Defining the Mechanisms by which Palmitoylation Regulates the Localization and Function of RGS4

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Regulator of G-protein signalling 4 (RGS4) modulates Gq and Gi signalling at the plasma membrane (PM). It has been demonstrated that the addition of palmitate to cysteine residues is an important regulator of RGS protein localization and function. The family of palmitate transferase enzymes shares a conserved Asp-His-His-Cys (DHHC) motif. We set out to establish the DHHC isoform(s) that affect RGS4 activity in HEK201 cells. Confocal microscopy revealed that overexpression of DHHCs 3 and 7 mobilized RGS4 to the Golgi. Knockdown of either DHHC3 or DHHC7 attenuated RGS4 inhibition of Gαq-coupled Ca\(^2+\) release and reduced RGS4 PM localization. Consistent with a role in promoting RGS4 lipid bilayer targeting, dominant negative mutants of the five most highly expressed DHHCs in HEK201 cells also diminished RGS4 PM association. Together, these data suggest that members of the mammalian DHHC family regulate RGS4 localization and function, likely through palmitoylation of its target cysteine residues.
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HIP HOP HEATERY FOREVER!!!
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List of Abbreviations

2-BP – 2-bromopalmitate
AC – adenylyl cyclase
APT – acylprotein thioesterase
BFR - baseline fluorescent ratio
cAMP – cyclic adenosine monophosphate
cDNA – complementary deoxyribonucleic acid
CRD – cysteine-rich domain
DAG – diacyl glycerol
DNA – deoxyribonucleic acid
GAP – GTPase activating protein
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
GDP – guanosine diphosphate
eNOS – endothelial nitric oxide synthase
FR – fluorescent ratio
G-protein – guanine nucleotide-binding regulatory protein
GIRK – G-protein coupled inwardly-rectifying potassium channel
GPCR – G-protein coupled receptor
GRK – G-protein-coupled receptor kinases
GTP – guanosine triphosphate
HIP14 – huntingtin interacting protein 14
IP3 – Inositol 1,4,5-triphosphate
kDa - kilodalton
MAPK – mitogen-activated protein kinase
mRNA – messenger ribonucleic acid
nNOS – neuronal nitric oxide synthase
PAT – palmitoyl-acyl transferase
PCC – Pearson’s correlation coefficient
PD – Parkinson’s Disease
PFR – peak fluorescent ratio
PI3K – phosphatidylinositol 3-kinase
PKA – protein kinase A
PKC – protein kinase C
PLCβ – phospholipase C beta
PM – plasma membrane
PTX – pertussis toxin
RGS – regulator of G-protein signalling
RT – reverse transcriptase
RT-PCR – real time polymerase chain reaction
SAN – sinoatrial node
siRNA – small interfering ribonucleic acid
TMD – transmembrane domains
YFP – yellow fluorescent protein
Chapter 1

Introduction

1.a. Heterotrimeric G-protein signalling

1.a.i. Overview

G-protein coupled receptors (GPCRs) and their associated heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) act to transmit extracellular physiologic signals across the plasma membrane to the intracellular environment. In the quiescent state, G-proteins are heterotrimeric complexes composed of an α, β and γ subunit. Upon binding of a specific ligand to the GPCR, the G-protein can be activated to the cytosolic surface of the plasma membrane (PM), where they stimulate downstream effects (1).

1.a.ii. G-protein Coupled Receptors

Membrane-spanning GPCRs are important to both biological and clinical science. They bind various extracellular hormones, neurotransmitters, ions and photons, and trigger intracellular G-protein signalling. The extracellular ligand-binding surface of GPCRs makes them important therapeutic targets for the treatment of a wide array of disease conditions. Indeed, it is predicted that up to 50% of drugs available on the market target GPCRs (2). All GPCRs are characterized by 7 helical transmembrane domains (TMDs) which anchor them to the lipid bilayer. These α-helices are separated by alternating extracellular and intracellular loops, of which the latter form the G-protein binding domain (3). Binding of a ligand to the GPCR causes changes in the orientation of the α-helices, increasing affinity for the ligand-receptor-G-protein complex, while also catalyzing the exchange of GDP for GTP on the G-
protein α subunit (4). The GTP-bound G-protein then dissociates from the receptor and proceeds to activate its downstream signalling cascade.

Apart from the 7 TMDs and conserved sequence motifs found within GPCR subfamilies, GPCRs vary considerably in structure at the ligand-binding extracellular loops and at their cytoplasmic surface (5). Distinct variations between GPCRs were recently identified following the solution of the first crystal structures of ligand activated human β2 adrenergic receptors and human A2A adenosine receptors (6-9). In the cardiovascular system – the area of interest in our lab – β2 adrenergic receptors are coupled to both the Gαs and Gαi pathways in cardiac myocytes.

Desensitization of the G-protein signalling cascade is important for proper feedback control of signalling. A major desensitization pathway for GPCRs is phosphorylation by G protein-coupled receptor kinases (GRKs). GRK proteins bind preferentially to activated GPCRs, phosphorylating them on their intracellular loops and carboxyl-end tails (10). Phosphorylation by GRKs flags the GPCR for binding by arrestin proteins, which promote sequestration and internalization of surface-expressed receptors leading to termination of G-protein signalling. In this fashion, GRK phosphorylation and arrestin binding act as molecular switches, causing the GPCR to transfer from G-protein-dependent to G-protein-independent pathways (11;12). Thus feedback regulation of G-protein signal transduction is important for maintaining cellular homeostasis.

1.a.iii. G-protein Structure and Properties

16 genes encode for Gα subunits, 5 genes encode for Gβ, and 12 genes for Gγ (13). The β and γ subunits form a tightly linked heterodimeric complex, with the capability to interact with different Gα subunits. The β subunit is characterized by its WD-40 repeats, which form a 7-blade β-propeller structure (3;14). The γ subunit features an N-terminal coiled coil that
promotes its interaction with the β subunit. Gα subunits are characterized by their GTPase domain-containing sequence which binds and hydrolyzes guanosine triphosphate (GTP), and an N-terminal α-helical domain which buries the bound GTP within the core of the G-protein (15).

G-proteins are divided into four subfamilies based on the names of their associated Gα subunits: Gαs, Gαq/11, Gαi/o, and Gα12/13 (15). The four broad families transduce signals from a large number of extracellular agents to their respective downstream signalling cascades. The Gs pathway was the first G-protein pathway described, and from its study came the discovery of protein phosphorylation (16), signal transducers (17), and second messengers (18). This pathway’s major role is to stimulate adenyl cyclase (AC), triggering the production of cyclic adenosine monophosphate (cAMP), a molecule responsible for activating numerous signalling cascades, such as Protein Kinase A (PKA) and the mitogen-activated protein kinase (MAPK) cascade.

The Gi pathway is responsible for inhibition of AC activity, causing several physiological responses (19). Both the Gαi subunit and its associated βγ complex have the ability to trigger signalling events independent of AC regulation; Gαi and Gαo modulate signals from c-Src to signal transducer and activator of transcription 3 (STAT3); Gβγ directly activates to phospholipase C beta (PLCβ), G-protein regulated inward-rectifying potassium channels (GIRKs), and phosphatidylinositol 3-kinase (PI3K), for example.

The classical pathway of Gq involves stimulation of PLCβ, which triggers the production of diacylglycerol (DAG) and inositol 1,4,5 – trisphosphate (IP3), and subsequent activation of Protein Kinase C (PKC) and release of intracellular calcium, respectively (19). The Gαq subunit is also known to activate the transcription factor NF-κB via PYK2.

The G12 and G13 pathways are less defined. Gα12 is thought to stimulate c-Src phospholipase D, and PKC, but the mechanisms are unclear (19). Gα13 activates Rho through
direct interaction with the guanine nucleotide exchange factor p115RhoGEF, and it also thought to activate PYK2, but this mechanism is not yet elucidated.

1.a.iv. The GTPase Cycle

In the early 1990s, work was done to identify three conformational states of G-proteins (20). Activation of a G-protein by its GPCR triggers the release of GDP from the quiescent Ga subunit, allowing GTP to bind and free Ga from the heterotrimer. This is known as the active state (21). The activated Ga and βγ are free to interact with their respective downstream partners. The Ga subunit has a relatively slow GTPase activity, catalyzing the hydrolysis of GTP to GDP and weakening the interaction of Ga with its effectors. The GDP-bound form of Ga marks the inactive state. In this conformation, Ga has a higher affinity for βγ complexes than for effector molecules. Of note is the moment between GDP release and GTP binding, when the G-protein is said to be in an “empty pocket” state, referencing a lack of guanine nucleotide in the pocket between the two Ga domains (3). A G-protein stabilizing complex between the “empty” heterotrimer and its activated GPCR is predicted to form, but the nature of this complex remains poorly understood.

The Ga subunit undergoes a variety of conformational changes throughout the GTPase cycle (21;22). Binding of GTP gives the α subunit a more ordered orientation and lower affinity for the βγ complex. Three flexible regions of Ga, designated Switches I, II, and III, are the players in this conformation. The most important of the three appears to be Switch II, the helical portion of the Ras-like domain that interacts with the γ-phosphate of GTP. Switch II appears disordered in the GDP-bound state and is sequestered when the heterotrimer is inactive, but when activated, Switch II forms extensive contacts with effectors (23).

The rate limiting step in the GTPase cycle is the hydrolysis of GTP and its subsequent release from the Ga binding pocket. While the Ga subunit has intrinsic GTPase activity, the
rates of reaction of hydrolysis and GDP release are relatively slow (20;24;25). As will be discussed below, proteins that can catalyze Ga-GTP hydrolysis are required for ensuring proper resetting of the GTPase cycle and maintenance of downstream G-protein signalling activity.

1.a.v. G-protein Localization and Trafficking

G-proteins elicit many of their effects at the PM. Previous work has shown, however, that G-proteins can shuttle rapidly between the PM and endomembranes in their basal state, at a t½ of <1 minute (26). To facilitate their membrane interaction, Ga subunits undergo a variety of lipid modifications (27). Myristoylation, the co-translational attachment of a 14-carbon myristate by amide linkage to an amino terminal glycine residue, was the first covalent addition to be identified as promoting G-protein attachment to the PM (28;29). Some G-proteins, however, attach firmly to the PM without N-myristoylation. This led to the hypothesis that G-proteins must undergo more than one lipid modification. It was discovered that for Ga subunits, the presence of a myristate group acted as a prerequisite for palmitoylation, the post-translational attachment of a 16-carbon palmitate group to a cysteine residue, via thioester bond (30). Both lipid modifications occur at the N-terminus of Ga subunits, acting as membrane binding signals for the G-protein (31).

There has been debate as to whether myristoylation and palmitoylation play independent, redundant, or synergistic roles in G-protein PM targeting. One prevailing theory describes myristoylated Ga subunits as transiently interacting with the PM and other cellular bodies, until they become palmitoylated, allowing them to anchor specifically onto the PM (31-34). However, 2-bromopalmitate (2-BP), a palmitoylation inhibitor, has been shown to block rapid G-protein shuttling from the PM to endomembranes (26), suggesting a role of palmitoylation in shuttling and not exclusively in membrane selectivity.
The βγ complex also regulates G-protein PM targeting. Following disruption of Ga subunit lipid modifications, overexpression of specific βγ subunits has been shown to recover G-protein PM localization (35). These findings implicate βγ as a critical membrane attachment signal for GaS and Gaq. Other molecular signals have also been found to promote PM targeting and binding of the α subunit, including N-terminal polybasic motifs and interaction with other membrane-binding proteins (36;37). Despite all that is known, the precise order that lipid modifications and protein signalling target the G-protein to the PM is yet to be discovered.

1.b. Regulators of G-protein Signalling

1.b.i. Discovery and Role in Signal Transduction

The amplitude and duration of G-protein signalling events are determined by the lifetime of GTP bound to active Ga, hence the rate limiting step of G-protein signalling is the release of GDP following GTP hydrolysis. However, this intrinsic hydrolysis is too slow to account for the rapid termination of signalling that occurs endogenously. Purified Ga subunits demonstrate extremely slow rates of GTP hydrolysis, in the range of 0.1 to 0.3 Pi/mol Ga/min (38). In GPCR-G-protein purified systems, there is an improvement in α subunit hydrolysis rate, but only by 10-fold (39). In contrast, the rate of G-protein deactivation in cells has been measured at 1000-fold faster than in vitro. For example, G-protein regulated phototransduction in intact rod cells, and G-protein mediated potassium and calcium channel currents demonstrated activation and termination of signalling on the order of milliseconds, grossly faster than was previously found in purified protein models (40;41). It was hypothesized that a series of factors must exist to regulate activation and deactivation of Ga subunit hydrolysis to help maintain signalling homeostasis. We now know these “factors” to be the Regulators of G-protein Signalling (RGS) superfamily.
The first evidence of intracellular regulators of G-protein signalling arose through studies in yeast, when it was discovered that a loss-of-function mutation in the gene \( \text{sst2}p \) caused a hypersensitive response to G-protein-mediated pheromone release (42). Studies also showed that gain-of-function mutations to this gene caused greater feedback inhibition on \( \Gamma_{\alpha} \) subunits (43). Independent of these studies, novel genes were discovered in mammals: \( \text{G0S8} \) was found to be upregulated in stimulated monocytes (44); a human protein termed GAIP was found to bind activated \( \Gamma_{\alpha_{13}} \) with great affinity (45); the B-cell specific BL34/1R20 was markedly upregulated by mitogenic stimuli (46). Through sequence analysis from a brain cDNA library, it was discovered these genes shared a ~130 amino acid core domain with Sst2p (47-49). This led to the understanding that this group of proteins belonged to a much larger protein superfamily. The superfamily was dubbed the Regulators of G-protein Signalling, with the core domain being named the RGS domain. BL34/1R20 and G0S8 were renamed RGS1 and RGS2, respectively.

Following their discovery, several studies were performed to demonstrate that RGS proteins act as GTPase-activating proteins (GAPs) (Figure 1) to increase the rate of \( \Gamma_{\alpha}-\text{GTP} \) hydrolysis by up to 1000-fold (50-52). RGS proteins are known to selectively bind to any of active \( \Gamma_{\alpha_6}, \Gamma_{\alpha_9}, \) and \( \Gamma_{\alpha_{12/13}} \), but not \( \Gamma_{\alpha_5} \), \textit{in vitro} (50). RGS proteins also display highest affinity for the GDP-Mg\(^{2+}\)-AlF\(^{4-}\)-\( \Gamma_{\alpha} \) complex, representing the transition state of \( \Gamma_{\alpha} \) hydrolysis (39). In this sense, RGS proteins can be said to have a catalytic effect on \( \Gamma_{\alpha} \) hydrolysis by possessing the ability to stabilize the transition state (53). Additionally, RGS proteins have been shown to inhibit G-protein signalling in a GAP-independent manner, through binding to the \( \Gamma_{\alpha} \) subunit (54) and also through physical interaction with downstream effector molecules, such as GIRKs (55). This alternative method of G-protein inhibition demonstrates a regulatory mechanism for RGS family members with low GAP activity.
**Figure 1.** RGS activity in the GTPase cycle. The inactive G-protein exists as a heterotrimer with GDP bound to the Gα subunit. Activation by an associated GPCR facilitates GDP release and GTP binding to Gα, causing a dissociation of Gα from the βγ complex. Each goes on to activate their respective downstream partners. Inactivation of G-protein signalling occurs through hydrolysis of GTP by Gα. However, this intrinsic GTPase activity is slow; RGS proteins catalyze this hydrolysis step, allowing for effective deactivation of the G-protein heterotrimer and reassociation with the GPCR.
1.b.ii. The RGS Superfamily

More than 30 RGS proteins exist, all of which are classified into six subfamilies: four subfamilies in which all members display GAP activity, and two subfamilies – the RGS-like subfamily and Axin-like subfamily – consisting of weakly homologous RGS domain identity and relatively poor Gα recognition (56), and as such will not be discussed further.

