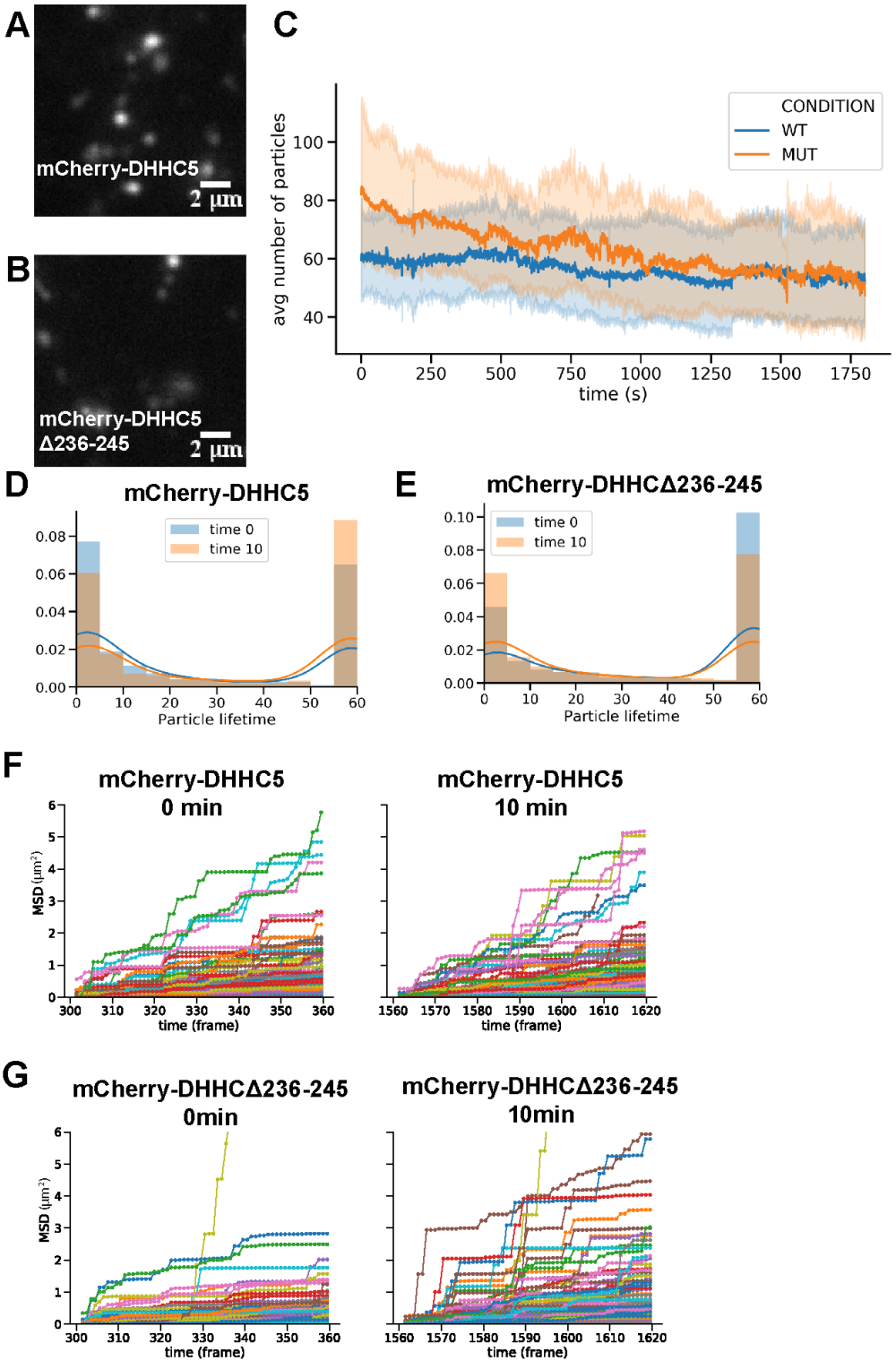

Figure 18 Palmitoylation of the C-terminal tail of DHHC5 regulates stimulation-dependent plasma membrane motility

