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REDEFINING THE SCOPE OF PRENYLATION: DISCOVERY OF "FORBIDDEN" SUBSTRATE RECOGNITION AND DEVELOPMENT OF METHODS UTILIZING PRENYLATED PROTEINS

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ABSTRACT

Post-translational modifications play a central role in controlling biological function and cell behavior through changes in protein structure, activity, and localization. Prenylation is one such modification wherein a 15- or 20-carbon isoprenoid group is attached to a cysteine residue near the C-terminus of a substrate protein by one of three enzymes: protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase-I) or protein geranylgeranyltransferase type II (GGTase-II, also known as Rab GGTase). These covalent modifications can aid in protein association with cellular membranes, with this localization necessary for function of many prenylated proteins. FTase and GGTase-I have been proposed to recognize a four amino acid "Ca_1a_2X" C-terminal sequence based on biochemical, structural, and computational studies of these enzymes. However, recent genetic screening studies in yeast suggest the potential for FTase to prenylate sequences of the form -$C(x)_3X$, with four amino acids downstream of the cysteine residue to be prenylated. The work herein begins to define the sequence scope for this -$C(x)_3X$ motif, establishes the biological relevance of this new class of prenyltransferase substrates in cells, and supports future investigation of the impact of these non-canonical prenylated proteins on cell behavior and biological function.

With the discovery of new -$C(x)_3X$ recognition motifs in prenylation, new methods with which to identify proteins capable of being prenylated are required. To this end, we have explored the use of engineered FTase variants, specifically RL FTase, selected for the ability to prenylate substrate sequences that are unreactive with WT FTase. Combining this engineered FTase variant with functionalized FPP analogues yields a bioorthogonal selective technique for isolating target proteins, even in the presence of other prenyltransferase substrate proteins in cell lysates. The value of this method is demonstrated by selective pulldown of model fluorescent

proteins in bacterial lysates in the presence of competitor proteins. The selectivity of FTase-catalyzed prenylation and the minimal size of the C-terminal FTase recognition motif render this approach applicable to a wide range of target proteins.

A second quantitative method introduced here is Protein-Lipidation Quantitation (PLQ); a new method that can simultaneously measure the amounts of a non-lipidated substrate protein and its lipidated product in a cellular context. In PLQ, use of a fluorescent protein fused to the substrate under investigation allows for quantitative detection of both the non-lipidated substrate and the lipidated product. Upon prenylation in cells, the substrate and the product in these cell lysates are separated by surfactant-mediated capillary electrophoresis (CE) and quantitated by integrating fluorescence intensity over respective CE peaks. This work demonstrates the usefulness of PLQ both in principle and in application with its ability to confirm a link between a mutation in the p53 tumor suppressor gene and cellular prenylation activity. The quantitative capabilities of PLQ will allow researchers to address previously unanswered hypotheses regarding protein lipidation and its roles in cellular regulation and biological function.

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Chapter 3: Chemoenzymatic protein labeling and isolation from eukaryotic cell lysates using enzymes with reengineered substrate selectivity

LIST OF ABBREVIATIONS

Ab	Antibody
AFOH	Anilinofarnesol
AGOH	Anilinogeraniol
AGPP	Anilinogeranyl diphosphate
BGPP	Biotin-labeled geranyl diphosphate
BSA	Bovine serum albumin
C15AlkOPP	Alkyne-modified farnesyl pyrophosphate
cAMP	Cyclic adenosine monophosphate
CE	Capillary electrophoresis
CFU	Colony forming unit
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Da	Dalton
DEAE	Diethylaminoethyl
Dff	2',7'-difluorofluorescein
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
Dns	Dansyl fluorophore
dNTP	Deoxyribonucleotide triphosphate
Dox	Doxycycline
E.coli	*Escherichia coli*
eGFP	Enhanced green fluorescent protein
EOF	Electroosmotic flow
ER	Endoplasmic reticulum
ESI	Electrospray Ionization
FB	Fast Break reagent
FBS	Fetal bovine serum
FCP	FYVE-containing coiled-coil protein
FDR	False discovery rate
FPLC	Fast protein liquid chromatography
FPP	Farnesyl pyrophosphate
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
GDP	Guanosine diphosphate
GGPP	Geranylgeranyl pyrophosphate
GGTase-I	Geranylgeranyltransferase type I
GGTase-II	Geranylgeranyltransferase type II

GGTI	Geranylgeranyltransferase inhibitor
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
GTPase	GuanosineTriphosphatase
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEPPSO	N-(2-hydroxyethyl)piperazine N′-(2-hydroxypropanesulfonic acid)
HPLC	High performance liquid Chromatography
HRP	Horseradish peroxidase
Hsp40	heat shock protein 40 kD
ICMT	Isoprenylcysteine methyl transferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LC	Liquid chromatography
LIF	Laser induced fluorescence
MAT	Mating type
MEKC	Micellar Electrokinetic Capillary Electrophoresis
m-PDA	m-phenlenediamine
mRNA	Messenger RNA
MS	Mass spectrometry
MTO	Multiple turnover
MVLA-CE	High Throughput Multiple Locus Variable Number of Tandem Repeat Analysis
NFM	Non-fat milk
OD	Optical density
PAGE	Polyacrylamide gel electrophoreisis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PLQ	Protein-Lipidation Quantitation
PMSF	Phenylmethanesulfonyl fluoride
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
Q sepharose	Quaternary sepharose
Rce1	Ras converting enzyme 1
RL	Arginine/lysine
RNA	Ribonucleic acid
RP-HPLC	Reverse-phase high performance liquid chromatography
S. aureus	*Staphylococcus aureus*
SC	Synthetic complete media

SD	Synthetic defined media
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
Ste24	CAAX prenyl protease 1
STO	Single turnover
TagRFP	Tag Red Fluorescent Protein
TAE	Tris-acetate/EDTA
TAMRA	Carboxytetramethylrhodamine
TBST	Tris buffered saline-Tween 20
TCEP	tris(2-carboxyethyl)phosphine
TMT	Tandem mass tag
UTR	Untranslated region
WT	Wild type
VNTR	Variable number tandem repeat
YDJ1	Yeast dnaJ
YPD	Yeast peptone dextrose
ZMPSTE24	Zinc metallopeptidase STE 24

Chapter 1: Introduction

1.1 Post-translational modifications

It has been predicted that while the human genome consists of between 20,000-25,000 genes, the human proteome contains a level of complexity far vaster than its genomic counterpart (Figure 1.1).[1-2] The ability of single genes to encode for many distinct proteins demonstrates the importance of protein modifications in the cell. These modifications can occur during transcription through use of multiple promoter and termination sites, through mRNA splicing, or recombination, but can also occur after translation through post-translational modifications.[3-4]

Post-translational modifications are the selective covalent changes to proteins which have crucial effects on protein structure, localization, stability, and protein-protein interactions. Protein lipidation is one specific PTM in which proteins are altered through the addition of one or more lipid groups to recognition motifs within the protein. Lipidation is often an important regulator of protein trafficking to cellular membranes but also has some roles in protein-protein interactions and stablility.[5-7] Lipidation can occur in tandem with other modifications, such as proteolysis, or the addition of the lipids to amino acids. The various lipid groups involved in this modification include fatty acids, sterols, phospholipids, isoprenoids, and glycosylphosphatidylinositol (GPI) anchors.[5]

Three types of lipid modification occur on the inner leaflet of the plasma membrane: palmitoylation, myristoylation, and prenylation (Figure 1.2). These PTMs aid in localizing modified proteins to the cytosolic side of the plasma membrane and play a significant role in protein trafficking and regulation.[9] Palmitoylation is the formation of a thioester linkage between a cysteine residue and a palmitic acid containing a 16 carbon chain, although other amino acids,

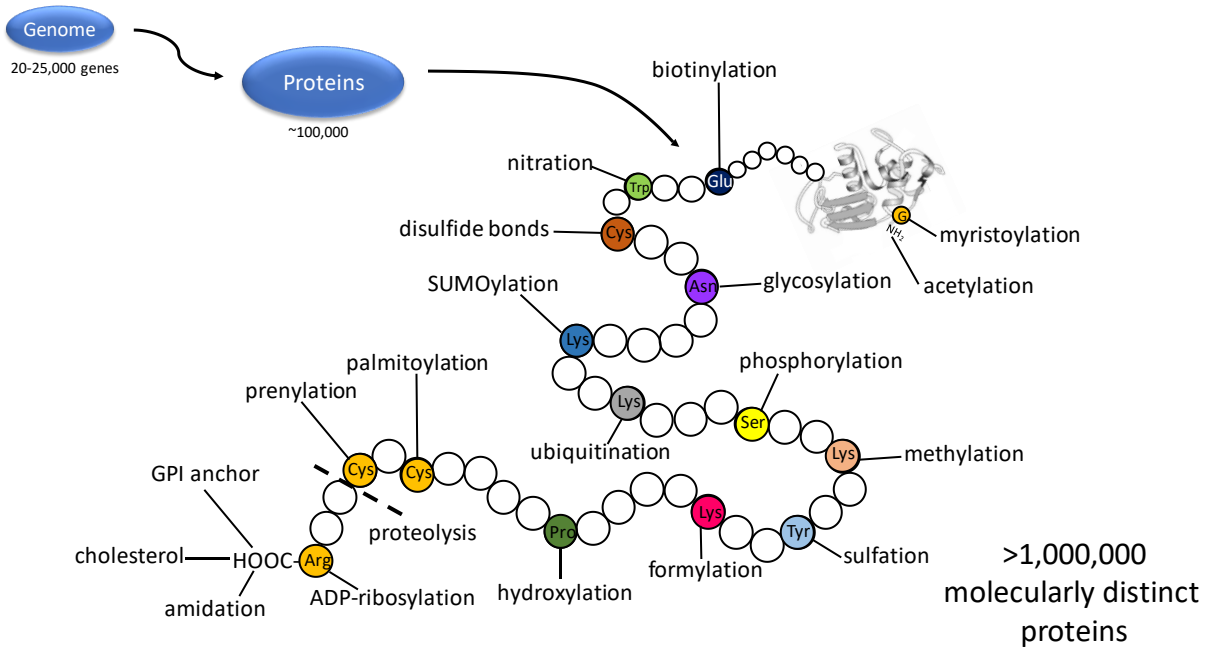

Figure 1.1 The human proteome is much larger than the genome from which it originates. Post-translational modifications create more than 1 million molecularly distinct proteins from just 20-25,000 genes. Representative modifications shown here are not represented with required amino acid motifs or downstream processing.

namely serine and threonine, are capable of undergoing the modification.[7, 10-12] This lipidation is unique in that it is reversible through thioester hydrolysis, allowing for palmitoylation to play important roles in protein trafficking and stability.[10, 13-15] Myristoylation occurs at an N-terminal glycine residue through the addition of a 14 carbon myristic acid moiety.[9, 16-18] This occurs through the formation of an amide bond and plays a key role in protein signaling, with many kinases undergo N-myristoylation.[19] Prenylation is the irreversible attachment of an isoprenoid chain 15- or 20-carbons in length. This modification is targeted to the C-terminal cysteine of a protein and is found to be widespread among yeast and mammals.[5-6, 20-23] Prenylation traffics proteins to the plasma membrane where they are involved in cell signaling cascades and protein-protein interactions.[9]

Two other types of lipid modification, glycophosphatidylinositol (GPI) anchors and cholesterol attachment, occur in the endoplasmic reticulum (ER) and Golgi lumen before trafficking of proteins to the outer leaflet of the plasma membrane (Figure 1.3).[24-25] A GPI moiety is attached to the C-terminus of a protein through its ethanolamine functional group and is involved in cell signaling, prion disease, and protein incorporation into lipid rafts.[24, 26-29] Cholesterol attachment to proteins also occurs at the C-terminus of proteins through an ester bond.[25, 30-31] This modification is important in tissue repair, organ and stem cell development or maintenance, and mainly occurs in the Hedgehog protein family.[30-32]

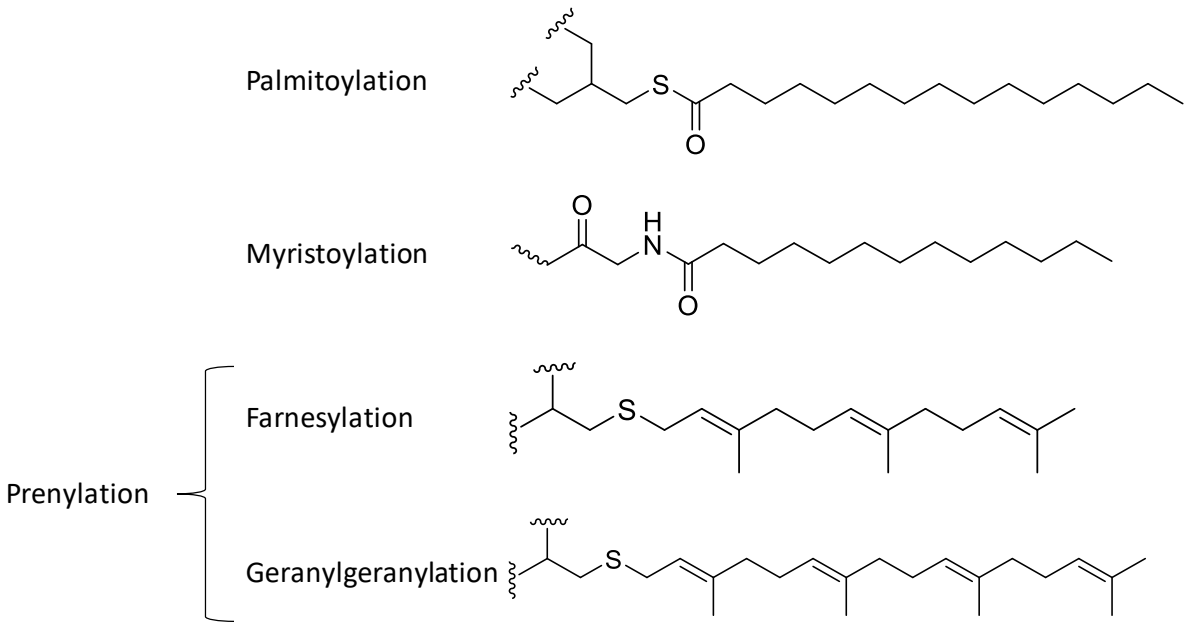

Figure 1.2: Post-translational lipidation targeting proteins to the inner leaflet of the plasma membrane. Palmitoylation occurs on a cysteine residue through a thioester linkage; Myristoylation forms an amide bond to the N-terminal amino group of proteins; Prenylation has two subgroups of modification: farnesylation (15 carbons) and geranylgeranylation (20 carbons). It involves the formation of a thioester bond on a cysteine residue at the C-terminus.