1.b.ii.1. RGS7-like (R7) Subfamily

The R7 subfamily consists of RGS6, RGS7, RGS9, RGS11, and an assortment of splice variants. Several subdomains occur across the family, such as the 64 amino acid G-protein γ-subunit-like (GGL) domain, a Dishevelled/EGL-10/Pleckstrin (DEP) domain, and an R7 homology domain (57). The GGL domain has been shown to direct binding to Gβ5 subunits which contributes to protein stability (58), while the DEP domain confers direct interaction between the RGS protein and other signalling molecules within cells (59).

1.b.ii.2. RGS12-like (R12) Subfamily

The R12 subfamily contains three members, RGS10, RGS12 and RGS14, and similar to the R7 subfamily, also features alternatively spliced variants. The R12 family members vary considerably in size with RGS10 containing 173 residues and RGS12 containing 1447 residues. However, along with the R7 subfamily, R12 members are larger than those in the Rz and R4 subfamilies. Just like the R7 subfamily, these R12 members share subfamily domains, namely, a Rap1/2-binding domain and a C-terminal GoLoco motif. The former exists between RGS12 and 14, and directs interaction with the small GTPases Rap1 and Rap2, while the latter – also existing between RGS12 and 14 – give the RGS proteins affinity for Gαi1-3 in their GDP-bound state (60-62).
1.b.ii.3. The Rz-like (Rz) Subfamily

The members of the Rz subfamily are encoded by the genes Rgs17, Rgs19 and Rgs20. Again, alternative splicing results in several protein isoforms. The function of the Rz subfamily members varies from other RGS proteins in that their action is dependent on phosphorylation of themselves and their binding partners. For instance, phosphorylation of Gαz and RGS19 can each play a role of reducing RGS-Gα affinity, and thus, RGS GAP activity (63;64).

1.b.ii.4. The RGS4-like (R4) Subfamily

The members of the R4 subfamily are the smallest in terms of structure, composed of an RGS domain flanked by minimal N- and C-termini. The subfamily also contains the most members: RGS1, 2, 3, 4, 5, 8, 13 and 16. All members are 20-30 kDa in size, and with the exception of RGS2, all possess the ability to non-specifically inhibit Gαi and Gαq (65). The prevailing idea on R4 members is that the RGS domain is the specific modulator of G-protein signalling events, while the N- and C- termini confer subcellular localization and protein and effector specificity (66). Consistent with this idea, it has been shown that the N-terminus of RGS4 conveys higher muscarinic receptor affinity and selectivity in mediating Gq-coupled calcium release in rat pancreatic acinar cells (67). This specificity is attributed to the variation that exists within the N-termini of each R4 subfamily member. The N-terminus of all members of the R4 subfamily, with the exception of RGS3, forms an amphipathic α-helix (68). It has been demonstrated that this N-terminal helix is important for plasma membrane association and proper function of members of the R4 subfamily (65;69;70). Our lab has demonstrated that the N-terminal helix gives RGS2 greater PM localization and increased Gq modulatory function relative to RGS5, due to hydrophobic residues specific to RGS2 within the helix (71).
1.b.ii.4.a. RGS4

The focus of this thesis is RGS4, the prototypical member of the R4 subfamily that has been demonstrated to show GAP activity for both the Ga\(_i/o\) and Ga\(_q\) subunits. The Rgs4 gene was first identified in a rat cDNA library screen, in which Rgs4 suppression presented a phenotype associated with the deletion of the sst2p gene in yeast (49). To date, RGS4 is probably the most extensively studied RGS protein. It is highly expressed in the central nervous system (CNS) and heart, in both human and mouse models.

1.b.ii.4.a.i. RGS4 in the Central Nervous System

During embryonic development in mice, RGS4 is transiently expressed in several CNS cell types, such as sympathetic ganglia and cranial sensory and motor neurons (72). In an extensive study using bacterial artificial chromosome analysis of the Rgs4 gene in the mouse brain, it was demonstrated that RGS4 expression occurs widespread in most cortical neuronal layers at all stages of development (73). The same study revealed a strong correlation between location of RGS4 expression and acetylcholinesterase, which suggested a role of the RGS protein in muscarinic receptor signalling regulation. RGS4 has also been documented in several subcortical regions, most abundantly in the striatum and amygdala (74).

While there have been varying reports of RGS4 involvement in opioid signalling in the brain (68,75), RGS4 has been implicated as a player in Parkinson's disease (PD). Depletion of dopamine in the striatum, as in PD, resulted in a variety of feedback responses, including an attenuation of M4 muscarinic receptor signalling attributed to upregulated Rgs4 levels (76). In addition to PD, RGS4 has also drawn consideration as a potential genetic factor in the development of schizophrenia. The Rgs4 gene resides in the schizophrenia susceptibility locus, and RGS4 expression is decreased in several frontal cortex regions in schizophrenics (77,78). However, functional studies have not sufficiently demonstrated a role for RGS4 in this
neurological disorder. The only behavioural study conducted presented no abnormalities in 
Rgs4-null rodents that would suggest a schizophrenic phenotype (79).

1.b.ii.4.a.ii. RGS4 in the Cardiovascular System

RGS4 is expressed in both atrial and ventricular myocytes, and is found to be upregulated – 2 to 3-fold higher human mRNA and protein levels – in times of pathophysiologic cardiac stress (80;81). Important studies have presented a role for RGS4 regulation on Gq signalling in ventricular hypertrophy. Pulmonary artery- and aortic-banded mice demonstrated increased Rgs4 mRNA levels in hypertrophied ventricular tissue, relative to normal tissue (81-83). Muslin and co-workers (84) demonstrated that RGS4 overexpression in rat cultured cardiomyocytes inhibited phenylephrine- and endothelin-induced myofilament reorganization and cell growth related to hypertrophy. The group was also able to block Gαq- and aortic banding-induced hypertrophy in mouse cultured cardiomyocytes through overexpression of RGS4 (85;86). These findings were augmented by the report that RGS4 overexpression inhibited hypertrophic signalling *in vivo*, via regulation of Protein Kinase G (83). However, the questions remains whether endogenous RGS4 levels are necessary and sufficient to provide the same cardioprotective mechanisms (87).

Recent work has implicated RGS4 as a player in chronotropic regulation of the heart. RGS4 expressed in isolated atrial myocytes has been shown to selectively associate with acetylcholine muscarinic 2 receptors (M2Rs) and GIRK channels, and its ability to act as a modulator for GIRK channel activation has been well described (88;89). Gαi/o-coupled M2R activation in atrial myocytes leads to acetylcholine-activated potassium currents (*I*<sub>Kach</sub>) through GIRK channels, an important hyperpolarizing current induced by parasympathetic signalling. Hence, due to its close association with this signalling pathway, RGS4 has been predicted to be a potential regulator of parasympathetic-linked dysrhythmias in atrial
myocytes (55;88;90). Recent work from our lab has strengthened this hypothesis. We have shown \textit{Rgs4} to be much more highly expressed in sinoatrial node (SAN) myocytes than in the myocardium (91). The RGS4 effect on M2-mediated parasympathetic signalling was shown at the \textit{in vivo, ex vivo, and in vitro} levels. \textit{Rgs4}-null mice demonstrated increased bradycardic response to M2-mediated parasympathetic agonists and lowered baseline heart rates than wild type. Retrograde perfused \textit{Rgs4}-null hearts showed enhanced negative chronotropic response to carbachol (CCh). Finally, SAN myocytes from \textit{Rgs4}-null mice demonstrated greater sensitivity to M2-mediated reduction in action potential firing, decreased GIRK channel desensitization to parasympathetic stimulus, and altered $I_{KACch}$ kinetics. Current efforts by our lab include examining the role of RGS4 in protecting against GIRK-mediated atrial fibrillation (in press).

\textbf{1.b.ii.4.a.iii. RGS4 Localization and Trafficking}

Like most RGS proteins, RGS4 is believed to exist in a soluble state, capable of residing either in the cytosol or bound to the PM (69;92;93). As regulators of G-proteins, RGS proteins must be recruited to the cytoplasmic face of the PM to elicit their effects. Indeed, RGS4 has been shown to localize to the PM following activation of $G_{\alpha_1}$ and $G_{\alpha_1/o}$ subunits (92), although its relative distribution in membrane and cytosolic pools under different signalling conditions is a target of ongoing research. The N-terminus of RGS4 is thought to direct PM targeting and localization, presumably due to formation of an amphipathic $\alpha$-helix, as mentioned above (69;94). The 33-amino acid N-terminal cationic helix features hydrophobic residues thought to increase affinity for the non-polar interior of the lipid bilayer membrane, and also features a cluster of positively charged amino acids at positions 13-29, which interact with the anionic environment created by phospholipid head groups on the inner leaflet of the PM (69). This dependency on the N-terminus for PM binding has been shown \textit{in vitro} and \textit{in vivo}. It has also
been shown that in reconstituted lipid vesicle systems, the N-terminus alone can bind with great affinity to PM in the absence of GPCRs, Gα subunits and Gβγ subunits, demonstrating a necessity and sufficiency of the N-terminus for RGS4 PM localization (94). Complementary to these findings, Gα and βγ subunit activity can recruit RGS4 to the membrane, but this is not necessary for RGS4 PM localization (95). Taken together, it can be said that the N-terminus, by way of the amphipathic α-helix, allows RGS4 to spontaneously bind to the PM, and any recruitment caused by G-protein subunit signalling enhances RGS4’s GAP activity (94), but is not crucial for RGS4 PM binding. Lastly, the N-terminus has also been shown to convey receptor selectivity of RGS4 in in vitro Gαq-coupled M1 driven calcium release experiments (67).

Other features of the N-terminus have been implicated in RGS4 trafficking. For example, RGS4 can be reversibly palmitoylated at cysteine residues 2 and 12 in its N-terminus (69). Membrane-bound RGS4 has been shown to be palmitoylated at these two residues, while cytosolic fractions of RGS4 are not (96). Models of the N-terminal α-helix demonstrate an elongation of the hydrophobic nature of RGS4 when palmitoylated at C2 and C12. This could suggest that palmitoylation is important for anchoring the protein onto the PM. This thesis will examine the role – if any – of palmitoylation on the subcellular trafficking and eventual PM localization of RGS4.

1.c. Palmitoylation

1.c.i. Definition and Properties

Proteins undergo several lipid modifications. The best documented and also most common forms of protein lipidation are myristoylation, prenylation, and palmitoylation. As mentioned above, myristoylation involves covalent addition of a 14 carbon myristate to an N-terminal glycine residue via amide linkage. Prenylation, or isoprenylation as it is also known,
occurs on cysteines of proteins ending in a CAAX (cysteine-aliphatic residue-aliphatic residue-any residue) motif. This multistep process results in the addition of a 15 carbon farnesyl or 20 carbon geranylgeranyl group via thioether linkage. In the study of RGS proteins, the most important lipid modification is palmitoylation, the post-translational addition of a 16 carbon palmitate to cysteine through a thioester bond. This can occur in two forms, namely, N- and S-palmitoylation. The former involves cysteine binding as an intermediate, followed by rapid rearrangement and conversion to an amide group (97); the latter, and the form discussed herein, simply ends at thioester bonding. What separates S-palmitoylation (referred to as "palmitoylation" henceforth) from other lipid modifications is its reversible nature, unlike the stable additions of myristate and isoprenoids (98;99).

Palmitoylation occurs on a wide variety of proteins, thus providing several diverse roles, depending on the location of palmitate incorporation on the protein sequence, the function of the substrate in question, and the particular subcellular localization of said protein substrate. Interestingly enough, unlike other lipid modifications, there is no required sequence motif within a protein for palmitoylation to occur, other than the presence of a cysteine residue. Thus, the location of palmitoylation on the protein sequence often dictates the function of the palmitate group (97). However, the lack of universal consensus sequence makes it difficult to predict whether a protein is a substrate for palmitoylation, and hence, building the palmitoylproteome requires extensive research.

Construction of the palmitoylproteome began following the discovery in 1979 that viral glycoproteins underwent a post-translational modification involving a methyl-ester palmitic acid (100). Since this time, the size of the palmitoylproteome has grown to over 50 proteins, many having been identified via systematic screening in yeast by Davis and co-workers (101). This analysis identified new members of substrate families already known to feature protein lipidation. For example, GTPases had previously been documented as having
palmitoylated members, and newly identified lipidated species were GTPases Rho2 and Rho3. Also identified was a new TMD-containing family of amino-acid permeases for whom function has yet to be elucidated, 8 trans-Golgi bound members of the SNARE protein family, and single TMD mannosyltransferases (101). This study aided in identifying sequence-specific patterns for palmitoylation targeting, particularly in translating to a mammalian system.

Palmitoylated proteins can be divided into two categories: proteins translated on free ribosomes, and those containing TMDs. Proteins with TMDs are often palmitoylated at cysteine residues close to the PM interface or in cytoplasmic C-terminal tails (97). The freely-synthesized proteins can go on to associate peripherally with the PM, either by single palmitoylation or palmitoylation alongside another lipid modification (dual lipidation) (98). The latter is commonly seen in the Goi subunit family and in the monomeric GTPases N-Ras and H-Ras.

The subsequent sections will discuss several roles of palmitoylation in the cell. The following is not a comprehensive list, but rather a discussion of the best documented functions of palmitoylation. In addition to the roles explained below, palmitoylation has been implicated in such tasks as triggering protein-protein interactions, promoting and/or preventing protein aggregation, and predetermining substrates necessary for the assembly of signalling complexes (99;102).

1.c.i.1. Palmitoylation Promotes Localization and Trafficking

The most common role of palmitoylation is to increase the affinity of a soluble protein for membranes, thereby regulating that protein's localization and function (97). The small GTPases H- and N-Ras are taken as an example. Ras proteins undergo farnesylation, then proteolytic cleavage of the C-terminal −AAX region followed by carboxymethylation on the farnesylated cysteine (103). From this point, family member K-Ras can traffic to the PM
without further modifications, due to the presence of an N-terminal polybasic motif (104). H- and N- Ras, however, must secrete to the Golgi, where cysteine residues are palmitoylated to allow for PM targeting and localization (105).

A couple insights come from this model of Ras trafficking. First, site-specific palmitoylation plays a key role in subcellular localization. N-Ras is palmitoylated at a single cysteine residue, conferring upon it adequate ability to shuttle to the PM; H-Ras, however, has potential for the palmitate addition at cysteines 181 and 184. Dual palmitoylation and palmitoylation of C184 results in greater proportion of Golgi-localized H-Ras, with the PM fraction undergoing GTP-dependent lateral diffusion to microdomains called lipid rafts (105). In contrast, sole palmitoylation of C181 leads to efficient H-Ras PM localization, similar to that of N-Ras. The second point of interest is the apparent role of palmitate removal. PM-bound Ras can undergo states of depalmitoylation, which shuttle it back to the Golgi or Endoplasmic Reticulum (ER), where repalmitoylation can occur, restarting the cycle (97;105). However, chemical replacement of the reversible palmitate group on N-Ras with a non-cleavable palmitate analogue prevented rapid exchange of the protein to the Golgi, keeping it membrane-bound (106). This finding presents the idea that depalmitoylation is also required for Ras trafficking and subcellular shuffling.

Studies presenting a cyclical acylation program, like that seen in Ras trafficking, coupled with biophysical in vitro studies of lipidated peptides, have given rise to a kinetic trap model of palmitoylation (33). The proposed model states that non-reversible lipidations, such as N-myristoylation and prenylations, allow proteins to transiently interact with various subcellular membranes in a vesicle-independent manner. The role of palmitoylation is to provide the protein with a second lipid modification, securing it to a specific membrane. The protein can still move through compartments, but only through vesicle-mediated mechanisms. Depalmitoylation of the protein returns it to its previous membrane-
promiscuous state. In this model, palmitoylation is proposed to play more of an anchoring role than a target-directing one.

1.c.i.2. Palmitoylation Plays a Role in Quality Control

Palmitoylation has also been proposed to aid in maintaining cell stability by ensuring correct protein composition and production. For example, the palmitoylation of yeast chitin synthase Chs3 appears to indicate proper folding of the multiple TMD protein. When palmitoylation is blocked, Chs3 no longer shuttles to the PM, accumulating at the ER (107). This stoppage of palmitoylation ensures only correctly translated proteins arrive at their intended cellular destination.