TIRF images of live NRVM transfected with mCherry-DHHC5 (A) and mCherry-DHHC5Δ236-245 (B). (C) Average number of particles in the TIRF planes over the 10 minute experiments for mCherry-DHHS5 (blue) and mCherry-DHHC5Δ236-245 (orange). 95% confidence intervals are shown as corresponding shaded areas and there is no difference because of the overlaps. (D-E) Histogram with kernel density estimation (KDE) of the life-time of particles at 0 (blue) and 10 min (orange) after isoproterenol (ISO) stimulation. (F-G) Mean-squared displacement (MSD) shown in 30 second windows of particles that exist continuously from 0 to 10 min of ISO stimulation. Each line represents one particle. n=377 for mCherry-DHHC5 (D and F) and n=221 for mCherry-DHHC5Δ236-245 (E and G).

The C-terminal tail of DHHC5 is phosphorylated in response to stimulation

In addition to palmitoylation in the C-terminal tail, a previous study that performed *in vivo* phosphoproteomics analysis in murine hearts also identified multiple potential phosphorylation sites in DHHC5, including Ser at position 380, 425, 432, 554, and 636 [260]. Additionally, they observed that the phosphorylation of Ser 636 is up regulated in response to β_1AR stimulation, potentially targeted by PKA [260]. Therefore, Dr. Marsden and I investigated whether the C-terminal tail of DHHC5 is phosphorylated in NRVM after β-AR stimulation. We used ISO and forskolin, a direct activator of ACs [261], and found that endogenous DHHC5 proteins showed increased phosphorylation levels after both stimulations (**Figure 19A**). The C-terminal tail of DHHC5 has 79 serine residues. To narrow down the potential phosphorylation sites, we created truncation mutants of mCherry-DHHC5 that change Ser 584 and 376 (highlighted in **Figure 15**) to stop codons. Overexpression of the mutant proteins in NRVM after ISO stimulation showed that DHHC5 is phosphorylated between position 376 and 583 (**Figure 19B**). There are 38 serines in between position 376 and 583. Therefore, further studies are still required to pinpoint the positions of phosphorylated Ser site(s) on DHHC5, identify the kinase(s) and phosphatase(s) that regulate its phosphorylation levels, and how phosphorylation affects DHHC5 activity in NRVM.

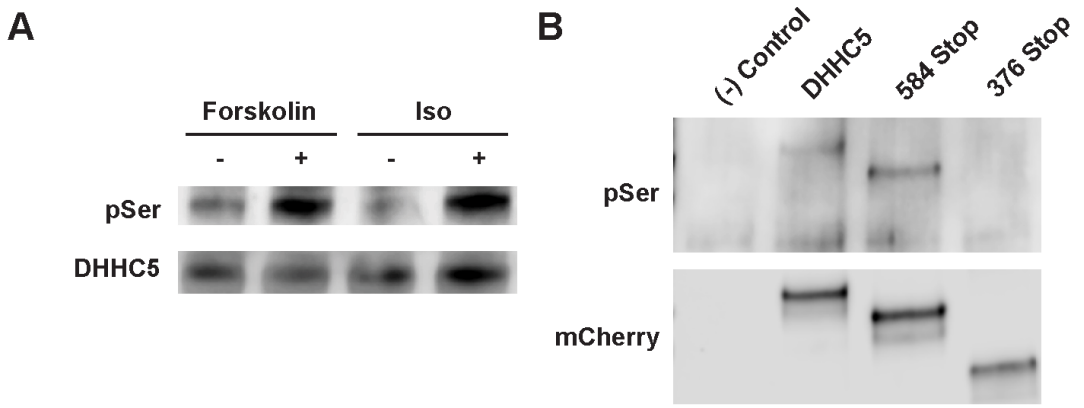

Figure 19 DHHC5 is phosphorylated in response to β-AR stimulations

(A) Western blot analysis using phospho-serine specific antibodies (pSer) on immuno-precipitated endogenous DHHC5 proteins from NRVMs stimulated with 10 μM forskolin or isoproterenol (Iso) for 5 minutes. (B) NRVMs are not transfected (-) Control, or transfected with wild-type mCherry-DHHC5 (DHHC5), truncated mCherry-DHHC5 where Ser 584 (584 Stop) or Ser 376 (376 Stop) is mutated to a stop codon. After Iso stimulation, mCherry-DHHC5 proteins are immunoprecipitated and analyzed using pSer antibodies. mCherry antibodies were used to detect total protein levels.