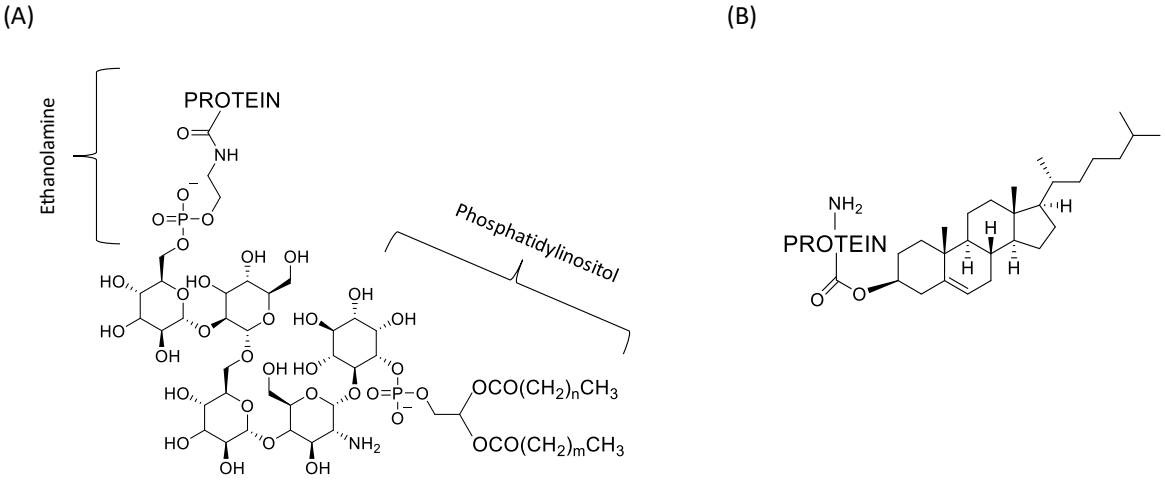

Figure 1.3 Post-translational lipidation targeting proteins to the outer leaflet of the plasma membrane. (A) Glycophosphatidylinositol (GPI) anchors consist of an ethanolamine group, a sugar chain (D-mannose and D-glucosamine), and a phosphatidylinositol group. Mannose hydroxyl groups can also be various substitutions of long chain fatty acids.[26] (B) A protein-cholesterol ester bond.[25]

1.2 The prenylation pathway

Prenylation is a post-translational modification in which a hydrophobic isoprenoid, either a farnesyl or geranylgeranyl group, is transferred to the C-terminal cysteine of a target protein.[6, 20-22] Prenylation can be catalyzed by three different enzymes depending on the sequence to be prenylated and the isoprenoid substrate to be attached: protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase-I), and protein geranylgeranyltransferase type II (GGTase-II).[33] FTase catalyzes the addition of a 15-carbon isoprenoid group from farnesyl pyrophosphate (FPP), while both GGTase-I and -II catalyze the addition of a 20-carbon isoprenoid group from geranylgeranyl pyrophosphate (GGPP).[6, 20] GGTase-II is unique in that it can modify a variety of motifs from the Rab family of proteins including CC, CXC, CCXX, CCXXX, where C is a cysteine which undergoes modification.[34-39] GGTase-II also requires a Rab escort protein in order to prenylate Rab proteins.[40]

For several decades, the prenylation pathway has been defined to follow a specific set of modifications before the protein is trafficked to the cell membrane, with the first step comprising cysteine alkylation by the FPP or GGPP prenyl donor. FTase or GGTase-I catalyze the addition of the farnesyl or geranylgeranyl group via FPP or GGPP, respectively, to a cysteine near the protein C-terminus.[6, 21] This cysteine is historically part of the Ca_1a_2X sequence, recognized by these two enzymes, in which 'C' is the cysteine to be prenylated, 'a' is any aliphatic amino acid, and 'X' is an amino acid which determines which type of isoprenoid will be attached to the substrate protein.[20, 33, 41-42] FTase substrates typically have an X residue of serine, methionine, alanine, or glutamine while GGTase-I usually recognizes leucine or phenylalanine at the X residue position.[6, 43-46] Some substrates may also be recognized by both enzymes as targets for farnesylation and/or geranylgeranylation.[21, 47-48]

Following addition of a farnesyl or geranylgeranyl group to the cysteine, most prenylated proteins then undergo two additional modifications before transport to the cell membrane. The last three amino acids of the Ca_1a_2X sequence are removed by the "CaaX" proteases Rce1p or Ste24p, with the resulting carboxylate undergoing methylation by the S-adenosylmethionine dependent isoprenylcysteine methyltransferase (ICMT) to form a prenylcysteine methyl ester C-terminus on the prenylated protein (Figure 1.4).[49-58] All three modification steps are necessary for function of most prenylated proteins.[50, 54-55] Recently, it has been found that certain proteins in yeast undergo prenylation but not proteolysis or methylation in what has been deemed the "shunt pathway", with evidence of these processing steps actually being deleterious to the protein's function.[8]

Once processed, prenylated proteins are shuttled to the cell membrane by one of two proposed mechanisms. For example, the majority of small GTPases in the Ras family contain a second upstream C-terminal amino acid sequence which serves as a signal to aid in membrane association. The isoform K-Ras4B contains a polybasic region which acts as a localization signal with the amino acid sequence (**K**MS**K**DG**KKKKKK**S**K**T**K**CVIM).[59-61] The positively charged lysine residues (bolded in sequence) aid in localization of K-Ras to the cell membrane via electrostatic interactions. A second upstream signal is observed in isoforms H-Ras and N-Ras where cysteine residues near the C-terminus under palmitoylation in the Golgi apparatus, increasing protein association with cell membranes.[60-62] Palmitoylation results in a reversible thioester bond which it believed to act as an "on/off switch" to control membrane association of these proteins (Figure 1.5).[63-64]

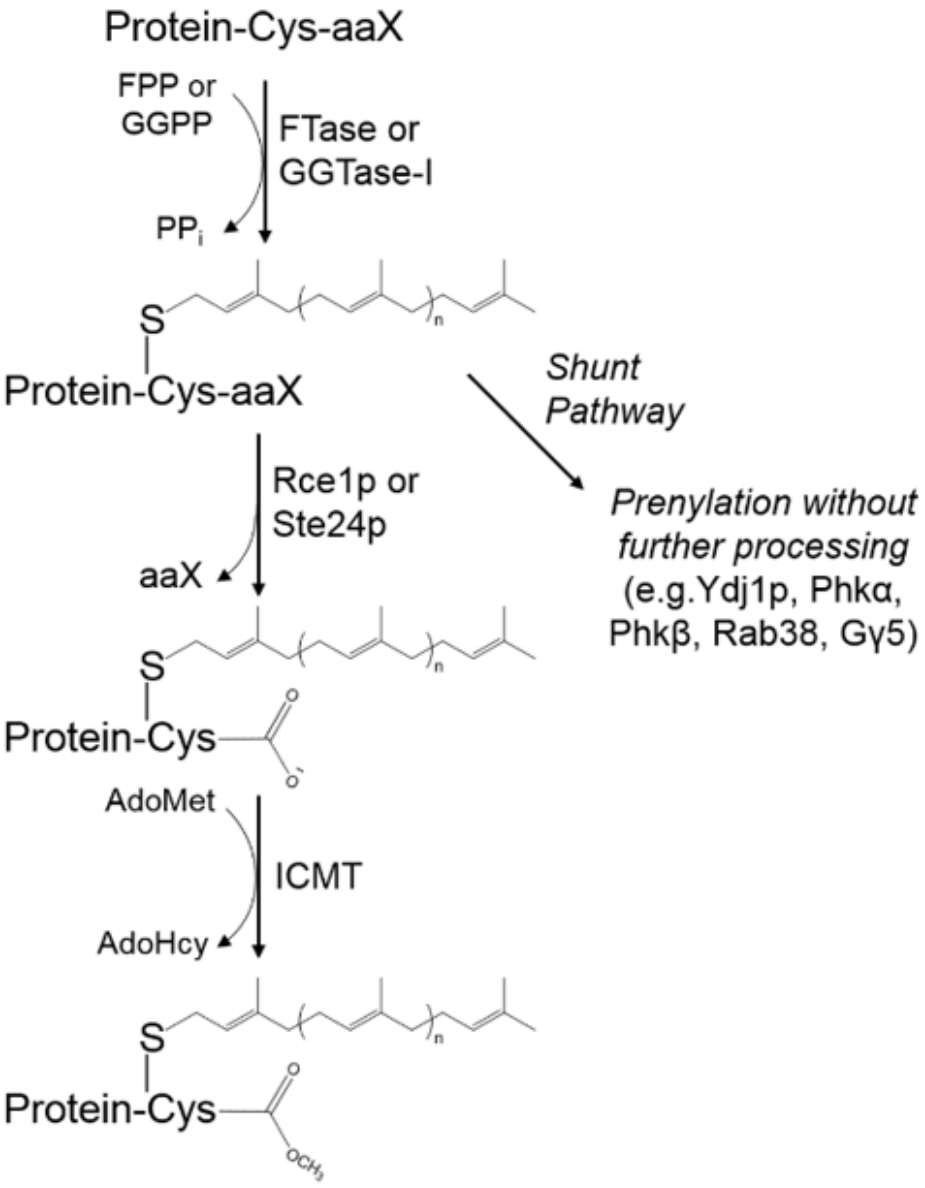

Figure 1.4 The prenylation pathway. Modification steps observed within the prenylation pathway, including prenylation, proteolysis, and methylation. A shunt pathway for proteins undergoing only prenylation without subsequent proteolysis is evidenced in some proteins in yeast[8] This figure has been reused with permission from reference 8 (Appendix V)

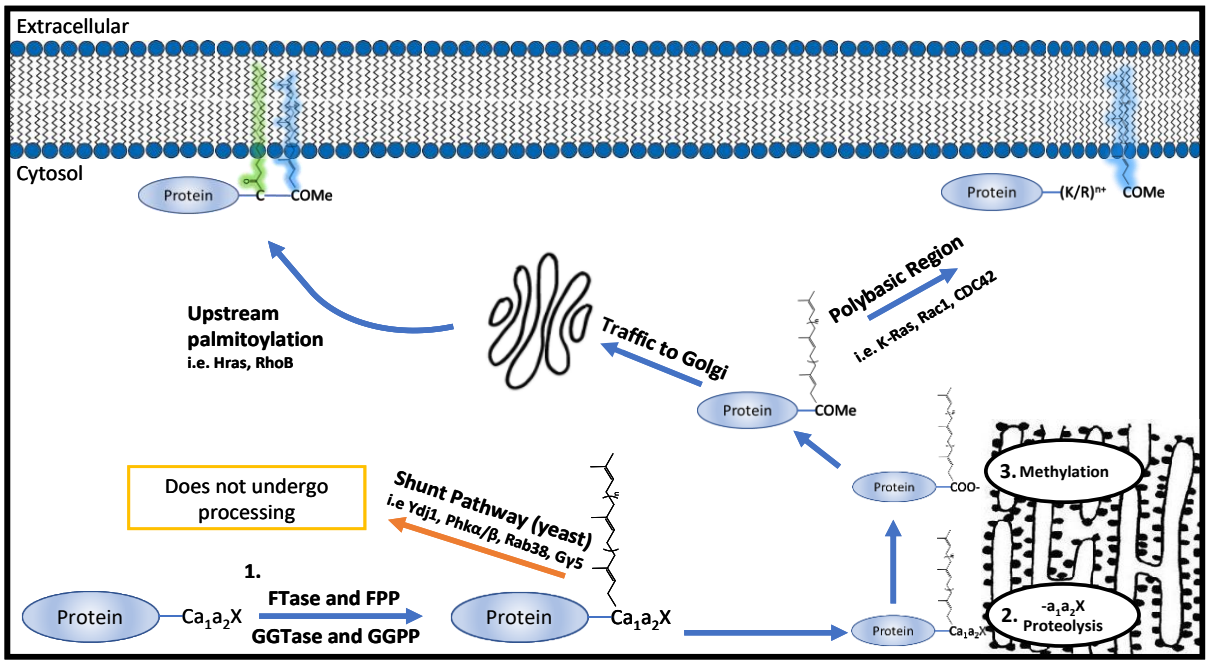

Figure 1.5 Processing and trafficking of prenylated proteins occurs at several locations within the cell. Prenylation occurs within the cytoplasm with subsequent proteolysis and methylation occurring at the rough endoplasmic reticulum. Trafficking of the processed proteins to the cell membrane can occur in one of two proposed ways. Yeast proteins which do not undergo processing remain in the cytoplasm via the shunt pathway.

1.3 Interactions and specificity between prenyltransferases and target proteins

FTase and GGTase-I are similar in structure, both being heterodimeric metalloenzymes with their active site located at the interface of their α and β subunits (Figure 1.6).[65] With a common α subunit, distinctions in their specificity arise from their different, homologous β subunits.[66-67] The proposed mechanism for both enzymes is alike in that the isoprenoid is proposed to bind first followed by the substrate to be prenylated. In FTase, the negatively charged diphosphate group of the isoprenoid is stabilized during catalysis through interaction with a bound Mg^{2+} ion (Figure 1.7).[66] In GGTase-I this ion is not present, with lysine 311β taking on the role of stabilization.[66] Both enzymes use a catalytic Zn^{2+} ion for activation of the cysteine to be prenylated through a lowering of the thiol group of cysteine to a pKa near physiological pH.[66] This newly formed thiolate anion undergoes a nucleophilic attack on C1 of FPP or GGPP, forming a new thioether bond on the cysteine residue.[68-69]

In addition to the interactions of the Zn^{2+} and Mg^{2+} ions described above, structural models of FTase and GGTase-I propose specific contacts within the active site with the cysteine and the carboxylate group of the Ca_1a_2X sequence that explain the apparent preference for the four-amino acid Ca_1a_2X substrate sequence (Figure 1.8).[70] The Ca_1a_2X motif was first described more than 30 years ago, when yeast mating factors, Ras GTPases, and nuclear lamins were found to be lipid-modified.[71-76] Since its description, studies of FTase and GGTase-I preference for amino acids within the Ca_1a_2X motif have focused on computational and biochemical techniques, such as single amino acid mutations at each position of the Ca_1a_2X sequence and mutations of FTase and GGTase-I to alter substrate selectivity.[21, 47, 77]

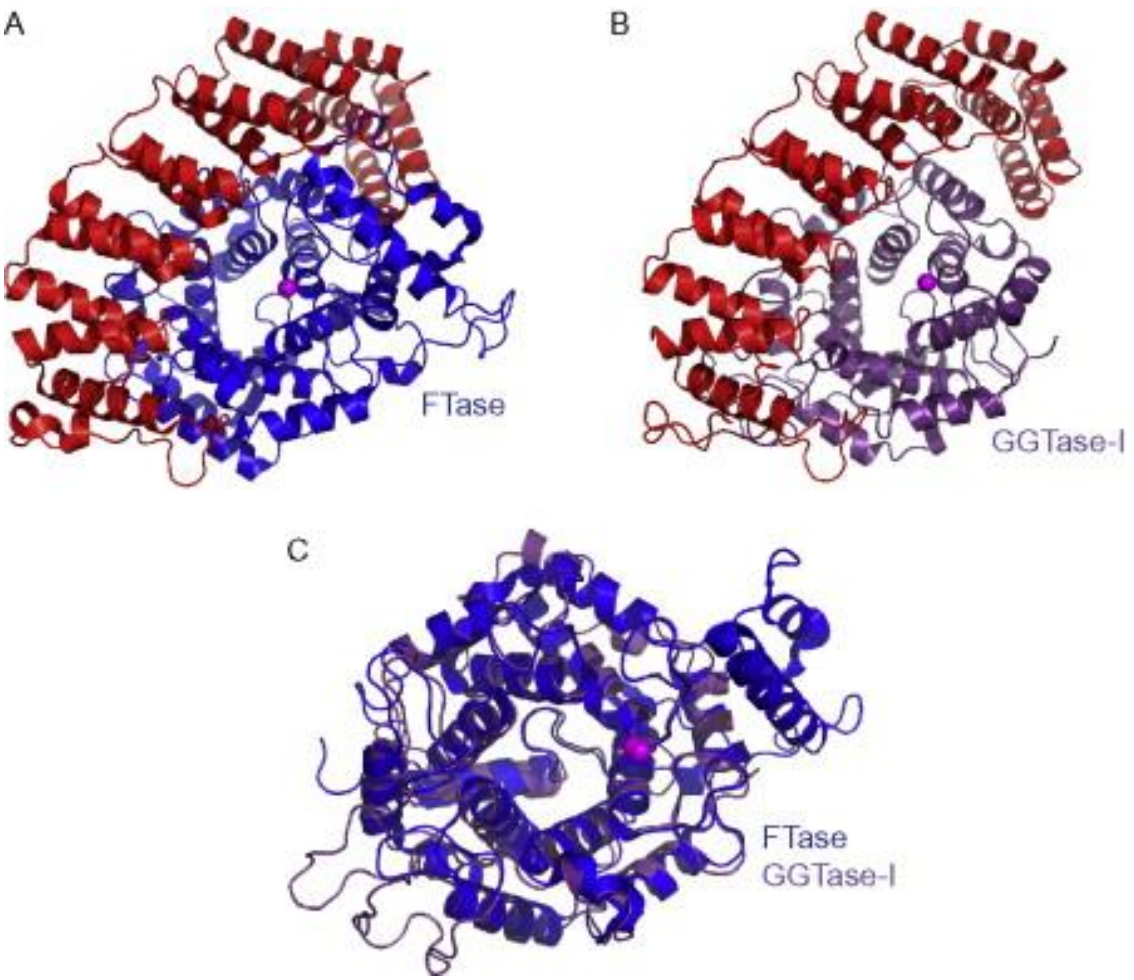

Figure 1.6 Structural homology of FTase and GGTase-I. (A) FTase with α subunit, red; and β subunit, blue; catalytic Zn^{2+}, pink. (B) GGTase-I. The α subunit is shared with FTase; β subunit, purple. (C) Superposition of the β subunits of FTase and GGTase-I, blue and purple, respectively. This figure has been reused with permission from reference 65 (Appendix VI).

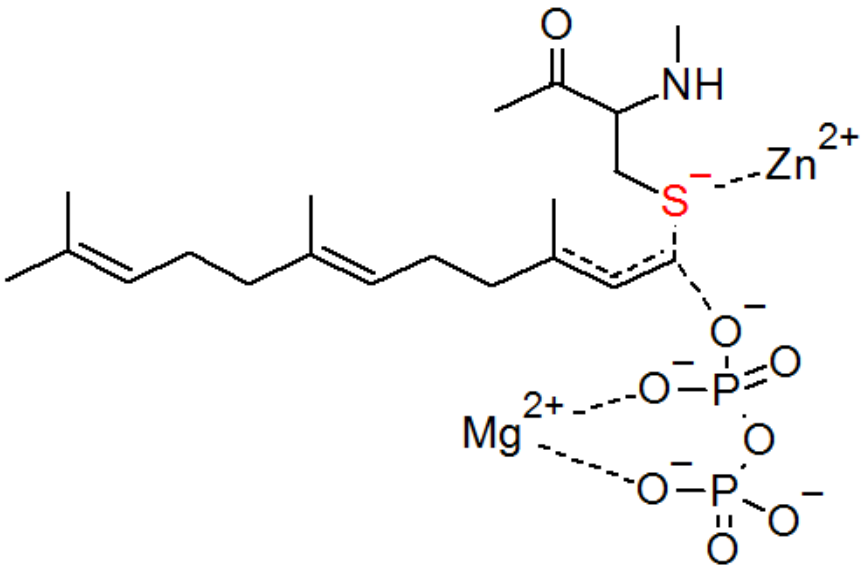

Figure 1.7 The role of metal ions in farnesylation. Magnesium, Mg^{2+}, ions serve to activate the pyrophosphate group on FPP as the leaving group while zinc, Zn^{2+}, lowers the thiol pKa to activate the sulfur. (Sulfur of cysteine to be prenylated shown in red.)