1.c.i.3. Palmitoylation Regulates Protein Degradation

Recent studies of SNARE proteins in yeast depict a role of protein palmitoylation in protecting against ubiquitylation, and eventually protein degradation. The SNARE protein Tlg1 in *S. cerevisiae* is subject to dual palmitoylation adjacent to its TMD, present for the protein’s entire lifespan. Tlg1 is then promoted to the ER membrane, where it aids in vesicular fusion with the late Golgi body. However, when Tlg1 is mutated at its cysteine residues, the protein becomes ubiquitylated by E3 ligases and is targeted to the vacuole for lysosomal fusion and degradation (108). It is proposed that the palmitoylated cysteine residues promote correct orientation of the Tlg1 TMD to the lipid bilayer, and lack of palmitoylation exposes acidic residues near the TMD, possibly flagging the protein for ubiquitylation by E3 ligases (108).

Not to be classified as strictly protective, palmitoylation has also been shown to promote protein degradation. The yeast sphingoid long-chain base kinase Lcb4 is palmitoylated at two internal cysteine residues. In the normal condition, Lcb4 becomes
downregulated once the cells enter the stationary phase. When palmitoylation is blocked via
cysteine mutation, the half-life of Lcb4 is greatly increased, promoting its proliferation (109).

1.c.ii. Palmitoylation of G-proteins

Palmitoylation of Gα subunits has been well documented (26-30). It occurs at the N-
terminus of Gα subunits, on cysteine residues close to N-myristoylated glycines in the Gαi
subfamily, and as the only lipid modification in the Gαs, Gαq, and Gα12/13 subfamilies (15). As
discussed earlier, there are conflicting reports on the role of palmitoylation in G-proteins;
some studies attribute kinetic trapping to palmitate incorporation (31-33), while others
suggest palmitoylation triggers constant cycling of proteins from the PM to various
endomembranes (26). Consistent with the kinetic trapping model, myristoylation and
palmitoylation have been shown to work cooperatively to promote Gαi PM localization
(30;34). Myristoylation occurs first, drawing the protein to the membrane, while
palmitoylation is thought to firmly anchor it there (33). Palmitoylation may also be the signal
that confers specificity of G-proteins to the PM rather than other intracellular membranes
(27;34;110). An important caveat, however, is that myristoylation appears to be sufficient but
not necessary for palmitoylation and thus PM localization. It was shown through COS cell
transfection that Gαi subunits mutated at N-terminal myristoylation sites and co-expressed
with βγ subunits were still able to incorporate palmitate groups to its N-terminus and traffic
to the PM (111). This suggests that the βγ complex possesses a robust PM-targeting
mechanism, and that PM localization is enough for palmitate incorporation to occur.

For Gα subunits that only undergo palmitoylation, mutation of key cysteine residues
greatly attenuates PM localization (112-114). Gilman and co-workers demonstrated that
mutation of both N-terminal candidate cysteines on Gαq impaired, but did not abolish, M1
muscarinic receptor association and PLCβ activation (115).
Finally, palmitoylation also appears to regulate protein-protein interactions of G-proteins. It increases the affinity of Goα for the βγ complex and also renders the Gs heterotrimer more resistant to thioesterase cleavage of palmitate (116). As well, palmitoylation can inhibit protein-protein association, as it does for Goα with the RGS protein GzGAP (117).

1.c.iii. Palmitoylation of G-protein Coupled Receptors

The only lipidation GPCRs are subject to is palmitoylation, with the sole exception being isoprenylation of prostacyclin receptors (118). Palmitoylation usually occurs at C-terminal cysteine residues located 10 to 14 amino acids downstream of the seventh TMD (119).

The first GPCR discovered to be targeted by palmitoylation was Rhodopsin in bovine retinas (120). The membrane-spanning region of a GPCR creates 3 extracellular loops and 3 intracellular loops alternately; the location of the palmitate group was predicted to integrate into the lipid bilayer, creating a fourth cytoplasmic loop, which was confirmed using a fluorescent-tagged analogue (121). The location of the cysteine residue in rhodopsin is analogous in 80% of all GPCRs (118), suggesting palmitoylation to be a general occurrence in this type of receptor. However, palmitoylation is not limited to this domain. Incorporation of radiolabelled palmitate has been detected in GPCRs mutated at all C-terminal cysteine residues, both in GPCRs known and not known to undergo palmitoylation in the characteristic region (122;123).

Palmitoylation plays multiple roles in GPCRs. It appears to be important in directing delivery of proteins to the cell surface. This is evidenced by the fact that some cysteine-mutated GPCRs remain bound to intracellular membranes, particularly the ER (124;125). This finding is consistent with the notion that palmitoylation of GPCRs occurs at the ER, or
somewhere within the ER-Golgi intermediary complex. Aside from PM targeting, GPCR palmitoylation has also been implicated in regulation of degradation, as evidenced in human A1 adenoceptors and the chemokine receptor CCR5 (124;126). This may be due to non-acylated cysteine sulhydryl groups triggering misfolding and mislocalization, and thereby degradation, of these proteins.

Because of the integration of palmitate groups into PMs causing formation of the extra cytoplasmic loop, and also due to its rapid turnover rate, the palmitoylation/depalmitoylation cycle plays a great hand in altering the conformation of membrane-bound GPCRs. As such, it acts to regulate GPCR-protein interactions, and is itself regulated by ligand binding (99). This activation-dependency of palmitoylation has been demonstrated in M2 receptors, β2 and α2A adrenoceptors, 5-HT4a serotonin receptors, and V1a vasopressin receptors (123;127-130). Agonist activation of GPCRs can increase or decrease palmitate turnover, but what is implicit in both cases is a dynamic pool of depalmitoylated receptors at the PM whose function is unknown.

GPCR palmitoylation acts to regulate several protein-protein interactions. Mutagenesis of a palmitoylation site can uncouple the GPCR from its associated G-protein and impair its signalling, as depicted in β2 adrenergic receptors and D1 dopamine receptors (131;132). However, this phenotype is not conserved in all GPCRs. GPCR palmitoylation also appears to counteract the effects of receptor-desensitization by phosphorylation. In β2 adrenoceptors again, palmitate groups appears to obstruct phosphorylation sites, hence impeding the GPCR interaction with PKA (131). Taken into context with the palmitate turnover cycle, it can be speculated that the pool of depalmitoylated receptors are more readily phosphorylated, leading to desensitization and downregulated GPCR signalling. Finally, GPCR palmitoylation has been found via mutagenesis to regulate receptor-GRK
interactions, thus mediating a second phosphorylation pathway and modulating binding by arrestin proteins (133).

1.c.iv. Palmitoylation of RGS Proteins

For the most part, RGS proteins do not contain the required sequence motifs for irreversible lipid modifications like myristoylation and prenylation (56). However, plenty of exposed cysteine residues on RGS proteins act as targets for rapid, reversible palmitoylation. This palmitoylation was first discovered at the N-terminal cysteine string motif in GAIP/RGS19, using radiolabelled palmitate (134). Several RGS proteins have been predicted to undergo palmitoylation based on sequence homology to GAIP. Today it has been shown that palmitoylation occurs at various sites in the RGS peptide sequence, conferring an array of regulatory functions.

Palmitoylation at the N-terminal domain has been well described in RGS proteins; RGS3, RGS4 and RGS16 localize to several membrane targets in a manner that appears to be influenced by the reversible palmitoylation that occurs at more than one N-terminal cysteine residue (69;70;92;96). RGS16, for instance, has two cysteine residues in its N-terminus, C2 and C12, where radiolabelled palmitate incorporation is blocked upon cysteine mutation (135). This mutation, however, did not block complete translocation of RGS16 to the PM (136). Rather, removal of palmitoylation at the N-terminal sites appeared to prevent RGS16 localization to the membrane microdomains of lipid rafts (135). As well, RGS16 GAP activity on the Gi and Gq is severely inhibited when palmitoylation is prevented (135;136). Also discovered was the fact that palmitoylation at the N-terminal sites of RGS16 triggered the palmitoylation of an internal cysteine, C98 (135).

Internal palmitoylation at the RGS domain appears to regulate the RGS GAP activity. As demonstrated in RGS16, palmitoylation at C98 is critical for GAP activity and regulation of Gαi.
dependent signalling in mammalian cells (137). This C98 is conserved in RGS10 at C66. When palmitoylated in vitro, RGS10 failed to elicit GAP activity toward either Gαi or Gαz (96). However, in reconstituted receptor-G-protein proteoliposomes, a more physiologically relevant system, wild type RGS10 GAP activity actually increased greater than 20-fold. This finding demonstrates that in cells, RGS10, a hydrophilic cytoplasmic protein that does not have an amphipathic α-helix, overcomes the inhibitory effects of RGS domain palmitoylation to utilize palmitate's membrane targeting characteristics (94). Palmitoylation in the RGS domain of RGS2 has also been recently explored (138). Three cysteine residues, C106, C116 and C199, exist as palmitoylation targets in this RGS protein. RGS2 is the only R4 subfamily member to demonstrate selective GAP activity towards Gαq; when palmitoylated at its cysteine sites, this GAP activity was inhibited in vitro, and circular dichroism experiments showed that palmitoylation changed the conformational state of RGS2.

Palmitoylation in RGS proteins has not been demonstrated in any RGS family members other than those discussed above. Palmitoylation in RGSZ-like subfamily members is predicted to occur due to the presence of N-terminal cysteine string motifs (134) which do not exist in the R4 subfamily, but other than in GAIP, this has not yet been demonstrated (139). The only other RGS member where palmitoylation has been exhibited is RGS7 (140). RGS7 exists in a heterodimer with the Gβ5 subunit, and membrane fractions of RGS7 have been shown to possess palmitate groups. The soluble dimer exists in the cytosol when transfected in cells, and activation of Gαo subunits appears to trigger RGS7/β5 palmitoylation at several sites in the RGS domain and promote dimer translocation to the PM (141).

1.c.iv.1 Palmitoylation of RGS4

RGS4 is localized to both the cytosol and plasma membrane. Membrane-bound RGS4 has been shown to be palmitoylated at C2 and C12 in the N-terminus, and C95 in the RGS
domain; cytosolic fractions of RGS4 are not palmitoylated (96). C95 is the residue conserved in RGS10 (C66) and RGS16 (C88), as discussed above. Mutation of C95 prevents radiolabelled palmitate incorporation into the RGS box of RGS4 (96). However, there are conflicting reports about the effects of palmitoylation at this site on RGS4 activity. Ross and co-workers demonstrated that palmitoylation at C95 blocked 80-100% of RGS4 GAP activity towards G\(\alpha_i\) in solution-based assays (96). In contrast, Druey and co-workers showed that blockage of palmitoylation at C95, via site-directed mutagenesis, resulted in reduced GAP activity on G\(\alpha_{i1}\) and diminished adenylyl cyclase inhibition (137).

As discussed previously, the presence of an N-terminal amphipathic \(\alpha\)-helix directs RGS4 localization to the PM, and palmitoylation at C2 and C12 appears to enhance this membrane binding, in addition to increasing GAP activity (56;69). However, mutation of these cysteine residues has not been shown to completely block PM targeting and translocation of RGS4, nor did it impair RGS4 function, in yeast (92). Rather, blocking palmitoylation at these sites prevents palmitoylation on the internal cysteine, C95 (56;135).

A potential role of palmitoylation at the N-terminal cysteines in RGS4 is as follows. When expressed in cell lines, RGS4 has a half-life of less than one hour, which is a result of the protein being targeted for degradation by the N-end rule pathway (142). The presence of an aspartate, glutamate, or cysteine at position 2 – as in RGS4 – increases arginylation at this residue, followed by ubiquitination and subsequent degradation. The role of fatty-acid acylation at the N-terminus would be to prevent degradation by interfering with arginylation at C2, thus preserving RGS4 levels within the cell (56).

1.d. DHHC Enzymes

1.d.i. The Case for Enzymatic Palmitoylation
Palmitoylation in cells is rapid and reversible, with substrates undergoing cycles of palmitate addition and removal. The palmitoylation turnover cycle can be likened to the reversible nature of protein phosphorylation, which features phosphate group addition and removal by families of protein kinases and phosphatases, respectively. Following this paradigm, palmitoylation also has two families of enzymes catalyzing palmitate attachment and hydrolysis. The family catalyzing the addition of palmitate groups to cysteine residues are termed palmitoyl-acyl transferases (PATs), and the family which cleaves the thioester bonds are called acylprotein thioesterases (APTs). Here, the discovery, structures and functions of the family of mammalian PATs are discussed in detail.

The development of the PAT enzyme field was delayed due to the demonstration that protein substrates can undergo thioacylation without the requirement of enzymes in vitro (143-145). However, a theory against this autoacylation in vivo comes from the consideration that in cells, acyl-CoAs – the acyl-donating molecules in spontaneous acylation reactions – are bound to acyl-CoA binding proteins (98). This effectively reduces the pool of fatty acyl groups available for palmitoylation and other lipidations. Silvius and co-workers showed that inclusion of acyl-CoA binding proteins in protein palmitoylation assays in vitro decreases the rate of autoacylation in a concentration-dependent manner (146). The same group calculated that in the presence of physiological concentrations of lipids, acyl-CoA, and its binding protein, autoacylation would have a half-life on the order of days (146), as opposed to the actual half-life of hours seen in some cases in vivo (147). The final case for enzymatic palmitoylation in cells came with the discovery of PATs, first in yeast, and then in mammals.

1.d.ii. Discovery of DHHC Proteins in Yeast

The first evidence of PATs in vivo was discovered in S. cerevisiae by two separate groups (148;149). First, Deschenes and co-workers identified a Ras mutant in yeast that relied
upon palmitoylation to maintain cell viability (150). Thus, using this palmitoylation-dependent Ras, called Ras2, they screened the yeast genome, creating mutations that would result in a reduction of palmitate incorporation into the protein and thus result in yeast death. From this screen, two genes of interest arose, ERF2 and ERF4. It was found that mutations to either of the gene products Erf2 or Erf4, integral membrane proteins known to complex together, caused a reduction in Ras2 palmitoylation and removal of Ras2 from the PM to endomembranes (150). The group later showed that the Erf2-Erf4 complex has PAT activity for the yeast Ras2 protein in vitro (148). Concurrent studies performed by Davis and co-workers identified the yeast ankyrin-repeat protein Akr1, another integral membrane protein, as a PAT for yeast casein kinase 2 (149). As a result, Erf2-Erf4 and Akr1 make up the first PATs identified and confirmed by genetic and biochemical methods.

Conserved in both Erf2 and Akr1 is a cysteine-rich domain (CRD), within which resides an aspartate-histidine-histidine-cysteine (DHHC) motif. The cysteine in this DHHC motif was found to undergo autoacylation upon incubation with acyl-CoA, and mutation of this residue resulted in inhibition of autoacylation and blockage of palmitate transfer to a substrate, demonstrating the PAT activity within the DHHC motif (148;149). Erf2 was found to require Erf4 binding in order to confer PAT activity (97).

Using sequence homology to Erf2, the mammalian gene ZDHHC9 was the first gene selected to examine PAT function in mammals. However, without a mammalian ortholog of Erf4, DHHC9 was found to be non-functional. Thus, through BLAST searches, the Golgi-localized GCP16 was identified, and it was demonstrated that DHHC9 and GCP16 together are functional orthologs of Erf2 and Erf4, palmitoylating both H-Ras and N-Ras in vitro (151).

1.d.iii. Structural Characteristics of DHHC Proteins
The DHHC-CRD motif was used to identify analogues of both the yeast and human PAT families. To date, there are 7 DHHC family members in yeast and 23 in humans (152). All are polytopic membrane proteins, with between 4 and 6 TMDs and the DHHC-CRD region residing on the cytoplasmic face usually between TMD2 and TMD3 (153). The consensus sequence of the DHHC-CRD in PATs is as follows (154):

\[ C \_2 C \_9 H C \_2 C \_4 D H H C \_5 C \_4 N \_3 F \]

This motif mimics a zinc-finger domain, and while it is an excellent candidate for zinc binding, this has not yet been shown (155). This region is robustly conserved in yeast and mammalian DHHC proteins, with a few exceptions: Akr1, Akr2 and Pfa5 in yeast, and DHHC22 in humans, are missing select cysteine or histidine residues within the DHHC-CRD motif (152). The missing amino acids do not affect Akr1 PAT activity, and the other candidates have not yet been tested.