Discussion

Lipid subdomains and beta-adrenergic signal transduction in the heart

Lipid subdomains have been proposed to create signaling centers to facilitate both signal propagation and inhibition. One of the lipid subdomains present within many cell types is caveolae. Within cardiomyocytes, caveolae are composed of caveolin-3, cholesterol, and sphingolipids. Caveolin proteins have been proposed to create a scaffold, allowing signaling complexes to form at the membrane. Multiple components of β-AR signaling have been identified in caveolae, such as β2-AR, Gαs, AC5/6 and Gαi [149, 251, 252], indicating that caveolae are central organizers of this pathway. DHHC5 itself has been shown to be present in the caveolae, allowing for direct palmitoylation of phospholemman [194]. Proteomic studies identified that palmitoylated proteins are predominantly found in caveolae, suggesting that this is a better predictor of caveolae localization than caveolin binding motifs [32]. My findings that DHHC5 is palmitoylated in the C-terminal tail upon stimulation and this modification is crucial for downstream cAMP production in cardiomyocytes implies that DHHC5 as well as its targets are likely enriched in caveolae to allow efficient signaling transduction.

In cardiomyocytes, not much is known about DHHC enzyme regulation. My findings highlight the central role of the C-terminal tail in modulating DHHC5 function in cardiomyocytes. In particular, we found that palmitoylation of the tail had dramatic effects on plasma membrane retention after activation of the β-AR pathway.

DHHC5 phosphorylation and beta-adrenergic signaling

The tail region is highly variable in the DHHC family of enzymes, indicating this region may be a central mediator of isoform-specific function and substrate recognition. We identified that DHHC5 is phosphorylated at one or multiple serine(s) between amino acids 376 and 583 in response to β-AR stimulation. Our group found a positive feedback mechanism in T cells where Lck is palmitoylated rapidly by plasma membrane localized DHHC21, which is activated by downstream Ca^{2+} release [113]. Therefore, we hypothesize that a similar mechanism may be present in cardiomyocytes where the phosphorylation of DHHC5 is mediated by PKA, a kinase that is activated downstream of β-AR. My results combined with a previous proteomic study [260] suggest that serines at positions 380, 425, 432, and 554 are all likely phosphorylation sites. Future studies will need to address how phosphorylation affects DHHC5 functions and downstream signaling.

Chapter VI Conclusion and Future Directions

β-AR signaling pathways are among the most well-studied pathways in the heart under both physiological and pathological conditions. In this study, I focused on the rapid changes mediating contractile functions after stimulation of the β-ARs in primary cardiomyocytes. Though Gα proteins have been previously identified as palmitoylated proteins with relatively fast turnover, relatively little is known about how the rapid changes of endogenous palmitoylation levels impacts cardiomyocytes physiology. In this study, I examined the functions of cardiac DHHC5 and its direct targets Gα proteins in mediating ISO-induced changes in contractility.

Distinctive palmitoylation profiles of β-AR signaling pathway in various cardiac cells

The rat myoblast cell line H9c2 has been used in various studies that examine the β-AR signaling pathways [231, 232]. Since β-ARs and downstream proteins are expressed in H9c2 cells and there are reports of ISO-induced cAMP production [230], I decided to use H9c2 cells as one of the models in my study because they are easier to maintain and perform molecular studies when compared to primary cardiomyocytes. The majority of my study is carried out in neonatal rat ventricular myocytes (NRVMs) because there are well established methods to monitor multiple downstream effects of β-AR signaling, including cAMP production and rate of spontaneous beating [200]. Despite the wide use of

NRVM as a model for studying β-AR signaling, it is often criticized for not being as fitting a model for adult cardiomyocytes, especially in studies related to pathological conditions such as heart failure. Therefore, at the beginning of my study (**Chapter III**), I decided to identify the palmitoylated proteins in the β-AR signaling pathways in all three cell types. I found that NRVM and adult rat ventricular tissue shared similar palmitoylation protein targets, while H9c2 cells showed GRK2 as an additional palmitoylated protein. Later I found that GRK2 can also be palmitoylated in NRVM, but only after ISO stimulation. My data indicate that the kinetics of proteins palmitoylation vary between different cardiac tissues, but the overall palmitoylated protein targets are similar among all three cell types examined. It should be noted that the adult rat ventricular tissue contains many cell types, of which myocytes comprise a minority. To fully understand the roles of palmitoylation on signaling proteins downstream of β-ARs in adult cardiomyocytes, future studies should be carried out in primary adult rat ventricular myocytes (ARVMs).