Proteomic studies, bioinformatics analysis, and computational/docking approaches have also been commonly utilized to predict which Ca_1a_2X motifs likely serve as substrates for FTase and/or GGTase-I with sequences longer than four amino acids found to be consistently forbidden for recognition by the enzymes.[78-81] These studies have led to our current understanding of prenyltransferase selectivity at each amino acid position within the Ca_1a_2X sequence but little to no work has been published on the length requirements for this motif. Despite this variety of approaches, there has been limited research on recognition sequences less than four amino acids in length (-CXX) and no published investigation into a motif longer than four amino acids (-CXXXX).[82]

In building to our current understanding of the interactions between prenyltransferases and their substrate, crystallographic studies have provided evidence for specificity of FTase and GGTase-I to only accept four amino acid motifs, providing a "molecular ruler" of the size and type of amino acid which could be accepted at each position and generating a list of potential proteins in the human genome that are targets of prenylation.[35, 70, 83-86] An example of these studies from Reid, T.S. et al. in 2004 provides a table of rules that govern the peptide specificity for FTase and GGTase-I through a study of several differing amino acid sequences. They describe the peptide binding site of the enzymes to be "rigid" with fixed anchor points at both the cysteine to be prenylated and at the "X" position through hydrogen bonding. While these structural studies provide a foundation for future biological studies of these enzymes, these studies were restricted to four amino acid peptides.

Computational studies have also been designed to further understand the recognition, selectivity, and restrictions of the Ca_1a_2X box on FTase and GGTase-I.[79-80] An example is illustrated by Maurer-Stroh in the development of the algorithm PrePS, a prenylation prediction

tool.[80] PrePS uses experimentally derived motifs as well amino acids just upstream of the cysteine to be prenylated to predict the likelihood of a motif acting as a substrate for farnesylation or geranylgeranylation. Due to the experimentally based nature of the algorithm's development, PrePS has the potential for many false negatives for a given set of motifs, restricting the number of motifs being determined likely as substrates for prenylation.

A second computational approach by London and coworkers in 2011 involves a structure-based modeling approach used to determine all potential Ca_1a_2X motifs that can undergo prenylation, including a new class of farnesylation targets.[79] This tool known as FlexPepBind was derived from knowledge of the enzyme's structure, using molecular dynamics, to determine binding energy of Cxxx substrates. FlexPepBind is a more powerful tool than PrePS as it has predicted novel substrates through docking of all possible Cxxx motifs into the active site and determining the minimal binding energy involved in the interaction.

Yet another approach in investigating Ca_1a_2X motif specificity involves biochemical and cell-based studies, including single amino acid mutations at each position of the Ca_1a_2X sequence and reengineering of FTase and GGTase-I selectivity at the level of the enzyme.[21, 43, 58, 72, 77, 87-92] The first approaches to these biochemical studies employed radiolabeled ^3H FPP and GGPP for protein labeling, as well as upstream precursors of these prenyl donors.[93-95] In these studies, cells were grown in the presence of these radiolabeled compounds wherein the result was labeled prenylated proteins that could be harvested from cells. These original techniques, while fundamental to our understanding of prenylation, gave low yields of prenylated protein, were expensive, and time consuming.

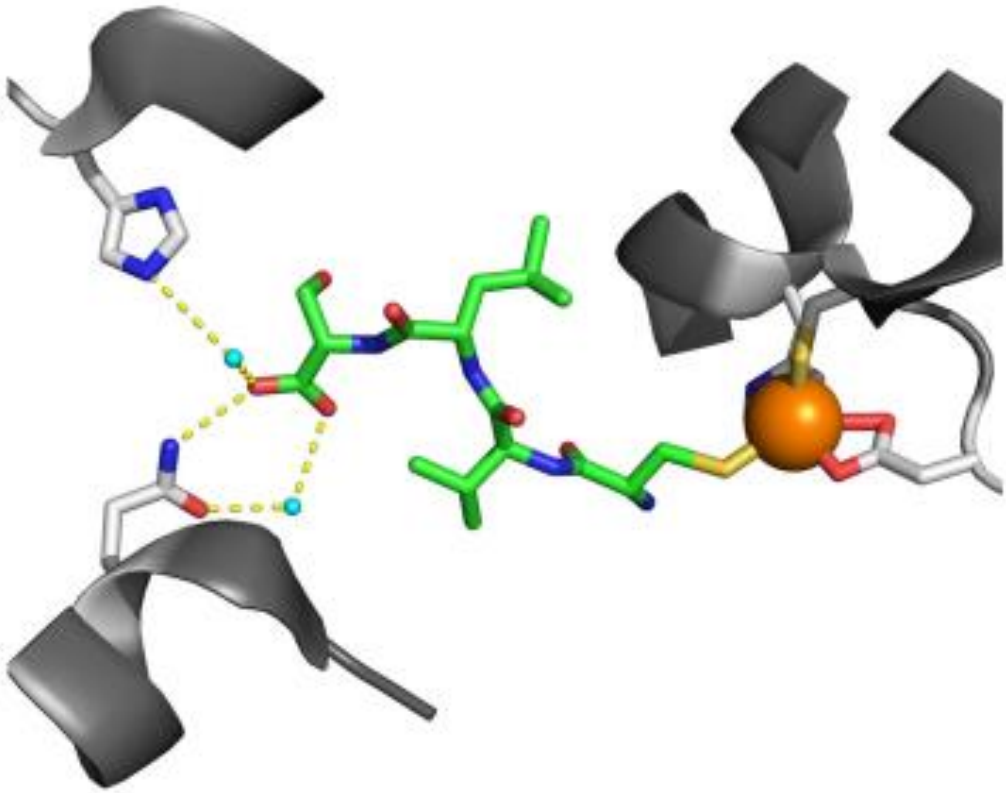

Figure 1.8 Structural model of FTase recognition of a substrate sequence. The binding of tetrapeptide (green) into the active site involves coordination of the cysteine thiol group to the catalytic zinc ion (orange sphere) and hydrogen bonding between the C-terminus and FTase residues (Q167) and (H149). (water, teal spheres) Image was generated from Protein Data Bank code 1TN8 using PyMOL reused with permission from reference 181 (Appendix V).

The use of reactivity series has also found use as a biochemical approach in identifying prenylation substrates. For example, Hougland and co-workers have used Ca_1a_2X peptide libraries of varying a_1, a_2, and X positions to determine amino acid preferences at each position for FTase and GGTase-I.[87, 91, 96] Their work revealed, for example, a preference against polar amino acids at the a_2 position, as this position is buried within the active site, and the notion that some sequences may undergo what is called "single turnover" reactivity. Under single turnover conditions, enzyme concentration is greater than that of substrate (STO, [E]>[S]). This state removes competition of substrates for enzyme as there is sufficient enzyme to prenylate all the substrate molecules without the need for product release (turnover) from the enzyme so that more substrate can bind. This is opposed to the more biological "multiple turnover" reactivity (MTO, [E]<<[S]) in which product must be released from the active site of the enzyme in order for more substrate to be converted to product. If sufficient product formation is shown under STO conditions but not MTO conditions, this evidences an issue in product release for a given substrate and enzyme. The determination of sequence dependence on STO vs. MTO conditions provides new insight into potential regulation of these prenylated proteins in the cell.

In recent years, the use of functionally labeled prenyl donors has gained popularity in investigation of FTase and GGTase-I substrate specificity. For example, alkyne and azide-tagged FPP and GGPP analogues (Figure 1.9) have been used to determine potential substrates for prenylation in which the functional group incorporation into prenylated proteins allows for detection and isolation of these proteins using a variety of techniques, such as fluorescent labeling with TAMRA-azide, biotinylation with subsequent isolation using streptavidin beads, and MS analysis following isolation.[97-108]

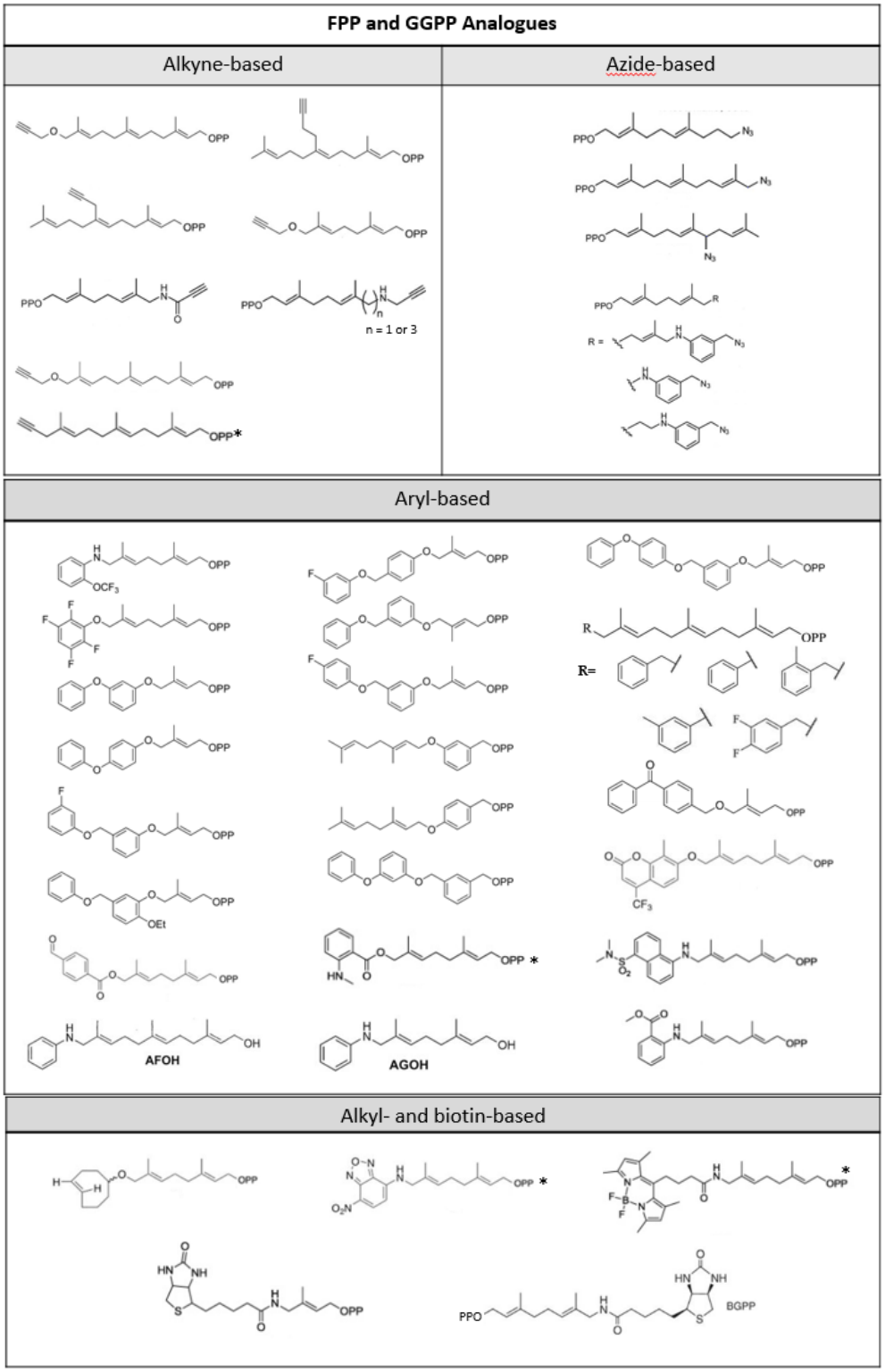

Figure 1.9 Examples of FPP and GGPP analogues used in investigations of prenyltransferase substrate specificity An (*) denotes a GGPP analogue is also available and was not included here for space. This list of analogues is not exhaustive and are derived from the references provided. [109-115]

Some specific examples include work by Spielman and coworkers in 2005 wherein an antibody-based detection of prenylated proteins from cells was achieved through use of an FPP analogue anilinogeraniol, AGOH (labeled in Figure 1.9).[116] This alcohol precursor is diphosphorylated in cells to yield AGPP, which is then transferred to substrates in a reaction comparable to that of FPP. In tandem with anti-anilinogeranyl antibody (polyAG-Ab) the use of AGOH analogue was one of the early successes in non-natural prenyl group incorporation that did not utilize radiolabeled isoprenoid donors to identify prenylated proteins in cells. This work using AGOH and western-blot analysis gave insight into the prenylation state of multiple proteins, such as Q61L oncogenic H-Ras, as well as investigated the effect of several FTI and GGTIs, such as lovastatin.[116-118] AGOH has since been examined in its role as an FTI itself in its ability to compete with endogenous FPP for substrate.[109]

A second example of work with FPP and GGPP analogues comes from Alexandrov and coworkers in 2009 in which a biotin-labeled geranylpyrophosphate (BGPP, Figure 1.9) was utilized by both natural RabGGTase and mutant FTase or GGTase-I.[115] Having all three enzymes at their disposal, this work demonstrated the identification and quantification of prenylation substrates and provided a more sensitive measure of the effect of prenyltransferase inhibitors on *in vivo* activity. An example from this work includes investigation of inhibitor BMS-3 which was confirmed to have no effect on GGTase-I in cells but was able to inhibit RabGGTase and FTase with a K_i of 7 and 50 nM, respectively.[116, 119] This inhibition was monitored in a simple and quick fashion via blotting with streptavidin-HRP after exposing cells to BGPP and laid ground for some of the first work on *in vivo* quantitation of prenyltransferase inhibition.

While the methods described above have provided invaluable insights into the structures, substrate specificity, and cellular recognition involved in prenylation, the best quantitative method

to understand of the role played by protein prenylation needs to simultaneously measure the amounts of both non-lipidated substrate and lipidated product. The BGPP analogue developed by Alexandrov provided a method for cellular quantitation of prenylated proteins, however, it could not simultaneously measure both substrate and product. Without the ability to measure modified and unmodified protein simultaneously, an accurate ratio between the two cannot be established. Many of these analogue methods also use gel electrophoresis/immunoblotting for their analysis with separation of proteins often being poor between substrate and product.

Computational methods have also provided valuable information as to the interactions between prenyltransferases and substrates within the binding site necessary to understanding substrate specificity and prediction of new substrates. These methods rely on known prenylation substrates determined experimentally and are limited to Ca_1a_2X motifs. Any recognition sequence outside the classic Ca_1a_2X could yield a false negative as being a potential substrate for prenyltransferases.

The importance of expanding our approaches to determining potential prenyltransferase substrates is highlighted in a study in which evidence for prenylation of non-canonical $C(x)_3X$ motifs was observed while studying the role of prenylation and its post-processing in the yeast heat-shock protein, Ydj1p, (see chapter 2).[8] This protein is a type I Hsp40 co-chaperone which ensures proper folding and stability of proteins during times of stress and must be prenylated in order to perform this function.[120-124] Unlike other proteins which undergo prenylation, Ydj1p does not undergo subsequent proteolysis and methylation and is therefore found in the cytosol rather than the cell membrane.[8] This genetic screening provided the capability to investigate prenylation motifs compatible with yeast FTase directly without the requirement for downstream processing. During this investigation, a unique observation occurred in that $C(x)_3X$ sequences appended to

Ydj1p, a product of randomized screening, showed evidence of prenylation through a thermotolerant phenotype in yeast. This finding highlights the importance to develop new, sensitive techniques which accurately predict and analyze sequences outside the traditional CaaX box.

The $C(x)_3X$ sequences emphasize the importance of developing new methods for studying prenylation. Use of fluorescence localization as a proxy for prenylation in cells would not be useful for prenylated proteins with a cytosolic role, for example, as the entire cell would exhibit fluorescence. Analogues could be useful in study of cytosolic prenylated proteins, however FPP and GGPP analogues like BGPP are bulky and can interfere with binding to the enzyme or do not bind with as high an affinity as natural substrates.[115] The binding of BGPP was remedied through mutation of the FTase and GGTase-I, a cumbersome process that does not yield quick results. Turning to computational methods resolves the challenges of working in cells but it currently does not use sequences longer than four amino acids in its algorithms and are likely to reject sequences outside this range. The development of a method which not only identifies both substrate and product simultaneously while providing a quantitative separation of the two species would change the way current research is done to confirm and expand upon prenylation in cells.

1.4 Biological significance of prenylation

Prenylation plays an important role in cell signaling, growth, differentiation and proliferation, and cell migration.[125-131] With prenylation being important in many cellular functions, these prenylated proteins can be found to play a role in a wide variety of diseases, the most prominent being cancer. The well-known GTPases H-Ras, K-Ras and N-Ras are involved in signal transduction and cell growth and have known oncogenic forms through amino acid mutations which keep the GTPase in an "always active" form, causing overgrowth of cells.[118, 132-134] Other proteins known to be prenylated, such as prelamin A, also play a role in diseases of cell growth and aging through an improper processing of the prenylated form of the protein.[135-137] These important roles in human disease have led to a robust interest in prenyltransferases as therapeutic targets for disease. Prenylation is not only an important modification in humans but also found in pathogenic organisms such as *Plasmodium falciparum*, *Candida albicans*, and *Legionella pneumophila*, in which prenyltransferase inhibitors are currently being investigated as potential therapeutics.[138-140] A challenge in studying pathogenic prenylation is the identification of the proteins undergoing the modification. It is known, for instance, that FTIs show promise as potential antimalarial drugs, but identification of the proteins they specifically target has only recently undergone investigation.[141-142] Recently, use of an alkyne FPP analogues and the algorithm PrePS identified several proteins which undergo prenylation in *Plasmodium falciparum* such as FYVE-containing coiled-coil protein (FCP) which may be the target of FTI therapeutics.[141] As discussed earlier, use of these techniques can be limiting in their ability to identify potential target proteins and highlights the biological significance in developing new methods to study prenylation.