Other domains appear to be significantly homologous across DHHC proteins. Mitchell and co-workers carried out a ClustalX alignment of the yeast and human DHHCs, which identified several amino acids highly conserved at amino acid positions nearby to the DHHC-CRD region (152). This led to the development of a longer, more convoluted consensus CRD region, albeit with more gaps in sequence homology between proteins:

\[ C \_2 C \_3 (R/K) P \_x R \_x \_2 H C \_x \_2 C \_x \_4 D H H C \_W (V/I) \_x N C (I/V) \_G X \_2 N \_X \_3 F \]

Also discovered were an aspartate-proline-glycine motif close to TMD2 and a threonine-threonine-x-glutamate motif near TMD4, though the functional significance of these two regions has yet to be elucidated.
Sequence alignment of all DHHC proteins, human and yeast, demonstrates a close phylogenetic relationship between the two species. Six potential subfamilies are predicted, with at least one yeast DHHC in each group (152). Hence, it is tempting to hypothesize that the function of human DHHCs within a subfamily is analogous to the corresponding yeast DHHC subfamily member, as seen in some cases (151;156). In addition, some subfamilies demonstrate shared structural characteristics amongst members; all members of the subfamily featuring Akr1, Akr2, and the human DHHCs 13 and 17, for example, have ankyrin-repeats in their N-termini (153;156).

1.d.iv. Mechanism for DHHC-catalyzed Protein Palmitoylation

All DHHCs examined to date undergo autoacylation when incubated with palmitoyl-CoA prior to their enzymatic PAT activity (148;149;151;157-159). DHHCs use acyl-CoA as a lipid substrate, as they cannot incorporate lone palmitate groups (158). The autoacylation is disrupted by hydroxylamine, through an effect similar to that of the compound on cleaving thioester bonds. Autoacylation appears to occur on the cysteine of the DHHC motif, as mutation of this amino acid blocks palmitoylation (148;149). The auto-palmoylation is a rapidly-acting stoichiometric addition of palmitate that precedes an eventual transfer of the lipid group to substrate (151). Taken together, these suggest that acylated DHHC proteins act as an enzymatic intermediate required for the PAT reaction to occur. Additionally, the cysteine residue is not the only residue in the DHHC motif found to be important for protein palmitoylation; mutation of the first histidine in the DHHC domain of Erf2 has been shown to drastically decrease palmitate transfer to Ras2, while not affecting autoacylation (148).

1.d.v. Yeast DHHC Localization and Function
Of the seven yeast DHHC PATs, only three have been confirmed to elicit PAT activity biochemically (105). However, mutations to the genes of each DHHC protein has demonstrated decreased substrate incorporation of palmitate for many of the seven, and often each individual DHHC localization profile dictates which membrane the substrate will bind to. In essence, this further fuels the legitimacy of the kinetic trapping model described above (97).

Erf2 is one of the three yeast DHHCs proven, using biochemical techniques, to confer palmitate to the yeast Ras2 protein. It does so at the ER (148;150). Akr1, a Golgi-localized PAT, palmitoylates yeast casein kinase 2 and sphingoid Lcb4 in vitro and in vivo (109;149). The vacuole-targeted Pfa3 – the only vacuolar DHHC, yeast or human (160) – mediates Vac8 palmitoylation and localization (157;161). Two ER-localized yeast DHHCs – Pfa4 and Swf1 – appear to exhibit selectivity to specific substrate targets. The protein family of amino-acid permeases appears to only be palmitoylated by Pfa4 (107), while Swf1 seems to regulate SNARE protein acylation and any transmembrane proteins with juxtamembranous cysteine residues (108;162). Substrates for the PM-bound Pfa5 and Golgi-localized Akr2 have yet to be discovered (160).

1.d.vi. Mammalian DHHC Localization and Function

In humans, the DHHC genes are denoted ZDHHC1 to ZDHHC24, with ZDHHC10 not marked as a gene. The gene products of these 23 ZDHHCs feature several TMDs, various ankyrin repeats, and the characteristic DHHC-CRD domain amongst other conserved domains discussed previously (Figure 2).

Igarashi and co-workers compiled the only comprehensive analysis of all yeast and mammalian DHHC intracellular localization patterns and tissue-specific expression profiles (160). This study painted an expansive portrait of the various cellular membranes DHHCs are
integrated into and their differential tissue distribution. The group discovered that all human DHHCs are localized to the Golgi, ER, or PM. Other than DHHC2, 11, 15, 19 and 20, all DHHCs are ubiquitously transcribed in human tissue. DHHC11 and 19 mRNA were testis-specific, while DHHC20 mRNA was found in testis and placental tissue. DHHC2 and 15 mRNA expression was unconfirmed.

The first mammalian DHHC identified, DHHC9 – the ortholog of yeast Erf2 – has been shown to possess PAT activity towards H-Ras and N-Ras upon complex formation with the Golgi-bound GCP16 (151). In humans, both DHHC9 and GCP16 are expressed in many vital tissues, such as the brain, heart, kidneys, liver and lungs; in the colon, however, DHHC9 is expressed alone. This raises the question whether the colon has DHHC9-singular PAT activity, or if a GCP16-analogue is expressed in this tissue.
**Figure 2. Human DHHC Enzyme phylogeny tree.** Structural characteristics of the 23 human DHHCs organized according to phylogeny based on ClustalX alignment of the 51 amino acid DHHC core sequence. All PATs feature the characteristic asp-his-his-cys (DHHC) motif within a cysteine-rich domain, as well as 4 or more TMDs, predicted here using TopPred II 1.1. DHHC17 (HIP14) and DHHC13 (HIP14-Like) carry ankyrin repeat motifs ahead of the DHHC-CRD. Adapted from figures in Ohno et al. (2006) (160) and Mitchell et al. (2006) (152).
1.d.vi.1. DHHC Proteins at the Synapse

Palmitoylation regulates a variety of neuronal scaffolding, tethering and signalling proteins, as well as several channels and receptors, at the synapse (98;163). Accordingly, mammalian DHHCs have been examined in this context. Palmitoylation of PSD-95, a synaptic scaffolding protein, is important in regulating trafficking, protein-protein interactions with the glutamate receptor and hence regulation of excitatory postsynaptic currents (164).
DHHC17, also known as Huntingtin Interacting Protein 14 (HIP14), has been demonstrated to palmitoylate the N-termini of PSD-95, SNAP-25, GAD65 and huntingtin, *in vitro* and in neurons, promoting perinuclear accumulation and synaptic clustering of these proteins (158). The role of HIP14 in huntingtin palmitoylation has implications in mediating Huntington's Disease. This neurological disorder is caused by the polyglutamine expansion of mutant huntingtin, resulting in uncontrolled protein aggregation within cells and eventual loss of synaptic function (105). It was found that mutated huntingtin showed reduced association with HIP14 and was less palmitoylated than wild type huntingtin (165). Due to mutant huntingtin being more deleterious for cells, regulation by HIP14 seems to play a neuroprotective role in the fight against Huntington's Disease.

Bredt and coworkers introduced an elaborate protocol for screening all 23 DHHCs for ability to induce PAT activity on specific substrates. Using PSD-95 as their first substrate, they found DHHC2, 3, 7 and 15 also preferentially palmitoylated the scaffolding substrate in culture (159). Further experiments involving DHHC15 mutated at its autoacylated cysteine (dominant-negative DHHC15) demonstrated the importance of DHHC15 in PSD-95 and glutamate receptor synaptic clustering *in vivo*.

DHHC3 (GODZ) is another player in regulation by palmitoylation in neuronal cells. GODZ is a 4-TMD Golgi-specific protein expressed ubiquitously in humans, and has been shown to sequester the GluRα1 glutamate receptor subunit to the Golgi when overexpressed
in culture (166). It has also been demonstrated to palmitoylate the SNARE protein SNAP25 and the γ2 subunit of GABA_A receptors (167;168). Recently, El-Husseini and co-workers utilized the acyl-biotin exchange assay to screen the PAT activity of four neuronal DHHCs. While DHHC8 was found to specifically modulate paralemmin-1 palmitoylation and HIP14 mediated huntingtin palmitoylation in COS7 cells, DHHC3 displayed a broader PAT activity profile (169). However, when the N-terminal ankyrin domain of HIP14 was fused to DHHC3, the DHHC3 fusion protein was able to bind and palmitoylate huntingtin to the same levels as wildtype HIP14. This demonstrates that regions outside the DHHC-CRD can contribute to substrate specificity.

1.d.vi.2. DHHC Proteins Regulate the NOS Family

The nitric oxide synthase (NOS) family of proteins is subject to regulation by DHHCs. A screen similar to that engineered by Bredt and coworkers was used to identify candidate PATs for endothelial NOS (eNOS). Residing on the cytoplasmic face of the Golgi, eNOS is subject to both myristoylation and palmitoylation, with inhibition of the latter shown to decrease nitric oxide release. The screen produced DHHC2, 3, 7, 8 and 21 – all Golgi-specific DHHCs – as candidates able to incorporate radiolabelled palmitate into eNOS (170). Interestingly, knockdown of DHHC21 in human endothelial cells, but not DHHC3, was able to inhibit palmitoylation of and NO release by eNOS.

A second NOS protein found to interact with DHHCs is neuronal NOS (nNOS). nNOS associates with DHHC23 [or nNOS-interacting DHHC domain-containing (NIDD)] at the postsynaptic density of neurons. Unique to this NOS family member is a PDZ domain at its N-terminus, thought to play a role in cellular targeting of nNOS. NIDD possesses a PDZ-binding motif at its C-terminus, and through PDZ interactions, is able to target nNOS to the synaptic PM to increase its activity (171). PAT activity by NIDD, however, has not been demonstrated.
This is an example of DHHC influence on protein trafficking via palmitoylation-independent mechanisms.

1.d.vi.3. DHHC Proteins in Genetically-linked Human Diseases

Studies of the DHHC family have produced several discoveries having to do with mutations in the human ZDHHC gene loci (97). ZDHHC2 was found to be downregulated in human colorectal cancer, a hepatocellular carcinoma, and a nonsmall lung cancer, in an analysis of 111 human tumors (172). On the schizophrenia susceptibility locus on chromosome 22q11, ZDHHC8 presented with the most single-nucleotide polymorphisms (173). As well, ZDHHC8-knockout mice exhibited behaviours characteristic of the schizophrenic phenotype, indicating that there may be a link between palmitate transferase activity and this psychiatric disease. In a screen of 18 patients with bladder cancer, ZDHHC11 was upregulated in the highest-grade cases, implicating DHHC11 as a predictor for bladder cancer progression (174). Finally, evidence exists for ZDHHCs in X-linked mental retardation. Analysis of lymphocytes from a patient of the disease revealed an absence of ZDHHC15 transcript variants (175). Frameshift and missense mutations were identified in the locus of ZDHHC9, particularly in the DHHC-CRD region, in families with X-linked mental retardation, making it a second susceptibility gene for the disease (176).

The mechanistic consequences and specific substrates of each DHHC involved in these genetic diseases have yet to be discerned. However, the above pathologies give insight to the need to further expand our knowledge of the regulation and function of enzymatic DHHC palmitoylation.

1.d.vii. DHHCs and G-Proteins
Although concrete evidence exists for G-protein subunit palmitoylation, very little is known about the enzymes responsible for this post-translational modification. As mentioned earlier, the DHHC9/GCP16 displays PAT activity to the small GTPases H-Ras and N-Ras (151). Very recently, DHHC19 was found to strongly co-localize with the trans-Golgi markers Gal-T and TGN38, increased palmitoylation of R-Ras in COS7 culture, and through this palmitoylation, increased the viability of NIH3T3 cells (177).

The most promising G-protein study came from Fukata and coworkers. Through systematic screening of the mammalian DHHC family, they identified DHHC3 and DHHC7 as candidate PATs for the Gαq subunit (178). Palmitoylation by these two DHHCs was shown to regulate Gαq rapid shuttling between the PM and the Golgi, and knockdown of these PATs blocked the Gαq-coupled α1-adrenergic receptor signalling pathway.

1.d.viii. DHHCs and RGS4

Despite the fact that palmitoylation is found to occur in numerous RGS proteins, no candidate PAT enzyme has been identified for any member of the RGS family, including RGS4.
1.e. Rationale

Palmitoylation is a key post-translational lipid modification of RGS proteins, demonstrated to regulate RGS protein trafficking, membrane association, protein-protein interactions, and GAP activity, to varying degrees (56;69;135;137;138;141;179). Specifically, RGS4 is palmitoylated at C2 and C12 in the N-terminus, and C95 in the RGS domain (96). Several roles of palmitoylation at these sites – some conflicting – have been proposed in the literature (69;92;96;137). Preliminary data from our lab has demonstrated that wild type RGS4 localizes to the PM, an effect that was completely abolished using the palmitoylation inhibitor 2-BP. Furthermore, mutation of cysteines 2 and 12 to alanine diminishes RGS4 PM translocation (Figure 3).

The family of 23 DHHC PAT enzymes are important for catalyzing palmitoylation on several mammalian proteins (97;105;168;180). However, the DHHC(s) responsible for RGS4 palmitoylation have not yet been identified. The aim of this work is to identify the member(s) of the DHHC family responsible for palmitoylating RGS4, and in the process, elucidate the contributions of palmitoylation to RGS4 localization and function.

1.f. Hypothesis

One or more of the 23 DHHC proteins is a PAT candidate for RGS4, and is instrumental in initiating RGS4 association with, and GAP function at, the PM.
FIGURE 3

FIGURE 3. Palmitoylation is important for RGS4 binding to the PM. HEK cells transiently transfected with human RGS4-YFP plasmid. Images captured using Olympus Fluoview 1000 confocal microscope, excitation wavelength 515 nm, 60x magnification. RGS4-WT-YFP localized to the PM (left), and this was abolished by co-transfection with the palmitoylation inhibitor, 2-bromopalmitate (2BP) (centre). Mutation of both C2 and C12 diminished, but did not eliminate, RGS4 translocation to the PM (right), suggesting that other cysteine residues may be involved in the RGS4-PM interaction via palmitoylation. This experiment and figure courtesy of Guillaume Bastin.
Chapter 2

Materials and Methods

2.a. Cell Culture

TSA201 (HEK201) cells stably expressing an SV40 temperature-sensitive T-antigen were grown in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1), supplemented with 10%(v/v) heat-inactivated fetal bovine serum, 2mM glutamine, 10μg/ml streptomycin, 100units/ml penicillin (complete media). M2-HEK cells were grown in complete media supplemented with 2μg/ml active Puromycin. HEK201 cells stably expressing an EYFP-tagged RGS4 were grown in complete media supplemented with 0.5mg/ml active G-418 (Geneticin, Sigma Aldrich). All cells were grown in 37°C incubators in a 5% CO₂ environment. Cells were transiently transfected with plasmid DNA using Fugene (Roche) or siRNA using Lipofectamine RNAiMAX (Invitrogen, catalogue no. 13778-075) according to manufacturers’ protocols.