The detected palmitoylated proteins in my study differ in some respects from previous reports. For example, the PKA regulatory subunit is reported to be palmitoylated in a proteomic study [217], but in my study I cannot conclusively determine if it is palmitoylated given the relatively low level of signal compared to the negative control. Additionally, I was also not able to detect AC6 palmitoylation, which was reported in another proteomic study [218]. This may be due to the limitations of ABE as my detection method. Future studies using other methods including Acyl-RAC or metabolic labeling can be helpful in confirming

whether PKA regulatory subunit and AC6 are indeed palmitoylated in cardiac cells. Another class of known palmitoylated proteins [165, 166] that I did not examine are β-ARs because they cannot be reliably detected using the ABE experimental settings as described in **Chapter III**. As an important technical consideration, I have found that the detection of palmitoylated proteins using ABE method is dependent on the type of detergent used to extract the proteins. For transmembrane proteins (such as DHHC5 in **Chapter V**), I found that DDM could not extract most of the transmembrane proteins and instead I used sodium dodecyl sulfate to lyse the cells before ABE.

The differences of protein palmitoylation between NRVM and H9c2 cells were more evident when we examine the rapid, stimulus-dependent palmitoylation of Gαs and Gαi. In **Chapter IV**, I concluded that H9c2 cells showed no significant changes in palmitoylation levels of both Gα proteins as well as downstream cAMP production or intracellular calcium levels. This could be partially explained by the lack of plasma membrane localized DHHC5 in H9c2 cells. There are three DHHC enzymes that were previously demonstrated to be localized at the plasma membrane: DHHC5, 20, and 21 [70]. DHHC enzymes have some level of substrate redundancy [71, 262] and therefore I cannot rule out the presence of other DHHC enzymes in H9c2 cells that palmitoylate proteins at the plasma membrane. Future studies are needed to identify the expression and localization of these DHHC enzymes in various cardiac cells. Therefore, I conclude that H9c2 cells are not suitable model for studying dynamic palmitoylation of signaling proteins downstream of β-ARs. However, H9c2 can

still be useful for some preliminary studies, including identifying the palmitoylation sites of proteins of interest.

Stimulus-dependent palmitoylation and β-AR signaling in cardiomyocytes

Protein palmitoylation is a common post-translational modification and the only reversible lipid modification. Proteomic studies suggested that at least 10% of the proteins encoded by the human genome are palmitoylated [188]. Protein palmitoylation results in various functional outcomes, including enhancing protein affinity to the membrane, shuttling proteins between different cellular membrane compartments, recruiting proteins to submembrane domains, regulating protein stability and protein-protein interaction (reviewed in [263, 264]). As mentioned in **Chapter I**, the recent development of non-radiolabeling methods to detect protein palmitoylation largely enhanced the understanding of dynamic protein palmitoylation and their roles in specific signaling pathways. Our group was one of the first to show that palmitoylation can occur with extremely rapid kinetics (within one minute) after extracellular stimulation, which recruits the palmitoylated proteins into lipid rafts to form a macromolecular complex that facilitate downstream signaling transduction [113]. In this study, I found a similar mechanism for β-AR signaling pathways in primary cardiomyocytes.

In **Chapter III**, I examined the changes in palmitoylation levels of endogenous proteins in primary cardiomyocytes stimulated by isoproterenol. I found a temporal correlation between the kinetics of Gαs and Gαi palmitoylation and downstream cAMP production and inotropic effects. After GPCR activation,

Gα proteins dissociate from the receptor and the Gβγ subunits [204]. I concluded that both Gαs and Gαi palmitoylation enhanced their localization to membrane subdomain caveolae, where the downstream effector adenylyl cyclase almost exclusively resides [149]. Gαs and Gαi have opposite effects on adenylyl cyclase. Under physiological conditions, the majority of the β-ARs in cardiomyocytes are coupled to Gαs. Therefore, I conclude that palmitoylation did not show any preference between Gαs and Gαi, but the abundance of functional Gαs leads to an overall positive inotropic effects. In this study, isoproterenol activates both Gαs-associated β_1AR and β_2AR, which can couple to both Gαs and Gαi [133]. Previous studies showed that β_1AR localizes throughout plasma membrane while β_2AR is enriched in caveolae [149]. Future studies using subtype-specific agonists are required to decipher whether stimulation of β_1AR or β_2AR leads to distinctive downstream palmitoylation level changes.