Despite its involvement in regular cellular functions, the involvement of prenylation in cell growth affords its role in cancer as the most extensively studied disease related to this modification. The connection between cancer and prenylation can be found in studying the family of Ras GTPase proteins such as H-Ras, N-Ras, and K-Ras, as well as members of the Rap family of proteins.[143-148] These small, prenylated GTPases serve to proliferate a signal from primary messengers, such as growth factors, to enzymes responsible for the formation of a secondary signal in the cell, such as cyclic AMP. This secondary messenger then sets off a cascade of changes, often through a series of kinases.[149-150] In order for GTPases to send a signal downstream of its effector, it must switch from an inactive GDP-bound form to an active GTP-bound form. In many types of cancer, Ras proteins are found to be "stuck" in their active GTP-bound form due to mutations in their genetic sequence.[151] With the inability to terminate its signal to downstream kinases for cell growth and proliferation, oncogenic GTPases are a primary target in cancer research.

With prenylation having a known role in cancer and tumor growth, several farnesyltransferase inhibitors (FTI) and geranylgeranyltransferase inhibitors (GGTI) have been explored as potential therapeutics.[152-156] Despite the wide range of developed FTIs and GGTIs, all have shown limited use at a clinical level for treating cancer but are still used widely at the research level.[152, 157-162] The low level of effectiveness for these inhibitors in cells and human patients stems from the observation that there is no correlation between inhibition of tumor growth and the mutations of Ras. While Ras is mutated and involved in cancer, the efficacy of these prenyltransferase inhibitors lie in their ability to inhibit multiple proteins.[163-164] For instance, K-Ras and N-Ras have been found to undergo geranylgeranylation when farnesyltransferase is subjected to an FTI.[165] This realization highlights the importance of identifying other proteins that

undergo prenylation in order to design new, better therapeutic inhibitors or improve upon previously developed ones.

Hutchinson-Gilford Progeria Syndrome is another disease with connections to prenylation of protein prelamin A.[166-167] In order for prelamin A to function properly, a processing step involving the removal of a 15-amino acid C-terminal peptide sequence by endoprotease ZMPSTE24 occurs after the prenylation and processing of the Ca_1a_2X C-terminus.[168] In progeria, prelamin A cannot be cleaved by ZMPSTE24 and farnesylated prelamin A aggregates on the nuclear envelope giving the affected person the appearance of rapid aging. Those with progeria have significantly shortened life expectancies with many challenges.

Farnesylation was confirmed as the cause of prelamin A aggregation in 2005 wherein a missense mutation on WT lamin A to produce a C-terminal SSIL was used to block prenylation of the protein.[169] No aggregation (nuclear blebbing) was seen with this mutation nor when the C-terminus was mutated to CSIL, which favors geranylgeranylation. As progeria is a farnesylation-specific disease, clinical trials for the use of FTIs as a therapeutic agent in humans began around 2012, after promising results in cells and mice.[170-175] The use of FTIs such as lonafarnib, pravastatin, and zoledronic acid increased life expectancy for children with progeria and improved their quality of life through measurement of weight gain and arterial density.[169, 175-179] One successful trial showed an improvement of 71% in weight gain for patients either treated with lonafarnib alone or with combined FTI therapies, suggesting no real benefit from treatment with multiple FTIs.[179] The use of FTIs was also shown to decrease neurological episodes such as seizures and headaches, of which these patients regularly suffer from.[177] While FTIs are promising treatment for progeria, they only treat symptoms and prolong life; they do not cure it. Research is

turning to gene therapy through stem cells and CRISPR/Cas9 gene editing as a way to possibly cure those who suffer from the disease.[180]

1.5 Importance and objectives

Prenylation is a vital modification in the normal functioning of many proteins within cells and also plays a role in a variety of diseases. With the estimation of over 1166 proteins in the human proteome containing a CaaX motif at their C-termini, having a clear representation of the actual number of proteins undergoing prenylation is an important step in understanding the roles played by prenylated proteins in the cell and in disease.[181] As stated previously, current investigations into prenylation using structural, computational, and biochemical techniques have been restricted in their length of the Ca_1a_2X motif to four amino acids only. With recent evidence for prenylation of non-canonical $C(x)_3X$ motifs in yeast through the study of heat-shock protein, Ydj1p, it is important to study the potential of these longer 5 amino acid motifs acting as prenylation substrates in the human proteome.[8]

This work aims to define a new motif recognized by both yeast and mammalian FTase in which we provide biochemical characterization at the peptide and protein level. Through study of the sequences found in the yeast genetic screening, we can determine sequences within the human genome capable of undergoing prenylation with a $C(x)_3X$ C-terminus and provide evidence of biological relevance for this new recognition motif. The main objective of the work herein is to characterize, identify, and expand upon the list of proteins that can be potentially prenylated both in vitro and in mammalian cells using a $C(x)_3X$ C-terminal motif, breaking a decades-old dogma for this post-translational modification's requirements.

The advent of this new research emphasizes the importance of developing new and more sensitive techniques to study prenylation, especially in the avenues of substrate identification,

specificity and quantitation at the cellular level. An ideal method for studying these areas should include the ability to clearly discern between substrate and product quantitatively, with minimal to no purification of cell lysates before analysis. This not only provides a method that is quick and easy to use, but also minimizes the loss of protein from the cells and therefore provides a more accurate picture of the total protein being studied.

One approach to effective protein isolation uses prenylation as a means of tagging any protein, not just ones which undergo prenylation, using the C-terminus CaaX motif as a tag. Herein we describe a means of using prenylation by a mutant farnesyltransferase (W102R W106L) to isolate protein using a non-natural CaaX sequence (-CVDS) and functionalized FPP analogue. Chemical labeling with a functionalized FPP analogue allows for immobilization of the target proteins via hydrazone ligation chemistry onto hydrazide beads prior to the release of the protein from the beads for analysis. Several labs have utilized the specificity of endogenous farnesyltransferase (FTase) in order to label proteins with FPP analogues with chemical reporter function groups.[182-189] However, these methods are limited to use in bacteria due to competition between the functionalized prenylation reaction and the endogenous prenylation that occurs in mammalian cells. Use of a mutant FTase and non-natural CaaX sequence eliminates the issue of competition from natural proteins, allowing for biorthogonal labeling and isolation of targets from mammalian cells.

While biorthogonal labeling in cells provides a new means of which to isolate proteins of interest, it does not provide a means of quantitation for both unmodified substrate and modified product. To meet this need, our work turned to development of Protein-Lipidation Quantitation (PLQ), the first method to measure a quantitative level of substrate and product in a biologically relevant context.[190] Through use of a fluorescent protein-target protein fusion and capillary

electrophoresis, PLQ is able to clearly separate lipidated product from its unmodified counterpart without need of prior purification from cell lysates. PLQ is applicable to multiple lipid donors and substrates and can be used to study prenylation's role in a variety of cellular contexts, including the presence of oncogenic proteins. Development of this new technique provides a new way of studying a variety of lipidation modifications in a quantitative and biologically relevant manner not possible until now.

1.6 References

1. Jensen, O. N., Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Current opinion in chemical biology* **2004,** *8* (1), 33-41.

2. Mann, M.; Jensen, O. N., Proteomic analysis of post-translational modifications. *Nature biotechnology* **2003,** *21* (3), 255-61.

3. Ayoubi, T. A.; Van De Ven, W. J., Regulation of gene expression by alternative promoters. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **1996,** *10* (4), 453-60.

4. Beck-Sickinger, A. G.; Mörl, K., Posttranslational Modification of Proteins. Expanding Nature's Inventory. By Christopher T. Walsh. *Angewandte Chemie International Edition* **2006,** *45* (7), 1020-1020.

5. Resh, M. D., Covalent Lipid Modifications of Proteins. *Current biology : CB* **2013,** *23* (10), R431-5.

6. Benetka, W.; Koranda, M.; Eisenhaber, F., *Protein Prenylation: An (Almost) Comprehensive Overview on Discovery History, Enzymology, and Significance in Physiology and Disease*. 2006; Vol. 137, p 1241-1281.

7. Nadolski, M. J.; Linder, M. E., Protein lipidation. *The FEBS journal* **2007,** *274* (20), 5202-10.

8. Hildebrandt, E. R.; Cheng, M.; Zhao, P.; Kim, J. H.; Wells, L.; Schmidt, W. K., A shunt pathway limits the CaaX processing of Hsp40 Ydj1p and regulates Ydj1p-dependent phenotypes. *eLife* **2016,** *5*.

9. Resh, M. D., Trafficking and signaling by fatty-acylated and prenylated proteins. *Nature chemical biology* **2006,** *2* (11), 584-90.

10. Resh, M. D., Palmitoylation of ligands, receptors, and intracellular signaling molecules. *Science's STKE : signal transduction knowledge environment* **2006,** *2006* (359), re14.

11. Smotrys, J. E.; Linder, M. E., Palmitoylation of intracellular signaling proteins: regulation and function. *Annual review of biochemistry* **2004,** *73*, 559-87.

12. Blaskovic, S.; Blanc, M.; van der Goot, F. G., What does S-palmitoylation do to membrane proteins? *The FEBS journal* **2013,** *280* (12), 2766-74.

13. Fukata, Y.; Fukata, M., Protein palmitoylation in neuronal development and synaptic plasticity. *Nature reviews. Neuroscience* **2010,** *11* (3), 161-75.

14. Roth, A. F.; Wan, J.; Bailey, A. O.; Sun, B.; Kuchar, J. A.; Green, W. N.; Phinney, B. S.; Yates, J. R., 3rd; Davis, N. G., Global analysis of protein palmitoylation in yeast. *Cell* **2006,** *125* (5), 1003-13.

15. Linder, M. E.; Deschenes, R. J., Palmitoylation: policing protein stability and traffic. *Nature reviews. Molecular cell biology* **2007,** *8* (1), 74-84.

16. Martin, D. D.; Beauchamp, E.; Berthiaume, L. G., Post-translational myristoylation: Fat matters in cellular life and death. *Biochimie* **2011,** *93* (1), 18-31.

17. Boutin, J. A., Myristoylation. *Cellular signalling* **1997,** *9* (1), 15-35.

18. Farazi, T. A.; Waksman, G.; Gordon, J. I., The biology and enzymology of protein N-myristoylation. *The Journal of biological chemistry* **2001,** *276* (43), 39501-4.

19. Towler, D. A.; Eubanks, S. R.; Towery, D. S.; Adams, S. P.; Glaser, L., Amino-terminal processing of proteins by N-myristoylation. Substrate specificity of N-myristoyl transferase. *The Journal of biological chemistry* **1987,** *262* (3), 1030-6.

20. Casey, P. J.; Seabra, M. C., Protein prenyltransferases. *The Journal of biological chemistry* **1996,** *271* (10), 5289-92.

21. Zhang, F. L.; Casey, P. J., Protein prenylation: molecular mechanisms and functional consequences. *Annual review of biochemistry* **1996,** *65*, 241-69.

22. Marshall, C. J., Protein prenylation: a mediator of protein-protein interactions. *Science (New York, N.Y.)* **1993,** *259* (5103), 1865-6.

23. Casey, P. J., Lipid modifications of G proteins. *Current opinion in cell biology* **1994,** *6* (2), 219-25.

24. Paulick, M. G.; Bertozzi, C. R., The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry* **2008,** *47* (27), 6991-7000.

25. Mann, R. K.; Beachy, P. A., Cholesterol modification of proteins. *Biochimica et biophysica acta* **2000,** *1529* (1-3), 188-202.

26. Ferguson, M. A., The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *Journal of cell science* **1999,** *112 (Pt 17)*, 2799-809.

27. Eisenhaber, B.; Maurer-Stroh, S.; Novatchkova, M.; Schneider, G.; Eisenhaber, F., Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. *BioEssays : news and reviews in molecular, cellular and developmental biology* **2003,** *25* (4), 367-85.

28. Nosjean, O.; Briolay, A.; Roux, B., Mammalian GPI proteins: sorting, membrane residence and functions. *Biochimica et biophysica acta* **1997,** *1331* (2), 153-86.

29. Tiede, A.; Bastisch, I.; Schubert, J.; Orlean, P.; Schmidt, R. E., Biosynthesis of glycosylphosphatidylinositols in mammals and unicellular microbes. *Biological chemistry* **1999,** *380* (5), 503-23.

30. Chen, X.; Tukachinsky, H.; Huang, C. H.; Jao, C.; Chu, Y. R.; Tang, H. Y.; Mueller, B.; Schulman, S.; Rapoport, T. A.; Salic, A., Processing and turnover of the Hedgehog protein in the endoplasmic reticulum. *The Journal of cell biology* **2011,** *192* (5), 825-38.

31. Ciepla, P.; Magee, A. I.; Tate, E. W., Cholesterylation: a tail of hedgehog. *Biochemical Society transactions* **2015,** *43* (2), 262-7.

32. Porter, J. A.; Young, K. E.; Beachy, P. A., Cholesterol modification of hedgehog signaling proteins in animal development. *Science (New York, N.Y.)* **1996,** *274* (5285), 255-9.

33. Yokoyama, K.; Goodwin, G. W.; Ghomashchi, F.; Glomset, J.; Gelb, M. H., Protein prenyltransferases. *Biochemical Society transactions* **1992,** *20* (2), 489-94.

34. Leung, K. F.; Baron, R.; Seabra, M. C., Thematic review series: lipid posttranslational modifications. geranylgeranylation of Rab GTPases. *Journal of lipid research* **2006,** *47* (3), 467-75.

35. Guo, Z.; Wu, Y. W.; Das, D.; Delon, C.; Cramer, J.; Yu, S.; Thuns, S.; Lupilova, N.; Waldmann, H.; Brunsveld, L.; Goody, R. S.; Alexandrov, K.; Blankenfeldt, W., Structures of RabGGTase-substrate/product complexes provide insights into the evolution of protein prenylation. *The EMBO journal* **2008,** *27* (18), 2444-56.

36. Khosravi-Far, R.; Clark, G. J.; Abe, K.; Cox, A. D.; McLain, T.; Lutz, R. J.; Sinensky, M.; Der, C. J., Ras (CXXX) and Rab (CC/CXC) prenylation signal sequences are unique and functionally distinct. *The Journal of biological chemistry* **1992,** *267* (34), 24363-8.

37. Stenmark, H.; Olkkonen, V. M., The Rab GTPase family. *Genome biology* **2001,** *2* (5), Reviews3007.

38. Anant, J. S.; Desnoyers, L.; Machius, M.; Demeler, B.; Hansen, J. C.; Westover, K. D.; Deisenhofer, J.; Seabra, M. C., Mechanism of Rab geranylgeranylation: formation of the catalytic ternary complex. *Biochemistry* **1998,** *37* (36), 12559-68.

39. Farnsworth, C. C.; Kawata, M.; Yoshida, Y.; Takai, Y.; Gelb, M. H.; Glomset, J. A., C terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. *Proceedings of the National Academy of Sciences of the United States of America* **1991,** *88* (14), 6196-200.

40. Shen, F.; Seabra, M. C., Mechanism of digeranylgeranylation of Rab proteins. Formation of a complex between monogeranylgeranyl-Rab and Rab escort protein. *The Journal of biological chemistry* **1996,** *271* (7), 3692-8.

41. Lamphear, C. L. Molecular recognition of substrates by protein farnesyltransferase and geranylgeranyltransferase-I. 2012.

42. Caplin, B. E.; Ohya, Y.; Marshall, M. S., Amino acid residues that define both the isoprenoid and CAAX preferences of the Saccharomyces cerevisiae protein farnesyltransferase. Creating the perfect farnesyltransferase. *The Journal of biological chemistry* **1998,** *273* (16), 9472-9.

43. Moores, S. L.; Schaber, M. D.; Mosser, S. D.; Rands, E.; O'Hara, M. B.; Garsky, V. M.; Marshall, M. S.; Pompliano, D. L.; Gibbs, J. B., Sequence dependence of protein isoprenylation. *The Journal of biological chemistry* **1991,** *266* (22), 14603-10.

44. Caplin, B. E.; Hettich, L. A.; Marshall, M. S., Substrate characterization of the Saccharomyces cerevisiae protein farnesyltransferase and type-I protein geranylgeranyltransferase. *Biochimica et biophysica acta* **1994,** *1205* (1), 39-48.

45. Reiss, Y.; Stradley, S. J.; Gierasch, L. M.; Brown, M. S.; Goldstein, J. L., Sequence requirement for peptide recognition by rat brain p21ras protein farnesyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* **1991,** *88* (3), 732-6.