2.b. Plasmid Constructs

RGS4 cloned into the NheI and AgeI cloning sites ahead of enhanced yellow fluorescent protein in pEYFP-C1 was used. pEF-Bos-HA-mouse DHHC clones, described previously (159), were a kind donation of the Fukata Lab (National Institute for Physiological Sciences, Okazaki). The mutants DHHS3, DHHS5, DHHS7, DHHS18 and DHHS21 were generated using site-directed mutagenesis. The plasma membrane-tethered ss-ECFP-TM- \( \alpha_{qi9} \), described previously(181), was a kind donation of the Lambert Lab (Medical College of Georgia, Augusta). The mutant ss-amber-TM- \( \alpha_{qi9} \) (Gqi9) was created using site-directed mutagenesis. The trans-Golgi marker TGN38-cerulean was a kind donation of the Lippincott-Schwartz Lab (National Institutes of Health, Bethesda).
2.c. siRNAs

All siRNAs were purchased from Dharmacon RNAi Technologies (part of Thermo Fisher Scientific). For human DHHC3 and DHHC7 knockdown, the following target sequences were used: DHHC3, CAUCGCGAGAGUUUACAGUUG; DHHC7, GCCCGUGGGUGAACAAUUG. The negative control siRNA used was siGENOME Non-Targeting siRNA #2 (catalogue no. D-001210-02-05).

2.d. Quantitative Real Time PCR and Analysis

Total RNA was extracted from HEK201 cells using TRIzol reagent (Invitrogen Life Technologies). All quantitative RT-PCR was performed using an ABI Prism 7900 HT (Applied Biosystems) using the Sybr Green detection system. 2μg of total RNA was reverse-transcribed with random hexamer primers using the Superscript III kit (Invitrogen Life Technologies) following the manufacturers protocols. cDNA was diluted to a final concentration of 1ng/μl. Two microliters of this mixture was used as template for real time PCR quantification. DHHC expression was evaluated in each cDNA sample as indicated. Primers were validated using primer validation curves (User Bulletin No. 2, Perkin Elmer Life Sciences). 5’ PCR primers for housekeeping genes GAPDH and 18S were used as normalizing controls. Refer to Table 1 for list of all 5’ PCR primers used. A no-RT and no template control sample were included for each primer set. Data were analyzed using the comparative CT method (User Bulletin No. 2, Perkin Elmer Life Sciences). The CT for each sample was manipulated first to determine the ΔCT [(average CT of sample triplicates for the gene of interest) – (average CT of sample triplicates for the normalizing gene)] and second to determine the ΔΔCT [(ΔCT sample)-(ΔCT for the calibrator sample)]. Values are expressed in log scale and the relative mRNA levels are presented by conversion to a linear value using $2^{-\Delta\Delta CT}$.
2.e. Confocal Microscopy

For dominant negative DHHC and overexpression DHHC experiments, live cells were plated in 35mm high-wall μ-Dishes (Ibidi, catalogue no. 81156). For DHHC knockdown experiments and all other experiments, Poly-L-Lysine coated 25mm circular #1 glass coverslips (VWR, catalogue no. 48380 080) were seeded with live cells in 6-well dishes and mounted in a modified Leyden chamber. Cells were plated 48 hours prior to assay so as to achieve 60% confluence on day of experiment. For dominant negative DHHC and overexpression DHHC studies, HEK201 cells stably expressing the YFP-tagged RGS4 construct were transfected with 1μg pECFP-C1 marker plasmid and 1μg of DHHC plasmid as indicated, 24 hours prior to assay. For DHHC knockdown studies, HEK201 cells stably expressing the YFP-tagged RGS4 construct were transfected with 30pmols of indicated DHHC siRNA (final concentration 10nM siRNA) 18 hours prior to assay. Cells were grown in 2ml complete media, which was replaced with 150mOsm hypotonic solution (62mM NaCl, 3mM KCl, 1mM MgSO₄, 1mM Na₂HPO₄, 1mM CaCl₂, 6.6 mM MOPS, 5mM HEPES, pH 7.3) immediately prior to assay. Confocal microscopy was performed on live cells at 37°C, 5% CO₂ using a FluoView 1000 laser scanning confocal microscope, under 60x oil objective. Images were analyzed using ImageJ, which assigns a gray value number corresponding to pixel intensity. For analysis, a line was drawn across a region of cell not in contact with other cells. PM fluorescence was chosen for the pixel on the line considered to be the outermost edge of the cell. Cytosolic fluorescence was calculated by averaging all cytosolic pixels on the line until two pixel distances away from “PM”. All figures present average PM:cytosol fluorescence ratios for a given condition, as well as the proportion of cells above a PM:cytosol of 1.1. Cells below this threshold were considered to have negligible PM fluorescence. For DHHC knockdown study, experimenter was blinded at time of data collection and image analysis.
2.f. Intracellular Calcium Imaging

M2-HEK cells were seeded onto Poly-L-Lysine coated #1 glass coverslips in 12-well dishes 48 hours prior to assay. For dominant negative DHHC studies, cells were transfected with 1μg YFP-tagged RGS4 plasmid and 1μg DHHS plasmid as indicated. For DHHC knockdown studies, cells were transfected with 1μg YFP-tagged RGS4 and 1μg Gqi9 plasmid; 6 hours later, media was removed and fresh media replaced, and cells were transfected with 12pmols of indicated DHHC siRNA (final concentration 10nM). 24 hours following transfection (18 hours following siRNA knockdown) coverslips were washed and incubated in calcium imaging buffer (11 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl2, 17 mM HEPES, and 1 mM CaCl2, pH 7.3) containing 5 μM Fura-2/AM ratiometric dye (Invitrogen) and 0.05% pluronic acid for 40 min at 37 °C. Fura-2-loaded cells were washed again and incubated for at least 5 min in calcium imaging buffer to allow hydrolysis of the acetoxymethyl ester. Coverslips were mounted in a modified Leyden chamber and imaged on an Olympus BX51WI upright microscope using a 40x water-dipping objective. Excitation light was provided by a DeltaRam V monochrometer (PTI, Lawrenceville, NJ). Fluorescence imaging was performed with ImageMaster imaging software (PTI). Images were acquired with a Photometrics Cascade 512B cooled charge-coupled device camera (Roper Scientific, Tucson, AZ).

YFP-expressing cells were identified using 510 ± 3nm excitation light in conjunction with a YFP filter set (Chroma Technology Corp., Brattleboro, VT) and selected as regions of interest within the ImageMaster software. For Fura-2 imaging, alternating excitation wavelengths (340 ± 5nm/380 ± 5 nm) were provided at ~1 excitation pair/s in conjunction with a 495-nm dichroic mirror and a 510 ± 20-nm emission filter (Chroma Technology Corp.), and paired images were collected after a 600-ms exposure. Fluorescent ratio (FR) values for the image pairs were determined for regions of interest previously selected on the basis of their YFP
expression. Baseline FRs (BFRs) (340nm/380nm) of nonstimulated cells (both transfected and non-transfected) were collected for 30 frames prior to the addition of 200 μM carbachol. Only cells with a baseline FR of less than 0.6 were selected for analysis. The fold-increase from BFR levels to the stimulated peak FR (PFR) was calculated only for cells with a relative YFP fluorescence of 4000 to 30,000. Identical excitation/emission conditions and data collection parameters were maintained for all individual experiments performed in this study.

2.g. Western Blotting

For DHHS dominant negative and DHHC overexpressor studies, identical cell samples were seeded alongside experimental cell samples, under same conditions, with identical amounts of DNA and/or siRNA transfected. Cell samples were lysed and protein extracts were digested with SDS loading buffer (10% v/v Glycerol, 0.004% SDS, 0.00125% bromophenol blue, 25mM Tris-HCl, pH 6.8, 0.00616% DTT) for 5 minutes on ice, sonicated for two 10-second pulses, boiled for 5 minutes, then electrophoresed on 12% poly acrylamide SDS-PAGE gels for 1.5 hours at 100V. Separated proteins were then transferred to nitrocellulose membrane (GE Healthcare, catalogue no. RPN303D) overnight on ice at 30V. Following transfer, membranes were blocked with 5% bovine serum albumin (BSA) in TBS-T (50mM Tris base, 150mM NaCl, 0.075% Tween-20) on a flat-bed shaker for 1 hour at room temperature. Blots were washed three times (15 minutes, 5 minutes, 5 minutes) with TBS-T and incubated with anti-HA mouse ascites primary polyclonal antibody, diluted 1:4000 in TBS-T with 5% BSA, for 2 hours at room temperature on a flat-bed shaker. After another three-step TBS-T wash (15 minutes, 5 minutes, 5 minutes), blots were incubated with anti-mouse secondary antibody conjugated with horseradish peroxidase, diluted 1:5000 in TBS-T with 5% BSA, for 1 hour at room temperature on a flat-bed shaker. Blots were then washed three times with TBS-T (15 minutes, 5 minutes, 5 minutes), incubated with ECL reagent
(Thermo Scientific, product no. 34080) and exposed on Bioflex MSI film (Clonex Corporation, catalogue no. CLMS810) for short (30 second) and long (5 minute) time courses.

2.h. Statistical Analysis

Data were analyzed using unpaired student’s t-test or one-way analysis of variance (ANOVA) with a Tukey’s Honestly Significant Difference (HSD) post-hoc test for multiple comparisons, where appropriate. Confocal data outlining percent of cells greater than the given PM:cytosol ratio were analyzed using Pearson’s Chi-Square Test. Unless otherwise stated, data are presented as mean +/- standard error of the mean (SEM). Significance for all data was taken at P values < 0.05. All data was analyzed using Microsoft Excel and SPSS.
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Table 1: 5’ PCR Primers used for mRNA quantification.
3.a. DHHCs 3, 5, 7, 18 and 21 are the mostly highly expressed DHHC proteins in HEK201 cells.

The literature demonstrates several cases where for specific tissues, the ZDHHC with the highest mRNA levels was a candidate PAT for a substrate of interest (159;170). Accordingly, to select potential DHHC candidates for RGS4 palmitoylation, we aimed to find the most highly expressed ZDHHCs in our cell type of interest. HEK201 cells were chosen due to their ease of culturing, high transfection efficiency, and relatively low endogenous RGS levels. As well, our previous data shows a robust, reversible PM localization profile of RGS4 when expressed in this cell type (Figure 3). Quantitative real-time polymerase chain reaction (qRT-PCR) experiments were performed in order to identify the DHHCs expressed at the highest levels (Figure 4). The following ZDHHC gene products were found to have the highest endogenous mRNA levels: DHHC3, DHHC5, DHHC21, DHHC18 and DHHC7. ZDHHC3 expressed highest of the group, at approximately 20 times greater than ZDHHC1 (ΔΔCT = 19.58 ± 2.67). ZDHHC7, approximately 8 times greater than DHHC1, was the lowest of the five candidates (ΔΔCT = 8.12 ± 0.64). As reference, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The mRNA levels of GAPDH were nearly 400 times greater than that of ZDHHC1 (ΔΔCT = 343.88 ± 97.90).
Figure 4. DHHCs 3, 5, 7, 18 and 21 are the mostly highly expressed ZDHHC genes in HEK201 cells. Expression profile of the 23 human ZDHHC genes, relative to ZDHHC 1, in HEK201 cells using quantitative RT-PCR. DHHCs 3, 5, 7, 18 and 21 were selected as target candidates based on their high relative levels of mRNA. Results represent mean of three experiments, triplicate 2ng RNA samples per experiment, normalized to GAPDH expression, ± SEM.
3.b. Overexpression of wild type DHHC candidates results in concentration of RGS4 at various subcellular organelles.

We next aimed to explore the influence of each candidate DHHC on RGS4 localization. We predicted that the RGS4-specific PAT would generate the greatest affinity of RGS4 for the PM via palmitoylation. To test this, we overexpressed HA-tagged wild type murine DHHC plasmid candidates, both individually and in combinations, in an HEK201 cell line stably expressing an enhanced yellow fluorescent protein (YFP)-tagged RGS4, and examined RGS4 localization patterns using confocal microscopy (Figure 5-A). Knowing that some of our candidate DHHCs were Golgi-localized in cells (160;178), we co-transfected our cells with the trans-Golgi marker TGN38, tagged with a cerulean fluorescence marker. We found that DHHC3 and 7 caused the greatest amount of intracellular RGS4 movement, but surprisingly, not to the PM. In these two conditions, RGS4-YFP was nearly completely abolished from the PM; in the DHHC3 condition, RGS4-YFP localization shown strong correlation with TGN-38. DHHC7 appeared to cause a weaker RGS4 co-localization with TGN-38. DHHCs 3 and 7 co-expressed also demonstrated a Golgi-localization of RGS4-YFP. No other DHHCs appeared to affect RGS4 localization.

We quantified this data by reporting the average Pearson's correlation coefficient (PCC), which represents the proportion of overlap between pixels of the two confocal channels (yellow and cerulean) used in this study (Figure 5-B). We selected the trans-Golgi globules, indicated by TGN38, as regions of interest. Indeed, the data confirmed that RGS4-YFP co-localized strongest to the trans-Golgi network upon overexpression with DHHC3, and this represented the largest change from wild type RGS4 expression patterns (PCC = 0.73 ± 0.04, p<0.05). DHHCs 3 and 7 co-overexpressed elicited a large change to RGS4 localization, albeit not as significant as DHHC3 alone (0.44 ± 0.11, p<0.05). No other DHHC condition elicited a significant movement of RGS4-YFP to the trans-Golgi; all of these conditions, other
than DHHC7 alone, had PCCs within between 0 and -1, indicating some level of mislocalization between RGS4-YFP and TGN-38. DHHC7, with a PCC of 0.16, demonstrated some level of colocalization, but was not statistically different from the RGS4-YFP control.

Finally, we confirmed the expression of our candidate DHHCs within each condition using Western blots (Figure 5-C). While all candidates were adequately expressed in their respective conditions, we noticed certain DHHCs expressed at higher levels than others, indicating variability in the levels of DHHC protein per condition.

We concluded that RGS4 traffics to the trans-Golgi network with great specificity upon overexpression of DHHC3. While DHHC7 undoubtedly shifted RGS4 proteins to a subcellular body – and it is tempting to assume similar effects by other DHHCs – we cannot make conclusions without the use of appropriate subcellular fluorescence markers.
FIGURE 5
A

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Figure 5. Overexpression of wild type DHHCs results in concentration of RGS4 at various subcellular organelles.  

A. HEK201 cells stably expressing RGS4-YFP, transiently co-transfected with DHHC and TGN38-cerulean, as indicated. By method of co-transfection, TGN38 represents DHHC uptake, except for control condition (“none”), which was transfected with TGN38 alone. Images captured using Olympus Fluoview 1000 confocal microscope, excitation wavelengths 450 nm (cerulean) and 515 nm (YFP), 60x oil magnification. Cells were incubated in 150 mOsm (hypotonic) buffer during image capture. RGS4-YFP was localized to the trans-Golgi network upon overexpression of DHHC3 alone and both DHHCs 3 and 7 together.  

B. Pearson’s correlation coefficient (PCC) for RGS4-YFP alone (control) and RGS4-YFP plus overexpression of DHHC as indicated. The PCC ranges from -1 to 1; 1 represents perfect overlap of pixels between channels, while -1 implies complete non-overlap. Trans-Golgi globules, represented by TGN38, were selected as regions of interest. At least 11 regions were selected for each condition. The data reveals that overexpression of DHHC3 alone, and DHHCs 3 and 7 together, resulted in the strongest RGS4-YFP co-localization with the trans-Golgi marker TGN38. These two conditions presented an RGS4 localization pattern statistically different from RGS4 alone (control). Results represent the mean PCC at the regions of interest for each condition ± SEM. Significance was determined using one-way ANOVA with a Tukey’s HSD post-hoc test for multiple comparisons, p<0.05. * = significant difference from control.  

C. Western Blot analysis from samples of RGS4-YFP alone (control), and each RGS4 + DHHC as indicated, demonstrating adequate expression of DHHC. Blots were incubated with antibodies against the HA-tag of DHHC constructs. Asterisks indicate DHHC protein band.
3.c. Effects of candidate DHHC knockdown on RGS4 regulation of G\textsubscript{q}-coupled intracellular Ca\textsuperscript{2+} release.

3.c.i. Validation of DHHC siRNA efficacy

The above experiment suggested that DHHCs 3 and 7 play a role in regulating RGS4 subcellular trafficking. These two DHHC enzymes have been implicated as the candidate PATs in regulating G\textsubscript{α\textsubscript{q}} subunit palmitoylation, PM targeting, and subsequent function (178).