A previous study has identified a single palmitoylation site on both Gαs and Gαi at Cys position 3 [170]. Mutation studies in cell lines showed that palmitoylation of Gαs and Gαi are essential for their membrane association and interaction with various RGS proteins [171, 172]. My study in primary cardiomyocytes showed only a temporal correlation between palmitoylation and downstream inotropic effects. Based on a previous study using membrane fractionation, palmitoylated Gαi proteins are shown to be enriched in lipid raft-like low-density membranes [265]. Therefore I conclude that palmitoylation of Gα proteins in cardiomyocytes leads to their localization to caveolae. Though my results from the DHHC5 knockdown cells proved the importance of palmitoylation

in β-AR signaling, future studies using the Cys 3 mutants of Gα proteins are needed. Since I concluded in **Chapter IV** that H9c2 cells are not a fitting model to study agonist-induced palmitoylation, we propose that mutating the endogenous Gα proteins using the CRISPR-Cas9 system in beating myocytes such as the human stem cell derived cardiomyocytes (method established in [266]) would be a useful model.

My study also identified GRK2 as a novel palmitoylation target. Other members of the GRK protein family, including GRK4 [179] and GRK6 [178], have been shown to have multiple palmitoylation sites. However, in **Chapter IV** I showed that GRK2 likely has only one palmitoylation site. Interestingly, I only observe palmitoylated GRK2 after ISO stimulation in NRVM. The kinetics of GRK2 palmitoylation is slower than that of Gα proteins, suggesting that the negative feedback of the β-AR signaling occurs at later time points. A previous study in adult rat ventricular myocytes showed that after 5 minutes of ISO stimulation, GRK2 showed plasma membrane translocation [267]. This timeline is consistent with my observation of the increase in palmitoylation level of GRK2. Previous studies also suggested that GRK2 localization to the plasma membrane is dependent on its binding to the Gβγ subunits [162, 163]. Future work is needed to reveal the functional outcomes of GRK2 palmitoylation.

My findings in **Chapter III** also showed that the palmitoylation levels of Gαs and Gαi went down while that of GRK2 remains the same after 30 minutes of stimulation. These findings suggest that there are enzymatic regulators that actively remove the palmitate moiety on both Gαs and Gαi. There are two well-

characterized acyl protein thioesterases (APT1/2) that are responsible for the removal of palmitate from proteins [74-77]. Additionally, the α/β-hydrolase fold (ABHD) family of serine hydrolases was identified as novel depalmitoylating enzymes [79, 80]. APT1 has been shown to remove palmitoylation groups on Gαs and Gαi *in vitro* [76]. However, further study on the expression of APT proteins and ABHD proteins are required to characterize their potential cardiac functions and to identify which proteins are responsible for the agonist-induced depalmitoylation of Gα proteins.

DHHC5 is a novel regulator of Gα palmitoylation and β-AR signaling in cardiomyocytes

In **Chapter III**, I established that DHHC5 directly palmitoylates Gαs and Gαi proteins and is crucial for signaling transduction downstream of β-ARs. A previous study showed that the Golgi-localized DHHC3 and 7 palmitoylates multiple Gα proteins [214]. In their study, Tsutsumi *et al.* first screened all 23 mammalian DHHC enzymes by overexpressing each one of them with tagged Gαq, Gαs, or Gαi2 in HEK 293T cell and then observed whether there is increased incorporation of [^{3}H]palmitate. Overexpression of DHHC3 and 7 enhanced palmitoylation of all three Gα proteins, and Gαi can also be palmitoylated by DHHC2 and 21. However, Tsutsumi *et al.* focused only on Gαq as the palmitoylated substrate in their knockdown study and *in vitro* palmitoylation assay. Therefore, they cannot conclusively rule out the possibility that other DHHC enzymes can efficiently palmitoylate Gαs and Gαi.