46. Omer, C. A.; Kral, A. M.; Diehl, R. E.; Prendergast, G. C.; Powers, S.; Allen, C. M.; Gibbs, J. B.; Kohl, N. E., Characterization of recombinant human farnesyl-protein transferase: Cloning, expression, farnesyl diphosphate binding, and functional homology with yeast prenyl-protein transferases. *Biochemistry* **1993,** *32* (19), 5167-5176.

47. Hartman, H. L.; Hicks, K. A.; Fierke, C. A., Peptide specificity of protein prenyltransferases is determined mainly by reactivity rather than binding affinity. *Biochemistry* **2005,** *44* (46), 15314-24.

48. Hicks, K. A.; Hartman, H. L.; Fierke, C. A., Upstream polybasic region in peptides enhances dual specificity for prenylation by both farnesyltransferase and geranylgeranyltransferase type I. *Biochemistry* **2005,** *44* (46), 15325-33.

49. Boyartchuk, V. L.; Ashby, M. N.; Rine, J., Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. *Science (New York, N.Y.)* **1997,** *275* (5307), 1796-1800.

50. Winter-Vann, A. M.; Casey, P. J., Post-prenylation-processing enzymes as new targets in oncogenesis. *Nature reviews. Cancer* **2005,** *5* (5), 405-12.

51. Choy, E.; Chiu, V. K.; Silletti, J.; Feoktistov, M.; Morimoto, T.; Michaelson, D.; Ivanov, I. E.; Philips, M. R., Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* **1999,** *98* (1), 69-80.

52. Bergo, M. O.; Wahlstrom, A. M.; Fong, L. G.; Young, S. G., Genetic analyses of the role of RCE1 in RAS membrane association and transformation. *Methods in enzymology* **2008,** *438*, 367-89.

53. Hollander, I.; Frommer, E.; Mallon, R., Human ras-converting enzyme (hRCE1) endoproteolytic activity on K-ras-derived peptides. *Analytical biochemistry* **2000,** *286* (1), 129-37.

54. Lau, H. Y.; Tang, J.; Casey, P. J.; Wang, M., Isoprenylcysteine carboxylmethyltransferase is critical for malignant transformation and tumor maintenance by all RAS isoforms. *Oncogene* **2017**.

55. Michaelson, D.; Ali, W.; Chiu, V. K.; Bergo, M.; Silletti, J.; Wright, L.; Young, S. G.; Philips, M., Postprenylation CAAX Processing Is Required for Proper Localization of Ras but Not Rho GTPases. *Molecular Biology of the Cell* **2005,** *16* (4), 1606-16.

56. Cansado, J., To finish things well: cysteine methylation ensures selective GTPase membrane localization and signalling. *Current genetics* **2018,** *64* (2), 341-344.

57. Yang, J.; Kulkarni, K.; Manolaridis, I.; Zhang, Z.; Dodd, R. B.; Mas-Droux, C.; Barford, D., Mechanism of isoprenylcysteine carboxyl methylation from the crystal structure of the integral membrane methyltransferase ICMT. *Molecular cell* **2011,** *44* (6), 997-1004.

58. Gutierrez, L.; Magee, A. I.; Marshall, C. J.; Hancock, J. F., Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *The EMBO journal* **1989,** *8* (4), 1093-8.

59. Chiu, V. K.; Bivona, T.; Hach, A.; Sajous, J. B.; Silletti, J.; Wiener, H.; Johnson, R. L., 2nd; Cox, A. D.; Philips, M. R., Ras signalling on the endoplasmic reticulum and the Golgi. *Nat Cell Biol* **2002,** *4* (5), 343-50.

60. Williams, C. L., The polybasic region of Ras and Rho family small GTPases: a regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. *Cellular signalling* **2003,** *15* (12), 1071-80.

61. Hancock, J. F.; Paterson, H.; Marshall, C. J., A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* **1990,** *63* (1), 133-9.

62. Lin, D. T. S.; Davis, N. G.; Conibear, E., Targeting the Ras palmitoylation/depalmitoylation cycle in cancer. *Biochemical Society transactions* **2017,** *45* (4), 913-921.

63. Goodwin, J. S.; Drake, K. R.; Rogers, C.; Wright, L.; Lippincott-Schwartz, J.; Philips, M. R.; Kenworthy, A. K., Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway. *The Journal of cell biology* **2005,** *170* (2), 261-72.

64. Rocks, O.; Peyker, A.; Kahms, M.; Verveer, P. J.; Koerner, C.; Lumbierres, M.; Kuhlmann, J.; Waldmann, H.; Wittinghofer, A.; Bastiaens, P. I., An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science (New York, N.Y.)* **2005,** *307* (5716), 1746-52.

65. Hast, M. A.; Beese, L. S., 13 - Structural Biochemistry of CaaX Protein Prenyltransferases. In *The Enzymes*, Tamanoi, F.; Hrycyna, C. A.; Bergo, M. O., Eds. Academic Press: 2011; Vol. 29, pp 235-257.

66. Lane, K. T.; Beese, L. S., Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *J Lipid Res* **2006,** *47* (4), 681-99.

67. Seabra, M. C.; Reiss, Y.; Casey, P. J.; Brown, M. S.; Goldstein, J. L., Protein farnesyltransferase and geranylgeranyltransferase share a common alpha subunit. *Cell* **1991,** *65* (3), 429-34.

68. Hightower, K. E.; Huang, C. C.; Casey, P. J.; Fierke, C. A., H-Ras peptide and protein substrates bind protein farnesyltransferase as an ionized thiolate. *Biochemistry* **1998,** *37* (44), 15555-62.

69. Saderholm, M. J.; Hightower, K. E.; Fierke, C. A., Role of metals in the reaction catalyzed by protein farnesyltransferase. *Biochemistry* **2000,** *39* (40), 12398-405.

70. Reid, T. S.; Terry, K. L.; Casey, P. J.; Beese, L. S., Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. *Journal of molecular biology* **2004,** *343* (2), 417-33.

71. Clarke, S.; Vogel, J. P.; Deschenes, R. J.; Stock, J., Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** *85* (13), 4643-7.

72. Hancock, J. F.; Magee, A. I.; Childs, J. E.; Marshall, C. J., All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **1989,** *57* (7), 1167-77.

73. Kitten, G. T.; Nigg, E. A., The CaaX motif is required for isoprenylation, carboxyl methylation, and nuclear membrane association of lamin B2. *The Journal of cell biology* **1991,** *113* (1), 13-23.

74. Powers, S.; Michaelis, S.; Broek, D.; Santa Anna, S.; Field, J.; Herskowitz, I.; Wigler, M., RAM, a gene of yeast required for a functional modification of RAS proteins and for production of mating pheromone a-factor. *Cell* **1986,** *47* (3), 413-22.

75. Vorburger, K.; Kitten, G. T.; Nigg, E. A., Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif. *The EMBO journal* **1989,** *8* (13), 4007-13.

76. Farnsworth, C. C.; Wolda, S. L.; Gelb, M. H.; Glomset, J. A., Human lamin B contains a farnesylated cysteine residue. *The Journal of biological chemistry* **1989,** *264* (34), 20422-9.

77. Hougland, J. L.; Hicks, K. A.; Hartman, H. L.; Kelly, R. A.; Watt, T. J.; Fierke, C. A., Identification of novel peptide substrates for protein farnesyltransferase reveals two substrate classes with distinct sequence selectivities. *Journal of molecular biology* **2010,** *395* (1), 176-90.

78. Cui, G.; Merz, K. M., Jr., Computational studies of the farnesyltransferase ternary complex part II: the conformational activation of farnesyldiphosphate. *Biochemistry* **2007,** *46* (43), 12375-81.

79. London, N.; Lamphear, C. L.; Hougland, J. L.; Fierke, C. A.; Schueler-Furman, O., Identification of a novel class of farnesylation targets by structure-based modeling of binding specificity. *PLoS computational biology* **2011,** *7* (10), e1002170.

80. Maurer-Stroh, S.; Eisenhaber, F., Refinement and prediction of protein prenylation motifs. *Genome Biology* **2005,** *6* (6), R55.

81. Maurer-Stroh, S.; Koranda, M.; Benetka, W.; Schneider, G.; Sirota, F. L.; Eisenhaber, F., Towards complete sets of farnesylated and geranylgeranylated proteins. *PLoS computational biology* **2007,** *3* (4), e66.

82. Schaber, M. D.; O'Hara, M. B.; Garsky, V. M.; Mosser, S. C.; Bergstrom, J. D.; Moores, S. L.; Marshall, M. S.; Friedman, P. A.; Dixon, R. A.; Gibbs, J. B., Polyisoprenylation of Ras in vitro by a farnesyl-protein transferase. *The Journal of biological chemistry* **1990,** *265* (25), 14701-4.

83. Lane, K. T.; Beese, L. S., Thematic review series: Lipid Posttranslational Modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *Journal of Lipid Research* **2006,** *47* (4), 681-699.

84. Ostermeier, C.; Brunger, A. T., Structural basis of Rab effector specificity: crystal structure of the small G protein Rab3A complexed with the effector domain of Rabphilin-3A. *Cell* **1999,** *96*.

85. Strickland, C. L.; Windsor, W. T.; Syto, R.; Wang, L.; Bond, R.; Wu, Z.; Schwartz, J.; Le, H. V.; Beese, L. S.; Weber, P. C., Crystal structure of farnesyl protein transferase complexed with a CaaX peptide and farnesyl diphosphate analogue. *Biochemistry* **1998,** *37* (47), 16601-11.

86. Stroupe, C.; Brunger, A. T., Crystal structures of a Rab protein in its inactive and active conformations. *Journal of molecular biology* **2000,** *304*.

87. Gangopadhyay, S. A.; Losito, E. L.; Hougland, J. L., Targeted reengineering of protein geranylgeranyltransferase type I selectivity functionally implicates active-site residues in protein-substrate recognition. *Biochemistry* **2014,** *53* (2), 434-46.

88. Gao, J.; Liao, J.; Yang, G. Y., CAAX-box protein, prenylation process and carcinogenesis. *American journal of translational research* **2009,** *1* (3), 312-25.

89. Hancock, J. F.; Cadwallader, K.; Paterson, H.; Marshall, C. J., A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *The EMBO journal* **1991,** *10* (13), 4033-9.

90. Hougland, J. L.; Gangopadhyay, S. A.; Fierke, C. A., Expansion of Protein Farnesyltransferase Specificity Using "Tunable" Active Site Interactions: Development of bioengineered prenylation pathways. *Journal of Biological Chemistry* **2012,** *287* (45), 38090-38100.

91. Hougland, J. L.; Lamphear, C. L.; Scott, S. A.; Gibbs, R. A.; Fierke, C. A., Context-dependent substrate recognition by protein farnesyltransferase. *Biochemistry* **2009,** *48* (8), 1691-701.

92. Ota, I. M.; Clarke, S., Enzymatic methylation of 23-29-kDa bovine retinal rod outer segment membrane proteins. Evidence for methyl ester formation at carboxyl-terminal cysteinyl residues. *Journal of Biological Chemistry* **1989,** *264* (22), 12879-12884.

93. Andres, D. A.; Crick, D. C.; Finlin, B. S.; Waechter, C. J., Rapid identification of cysteine-linked isoprenyl groups by metabolic labeling with [3H]farnesol and [3H]geranylgeraniol. *Methods in molecular biology (Clifton, N.J.)* **1999,** *116*, 107-23.

94. Gibbs, B. S.; Zahn, T. J.; Mu, Y.; Sebolt-Leopold, J. S.; Gibbs, R. A., Novel farnesol and geranylgeraniol analogues: A potential new class of anticancer agents directed against protein prenylation. *Journal of medicinal chemistry* **1999,** *42* (19), 3800-8.

95. Hancock, J. F., Reticulocyte lysate assay for in vitro translation and posttranslational modification of Ras proteins. *Methods in enzymology* **1995,** *255*, 60-5.

96. Flynn, S. C.; Lindgren, D. E.; Hougland, J. L., Quantitative Determination of Cellular Farnesyltransferase Activity: Towards Defining the Minimum Substrate Reactivity for Biologically Relevant Protein Farnesylation. *Chembiochem : a European journal of chemical biology* **2014,** *15* (15), 2205-2210.

97. DeGraw, A. J.; Palsuledesai, C.; Ochocki, J. D.; Dozier, J. K.; Lenevich, S.; Rashidian, M.; Distefano, M. D., Evaluation of alkyne-modified isoprenoids as chemical reporters of protein prenylation. *Chemical biology & drug design* **2010,** *76* (6), 460-71.

98. Jennings, B. C.; Danowitz, A. M.; Wang, Y. C.; Gibbs, R. A.; Distefano, M. D.; Fierke, C. A., Analogs of farnsyl diphosphate alter CaaX substrate specificity and reactions rates of protein farnesyltransferase. *Bioorganic & medicinal chemistry letters* **2016,** *26* (4), 1333-6.

99. Palsuledesai, C. C.; Ochocki, J. D.; Kuhns, M. M.; Wang, Y.-C.; Warmka, J. K.; Chernick, D. S.; Wattenberg, E. V.; Li, L.; Arriaga, E. A.; Distefano, M. D., Metabolic Labeling with an

Alkyne-modified Isoprenoid Analog Facilitates Imaging and Quantification of the Prenylome in Cells. *ACS Chemical Biology* **2016,** *11* (10), 2820-2828.

100. Rashidian, M.; Dozier, J. K.; Lenevich, S.; Distefano, M. D., Selective Labeling of Polypeptides Using Protein Farnesyltransferase via Rapid Oxime Ligation. *Chemical communications (Cambridge, England)* **2010,** *46* (47), 8998-9000.

101. Rashidian, M.; Kumarapperuma, S. C.; Gabrielse, K.; Fegan, A.; Wagner, C. R.; Distefano, M. D., Simultaneous dual protein labeling using a triorthogonal reagent. *J Am Chem Soc* **2013,** *135* (44), 16388-96.

102. Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D., Chemoenzymatic Reversible Immobilization and Labeling of Proteins without Prior Purification. *Journal of the American Chemical Society* **2012,** *134* (20), 8455-8467.

103. Turek-Etienne, T. C.; Strickland, C. L.; Distefano, M. D., Biochemical and Structural Studies with Prenyl Diphosphate Analogues Provide Insights into Isoprenoid Recognition by Protein Farnesyl Transferase. *Biochemistry* **2003,** *42* (13), 3716-3724.

104. Wang, Y.-C.; Dozier, J. K.; Beese, L. S.; Distefano, M. D., Rapid Analysis of Protein Farnesyltransferase Substrate Specificity Using Peptide Libraries and Isoprenoid Diphosphate Analogues. *ACS Chemical Biology* **2014,** *9* (8), 1726-1735.

105. Zhang, Y.; Blanden, M. J.; Sudheer, C.; Gangopadhyay, S. A.; Rashidian, M.; Hougland, J. L.; Distefano, M. D., Simultaneous Site-Specific Dual Protein Labeling Using Protein Prenyltransferases. *Bioconjug Chem* **2015,** *26* (12), 2542-53.

106. Berry, A. F.; Heal, W. P.; Tarafder, A. K.; Tolmachova, T.; Baron, R. A.; Seabra, M. C.; Tate, E. W., Rapid multilabel detection of geranylgeranylated proteins by using bioorthogonal ligation chemistry. *Chembiochem : a European journal of chemical biology* **2010,** *11* (6), 771-3.

107. Nguyen, U. T.; Guo, Z.; Delon, C.; Wu, Y.; Deraeve, C.; Franzel, B.; Bon, R. S.; Blankenfeldt, W.; Goody, R. S.; Waldmann, H.; Wolters, D.; Alexandrov, K., Analysis of the eukaryotic prenylome by isoprenoid affinity tagging. *Nature chemical biology* **2009,** *5* (4), 227-35.

108. Chan, L. N.; Hart, C.; Guo, L.; Nyberg, T.; Davies, B. S.; Fong, L. G.; Young, S. G.; Agnew, B. J.; Tamanoi, F., A novel approach to tag and identify geranylgeranylated proteins. *Electrophoresis* **2009,** *30* (20), 3598-606.

109. Chen, M.; Knifley, T.; Subramanian, T.; Spielmann, H. P.; O'Connor, K. L., Use of Synthetic Isoprenoids to Target Protein Prenylation and Rho GTPases in Breast Cancer Invasion. *PloS one* **2014,** *9* (2), e89892.

110. Temple, K. J.; Wright, E. N.; Fierke, C. A.; Gibbs, R. A., Exploration of GGTase-I substrate requirements. Part 1. Synthesis & biochemical evaluation of novel aryl-modified geranylgeranyl diphosphate analogs. *Bioorganic & medicinal chemistry letters* **2016,** *26* (15), 3499-3502.

111. Subramanian, T.; Pais, J. E.; Liu, S.; Troutman, J. M.; Suzuki, Y.; Leela Subramanian, K.; Fierke, C. A.; Andres, D. A.; Spielmann, H. P., Farnesyl Diphosphate Analogues with Aryl Moieties Are Efficient Alternate Substrates for Protein Farnesyltransferase. *Biochemistry* **2012,** *51* (41), 8307-8319.

112. Labadie, G. R.; Viswanathan, R.; Poulter, C. D., Farnesyl Diphosphate Analogues with ω-Bioorthogonal Azide and Alkyne Functional Groups for PFTase-Catalyzed Ligation Reactions. *The Journal of organic chemistry* **2007,** *72* (24), 9291-9297.