Because RGS4 is an activator of G\textsubscript{α\textsubscript{q}} GTPase activity \textit{in vivo}, we decided to explore the effects of DHHCs 3 and 7 on RGS4 function. We intended to knock down each DHHC of interest and examine the changes in RGS4 regulation of agonist-dependent Ca\textsuperscript{2+} signalling by the G\textsubscript{q}-coupled muscarinic receptor pathway.

Prior to experimentation, we validated each member of a pool of DHHC-specific siRNAs. Using quantitative RT-PCR, we tested the efficacy of siRNAs for DHHC3 and DHHC7 to knock down their intended gene, while observing any non-targeted DHHC mRNA knockdown that may occur (Figure 6). To this effect, DHHC3 siRNA number 4 and DHHC7 siRNA number 3 were selected, based on their ability to successfully knock down intended targets while minimizing fluctuations in family DHHC mRNA levels.

To further our validation, we tested the effects of pooling siRNA constructs (Appendix A), and time-dependent knockdown (Appendix B) on target transcript expression. Neither multiple siRNA duplexes nor increasing time of knockdown resulted in any added target gene knockdown, with both techniques causing further upregulation of non-target DHHC candidates.
Figure 6. Validation of DHHC3 and DHHC7 siRNA efficacy. Expression levels of five DHHC candidates, relative to non-knockdown control levels (dashed line), following knockdown with four siRNA target sequences each for (A) DHHC3 and (B) DHHC7. siRNAs were chosen on the basis of maximal knockdown of intended DHHC target, and minimal fluctuations of non-target DHHCs. Data presented are ΔΔCT values from triplicate 2 ng HEK201 mRNA sample measurements, normalized to GAPDH expression.
3.c.ii. TM-Gαqi9 is coupled to intracellular Ca\(^{2+}\) release in M2R stably-expressing HEK cells

We attempted to assess the effects of knocking down DHHCs 3 and 7 on RGS4 and its GAP activity for Gαq-coupled Ca\(^{2+}\) release from intracellular stores. Briefly, stimulation of M1 muscarinic receptors through binding of the agonist acetylcholine activates the Gαq subunit. Following binding of GTP and detachment from the βγ complex, Gαq initiates cleavage of phosphatidylinositol 4,5-bisphosphate into DAG and IP\(_3\) by the enzyme PLCβ. IP\(_3\) diffuses intracellularly and binds to IP\(_3\) receptors on the ER, allowing for an efflux of Ca\(^{2+}\), increasing the cytosolic Ca\(^{2+}\) concentration (19). The fact that DHHC3 and DHHC7 palmitoylate and regulate Gαq subunits (178) was an issue we had to address. Otherwise, any functional changes observed may not be a result of manipulating RGS4, but rather may be due to G-protein manipulation. Thus, we employed the use of a PM-tethered Gα chimera, first designed and characterized by the Bourne group (182) and utilized by Lambert and co-workers (181;183). Inactivation of G-proteins through RGS protein signalling, for example, causes a rapid transient dissociation of the heterotrimer from the PM. The construct CFP-TM-Gαqi9 (Gqi9) features a CFP marker (which we disabled via site-directed mutagenesis), a TMD that tethers the G-protein to the membrane even when the heterotrimer is inactive, and a Gα subunit chimera consisting of Gαq with nine C-terminal residues substituted for those found in Gαi. Gα subunits interact with GPCRs via their carboxy-tail (182). Hence, the Gqi9 subunit becomes activated by Gαi-coupled receptors, but signals downstream effectors normally triggered by Gαq/11.

In this experiment, we transfected Gqi9 into HEK cells stably expressing M2Rs, and validated our signalling system by showing presence of the Gα chimera significantly increased Gαq-coupled Ca\(^{2+}\) release via stimulation of M2Rs with the acetylcholine mimetic carbachol
(CCh) (PFR/BFR = 2.77 ± 0.11, p<0.05), when compared to YFP control alone (PFR/BFR = 2.30 ± 0.07) (Figure 7-B).

Finally, to ensure our palmitoylation-insensitive Gq mechanism was working up to theoretical standard, we showed that Gqi9-dependent Ca^{2+} release is not altered by knockdown of DHHCs 3 and 7 (see section 3.c.iii., Figure 8-A).
**FIGURE 7**

**A**

TM-Gα_{q\tilde{i}9} is coupled to intracellular Ca^{2+} release in M2R stably expressing HEK cells. 

A. Mechanism of the Gα subunit chimera TM-Gα_{q\tilde{i}9} (Gqi9), composed of the Gα_{q} subunit substituted at the last 9 C-terminus residues for those of Gα_{i}. This allows for Gαq effector molecules to be activated through stimulation of Gi-coupled receptors (M2Rs). This allows for bypassing of the endogenous Gq signalling pathway. In the absence of palmitoylation, important for Gα_{q} PM targeting, the subunit stays bound to the membrane via one TMD, making it palmitoylation-insensitive. 

B. Normalized fold intracellular Ca^{2+} increase (PFR/BFR) in M2R stably-expressing HEK cells transfected with YFP (control) or YFP and Gqi9. Cells were incubated in Fura 2-AM ratiometric dye for 40 minutes prior to experiment. Cytosolic calcium level was measured at baseline and at maximal release from intracellular stores following stimulation with carbachol (200\textmu M). Cells were visualized using Olympus BX51WI fluorescence microscope, excitation wavelengths 340nm and 380nm, 40x magnification. Presence of Gqi9 significantly augmented intracellular Ca^{2+} release (PFR/BFR = 2.77 ± 0.11) compared to control condition (2.30 ± 0.07). Results show mean fold Ca^{2+} increase ± SEM, with a minimum of 15 cells per condition, measured on one experimental day. Data was analyzed using unpaired student’s t-test, p<0.05.

**FIGURE 7.**
3.c.iii. Individual knockdown of DHHC3 or DHHC7 elicits better attenuation of RGS4 inhibition on Gqi9-dependent Ca^{2+} release than combination knockdown.

We next attempted to assess the effects of knocking down DHHCs 3 and 7 on RGS4 and its GAP activity for Gαq-coupled Ca^{2+} release from intracellular stores. We expected that transfection of RGS4 alone would significantly decrease the release of intracellular Ca^{2+} through RGS4 inhibition for Gαq signalling, and indeed, this was the case (YFP + Gqi9 PFR/BFR = 2.18 ± 0.05, RGS4 + Gqi9 PFR/BFR = 1.28 ± 0.04, p<0.05) (Figure 8-A). We hypothesized that knocking down each DHHC individually would attenuate RGS4 GAP activity, and knocking down both together would demonstrate an additive inhibitory effect. Confirming the initial part of this hypothesis, knockdown of DHHC3 alone (PFR/BFR = 1.54 ± 0.04) and DHHC7 alone (PFR/BFR = 1.57 ± 0.06) resulted in relatively similar attenuation of RGS4 GAP activity (p<0.05). When knocked down together (PFR/BFR = 1.36 ± 0.04), however, RGS4 GAP activity was surprisingly not significantly different from the RGS4 control condition.

We expressed each RGS4 signalling condition as a percentage of its respective control, to demonstrate the true effect of RGS4 within each condition (Figure 8-B). The effects of individual knockdown and dual knockdown of DHHC3 and DHHC7 were conserved, thus confirming that knockdown of each DHHC singularly had an RGS4-inhibiting influence, but RGS4 signalling was unaltered upon knockdown of both together.
Figure 8. Individual knockdown of DHHC3 and DHHC7 elicits better attenuation of RGS4 inhibition on Gq-dependent Ca\textsuperscript{2+} release than combination knockdown.  

A. Normalized fold intracellular Ca\textsuperscript{2+} increase (PFR/BFR) in M2R stably-expressing HEK cells transfected with YFP + Gqi9 (control) and RGS4 + Gqi9 and knocked down using siRNAs as indicated. Cells were incubated in Fura 2-AM ratiometric dye for 40 minutes prior to experiment. Cytosolic calcium level was measured at baseline and at peak release from intracellular stores following stimulation with carbachol (200\textmu M). Cells were visualized using Olympus BX51WI fluorescence microscope, excitation wavelengths 340nm and 380nm, 40x magnification. Knockdown of DHHC3 DHHC7 individually significantly attenuated RGS4 GAP activity on Gq-dependent Ca2+ release. RGS4 activity was not significantly inhibited upon knockdown of DHHC3 and DHHC7 together. Results show mean fold Ca\textsuperscript{2+} increase ± SEM, with a minimum of 45 cells per condition, measured over three experimental days. Results were normalized to fold calcium increase measured in untransfected control cells for the given experimental day. Data was analyzed using one-way ANOVA with a Tukey’s HSD post-hoc test for multiple comparisons, p<0.05.

B. Normalized fold calcium increase reported as a percent of control. Effect of DHHC3 and DHHC7 knockdown singularly and together was conserved when data was reported as a proportion of each condition’s individual control. Error bars represent % error of the mean * = significant difference from RGS4 + Gqi9.
3.c.iv. Knockdown of DHHC3 and DHHC7 alters the mRNA levels of other candidate DHHCs.

To confirm knockdown of DHHCs 3 and 7 in our calcium imaging experimental conditions, quantitative RT-PCR was performed on mRNA samples from each condition. This also gave us an opportunity to examine mRNA levels of all five highly expressed candidate DHHCs. The screen revealed that ZDHHC3 mRNA was knocked down by between 71% and 77% compared to non-knocked down (control) sample, while ZDHHC7 mRNA was down by between 79% and 81% compared to control (Figure 9-A,B). However, there appeared to be some non-specific knockdown of ZDHHC genes as well; ZDHHCs 3, 5, 7, 18 and 21 were reduced by as much as 40% compared to control in the presence of non-targeting siRNAs (Figure 9-C to E).
Figure 9. Knockdown of DHHC3 and DHHC7 alters the mRNA levels of other candidate DHHCs. mRNA levels for gene products (A) DHHC3, (B) DHHC7, (C) DHHC5, (D) DHHC18, and (E) DHHC21, relative to non-knocked down control (dashed line), for each calcium imaging condition, as indicated. DHHC siRNAs knocked down target genes by an average efficiency of approximately 80%. However, siRNAs also produced non-specific knockdown of other DHHC family members, by approximately 20-40% in some cases. Data presented are ΔΔCT values from triplicate 2 ng HEK201 mRNA sample measurements, normalized to GAPDH expression.
3.c.v. Non-targeting siRNA has little effect on both Gq-dependent Ca\(^{2+}\) release and transcript levels of candidate DHHCs.

Our calcium imaging data suggested that DHHC3 and DHHC7 each alter RGS4’s ability to regulate G\(\alpha_q\)-coupled release of Ca\(^{2+}\) from intracellular stores. However, the change in signalling observed could have been a general result of siRNA transfection. In order to control for possible siRNA-dependent effects, we repeated the calcium assay in the presence of non-targeting siRNA (Figure 10-A, B). This negative control siRNA did not significantly affect Ca\(^{2+}\) release following stimulation with CCh (PFR/BFR = 1.70 ± 0.12) when compared to release when RGS4 was transfected alone (PFR/BFR = 1.73 ± 0.15). These results suggest that the attenuation of RGS4 GAP activity on G\(\alpha_q\) by knockdown of either DHHC3 or DHHC7 was a genuine loss-of-function effect, and not due to an imbalance in steady-state cell signalling.

To ascertain whether the previous noted changes in mRNA levels to non-targeted DHHCs following knockdown may have also been due to a general siRNA-related disturbance in cell transcription machinery, samples from the negative control siRNA calcium study were run under quantitative RT-PCR. The findings demonstrated that a fluctuation of ZDHHC mRNA levels between 0-20% was created by the non-targeting siRNA, and thus attributed to non-specific siRNA effects (Figure 10-C). In the context of non-specific knockdown, it would appear that a majority of non-specific downregulation observed could be attributed to siRNA targeting multiple DHHC sequences, or global changes in transcriptional regulation.
FIGURE 10

A

Normalized Fold Ca2+ Increase (Peak FR/Baseline FR)

Gqi9

Gqi9 + non-targeting siRNA

N.S.

B

Normalized Fold Ca2+ Increase (% of Control)

Gqi9

Gqi9 + non-targeting siRNA

N.S.
Figure 10. Non-targeting siRNA has little effect on both Gq-dependent Ca$^{2+}$ release and transcript levels of candidate DHHCs. **A.** Normalized fold intracellular Ca$^{2+}$ increase (PFR/BFR) in M2R stably-expressing HEK cells transfected with YFP + Gqi9 (control) with and without non-targeting (negative) siRNA as indicated. Cells were incubated in Fura 2-AM ratiometric dye for 40 minutes prior to experiment. Cytosolic calcium level was measured at baseline and at peak release from intracellular stores following stimulation with carbachol (200μM). Cells were visualized using Olympus BX51WI fluorescence microscope, excitation wavelengths 340nm and 380nm, 40x magnification. Treatment with non-targeting siRNA had no significant effect on RGS4 signalling. Results show mean fold Ca$^{2+}$ increase ± SEM, with a minimum 15 cells per condition, measured on one experimental day. Results were normalized to fold calcium increase measured in untransfected control cells. Data was analyzed using one-way ANOVA with a Tukey’s HSD post-hoc test for multiple comparisons, p<0.05. N.S. = not significantly different. **B.** Fold calcium increase reported as a percent of control. Lack of effect of non-targeting siRNA on RGS4 GAP activity was conserved when data was reported as a proportion of each condition’s individual control. Error bars represent % error of the mean. N.S. = not significantly different. **C.** mRNA levels for gene products of the five candidate DHHCs, relative to siRNA untreated control (dashed line), following treatment with non-targeting siRNA. The negative siRNA induced negligible (~0-20%) average mRNA fluctuations of ZDHHC genes. Any further mRNA up- or down-regulation in previous experiments is presumably due to siRNA targeting of non-specific DHHC sequences. Data presented are ΔΔCT values from triplicate 2ng M2HEK mRNA sample measurements, normalized to ribosomal 18S expression.
3.d. Individual knockdown of DHHC3 or DHHC7 elicits greater inhibition on RGS4 translocation to the PM than combination knockdown.

We next examined the effects of knocking down DHHC3 and DHHC7 on RGS4 localization. We knocked down candidate DHHCs in the RGS4-YFP stable cell line and analyzed the cells using confocal microscopy (Figure 11-A). We quantitatively characterized which siRNA condition caused the greatest RGS4 change in PM-to-cytosol fluorescence ratio compared to that of RGS4-YFP alone. This analysis showed that siRNA inhibition of DHHC3 alone (PM:cytosol = 1.19 ± 0.02) and DHHC 7 alone (PM:cytosol = 1.13 ± 0.01) significantly reduced RGS4-YFP association with the PM compared to RGS4 control (PM:cytosol = 1.34 ± 0.04, p<0.05) (Figure 11-B). Knockdown of both DHHCs together also attenuated RGS4 PM localization (PM:cytosol = 1.25 ± 0.02), but similar to the functional data, not nearly as significantly as when knocked down individually. This finding, in PM regions free from cellular contact, also persisted at junctional membrane domains between cells (Appendix C).