A recent study showed that one DHHC enzyme can trans-palmitoylate another DHHC protein and alter its activity [190]. Therefore, overexpression of DHHC proteins in cells lines is not an ideal method to study their direct activities on substrates. My study is the first to show that Gαs and Gαi are direct substrates of DHHC5 through an *in vitro* palmitoylation assay. Most of the previous *in vitro* palmitoylation assays used [^{3}H]palmitoyl-CoA [124] or require purified DHHC proteins [186], I used a palmitoyl-CoA derivative conjugated to a dye for easy visualization of the palmitoylation signal and we used immunoprecipitated DHHC enzymes. I confirmed that the conjugated palmitoyl-CoA is added to the substrate *via* thioester bond. Additionally, I provided evidence that DHHC5 has substrate specificity in my assay conditions. My results do not exclude the possibility that other DHHC enzymes can palmitoylate Gαs and Gαi. It would be interesting to test whether other DHHC enzymes, especially the plasma membrane localized DHHC20 and 21, are able to palmitoylate Gα proteins. Another limitation of my method is that there can be other proteins, including activators or inhibitors of DHHC5, co-precipitating with DHHC5 in my *in vitro* assay. To exclude the potential influences by co-precipitated proteins, future studies using purified DHHC5 proteins are still needed.

In addition to palmitoylation, Gαi proteins are also myristoylated at the N-terminus [268]. Some studies suggested that N-myristoylation is required for subsequent palmitoylation [269, 270]. Other studies suggested that the palmitoylation status of Gαi is not dependent on myristoylation [271]. My *in vitro*

palmitoylation assay results using non-myristoylated recombinant Gαi proteins supported the latter theory. However, given that my assay is performed without the presence of any membrane systems, I cannot conclusively show that the myristoylation and palmitoylation are two independent events *in vivo*.

In **Chapter III**, I showed for the first time that DHHC5 is required for the signaling events downstream of β-AR stimulation in primary cardiomyocytes. As discussed in **Chapter I**, previous studies on the cardiac functions of DHHC5 focused largely on its substrate phospholemman and the subsequent massive endocytosis process. The existing DHHC5 hypomorphic mouse model shows severe cardiac developmental defects [227] and therefore may not be suitable for studying β-AR signaling mediated inotropic responses in physiological and pathological conditions.

A previous study in human patients with heart failure showed that decrease β-adrenergic responses to catecholamine is a major contributor to the disease [272]. It has been proposed that restoring the β-AR signaling responses is beneficial. For example, transgenic mice overexpressing β_2AR in heart showed improved cardiac contractility [273]. However, prolonged activation of the β-AR signaling pathway is deleterious and leads to the activation of GRK-mediated receptor internalization (reviewed in [274]). Transgenic mice overexpressing Gαs showed enhanced left ventricular functions at early stages and severe cardiomyopathy as they age [275]. Therefore, cardiac specific and inducible DHHC5 deficient or overexpression transgenic animal models will be useful to

understand the mechanism through which DHHC5 regulates β-AR signaling *in vivo*.

Palmitoylation of DHHC5 in the tail region regulates its function

In **Chapter V**, I reported that DHHC5 is palmitoylated at three Cys sites in the C-terminal tail. Mutation of one of the three sites is sufficient to abolish palmitoylation at all three sites. For many of the proteins with multiple palmitoylation sites including calnexin and β_2AR, mutating one cysteine is not sufficient to abolish palmitoylation on the other site(s) [168, 216]. In the case of calnexin where the palmitoylation sites are adjacent (Cys 502 and 503), the majority of calnexin proteins are either non-palmitoylated or dual-palmitoylated, and mutating either site is sufficient to change protein functions [215]. My data suggest a similar all or none model for DHHC5 tail palmitoylation.

My study is the first to show that there is stimulus-dependent dynamic palmitoylation in the DHHC5 tail. The kinetics of DHHC5 palmitoylation are similar to that of Gαs and Gαi, supporting my model that palmitoylation promotes the spatial proximity of signaling proteins at the plasma membrane to facilitate efficient signaling transduction. However, to confirm whether palmitoylation promotes the interaction between DHHC5 and Gα proteins, or whether palmitoylation promotes DHHC5 localization into caveolae, future studies using dual-color TIRF microscopy or other complementary techniques are required.