113. Jennings, B. C.; Danowitz, A. M.; Wang, Y.-C.; Gibbs, R. A.; Distefano, M. D.; Fierke, C. A., Analogs of farnsyl diphosphate alter CaaX substrate specificity and reactions rates of protein farnesyltransferase. *Bioorganic & medicinal chemistry letters* **2016,** *26* (4), 1333-1336.

114. Wang, Y.-C.; Distefano, M. D., Synthetic isoprenoid analogues for the study of prenylated proteins: Fluorescent imaging and proteomic applications. *Bioorganic Chemistry* **2016,** *64*, 59-65.

115. Nguyen, U. T. T.; Guo, Z.; Delon, C.; Wu, Y.; Deraeve, C.; Fränzel, B.; Bon, R. S.; Blankenfeldt, W.; Goody, R. S.; Waldmann, H.; Wolters, D.; Alexandrov, K., Analysis of the eukaryotic prenylome by isoprenoid affinity tagging. *Nature chemical biology* **2009,** *5*, 227.

116. Troutman, J. M.; Roberts, M. J.; Andres, D. A.; Spielmann, H. P., Tools To Analyze Protein Farnesylation in Cells. *Bioconjugate Chemistry* **2005,** *16* (5), 1209-1217.

117. Wojtkowiak, J. W.; Gibbs, R. A.; Mattingly, R. R., Working together: Farnesyl transferase inhibitors and statins block protein prenylation. *Molecular and cellular pharmacology* **2009,** *1* (1), 1-6.

118. Prior, I. A.; Lewis, P. D.; Mattos, C., A comprehensive survey of Ras mutations in cancer. *Cancer research* **2012,** *72* (10), 2457-2467.

119. Lackner, M. R.; Kindt, R. M.; Carroll, P. M.; Brown, K.; Cancilla, M. R.; Chen, C.; de Silva, H.; Franke, Y.; Guan, B.; Heuer, T.; Hung, T.; Keegan, K.; Lee, J. M.; Manne, V.; O'Brien, C.; Parry, D.; Perez-Villar, J. J.; Reddy, R. K.; Xiao, H.; Zhan, H.; Cockett, M.; Plowman, G.; Fitzgerald, K.; Costa, M.; Ross-Macdonald, P., Chemical genetics identifies Rab geranylgeranyl transferase as an apoptotic target of farnesyl transferase inhibitors. *Cancer Cell* **2005,** *7* (4), 325-336.

120. Caplan, A. J.; Douglas, M. G., Characterization of YDJ1: a yeast homologue of the bacterial dnaJ protein. *The Journal of cell biology* **1991,** *114* (4), 609-21.

121. Caplan, A. J.; Tsai, J.; Casey, P. J.; Douglas, M. G., Farnesylation of YDJ1p is required for function at elevated growth temperatures in Saccharomyces cerevisiae. *The Journal of biological chemistry* **1992,** *267* (26), 18890-5.

122. Kimura, Y.; Yahara, I.; Lindquist, S., Role of the protein chaperone YDJ1 in establishing Hsp90-mediated signal transduction pathways. *Science (New York, N.Y.)* **1995,** *268* (5215), 1362-5.

123. Lu, Z.; Cyr, D. M., The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding. *The Journal of biological chemistry* **1998,** *273* (10), 5970-8.

124. Qiu, X.-B.; Shao, Y.-M.; Miao, S.; Wang, L., The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and Molecular Life Sciences CMLS* **2006,** *63* (22), 2560-2570.

125. Beck, L. A.; Hosick, T. J.; Sinensky, M., Isoprenylation is required for the processing of the lamin A precursor. *The Journal of cell biology* **1990,** *110* (5), 1489-99.

126. Thissen, J. A.; Gross, J. M.; Subramanian, K.; Meyer, T.; Casey, P. J., Prenylation-dependent association of Ki-Ras with microtubules. Evidence for a role in subcellular trafficking. *The Journal of biological chemistry* **1997,** *272* (48), 30362-70.

127. Cox, A. D.; Der, C. J., The ras/cholesterol connection: implications for ras oncogenicity. *Critical reviews in oncogenesis* **1992,** *3* (4), 365-400.

128. Cox, A. D.; Hisaka, M. M.; Buss, J. E.; Der, C. J., Specific isoprenoid modification is required for function of normal, but not oncogenic, Ras protein. *Mol Cell Biol* **1992,** *12* (6), 2606-15.

129. Inglese, J.; Koch, W. J.; Caron, M. G.; Lefkowitz, R. J., Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature* **1992,** *359* (6391), 147-50.

130. Cox, A. D.; Der, C. J., Protein prenylation: more than just glue? *Current opinion in cell biology* **1992,** *4* (6), 1008-16.

131. Benetka, W.; Koranda, M.; Eisenhaber, F., Protein Prenylation: An (Almost) Comprehensive Overview on Discovery History, Enzymology, and Significance in Physiology and Disease. *Monatshefte für Chemie / Chemical Monthly* **2006,** *137* (10), 1241.

132. Alan, J. K.; Lundquist, E. A., Mutationally activated Rho GTPases in cancer. *Small GTPases* **2013,** *4* (3), 159-163.

133. Rajalingam, K.; Schreck, R.; Rapp, U. R.; Albert, Š., Ras oncogenes and their downstream targets. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **2007,** *1773* (8), 1177-1195.

134. Fernández-Medarde, A.; Santos, E., Ras in Cancer and Developmental Diseases. *Genes & Cancer* **2011,** *2* (3), 344-358.

135. Davies, B. S.; Fong, L. G.; Yang, S. H.; Coffinier, C.; Young, S. G., The posttranslational processing of prelamin A and disease. *Annual review of genomics and human genetics* **2009,** *10*, 153-74.

136. Davies, B. S. J.; Coffinier, C.; Yang, S. H.; Barnes, R. H.; Jung, H.-J.; Young, S. G.; Fong, L. G., Investigating the purpose of prelamin A processing. *Nucleus* **2011,** *2* (1), 4-9.

137. Worman, H. J., Prelamin A prenylation and the treatment of progeria. *Journal of Lipid Research* **2010,** *51* (2), 223-225.

138. Piispanen, A. E.; Bonnefoi, O.; Carden, S.; Deveau, A.; Bassilana, M.; Hogan, D. A., Roles of Ras1 membrane localization during Candida albicans hyphal growth and farnesol response. *Eukaryot Cell* **2011,** *10* (11), 1473-84.

139. Selvig, K.; Ballou, E. R.; Nichols, C. B.; Alspaugh, J. A., Restricted substrate specificity for the geranylgeranyltransferase-I enzyme in Cryptococcus neoformans: implications for virulence. *Eukaryot Cell* **2013,** *12* (11), 1462-71.

140. Hast, M. A.; Nichols, C. B.; Armstrong, S. M.; Kelly, S. M.; Hellinga, H. W.; Alspaugh, J. A.; Beese, L. S., Structures of Cryptococcus neoformans protein farnesyltransferase reveal strategies for developing inhibitors that target fungal pathogens. *The Journal of biological chemistry* **2011,** *286* (40), 35149-62.

141. Suazo, K. F.; Schaber, C.; Palsuledesai, C. C.; Odom John, A. R.; Distefano, M. D., Global proteomic analysis of prenylated proteins in Plasmodium falciparum using an alkyne-modified isoprenoid analogue. *Scientific Reports* **2016,** *6*, 38615.

142. Gisselberg, J. E.; Zhang, L.; Elias, J. E.; Yeh, E., The Prenylated Proteome of Plasmodium falciparum Reveals Pathogen-specific Prenylation Activity and Drug Mechanism-of-action. *Molecular & cellular proteomics : MCP* **2017,** *16* (4 suppl 1), S54-s64.

143. Di, J.; Huang, H.; Qu, D.; Tang, J.; Cao, W.; Lu, Z.; Cheng, Q.; Yang, J.; Bai, J.; Zhang, Y.; Zheng, J., Rap2B promotes proliferation, migration, and invasion of human breast cancer through calcium-related ERK1/2 signaling pathway. *Sci Rep* **2015,** *5*, 12363.

144. Bailey, C. L.; Kelly, P.; Casey, P. J., Activation of Rap1 promotes prostate cancer metastasis. *Cancer research* **2009,** *69* (12), 4962-8.

145. Scheffzek, K.; Ahmadian, M. R.; Kabsch, W.; Wiesmuller, L.; Lautwein, A.; Schmitz, F.; Wittinghofer, A., The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science (New York, N.Y.)* **1997,** *277* (5324), 333-8.

146. Malumbres, M.; Barbacid, M., RAS oncogenes: the first 30 years. *Nature reviews. Cancer* **2003,** *3* (6), 459-65.

147. Campbell, P. M.; Der, C. J., Oncogenic Ras and its role in tumor cell invasion and metastasis. *Seminars in cancer biology* **2004,** *14* (2), 105-14.

148. Bos, J. L., ras oncogenes in human cancer: a review. *Cancer research* **1989,** *49* (17), 4682-9.

149. Gschwind, A.; Fischer, O. M.; Ullrich, A., The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nature reviews. Cancer* **2004,** *4* (5), 361-70.

150. Schlessinger, J., Cell signaling by receptor tyrosine kinases. *Cell* **2000,** *103* (2), 211-25.

151. Shah, S.; Brock, E. J.; Ji, K.; Mattingly, R. R., Ras and Rap1: A Tale of Two GTPases. *Seminars in cancer biology* **2018**.

152. Ochocki, J. D.; Distefano, M. D., Prenyltransferase Inhibitors: Treating Human Ailments from Cancer to Parasitic Infections. *MedChemComm* **2013,** *4* (3), 476-492.

153. Nallan, L.; Bauer, K. D.; Bendale, P.; Rivas, K.; Yokoyama, K.; Horney, C. P.; Pendyala, P. R.; Floyd, D.; Lombardo, L. J.; Williams, D. K.; Hamilton, A.; Sebti, S.; Windsor, W. T.; Weber, P. C.; Buckner, F. S.; Chakrabarti, D.; Gelb, M. H.; Van Voorhis, W. C., Protein farnesyltransferase inhibitors exhibit potent antimalarial activity. *Journal of medicinal chemistry* **2005,** *48* (11), 3704-13.

154. Gelb, M. H.; Brunsveld, L.; Hrycyna, C. A.; Michaelis, S.; Tamanoi, F.; Van Voorhis, W. C.; Waldmann, H., Therapeutic intervention based on protein prenylation and associated modifications. *Nature chemical biology* **2006,** *2* (10), 518-28.

155. Sousa, S. F.; Fernandes, P. A.; Ramos, M. J., Farnesyltransferase inhibitors: a detailed chemical view on an elusive biological problem. *Current medicinal chemistry* **2008,** *15* (15), 1478-92.

156. Berndt, N.; Hamilton, A. D.; Sebti, S. M., Targeting protein prenylation for cancer therapy. *Nature reviews. Cancer* **2011,** *11* (11), 775-91.

157. Gibbs, R. A.; Zahn, T. J.; Sebolt-Leopold, J. S., Non-peptidic prenyltransferase inhibitors: diverse structural classes and surprising anti-cancer mechanisms. *Current medicinal chemistry* **2001,** *8* (12), 1437-65.

158. Zujewski, J.; Horak, I. D.; Bol, C. J.; Woestenborghs, R.; Bowden, C.; End, D. W.; Piotrovsky, V. K.; Chiao, J.; Belly, R. T.; Todd, A.; Kopp, W. C.; Kohler, D. R.; Chow, C.; Noone, M.; Hakim, F. T.; Larkin, G.; Gress, R. E.; Nussenblatt, R. B.; Kremer, A. B.; Cowan, K. H., Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **2000,** *18* (4), 927-41.

159. Peterson, Y. K.; Kelly, P.; Weinbaum, C. A.; Casey, P. J., A novel protein geranylgeranyltransferase-I inhibitor with high potency, selectivity, and cellular activity. *The Journal of biological chemistry* **2006,** *281* (18), 12445-50.

160. Sun, J.; Qian, Y.; Hamilton, A. D.; Sebti, S. M., Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation

but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. *Oncogene* **1998,** *16* (11), 1467-73.

161. Lobell, R. B.; Omer, C. A.; Abrams, M. T.; Bhimnathwala, H. G.; Brucker, M. J.; Buser, C. A.; Davide, J. P.; deSolms, S. J.; Dinsmore, C. J.; Ellis-Hutchings, M. S.; Kral, A. M.; Liu, D.; Lumma, W. C.; Machotka, S. V.; Rands, E.; Williams, T. M.; Graham, S. L.; Hartman, G. D.; Oliff, A. I.; Heimbrook, D. C.; Kohl, N. E., Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models. *Cancer research* **2001,** *61* (24), 8758-68.

162. Morgan, M. A.; Ganser, A.; Reuter, C. W., Therapeutic efficacy of prenylation inhibitors in the treatment of myeloid leukemia. *Leukemia* **2003,** *17* (8), 1482-98.

163. Sepp-Lorenzino, L.; Ma, Z.; Rands, E.; Kohl, N. E.; Gibbs, J. B.; Oliff, A.; Rosen, N., A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. *Cancer research* **1995,** *55* (22), 5302-9.

164. Kurzrock, R.; Kantarjian, H. M.; Cortes, J. E.; Singhania, N.; Thomas, D. A.; Wilson, E. F.; Wright, J. J.; Freireich, E. J.; Talpaz, M.; Sebti, S. M., Farnesyltransferase inhibitor R115777 in myelodysplastic syndrome: clinical and biologic activities in the phase 1 setting. *Blood* **2003,** *102* (13), 4527-34.

165. Whyte, D. B.; Kirschmeier, P.; Hockenberry, T. N.; Nunez-Oliva, I.; James, L.; Catino, J. J.; Bishop, W. R.; Pai, J.-K., K- and N-Ras Are Geranylgeranylated in Cells Treated with Farnesyl Protein Transferase Inhibitors. *Journal of Biological Chemistry* **1997,** *272* (22), 14459-14464.

166. Broers, J. L.; Ramaekers, F. C.; Bonne, G.; Yaou, R. B.; Hutchison, C. J., Nuclear lamins: laminopathies and their role in premature ageing. *Physiological reviews* **2006,** *86* (3), 967-1008.

167. Young, S. G.; Meta, M.; Yang, S. H.; Fong, L. G., Prelamin A farnesylation and progeroid syndromes. *The Journal of biological chemistry* **2006,** *281* (52), 39741-5.

168. Leung, G. K.; Schmidt, W. K.; Bergo, M. O.; Gavino, B.; Wong, D. H.; Tam, A.; Ashby, M. N.; Michaelis, S.; Young, S. G., Biochemical studies of Zmpste24-deficient mice. *The Journal of biological chemistry* **2001,** *276* (31), 29051-8.

169. Capell, B. C.; Erdos, M. R.; Madigan, J. P.; Fiordalisi, J. J.; Varga, R.; Conneely, K. N.; Gordon, L. B.; Der, C. J.; Cox, A. D.; Collins, F. S., Inhibiting farnesylation of progerin prevents the characteristic nuclear blebbing of Hutchinson-Gilford progeria syndrome. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (36), 12879-12884.

170. Mehta, I. S.; Eskiw, C. H.; Arican, H. D.; Kill, I. R.; Bridger, J. M., Farnesyltransferase inhibitor treatment restores chromosome territory positions and active chromosome dynamics in Hutchinson-Gilford progeria syndrome cells. *Genome biology* **2011,** *12* (8), R74.

171. Wang, Y.; Ostlund, C.; Worman, H. J., Blocking protein farnesylation improves nuclear shape abnormalities in keratinocytes of mice expressing the prelamin A variant in Hutchinson-Gilford progeria syndrome. *Nucleus (Austin, Tex.)* **2010,** *1* (5), 432-9.

172. Rivera-Torres, J.; Acin-Perez, R.; Cabezas-Sanchez, P.; Osorio, F. G.; Gonzalez-Gomez, C.; Megias, D.; Camara, C.; Lopez-Otin, C.; Enriquez, J. A.; Luque-Garcia, J. L.; Andres, V., Identification of mitochondrial dysfunction in Hutchinson-Gilford progeria syndrome through use of stable isotope labeling with amino acids in cell culture. *Journal of proteomics* **2013,** *91*, 466-77.

173. Yang, S. H.; Chang, S. Y.; Ren, S.; Wang, Y.; Andres, D. A.; Spielmann, H. P.; Fong, L. G.; Young, S. G., Absence of progeria-like disease phenotypes in knock-in mice expressing a non-farnesylated version of progerin. *Human molecular genetics* **2011,** *20* (3), 436-44.

174. Yang, S. H.; Chang, S. Y.; Andres, D. A.; Spielmann, H. P.; Young, S. G.; Fong, L. G., Assessing the efficacy of protein farnesyltransferase inhibitors in mouse models of progeria. *Journal of lipid research* **2010,** *51* (2), 400-5.

175. Young, S. G.; Yang, S. H.; Davies, B. S.; Jung, H. J.; Fong, L. G., Targeting protein prenylation in progeria. *Science translational medicine* **2013,** *5* (171), 171ps3.