To supplement this data, we identified the proportion of cells expressing RGS4 above a PM:cytosol threshold of 1.1 for each condition (Figure 11-C). Any cell below this threshold was said to have no PM localization. The proportion of cells above 1.1 when DHHCs 3 and 7 were knocked down together was found to be non-significantly different from the ratio of cells with DHHC activity intact (p<0.05). Knockdown of either DHHC3 or DHHC7 individually, however, significantly decreased the percentage of cells above 1.1 compared to control.
FIGURE 11

A

RGS4 YFP

RGS4 + D3, D7 siRNA

RGS4 + D3 siRNA

RGS4 + D7 siRNA

B

RGS4 PM:Cytosol Fluorescence Ratio

Control  DHHC3 siRNA  DHHC7 siRNA  DHHC3 + DHHC7 siRNA

*  *  *
Figure 11. Individual knockdown of DHHC3 or DHHC7 elicits greater inhibition on RGS4 translocation to the PM than combination knockdown. A. HEK201 cells stably expressing RGS4-YFP, transiently knocked down with the indicated DHHC siRNA(s). Images captured using Olympus Fluoview 1000 confocal microscope, excitation wavelength 515 nm, 60x oil magnification. Cells were incubated in 150 mOsm (hypotonic) buffer during image capture. Knockdown of DHHCs 3 and 7 singularly elicited a stronger reduction of RGS4-YFP at the PM than when knocked down in combination. B. PM:cytosol fluorescence ratios for RGS4-YFP alone (control) and RGS4 + siRNA(s), as indicated. Only regions of non-contact between cells were chosen as regions of interest. Minimum 75 cells per condition were selected. The data demonstrates all knocked-down conditions reduced RGS4 PM association to varying degrees (RGS4 control PM:cytosol = 1.34 ± 0.04, RGS4 + D3 sirna PM:cytosol = 1.19 ± 0.02, RGS4 + D7 sirna PM:cytosol = 1.13 ± 0.01, RGS4 + D3, D7 siRNAs PM:cytosol = 1.25 ± 0.02). Combined knockdown of DHHCs 3 and 7 displayed less RGS4-PM mislocalization than when DHHC7 was knocked down alone. Results represent mean PM:cytosol fluorescence ratio ± SEM, analyzed using one-way ANOVA with a Tukey’s HSD post-hoc test for multiple comparisons, p<0.05. * = significant difference from control, § = significant difference from D3 + D7 siRNA. C. Percentage of cells per condition presenting with a PM-to-cytosol fluorescence ratio greater than 1.1. Analysis reveals that the proportion of cells above 1.1 for DHHC 3 and 7 dual knockdown was not significantly different from the proportion of RGS4 control cells above this threshold. Minimum 75 cells per condition were selected. Data were analyzed using Pearson’s chi-square test between each condition individually, p<0.05. * = significant difference from control, § = significant difference from D3 + D7 siRNA.
3.e. Inhibition of each candidate DHHC through use of dominant negatives reduces RGS4 PM translocation.

The preceding data demonstrated that individual blockage of DHHC3 and DHHC7 at the mRNA level significantly inhibited RGS4 localization and function in HEK cells. We also wanted to test whether RGS4 was mislocalized from the PM when candidate DHHCs were inhibited at the protein level, through the use of dominant negative mutants. At the time of discovery of PATs in yeast, it was found that mutation of the cysteine residue in the DHHC motif conferred inhibition of palmitoylation (148,149). Subsequently, Lusher and co-workers showed that both DHHC3 and its dominant negative (DHHS3) worked by forming homomultimers and heteromultimers with DHHC7, which was consistent with their finding that overexpressed DHHS3 acted as a dominant negative for both DHHCs 3 and 7 (167). We created dominant negatives for our five candidate HA-tagged murine DHHCs using site-directed mutagenesis (Figure 12). We then transfected each DHHS into our RGS4-YFP cell line and analyzed the cells using confocal microscopy. As the DHHS plasmid constructs lacked a marker detectable by fluorescence microscopy, we co-transfected our cells with equal quantities of plasmid vector encoding the enhanced cyan fluorescent protein (CFP). This allowed us to correlate CFP expression with presence of DHHS protein. Unexpectedly, we found that all five DHHS mutants had a qualitative effect in reducing RGS4 PM localization to some degree (Figure 13-A).

Quantification of this data showed that RGS4-YFP alone was significantly more localized to the PM (PM:cytosol = 1.35 ± 0.03, p<0.05) than when under inhibition by any DHHC dominant negative (Figure 13-B). Surprisingly, DHHS18 alone demonstrated the greatest attenuation of RGS4 localization (PM:cytosol = 1.07 ± 0.01), while DHHS3 alone appeared to inhibit RGS4 translocation the least (PM:cytosol = 1.18 ± 0.04). We also tested the effects of inhibition by multiple DHHS proteins on RGS4 localization. A combination of
DHHS3 and DHHS7 demonstrated an additive result on RGS4 PM association (DHHS3 PM:cytosol = 1.18 ± 0.04, DHHS7 PM:cytosol 1.15 ± 0.02, DHHS3 + DHHS7 PM:cytosol = 1.13 ± 0.02). In contrast, DHHS18 and DHHS21 together demonstrated counteracting, or competitive, effects (DHHS18 PM:cytosol = 1.07 ± 0.01, DHHS21 PM:cytosol = 1.17 ± 0.03, DHHS18 + DHHS21 PM:cytosol= 1.10 ± 0.02). Neither of these results was statistically different from individual DHHS transfection conditions.

To confirm that the effect of each DHHS was internally conserved from cell-to-cell, and not due to anomalies by individual cells within a condition, we reported the percentage of cells above the PM:cytosol threshold of 1.1 (Figure 13-C). Indeed, this data mirrored the pattern seen in our average PM:cytosol data, indicating robustness of the experimental results.

Lastly, we utilized Western blotting to demonstrate the expression of DHHS mutants within a given condition (Figure 13-D). Similar to our DHHC overexpression microscopy study, we found DHHS protein levels to be variable between candidates.
**Figure 12. Generation of DHHC dominant negative mutants.** Sample chromatogram for a 75 base pair stretch of DHHC3 dominant negative mutant (DHHS3). Mutants were created via site-directed mutagenesis using murine DHHC plasmids and mutant DHHC primers. In order for DHHC enzymes to elicit their PAT activity, the cysteine residue of the DHHC region undergoes autopalmitoylation prior to substrate palmitoylation (148;149). Single base-pair mutations (indicated by arrow) converted this cysteine residue to serine, effectively eliminating the functionality of each DHHC. Formation of dimers and multimers between DHHS and DHHC block autoacylation and PAT activity of the wild type enzyme, allowing DHHS to act as a dominant negative mutant (167).
FIGURE 13

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FIGURE 13 cont.
Figure 13. Inhibition of each candidate DHHC through use of dominant negatives reduces RGS4 PM translocation. A. HEK201 cells stably expressing RGS4-YFP, transiently co-transfected with DHHS and pECFP as indicated. By method of co-transfection, CFP represents DHHS uptake, except for control condition (“none”), which was transfected with CFP alone. Images captured using Olympus Fluoview 1000 confocal microscope, excitation wavelengths 450 nm (CFP) and 515 nm (YFP), 60x oil magnification. Cells were incubated in 150 mOsm (hypotonic) buffer during image capture. RGS4-YFP alone localized to the PM. This effect was diminished by each dominant negative DHHC candidate, to various degrees. B. PM:cytosol fluorescence ratios for RGS4-YFP alone (control) and RGS4 + DHHS, as indicated. Only regions of non-contact between cells were chosen as regions of interest. Minimum 45 cells were chosen for each condition. The data demonstrates that all DHHS enzymes had a significant effect in attenuating RGS4-YFP trafficking to the PM. Results represent mean PM:cytosol fluorescence ratio ± SEM, analyzed using one-way ANOVA with a Tukey’s HSD post-hoc test for multiple comparisons, p<0.05. * = significant difference from control, § = significant difference from S3. C. Proportion of cells per condition presenting with a PM:cytosol ratio greater than 1.1. Once again, data mimicked average PM:cytosol ratios, showing that significantly less cells had reduced localization of RGS4 to the PM, in comparison to wild type RGS4 (control). Minimum 45 cells were chosen for each condition. Data were analyzed using Pearson’s chi-square test between control and each individual condition, p<0.05. * = significant difference from control. D. Western Blot analysis from a sample of RGS4 YFP alone (control), and each RGS4 + DHHS as indicated, demonstrating adequate expression of DHHS. Blots were incubated with antibodies against the HA-tag of DHHS constructs. Asterisks indicate DHHS protein band.
Chapter 4

Discussion

4.a. General Findings

RGS4 is expressed in cytoplasmic and membrane fractions in cells *in vitro*. The precise mechanisms behind RGS4 trafficking within the cell have yet to be elucidated. RGS4 has been demonstrated to undergo palmitoylation at three cysteine residues and the purposes of this palmitoylation have been both discussed and debated in the literature (94; 96; 135; 137; 184). The addition of the general palmitoylation inhibitor 2-BP has been shown to reduce the amount of RGS4 associated with plasma membrane in HEK201 cells. Here, our data suggests that members of the mammalian DHHC PAT family have an effect on RGS4 localization and function. Through mechanisms which may involve palmitoylation, DHHCs 3, 5, 7, 18 and 21 appear to regulate RGS4 attachment to the plasma membrane. In particular, removal of DHHC3 or DHHC7 individually has been shown to negatively affect RGS4 inhibitory activity for the Gαq subunit. Surprisingly, however, knockdown of these two PATs together appears to have no effect on RGS4 function, and minimal, if any, effect on RGS4 PM localization, suggesting DHHC functional regulation may be more complex than previously anticipated. Taken together, these data suggest that several DHHC enzymes may play a role in RGS4 localization and function.

4.a.i. Palmitoylation may play a role in RGS4 membrane anchoring and function.

Previous work asserting that palmitoylation at the N-terminus is not essential for RGS4 PM targeting was either carried out in yeast cells (92), or conducted on RGS16 in mammalian cells and predicted to occur in RGS4 due to sequence similarity (70; 136). If target
DHHCs studied herein do indeed regulate RGS4 through palmitate transfer, then our novel mammalian cell data might suggest the contrary. A consolidating point could be that palmitoylation may enhance membrane anchoring in a manner explained by the kinetic trapping model. That is, palmitoylation anchors RGS4 to the PM and creates time for the normally random coiled N-terminus to form its membrane-associating amphipathic helix. In this way palmitoylation, although not strictly required for membrane localization, may be expected to play a more significant role than previously appreciated in the localization and function of RGS4. Despite what has been shown by us and other groups, the precise order of events leading to RGS4 function at the PM – subcellular RGS4 trafficking, PAT recruitment, palmitoylation, N-terminal-directed PM targeting – is still undefined. Understanding this will help clarify the specific role(s) that palmitoylation plays in RGS4 localization and function.

4.b. Selection of candidate DHHC Enzymes

We chose to study the five DHHC enzymes with the highest mRNA levels in our cell type of interest. These were selected as candidate PAT enzymes for human RGS4 in HEK201 cells. This assumption narrows the field of DHHC candidates, based not on their ability to incorporate palmitate into a substrate, but rather on the amount of mRNA transcript present. Support for this approach comes from previous work showing that substrate-specific PAT enzymes are often the most highly expressed DHHC in tissues where the substrate itself is specifically expressed (159;170). This raises the question whether PATs are engineered to pair with specific proteins, or if substrates are palmitoylated by the DHHC enzyme most highly expressed in a given tissue. Further work is required to address this question.

Although selecting the top five DHHC expressors provided a good experimental starting point for our project, these studies may eventually need to be complemented by the examination of all DHHC family members.
4.c. Overexpression of wild type DHHCs directs RGS4 to specific subcellular DHHC pools.

Following selection of target DHHCs, we aimed to explore the effects of each PAT enzyme on RGS4 localization. Co-transfection of our RGS4-YFP cell line with individual and combination DHHCs and the trans-Golgi marker TGN-38 demonstrated that RGS4 was robustly directed towards the trans-Golgi network following transfection of DHHC3. DHHC7 appeared to cause a weakly colocalize RGS4 with TGN38, suggesting that DHHC7 may direct RGS4 to a region representing the cis- or medial-Golgi network. Because the literature demonstrates a subcellular localization profile of DHHCs 3 and 7 to the Golgi body (160), it is plausible that overexpression of candidate DHHCs attracts substrates to their specific subcellular domain. If so, it is unknown whether DHHC enzymes would initiate this substrate trafficking via palmitoylation-dependent trapping in the Golgi apparatus, or lipid-independent interactions with the substrate. Interestingly, several DHHC family members feature ankyrin repeats, SH3 domains, and PDZ motifs, whose function could be to recruit substrates and other regulatory binding partners to subcellular DHHC pools (171;180).

4.d. DHHC3 and DHHC7 modulate RGS4 regulation of Gqi9-dependent intracellular Ca\textsuperscript{2+} release

Our lab has previously demonstrated that RGS2 acts as a potent inhibitor of M1-coupled Ca\textsuperscript{2+} release using the same Ca\textsuperscript{2+} imaging assay employed here (71). We examined RGS4 function following siRNA knockdown of candidate DHHCs using a G\textsubscript{a,qi9}-stimulated Ca\textsuperscript{2+} release assay. We selected DHHCs 3 and 7 for candidates of RGS4 functional regulation based on our demonstration that they greatly mobilize RGS4, and also due to their PAT activity for G\textsubscript{a,q} (178). Our data demonstrated that RGS4 alone effectively inhibited intracellular Ca\textsuperscript{2+}
release initiated by the Gq signalling cascade in M2-HEK cells. Knockdown of either DHHC3 or DHHC7 significantly decreased the ability of RGS4 to attenuate this Gq-dependent Ca\textsuperscript{2+} release. This is the first demonstration that RGS protein function can be regulated by DHHC enzymes, presumably through their PAT activity on N-terminal cysteine residues.

**4.d.i. Orientation and stability conferred by palmitoylation may be important for RGS4 function.**

Ross and co-workers identified several characteristics of RGS4 interaction at the PM that are important for proper GAP activity (94). They found when RGS4 was reconstituted in phospholipid vesicles *in vitro* alongside G-proteins and GPCRs, it bound reversibly to the membrane, but this binding became permanent within 5 minutes. As well, RGS4 GAP activity was found to increase up to 3-fold over time, prior to the addition of GPCR agonist. The steady-state PM reorientation and surge in GAP function is caused by conformational changes that can be attributed to palmitoylation of RGS4 at the N-terminus (94). Perhaps the role of DHHC enzymes is to promote RGS4 affinity to the PM and increase its baseline GAP activity through the transfer of palmitate to its N-terminal cysteine residues. This would support the hypothesis that RGS4 does not require G-protein signalling to target it to the PM and is able to elicit its function at the cytosolic face of the membrane independent of secondary messenger activation. Further experimentation is required to better understand the separate roles lipidation at the N-terminus and RGS domain play in RGS4 stabilization and function at the PM.

**4.e. DHHCs regulate RGS4 association with the PM.**

Members of the R4 RGS subfamily require PM association to elicit their function (65;69;70). Our lab has shown that specific non-polar residues unique to RGS2 extend the
hydrophobic phase of its N-terminus amphipathic α-helix, giving RGS2 stronger PM localization and greater inhibition of Gq compared to other R4 members (71). Sequence alignment of RGS2 with RGS4 demonstrates that palmitoylation at the N-terminal cysteines of the latter would impart a hydrophobic extension similar to that found in the former. Indeed, we have found that disruption of the amphipathic α-helix with an L23D mutation (data not shown) and blockage of palmitoylation with 2-BP (Figure 3) both inhibit RGS4-YFP translocation to the PM in HEK201 cells. This preliminary data suggests the importance of palmitoylation to increased RGS4 localization. We propose that this palmitoylation is catalyzed by one or more of the target DHHC enzymes examined in this work.

To assess whether RGS4 localization would change following inhibition of candidate DHHCs, we suppressed DHHCs at both the mRNA and protein levels. Consistent with an important role for individual DHHCs in regulating RGS4 localization, both methods revealed reduction of RGS4-YFP fluorescence at the plasma membrane of HEK201 cells. In fact, dominant negative DHHS3 and DHHS7 each produced nearly identical RGS4 PM:cytosol ratios to their siRNA counterparts. Once again, these novel findings show regulation of RGS4 localization by PAT enzymes.

4.4. siRNA-induced inhibition of DHHCs may work through different mechanisms compared to dominant negative inhibition of DHHCs.

Surprisingly, combined siRNA inhibition of DHHCs 3 and 7 caused a different RGS4 localization phenotype compared to combined protein inhibition of these same PATs. RGS4 PM localization was nearly uninhibited when DHHCs 3 and 7 were knocked down together. This finding complemented our observations of combined knockdown on RGS4 functionality. In stark contrast to the siRNA localization data, transfection of both DHHS3 and DHHS7 together significantly reduced RGS4 PM translocation, more so than expression of either
dominant negative individually. In fact, all DHHS dominant negatives appeared to elicit some level of localization inhibition on RGS4. We propose that the counteracting phenotypes observed were a result of the utilization of separate mechanisms by each inhibition type, as discussed below.