It is clear from my TIRF microscopy data that palmitoylation stabilizes DHHC5 proteins at the plasma membrane after ISO stimulation in NRVMs. TIRF

microscopy has been used to study transmembrane protein dynamics after cellular stimulation in other studies [256-258]. For example, one study using Chinese hamster ovary (CHO) cells showed that γ-aminobutyric acid ($GABA_B$) receptors showed increased lateral mobility after agonist stimulation, while β-ARs showed no such changes [257]. However, few studies have linked dynamic changes observed at the plasma membrane using TIRF microscopy with downstream signaling events. In **Chapter V** I showed that DHHC5Δ236-245 mutant failed to rescue downstream cAMP production in DHHC5 knockdown cardiomyocytes. My data suggested that stabilization of DHHC5 at the plasma membrane by palmitoylation of the three Cys in the tail region is necessary for β-AR signaling. Since there are endogenous DHHC5 proteins present when I examine recombinant DHHC5 tail palmitoylation, I cannot conclude whether DHHC5 trans-palmitoylates or is regulated by another DHHC enzyme.

General conclusions and significance

Defects in β-AR signaling contribute to the pathogenesis of heart failure [276, 277]. Therefore, understanding the regulatory mechanisms of β-AR signaling is crucial for therapeutic intervention. S-palmitoylation is a reversible post-translational modification that plays an important role in regulating protein localization, trafficking, and stability. Multiple groups have studied the dynamic palmitoylation of Gα proteins in cell lines and *in vitro* models [120-122]. However, my study is the first to show that activation of β-ARs in cardiomyocytes leads to rapid DHHC5-mediated palmitoylation of Gα proteins to regulate contractile

functions (**Figure 20**). Mystudy also suggested that DHHC5 is an important mediator of myocytes contraction and a potential drug target under pathological conditions.

To date, there is no report of specific inhibitor for DHHC5 or any other single DHHC enzyme. The most commonly used inhibitor for palmitoylation is 2-bromopalmitate (2-BP). 2BP is a palmitate analog that irreversibly inhibits multiple cellular processes including lipid metabolism [278] and S-palmitoylation [279]. Interestingly, it is reported that 2-BP also inhibits the depalmitoylating enzyme APT1 [169]. By using a click-inducible 2-BP analog, one study found that it does not preferentially target DHHC enzymes [280]. These studies indicate that 2-BP is not an ideal inhibitor for protein palmitoylation. One recent study found a new class of compounds based on a bis-cyclic piperazine scaffold to be efficient and specific inhibitors for yeast Erf2 (homolog of the mammalian enzyme DHHC9). In the past decade, there has been growing interest in studying the reaction mechanisms of DHHC enzymes and the underlying structural biology [186], both of which are critical for the development of drugs targeting these proteins. Due to the potential involvement of these enzymes in a wide variety of pathological conditions such as cancer, cardiovascular, and neurological diseases, I believe that the development of inhibitors targeting specific mammalian DHHC enzymes is an important goal for improving human health.