176. Gordon, L. B.; Shappell, H.; Massaro, J.; D'Agostino, R. B., Sr.; Brazier, J.; Campbell, S. E.; Kleinman, M. E.; Kieran, M. W., Association of Lonafarnib Treatment vs No Treatment With Mortality Rate in Patients With Hutchinson-Gilford Progeria Syndrome. *Jama* **2018,** *319* (16), 1687-1695.

177. Ullrich, N. J.; Kieran, M. W.; Miller, D. T.; Gordon, L. B.; Cho, Y. J.; Silvera, V. M.; Giobbie-Hurder, A.; Neuberg, D.; Kleinman, M. E., Neurologic features of Hutchinson-Gilford progeria syndrome after lonafarnib treatment. *Neurology* **2013,** *81* (5), 427-30.

178. Gordon, L. B.; Kleinman, M. E.; Miller, D. T.; Neuberg, D. S.; Giobbie-Hurder, A.; Gerhard-Herman, M.; Smoot, L. B.; Gordon, C. M.; Cleveland, R.; Snyder, B. D.; Fligor, B.; Bishop, W. R.; Statkevich, P.; Regen, A.; Sonis, A.; Riley, S.; Ploski, C.; Correia, A.; Quinn, N.; Ullrich, N. J.; Nazarian, A.; Liang, M. G.; Huh, S. Y.; Schwartzman, A.; Kieran, M. W., Clinical trial of a farnesyltransferase inhibitor in children with Hutchinson-Gilford progeria syndrome. *Proceedings of the National Academy of Sciences of the United States of America* **2012,** *109* (41), 16666-71.

179. Gordon, L. B.; Kleinman, M. E.; Massaro, J.; D'Agostino, R. B., Sr.; Shappell, H.; Gerhard-Herman, M.; Smoot, L. B.; Gordon, C. M.; Cleveland, R. H.; Nazarian, A.; Snyder, B. D.; Ullrich, N. J.; Silvera, V. M.; Liang, M. G.; Quinn, N.; Miller, D. T.; Huh, S. Y.; Dowton, A. A.; Littlefield, K.; Greer, M. M.; Kieran, M. W., Clinical Trial of the Protein Farnesylation Inhibitors Lonafarnib,

Pravastatin, and Zoledronic Acid in Children With Hutchinson-Gilford Progeria Syndrome. *Circulation* **2016,** *134* (2), 114-25.

180. Compagnucci, C.; Bertini, E., The Potential of iPSCs for the Treatment of Premature Aging Disorders. *International Journal of Molecular Sciences* **2017,** *18* (11), 2350.

181. Blanden, M. J.; Suazo, K. F.; Hildebrandt, E. R.; Hardgrove, D. S.; Patel, M.; Saunders, W. P.; Distefano, M. D.; Schmidt, W. K.; Hougland, J. L., Efficient farnesylation of an extended C-terminal C(x)3X sequence motif expands the scope of the prenylated proteome. *The Journal of biological chemistry* **2018,** *293* (8), 2770-2785.

182. Duckworth, B. P.; Xu, J.; Taton, T. A.; Guo, A.; Distefano, M. D., Site-specific, covalent attachment of proteins to a solid surface. *Bioconjug Chem* **2006,** *17* (4), 967-74.

183. Xu, J.; Degraw, A. J.; Duckworth, B. P.; Lenevich, S.; Tann, C. M.; Jenson, E. C.; Gruber, S. J.; Barany, G.; Distefano, M. D., Synthesis and reactivity of 6,7-dihydrogeranylazides: reagents for primary azide incorporation into peptides and subsequent staudinger ligation. *Chemical biology & drug design* **2006,** *68* (2), 85-96.

184. Duckworth, B. P.; Zhang, Z.; Hosokawa, A.; Distefano, M. D., Selective labeling of proteins by using protein farnesyltransferase. *Chembiochem : a European journal of chemical biology* **2007,** *8* (1), 98-105.

185. Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D., Chemoenzymatic reversible immobilization and labeling of proteins without prior purification. *Journal of the American Chemical Society* **2012,** *134* (20), 8455-67.

186. Weinrich, D.; Lin, P. C.; Jonkheijm, P.; Nguyen, U. T.; Schroder, H.; Niemeyer, C. M.; Alexandrov, K.; Goody, R.; Waldmann, H., Oriented immobilization of farnesylated proteins by the thiol-ene reaction. *Angewandte Chemie (International ed. in English)* **2010,** *49* (7), 1252-7.

187. Palsuledesai, C. C.; Distefano, M. D., Protein prenylation: enzymes, therapeutics, and biotechnology applications. *ACS Chem Biol* **2015,** *10* (1), 51-62.

188. Khatwani, S. L.; Kang, J. S.; Mullen, D. G.; Hast, M. A.; Beese, L. S.; Distefano, M. D.; Taton, T. A., Covalent protein-oligonucleotide conjugates by copper-free click reaction. *Bioorganic & medicinal chemistry* **2012,** *20* (14), 4532-9.

189. Gauchet, C.; Labadie, G. R.; Poulter, C. D., Regio- and chemoselective covalent immobilization of proteins through unnatural amino acids. *J Am Chem Soc* **2006,** *128* (29), 9274-5.

190. Shala-Lawrence, A.; Blanden, M. J.; Krylova, S. M.; Gangopadhyay, S. A.; Beloborodov, S. S.; Hougland, J. L.; Krylov, S. N., Simultaneous Analysis of a Non-Lipidated Protein and Its Lipidated Counterpart: Enabling Quantitative Investigation of Protein Lipidation's Impact on Cellular Regulation. *Analytical chemistry* **2017,** *89* (24), 13502-13507.

Chapter 2: Investigation of -C(x)$_3$X sequence recognition by FTase and GGTase-I in vitro and in cell

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Co-author contributions include: KFS performed mass spectrometry and in-gel fluorescence analysis of in vitro farnesylated peptides and proteins, and proteomic analysis. ERH, DSH, WPS, MP, and WKS performed the yeast screens and yeast-based biological assays.

2.1 Introduction

Protein lipidation (e.g. prenylation , palmitoylation, myristoylation) is a post-translational modification that plays a direct role in protein trafficking to cellular membranes, as well as protein-protein interactions.[1-10] One type of lipidation, prenylation, involves the covalent attachment of a hydrophobic isoprenoid group, either a 15-carbon farnesyl or 20-carbon geranylgeranyl group, to a cysteine thiol side chain near or at the C-terminus of certain proteins. This cysteine is part of a C-terminal CaaX recognition sequence recognized by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase type I (GGTase-I).[11-14] Geranylgeranyltransferase type II (GGTase-II) is a third enzyme of this class in which a cysteine present in a C-terminal CC or CxC motif can be recognized for prenylation. GGTase-II is also known as Rab GGTase.[15-18] Cysteine prenylation alters the properties of proteins in several well-studied cases (e.g. Ras and Ras-related GTPases), resulting in protein association with cell membranes where these proteins are involved in cell signaling pathways.[19-23]

The prenylation pathway, as previously described, consists of number of modification steps that can precede substrate protein localization to cellular membranes (Chapter 1, Figure 1.3). In The pathway begins with the enzymatic alkylation of a cysteine by FTase or GGTase-I with a farnesyl or geranylgeranyl group using farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP), respectively. Following modification by FTase or GGTase-I, prenylated proteins CaaX often undergo additional modifications involving proteolysis and methylation of the CaaX sequence.[24-28] Proteolytic removal of the last three -aaX amino acids by membrane-associated proteases Rce1p or Ste24p results in a C-terminal prenylcysteine residue bearing a negatively charged carboxylate group. This carboxylate is then methylated by the SAM-dependent enzyme isoprenylcysteine carboxyl methyltransferases (ICMT). These modification steps are necessary for

most prenylated proteins to function, although there can be some exceptions to the extent of and necessity for these modifications.[29-30] Recently, a shunt pathway for prenylated proteins in yeast has been reported by Hildebrandt and coworkers in which the CaaX motif undergoes prenylation but no subsequent processing occurs. In this case, post-prenylation processing is not only not required, it is actually deleterious to protein function.[31]

The CaaX motif classically thought of as being required for prenylation was first described over three decades ago when studies of yeast mating factor, Ras proteins, and nuclear lamins were being investigated for their ability to be palmitoylated.[32-38] It was found that these different proteins all contained a similar C-terminal C-X-X-X motif to which a lipid was bound that was smaller than that of a palmitoyl group. Upon the discovery of an isoprenoid group as the lipid donor for this modification, it was determined that a four amino acid C-terminus was required for prenylation through a top-down approach, with all the proteins being studied for their ability to undergo prenylation having this same motif and a general acceptance of this requirement was established. While there were limited early investigations into shorter Cxx motifs,[39] investigation of the CaaX sequence has predominately focused on amino acid selectivity within the motif and not studied for length requirements in recognition by FTase and/or GGTase-I. Biochemical studies which include single amino acid mutations at each position of the CaaX, random library screening, and in vivo studies all determined general rules for recognition and selectivity at each amino acid position for prenylation.[14, 39-46] Crystallographic studies have provided further evidence for specificity of FTase and GGTase-I to only accept four amino acid motifs, providing a "molecular ruler" of the size and type of amino acid which could be accepted at each position and generating a list of potential proteins in the human genome that are targets of prenylation.[47-48] These works have greatly influenced the understanding in how the CaaX motif interacts with prenyltransferases at each end

of the recognition sequence. An example of these studies from Reid, T.S. et al. in 2004 provided a table of rules that govern the peptide specificity for FTase and GGTase-I through a study of several differing amino acid sequences. This work describes the peptide binding site of the enzymes to be "rigid" with fixed anchor points at both the cysteine to be prenylated and at the "X" position through hydrogen bonding. Proteomics studies utilizing chemically modified prenyl donors, bioinformatics analysis, and computational/docking approaches have further extended our ability to identify and predict which CaaX motifs likely serve as substrates for FTase and/or GGTase-I.[47, 49-52] An example of which is illustrated by London and coworkers in 2011 in which a structure-based modeling approach was used to determine all potential CaaX motifs that can undergo prenylation, including a new class of farnesylation targets. This tool known as FlexPepBind was derived from the structural features determined from previous crystallographic studies in which a four amino acid CaaX box was used.

Recently, a novel thermotolerance screen for protein prenylation in yeast has expanded our capability to identify prenyltransferase substrate sequences in yeast through use of genetic screenings.[31] This screen is based on the yeast protein Ydj1p, a type I Hsp40 co-chaperone that is required for high-temperature growth. This protein is a type I Hsp40 co-chaperone which ensures proper folding and stability of proteins during times of stress, such as elevated temperature, and must be prenylated in order to perform this function.[53-57] Unlike other proteins which undergo prenylation, Ydj1p does not undergo subsequent proteolysis and methylation and is therefore found in the cytosol rather than the cell membrane.[31] This unique property of Ydj1p allows for direct investigation of protein prenylation without the requirement for concurrent sequence compatibility with Rce1p or Ste24p for subsequent proteolysis.

The work herein supports the discovery of a five amino acid $C(x)_3X$ sequence motif recognized by both yeast and mammalian FTase orthologs.[58] Multiple $C(x)_3X$ peptide sequences, originally identified by genetic screening in yeast, can be efficiently farnesylated in both peptide and protein substrates. We demonstrate that several additional $C(x)_3X$ sequences derived from human proteins are efficiently prenylated by rat FTase, and that a reporter protein terminating in a $C(x)_3X$ sequence exhibits sufficient reactivity to be farnesylated under biologically relevant conditions within a human cell.

2.2 Genetic screening in yeast suggests prenylation of C(x)$_3$X sequences by yeast FTase

Our investigation into the prenylation of C(x)$_3$X sequences began in part by the observation that the CGGDD sequence of human annexin A2 has been identified as potentially prenylated in several studies using in vivo farnesylation probes.[51, 59-62] This observation suggests prenylation and associated processing may be compatible with sequences of non-canonical length. To examine this possibility, we initially used yeast a-factor as a genetic reporter to identify whether C(x)$_3$X sequences could serve to be recognized by farnesyltransferase. Farnesylated a-factor is a secreted, diffusible signaling molecule produced by MATa haploid yeast that temporarily triggers cell cycle G1 arrest in nearby MATα haploid yeast so that mating can occur; this arrest is enhanced in certain mutant backgrounds (e.g. MATα sst2-1).[63] Using this a-factor induced growth arrest phenotype, a plasmid-based library of a-factor mutants with C-terminal C(x)$_3$X sequences was produced to observe evidence of prenylation through the growth arrest assay. A plate containing a population of MATa colonies expressing mutants was printed onto a thin lawn of MATα sst2-1 yeast, the printed plate incubated for a period of time, and individual colonies surrounded by a zone of MATα growth inhibition, known as a halo, were scored as positive hits; associated plasmids were recovered and sequenced. We estimate 22.5% of the possible C(x)$_3$X combinations were evaluated (i.e. ~40,000 colonies; see Section 2.10 for description of coverage estimate).[64] With this limited coverage of the potential sequence space, the a-factor screen yielded two C(x)$_3$X hits (Table 1). The two C(x)$_3$X sequences were retested with other a-factor mutant sequences using the halo assay (Figure 2.1). The halos associated with the C(x)$_3$X sequences were qualitatively smaller than that those associated with wild type a-factor (CVIA C-terminal sequence). Unexpectedly, the CGGDD sequence did not produce a halo; this was true even when encoding the mutant in an over-expression vector.

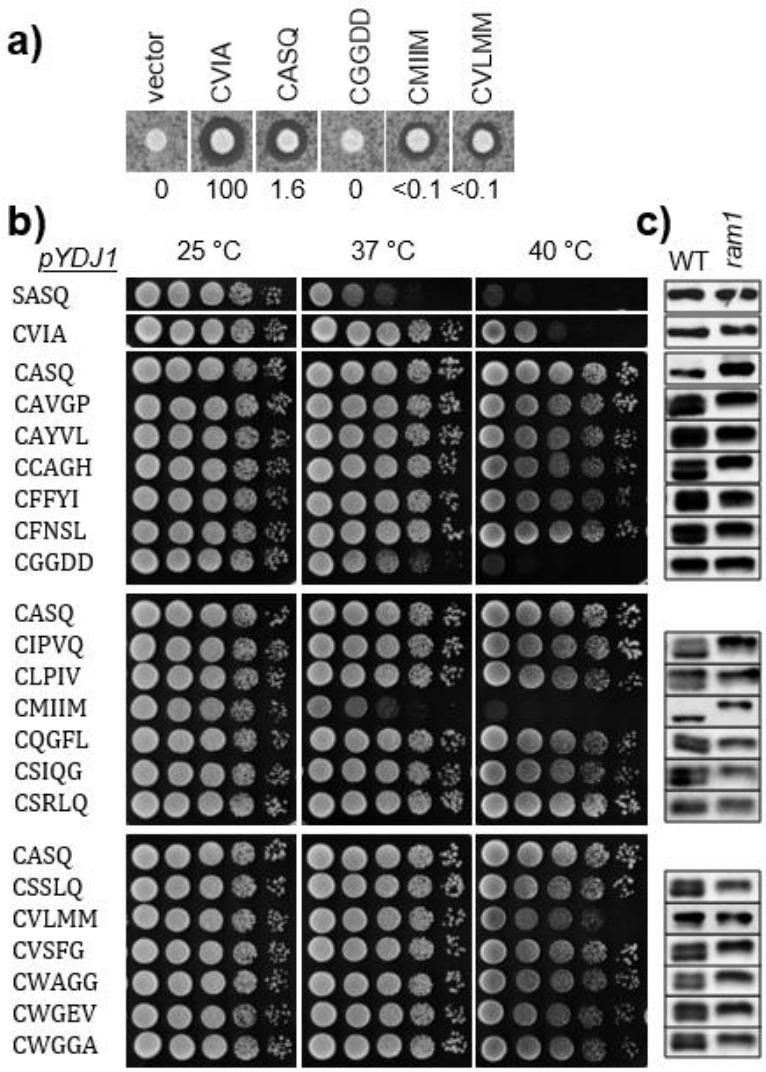


Figure 2.1 Phenotypes and isoprenylation status of $C(x)_3X$ motifs identified by yeast-based screening. a) Plasmid-encoded **a**-factor $C(x)_3X$ variants were evaluated for their ability to produce **a**-factor in SM2331 (*MAT***a** *mfa1 mfa2*) using a spot halo assay. Strains were spotted onto YPD, cultured for 48 hours at 30 °C, and replica transferred onto a thin lawn of RC757 (*MAT*α *sst2-1*). Plates were imaged after 16 hours incubation at 30 °C. The values represent the results of quantitative mating analyses and are reported as percent relative to control (CVIA). b) Plasmid encoded Ydj1p $C(x)_3X$ variants were evaluated for their ability to rescue growth of yWS304 (*ydj1*Δ) at indicated temperatures. Each set of spots represents a 10-fold dilution series prepared from a saturated culture grown in selective media that was spotted onto YPD. Images are representative of data from 2 separate experiments in which at least 2 replicates of each strain were evaluated. c) Immunoblot of lysates from strains containing the indicated Ydj1p $C(x)_3X$ variant. Farnesylated Ydj1p has increased mobility compared to unmodified Ydj1p. The strains used were yWS304 (WT) and yWS1632 (*ram1*); *RAM1* encodes the FTase α subunit. This figure has been reused with permission from reference 58 (Appendix V). This data was collected and analyzed by our collaborator Walter K. Schmidt (University of Georgia).