4.f.i. Proposed mechanisms of siRNA-induced DHHC inhibition

4.f.i.1. Combined knockdown of DHHCs 3 and 7 may trigger a compensatory PAT response, affecting RGS4 localization and function

Dual inhibition of our target PAT candidates produced some unexpected results. Combined knockdown of DHHCs 3 and 7 did not attenuate RGS4 GAP activity for Gαq, and produced nearly negligible reduction of RGS4 PM translocation. This phenomenon could be explained by compensation of PAT activity known to exist within the DHHC family. Sessa and co-workers demonstrated that knockdown of DHHC21, but not DHHC3, in endothelial cells resulted in less eNOS palmitoylation and NO production, despite DHHC3 palmitoylating eNOS in vitro (170). In fact, substantial DHHC21 knockdown resulted in only 50% palmitoylation reduction, suggesting that other DHHC enzymes may be compensating for the loss of DHHC21. PSD-95 in neurons can be palmitoylated in vivo by both HIP14 (DHHC17) and DHHC15 (158;159), and the Gαq subunit is palmitoylated by DHHC3 and DHHC7 (178). In yeast, deletion of the gene for either Erf2 or Erf4 did not eliminate Ras2 palmitoylation (150), and Vac8 still underwent palmitate incorporation when the gene for its candidate PAT, Pfa3, was deleted (101). In short, a functional redundancy of DHHC proteins seems to exist, whereby multiple DHHCs can target the same substrate. Knockdown of DHHC3 and DHHC7 together may produce enough of a physiologic effect on the cell to trigger a compensatory upregulation of another target DHHC, thus preventing attenuation of RGS4 inhibition of Gαq signalling.
4.ii.2. Combined knockdown of DHHCs 3 and 7 may alter the function of other PAT substrates involved in the GPCR signalling cascade.

Several players involved in G-protein signalling are subject to palmitoylation, including GPCR-regulatory proteins, G-proteins, and GPCRs themselves. Mutation of palmitoylation sites in β2 adrenergic receptors, for example, uncouples them from their associated G-protein and leaves several phosphorylation sites exposed to potential activity by PKA and subsequent targeting for degradation (131). Similar mutations in A1 adenosine receptors and CCR5 chemokine receptors causes accumulation of these GPCRs in intracellular stores and increases their respective proteolysis (124;126). Most important for this study, however, are the experiments on M2R palmitoylation by Hayashi and Haga (1997). Their findings revealed that M2R palmitoylation was promoted through conformational changes induced by agonist binding, and mutation of the target cysteine residue on M2Rs prevented palmitate incorporation, resulting in reduced coupling of M2Rs with G proteins (128). If DHHC3 and DHHC7 are the candidate PATs for M2Rs, or if knockdown of these PATs is sufficient to prevent palmitoylation of the GPCR, it could suggest that the lack of Ca2+ release we observed following dual DHHC knockdown might not be a result of active RGS4, but rather a lack of M2-coupled Gqi9 activation at the PM.

4.ii.3. siRNAs elicit significant off-target effects in HEK201 cells.

siRNAs are known to trigger innate immune responses in human tissue (185). Induction of high levels of cytokines such as interleukin-6, tumor necrosis factor α, and interferons, as well as Toll-like receptors, would trigger an inflammatory response and protection of the host cell from foreign double-stranded RNA; this process also increases the cell’s risk of cytotoxic death (185;186). Innate immunity potentially triggered in our
experiments would prevent the dually-transfected siRNAs from having any gene-altering effects, while also initiating a global downregulation of transcriptional machinery.

When we conducted siRNA knockdown confirmation experiments using quantitative RT-PCR, we observed a 20-40% downregulation of non-targeted ZDHHCs, and testing mRNA levels in cells transfected with non-targeting siRNA suggested that 0-20% of this reduction, depending on the ZDHHC in question, was a global downregulation attributed to general siRNA uptake. This was confirmed by the fact GAPDH mRNA was altered within the same negligible 0-20% range regardless of whether DHHC-targeting or non-targeting siRNA was used (data not shown).

The fraction of mRNA attenuation not linked to global siRNA effects could be caused by off-targeting knockdown, which results in downregulation of unintended sequences through mechanisms such as partial sequence complementarity within the 3’ UTR of siRNAs (187). It has been shown that non-targeted transcripts with as few as 11 continuous identical nucleotides can become targets for sequence-specific siRNA knockdown (186). Examination of the target sequences of our candidate DHHCs will likely provide insight into off-target gene changes that may arise. Knockdown of DHHC3, for example, produced the most off-target downregulation of DHHC7, and vice versa. Indeed, BLAST queries revealed that 15 out of 19 nucleotides of the DHHC3 siRNA target sequence were conserved in a specific ZDHHC7 sequence, representing the most similarity compared to the other candidate DHHCs; the same level of sequence identity is true for the DHHC7 siRNA target region and a specific sequence of ZDHHC3.

4.f.ii. Proposed mechanisms of dominant negative DHHC inhibition

4.f.ii.1. DHHC enzymes and DHHS mutants form homo- and hetero-multimers in a potentially competitive manner.
In the literature, immunoprecipitation of a GFP-tagged DHHC3 resulted in substantial coimmunoprecipitation of FLAG-tagged DHHC3, demonstrating an ability for DHHC3 to homodimerize prior to eliciting PAT function (167). DHHC3 also formed heteromultimers with other DHHCs (167). This same paradigm applied to the dominant negative isoform, DHHS3. If RGS4 palmitoylation is compensated by other DHHCs when RGS4-specific PATs are lost, the ability of DHHS mutants to form heteromultimers provides this PAT family several levels of inhibition, thereby explaining the strong RGS4 mislocalization observed in the presence of both DHHS3 and DHHS7.

Our data demonstrated that dominant negative DHHCs may not always display additive inhibition. Transfection of DHHS18 and DHHS21 together elicited more inhibition on RGS4 translocation than DHHS21 alone, but less than DHHS18 alone. If DHHC multimerization occurs in all family members, then it is likely that competition exists between DHHS mutants and DHHCs. Thus, the dynamic nature of DHHC-DHHS multimer formation might explain the differences seen with different DHHS combinations.

It should be noted that siRNA inhibition of DHHCs may trigger different effects on multimer formation compared to those induced by dominant negative inhibition, further explaining the differences in our data sets. However, due to the relative newness of the field of DHHCs, little is known about inhibitory mechanisms and heteromultimer formation, and discussions on multimer competition are purely speculative.

4.g. Limitations

The most significant limitation to our current findings was the absence of biochemical data proving that RGS4 palmitoylation can be directly altered under the experimental conditions tested herein. Thus, it remains possible that the RGS4 phenotypes we observed are due to changes in palmitoylation of other molecules in a way that may affect RGS4 localization.
and function. The rapid clearance of RGS4 from live cells (142) has made traditional $^3\text{H}$-palmitate labelling studies difficult and thus we are currently collaborating with the Berthiaume group at the University of Alberta to develop a more sensitive assay with which to examine potential changes in RGS4 palmitoylation in the presence and absence of candidate DHHCs.

For confocal microscopy studies, we did not possess fluorescent-labelled DHHS plasmids to indicate the uptake of dominant negatives by live cells. Thus, we co-transfected equivalent micrograms of pECFP plasmid to identify cells that expressed DHHS proteins. This requires the assumption that cells uptake plasmid in a 1:1 manner, but we compensated for this by analyzing large sample numbers. In the case of siRNA knockdown, the experimenter was blinded to the conditions throughout data collection and analysis to prevent innate bias or conjecture. Large sample sizes were also analyzed. For our wild type DHHC overexpression studies, we assumed based on the literature that DHHC7 is Golgi localized, albeit to a different Golgi compartment than DHHC3. While we state that overexpression of DHHCs likely targets RGS4 to their respective subcellular compartments, the appropriate organelle markers are necessary to verify this claim. Lastly, for both wild type DHHC and mutant DHHS confocal microscopy studies, we found that PAT candidate protein expression varied widely between the different isoforms. This could be problematic if the expression of low-level DHHCs was below the threshold of PAT activity required to elicit an effect on RGS4 localization. This might explain the strong RGS4 subcellular trafficking observed with the highly expressed DHHCs 3 and 7, while lesser expressed DHHCs 18 and 21 did not appear to target RGS4 to their respective organelles of origin.

Calcium imaging studies required extensive validation of siRNA constructs prior to their utilization. Balance had to be found between siRNA efficacy and overall cell health. Knockdown time was reduced to 18 hours – 6 less hours than the optimal time – to conserve
adequate cell state and behaviour. Even the shortened knockdown period resulted in off-target ZDHHC alterations.

During validation, we demonstrated that pooling two siRNA constructs for an individual DHHC did not increase target knockdown, and instead caused upregulation of non-target ZDHHC mRNAs compared to control (Appendix A). We also found that treatment of siRNA for 48 hours created a global upregulation of all genes in question when compared to 24 hour siRNA treatment (Appendix B). Even with optimized protocols, we were only able to achieve a maximum 80% knockdown efficiency with siRNA. Typical knockdown experiments aim to achieve > 90% knockdown to minimize the possibility that residual target activity may confound the analysis. Thus, although we observe marked phenotypes with 80% knockdown, we cannot exclude the possibility that some minimal target DHHC activity remained. Lastly, because of the relatively new nature of this field, we could not get access to DHHC antibodies to demonstrate that target DHHC protein expression was attenuated following DHHC mRNA knockdown.

Isolating the relative intracellular Ca$^{2+}$ fraction released by Gqi9-coupled M2R stimulation from that of endogenous receptors would have allowed us to identify the absolute contribution of RGS4 signalling to our pathway of interest. We initially eliminated endogenous M1 coupled-Ca$^{2+}$ release with treatment of the selective M1 blocker VU0255305. However, we abandoned this protocol and opted to use untreated cell PFR/BFR as a control, due to a reduced cell viability caused by combining drug treatment with DNA transfection and siRNA knockdown.

The data from the Ca$^{2+}$ imaging functional assay demonstrated a reduced fold Ca$^{2+}$ increase in the control (YFP + Gqi9) (Figure 8) compared to the same control during Gqi9 validation (Figure 7). This suggests a day-to-day variability in the assay which could arise from several biological or technical factors. By not including a control without Gqi9 in our
functional assay, we could not demonstrate the contribution of the PM-tethered $G_\alpha_q$ chimera to the overall $Ca^{2+}$ signal on any given experimental day.

Finally, owing to the fact that palmitoylation occurs in several target proteins in our Gqi9-coupled $Ca^{2+}$ release pathway (92;128;178), it is possible that knockdown of DHHCs 3 and 7 could prevent palmitoylation and disable function of endogenous $G_\alpha q$ or any other undiscovered candidate substrates in the Gq-coupled signalling cascade, in addition to RGS4. This would suggest a false positive for the RGS4 functional phenotypes we observed.

4. h. Future Directions

The data we present here effectively demonstrates that multiple DHHC enzymes appear to play a role in regulating intracellular RGS4 trafficking and functionality. The next step would be the establishment that palmitoylation is the mechanism behind this regulation. This would involve showing that RGS4 forms thioester bonds with palmitate at its target cysteine residues \textit{in vitro}.

Secondly, the surprising result that arose from our DHHC dominant negative microscopy study – DHHS18 imparts the greatest inhibition of RGS4 PM localization – is worth exploring further. For DHHC18 to be identified as the RGS4-specific PAT, it would have to be demonstrated that knockdown of this enzyme results in a similar decrease of RGS4 PM localization as well as attenuation of RGS4 function in an assay such as the one used in our calcium release experiments.

The eventual goal of the work on RGS4 is to utilize it, as well as its candidate PATs, as therapeutic targets to treat human pathologies. For example, if \textit{Rgs4} upregulation can indeed block $G_\alpha q$-triggered ventricular hypertrophy, as suggested in several studies (84-86), then it would be interesting to see whether upregulation of candidate \textit{ZDHHC}s can increase endogenous RGS4 functionality and produce the same cardioprotective effects. A proposed
study would be to repeat several protocols of this thesis in cultured cardiomyocytes. Knockdown of the most highly expressed DHHCs in this tissue could be performed to elucidate whether this action blocks RGS4 inhibition of Gq-coupled cell growth through prevention of palmitoylation.

Recent work from our lab reveals that Rgs4-deficient mice demonstrate alterations in ECG recordings – loss of P waves and irregular action potential firing – characteristic of the atrial fibrillation phenotype, when treated with CCh (in press). Coupled with our finding that RGS4 regulates parasympathetic signalling at the SAN (91), we propose that loss of RGS4 renders the heart more susceptible to atrial fibrillation. Palmitoylation has been shown to occur in cardiac myocytes (188), but the contribution of palmitoylation to RGS4 function in SAN myocytes, and the role of DHHC enzymes in this context, is unknown. Demonstrating that candidate DHHCs can govern RGS4 modulation of G_i-coupled M2R signalling and GIRK activation would be instrumental in establishing a second G-protein pathway on which palmitoylation may regulate RGS4 activity, and would also propose a clinical application for the use of DHHCs as therapeutic targets.
Chapter 5

References


Chapter 6

Appendices
Appendix A. Effects of pooling siRNA constructs on knockdown efficacy. Expression levels of five DHHC candidates, relative to non-knockdown control levels (dashed line), following knockdown of DHHC3 mRNA using multiple siRNA target sequences. Pooling of siRNA constructs elicited no additional target gene knockdown compared to single siRNAs, and all combinations resulted in off-target gene upregulation. Data presented are ΔΔCT values from triplicate 2ng HEK201 mRNA sample measurements, normalized to GAPDH expression.
**APPENDIX B**

**Appendix B. Time-dependent effects on siRNA knockdown efficacy.** Expression levels of five DHHC candidates, relative to non-knockdown control levels (dashed line), following knockdown of DHHC3 mRNA for 24 and 48 hours, using DHHC3 siRNA constructs (A) 2, (B) 3, and (C) 4. Increasing the knockdown period to 48 hours resulted in a general upregulation of both targeted and non-targeted ZDHHCs. Data presented are ΔΔCT values from triplicate 2ng HEK201 mRNA sample measurements, normalized to GAPDH expression.
APPENDIX C

Appendix C. Knockdown of DHHCs 3 and 7 inhibits RGS4 PM localization at junctional membrane domains between cells. PM:cytosol fluorescence ratios for RGS4-YFP alone (control) and RGS4 + siRNA(s), as indicated. Regions of cell contact (cell-cell borders) were chosen as regions of interest. Minimum 95 regions per condition were selected. The data demonstrates all knocked-down conditions reduced RGS4 PM association to varying degrees (RGS4 control PM:cytosol = 1.99 ± 0.03, RGS4 + D3 sirna PM:cytosol = 1.78 ± 0.03, RGS4 + D7 sirna PM:cytosol = 1.78 ± 0.02, RGS4 + D3, D7 siRNAs PM:cytosol = 1.88 ± 0.03). Combined knockdown of DHHCs 3 and 7 displayed less RGS4-PM mislocalization than when DHHCs were knocked down individually. These data supplement the effect of inhibited target DHHCs on RGS4 PM localization at regions where cells have no inter-cellular contact (Figure 11-B). Results represent mean PM:cytosol fluorescence ratio ± SEM, analyzed using one-way ANOVA with a Tukey’s HSD post-hoc test for multiple comparisons, p<0.05. * = significant difference from control.
Appendix D. **DHHC3 shRNAs elicit less target knockdown than DHHC3 siRNAs.** Expression levels of five DHHC candidates, relative to non-knockdown control levels (dashed line), following knockdown with five shRNA target sequences for DHHC3. shRNAs were found to knock down target DHHCs less effectively (average knockdown ~70%) compared to siRNAs for the same target DHHCs (average knockdown ~80%). In addition, the most effective DHHC3 shRNA produced greater non-target DHHC mRNA fluctuations compared to the most effective DHHC3 siRNA. Data presented are ΔΔCT values from triplicate 2ng HEK201 mRNA sample measurements, normalized to GAPDH expression.