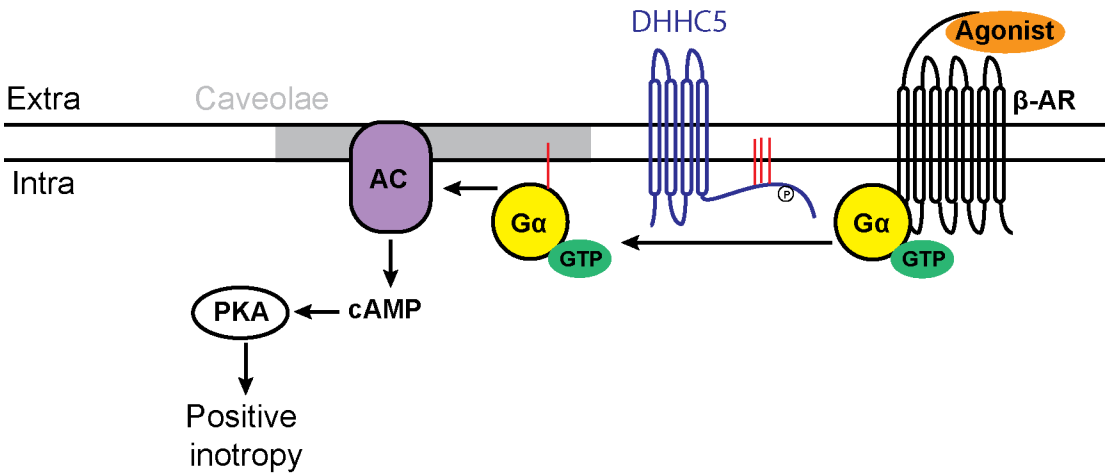

Figure 20 Model of DHHC5-mediated palmitoylation of Gα proteins in cardiomyocytes

Agonist stimulation of beta-adrenergic receptors (β-ARs) leads to palmitoylation (red lines) of Gα proteins and their recruitment into caveolae (grey) where they activate adenylyl cyclase (AC). Subsequently cAMP is produced and protein kinase A (PKA) is activated. PKA phosphorylates downstream substrates and leads to positive inotropy. DHHC5 is palmitoylated at three sites in the C-terminal tail. It is also phosphorylated at one (or multiple) serine(s) between amino acid 376 and 583. Extra=extracellular; Intra=intracellular; P=phosphorylation

Future perspectives of palmitoylation and its enzymatic regulations

Protein palmitoylation is one of the most crucial post-translational modifications that regulate a wide variety of cellular processes in all cell types. Recent advances in techniques enable the discoveries of novel palmitoylated proteins and their palmitoylated Cys sites using proteomic approaches [109, 110, 112, 281]. The functional outcomes of protein palmitoylation include anchoring cytosolic proteins to the plasma membrane, targeting proteins to membrane subdomains, regulating protein stability and interactions. My study and other on-going projects from our lab aim to understand how palmitoylation of signaling proteins is regulated and how protein palmitoylation spatially and temporally coordinates with downstream events in specific cell types. Here are some of the major questions in the field of palmitoylation and its enzymatic regulations that remain to be addressed in the near future.

What is the physiological relevance of protein palmitoylation? With recent advances in genomic editing, building various tissue-specific animal models will be helpful in understanding the functional importance of palmitoylation. Multiple approaches, including mutating the palmitoylated Cys residues on target proteins and knocking out one (or multiple due to potential functional redundancy) of the DHHC enzymes, are needed to study the physiological processes regulated by protein palmitoylation.

Is palmitoylation of specific subsets of proteins disrupted or enhanced in various diseases? Studies using well-established *in vivo* animal models, such as surgical, toxin-induced, or genetic models for heart failure, as well as human

patient samples are needed to determine how palmitoylation is regulated differently compared to normal conditions.

How does palmitoylation potentially affect other posttranslational modifications on target proteins? Many palmitoylated proteins, including Gα and DHHC5 in my study, undergo other types of lipidation or phosphorylation. However, the level of cross-talk and the regulatory mechanisms between palmitoylation and other modifications remain largely unsolved.

How are depalmitoylation enzymes regulated? Compared to the amount of studies on DHHC enzymes, the understanding of depalmitoylating enzymes APTs and ABHD proteins is still rudimentary. Future studies need to focus on the substrate specificity and spatiotemporal regulations of depalmitoylation enzymes.

My project is one of the first that studies dynamic palmitoylation of target proteins by a specific DHHC enzyme in cardiomyocytes. Some of the techniques developed in my study will be useful for future investigations of cellular functions of protein palmitoylation. As listed in this chapter, there are still many questions remain to be answered regarding the molecular, cellular, and physiological functions of protein palmitoylation.

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Vita

Jie (Jessica) Chen was born in Shanghai, China in 1991, the daughter of Wei Chen and Jin Huang. After completing her work at Shanghai No.3 Girls High School, Shanghai, China in 2009, she entered Washington University in St. Louis, Missouri. She received the degree of Bachelor of Arts with a major in Neuroscience from Washington University in St. Louis in May 2013. For the next two years, she worked as a research assistant in the Department of Genetics at MD Anderson Cancer Center. In August of 2015, she entered The University of Texas MD Anderson Center UTHealth Graduate School of Biomedical Sciences.

Permanent address:

5925 Almeda Road

Houston, TX 77004