2.3 $C(x)_3X$ peptide substrates are substrates for mammalian protein farnesyltransferase *in vitro*

Evidence for the activity of Ydj1p with non-canonical $C(x)_3X$ sequences in yeast led to the design of an *in vitro* system to test the activity of these motifs in a reaction with synthetic peptides and using purified mammalian FTase and GGTase-I in cell-free prenylation assays. This system addressed the ambiguity presented by potential proteolysis by a carboxypeptidase in cells, producing a typical CaaX motif. Mammalian FTase was also used to test whether prenylation of these $C(x)_3X$ sequences was exclusive to yeast. For these assays, fifteen $C(x)_3X$ sequences identified by yeast screening were synthesized as 6-mer peptides with each $C(x)_3X$ sequence preceded by a glycine residue. Each peptide was appended by an N-terminal dansyl fluorophore producing a peptide design of dns-$GC(x)_3X$ (Table 2.1). As demonstrated in previous studies using canonical CaaX sequence peptides, attachment of the environmentally sensitive dansyl fluorophore allows real-time monitoring of prenylation through dansyl group fluorescence enhancement upon addition of the hydrophobic farnesyl group on cysteine to monitor activity over time.[65-68] Using this assay, incubation with purified rat FTase and FPP resulted in significant fluorescence enhancement (>5-fold, relative to negative "-FPP" control) with 5 of the 15 sequences (Figure 2.2 and Table 2.1). Among these sequences, two peptides (dns-GCMIIM and dns-GCAVGP) exhibited a leveling off of fluorescence over time consistent with complete farnesylation within 4 hours. (Figure 2.2b). In contrast with GGTase-I and GGPP, none of the dns-$GC(x)_3X$ peptides exhibited an increase in fluorescence as compared to controls, suggesting no geranylgeranylation activity. The annexin CGGDD sequence was also evaluated and observed to be unreactive under all conditions tested.

Table 2.1 Mammalian FTase reactivity with C(x)$_3$X sequences derived from yeast mating and thermotolerance screening.

Peptide sequence	Reporter / source	Fluorescence enhancement of dns-GC(x$_3$)X[a]	HPLC detection of farnesylated dns-GC(x$_3$)X
CGGDD	(59)	-	-
CMIIM	**a**-factor	+	+
CVLMM	**a**-factor	*not determined*	*not determined*
CAVGP	Ydj1	+	+
CAYVL	Ydj1	+	+
CCAGH	Ydj1	*not determined*	*not determined*
CFFYI	Ydj1	+	+
CFNSL	Ydj1	-	+
CIPVQ	Ydj1	-	+
CLPIV	Ydj1	-	+
CQGFL	Ydj1	-	+
CSIQG	Ydj1	+	+
CSRLQ	Ydj1	-	+
CSSLQ	Ydj1	-	+
CVSFG	Ydj1	-	+
CWAGG	Ydj1	-	+
CWGEV	Ydj1	-	+
CWGGA	Ydj1	-	+

[a] Activity in the fluorescence-based screening determined by >5-fold enhancement of prenylation reaction compared to a negative control reaction, as described in Experimental Procedures.

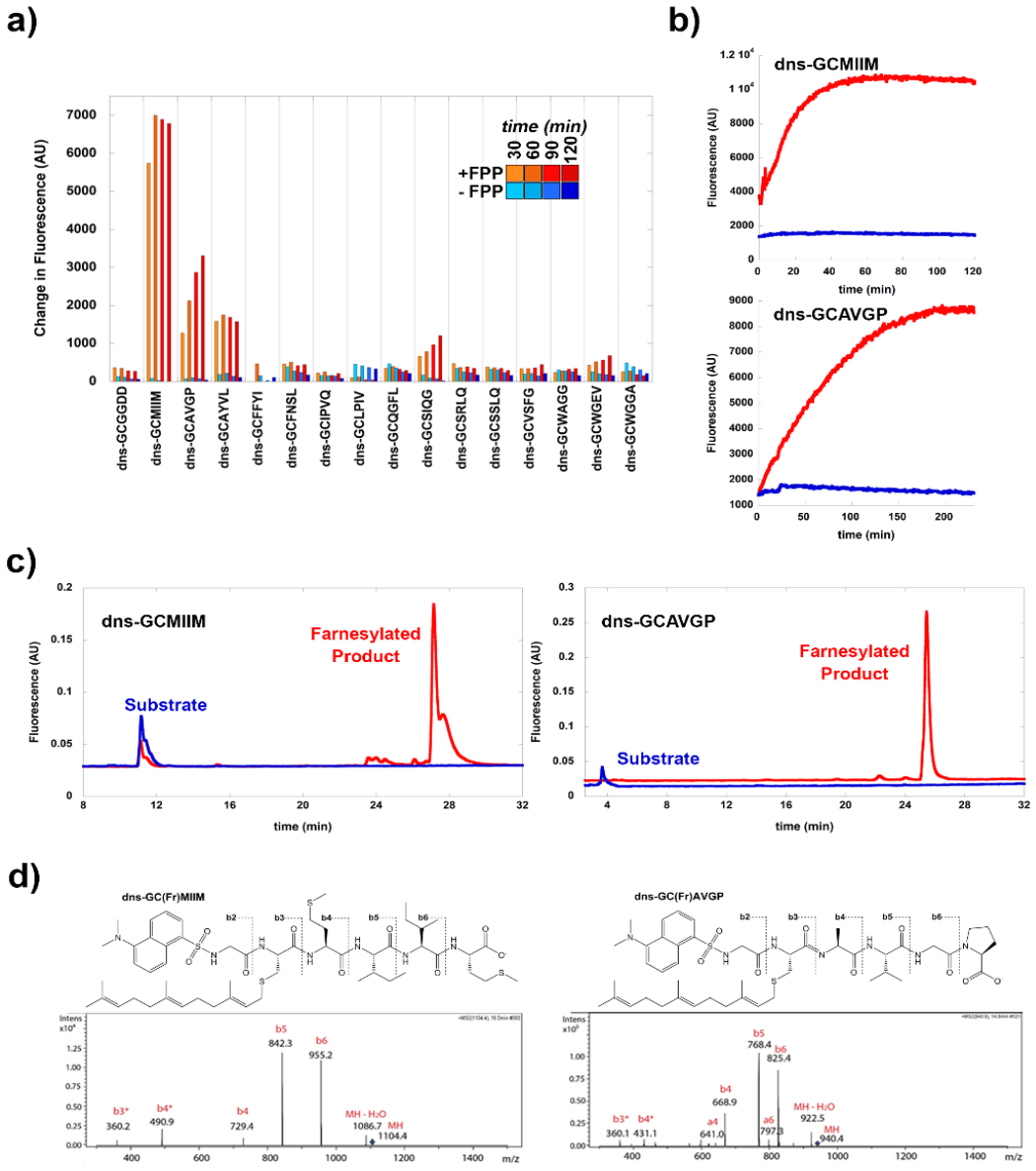


Figure 2.2 Dansyl-GC(x)$_3$X peptides can be efficiently farnesylated by mammalian FTase. a) Fluorescence-based screening for FTase-catalyzed farnesylation of dns-GC(x)$_3$X peptides. b) Farnesylation of dns-GCMIIM (top) and dns-GCAVGP (bottom) by FTase as monitored by fluorescence enhancement. Red trace, farnesylation reaction; blue trace, control reaction lacking FPP. c) Reverse phase HPLC analysis of FTase-catalyzed farnesylation of dns-GCMIIM (left) and dns-GCAVGP (right); substrate and farnesylated product peaks are labeled. Red trace, farnesylation reaction; blue trace, control reaction lacking FPP. d) ESI MS/MS analysis of farnesylated dns-GCMIIM (left) and dns-GCAVGP (right). Reactions were performed and analyzed as described in Section 2.10; tables of fluorescence screening data and ESI MS/MS ion assignments are included in Appendix I. This figure has been reused with permission from reference 58 (Appendix V) with ESI MS/MS analysis performed by Kial Suazo, Univeristy of Minnesota.

To confirm that the increase in dansyl fluorescence was due to prenylation, reverse-phase HPLC was used to directly detect product formation by monitoring a shift in retention time compared to the unmodified substrate. Under the same reaction conditions as the fluorescence plate reader assay, dns-GCGGDD was the only peptide which displayed no evidence of activity (Table 2.1 and Appendix I). As expected, dns-GCMIIM and dns-GCAVGP exhibited the complete or near complete disappearance of the substrate peak with a new product peak forming at 27 and 25.5 min, respectively (Figure 2.2c). Also showing evidence of near-complete prenylation were dns-GCWGEV, and dns-GCQGFL. All other sequences presented varying levels of activity as evidenced by the prescence of both substrate and product peaks (Appendix I). Of note is the difference in sensitivity of the two methods used to determine peptide activity. Sequences that did not show an enhancement of the dansyl fluorophore and were comparable to their -FPP or -FTase control counterparts, were still found to be active when analyzing peptides via direct product detection using HPLC. These false negatives in the fluorescence assay highlighted the importance of confirming dns-GC$(x)_3$X peptide reactivity by RP-HPLC.

Farnesylation of dns-GCMIIM and dns-GCAVGP was further confirmed by ESI MS/MS, with peaks at 1104.4 Da and 940.5 Da for dns-GCMIIM and dns-GCAVGP, respectively, corresponding to their predicted masses when modified with a 205 Da farnesyl group. There was no detection of the non-farnesylated substrates (dns-GCMIIM, 899.5 Da; dns-GCAVGP, 735.3 Da) (Figure 2.2d). The observed MS/MS fragments were consistent with the calculated a- and b-type ions of the farnesylated peptides (Appendix I). In addition, the characteristic fragments from the loss of thiofarnesyl moiety (b3* and b4*) were detected. This is a result of the side chain cleavage between the cysteine β-carbon and sulfur atom, indicating the farnesyl group was indeed appended to cysteine.[69]

2.4 Steady-state kinetic analysis of dns-GC(x)$_3$X peptide reactivity with FTase and the effects of FTase inhibition by tipifarnib

While HPLC analysis was found to be more consistent in determining the reactivity of C(x)$_3$X sequences with FTase, use of the fluorescent plate reader assay allowed for determination of steady-state kinetic analysis of the two peptides exhibiting the largest fluorescence enhancement upon farnesylation (Figure 2.3, Table 2.2). We compared the activities of dns-GCMIIM and dns-GCAVGP with canonical sequences known to undergo prenylation such as dns-GCVLS derived from H-Ras.[70] To study the effect of the additional amino acid in the C(x)$_3$X motif on k_{cat}, K_m, or both parameters, the 5-mer peptides dns-GCMII and dns-GCAVG were also examined (Table 2.2). The dns-GCMIIM and dns-GCAVGP peptides exhibit similar values for k_{cat}, with the K_m for dns-GCMIIM approximately 10-fold lower than that for dns-GCAVGP. Compared to dns-GCVLS derived from the sequence of H-Ras (k_{cat} = 0.3 s^{-1}, K_m = 1.5 μM),[70] k_{cat} values for both dns-GC(x)$_3$X peptides are reduced ~30-fold while K_m increases ~4-fold for dns-GCAVGP and decreases ~3-fold for dns-GCMIIM (Table 2.2). When compared to dns-GCMII and dns-GCAVG, k_{cat}/K_m was increased 25-fold for dns-GCAVG relative to dns-GCVAGP and, surprisingly, decreased 2-fold for dns-GCMII relative to its non-canonical counterpart, dns-GCMIIM (Table 2.2). With this change in activity arising from both k_{cat} and K_m, it can be inferred that the last amino acid of these C(x)$_3$X motifs affect both binding and catalytic turnover. Only dns-GCMII serves as a GGTase-I substrate with a k_{cat}/K_m value comparable to dns-GCMIIM with FTase (1.4 +/- 0.1 x 10^4 $M^{-1}s^{-1}$, Table 2.2).

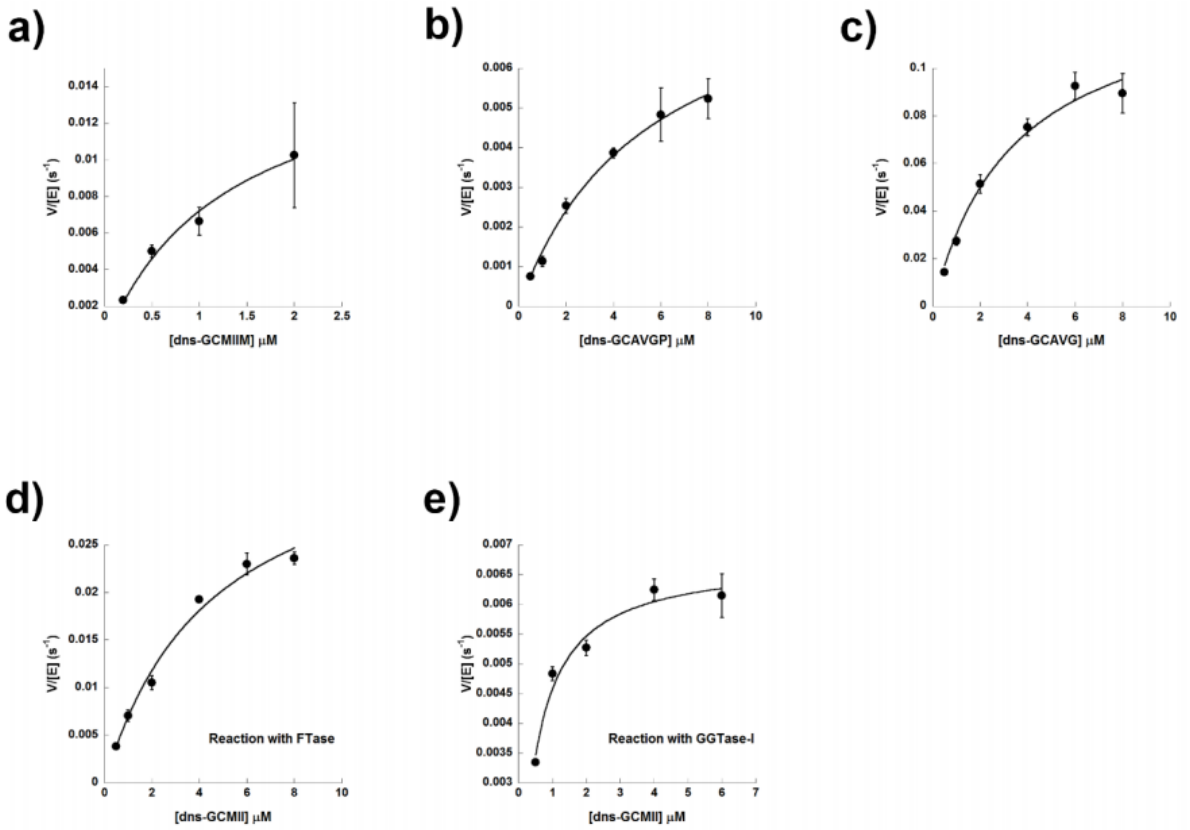


Figure 2.3 Steady state characterization of the reactivity of dns-GC(x)$_3$X and dns-GCaaX peptides with FTase. a-d) Dependence of farnesylation activity on peptide substrate concentration catalyzed by FTase: a) dns-GCMIIM; b) dns-GCAVGP; c) dns-GCAVG; d) dns-GCMII; e) Dependence of geranylgeranylation activity on dns-GCMII peptide substrate concentration catalyzed by GGTase-I. The curve represents the best fit to the Michaelis-Menten equation. Reactions were performed and analyzed as described in Section 2.10. Error bars represent the standard deviation from a minimum of three replicates. This figure has been reused with permission from reference 58 (Appendix V).

Table 2.2 Steady-state kinetic parameters: peptide reactivity with mammalian FTase and GGTase-I[a]

	Reactivity with FTase				Reactivity with GGTase-I		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} / K_m ($M^{-1}s^{-1}$)		k_{cat} (s^{-1})	K_m (μM)	k_{cat} / K_m ($M^{-1}s^{-1}$)
dns-GCVLS[b]	0.3	1.5	2×10^5		*Not reported*	*Not reported*	*Not reported*
dns-GCAVGP	0.009 ± 0.001	5.6 ± 1.0	$1.6 \pm 0.3 \times 10^3$		*No activity*	*No activity*	*No activity*
dns-GCAVG	0.14 ± 0.01	3.5 ± 0.3	$4.0 \pm 0.2 \times 10^4$		*No activity*	*No activity*	*No activity*
dns-GCMIIM	0.009 ± 0.001	0.5 ± 0.1	$1.9 \pm 0.6 \times 10^4$		*No activity*	*No activity*	*No activity*
dns-GCMII	0.040 ± 0.002	4.6 ± 0.6	$8.6 \pm 0.6 \times 10^3$		0.0070 ± .0003	0.50 ± 0.07	$1.4 \pm 0.1 \times 10^4$

[a] Steady-state parameters were determined at saturating FPP (10 μM) or GGPP (10 μM) and varying peptide concentrations under conditions described in the Experimental Procedures. Errors represent the standard deviation from a minimum of three replicates.
[b] reference 69

There have been several studies in the development of inhibitors of FTase as a target of cancer treatment.[71-72] One such inhibitor is Tipifarnib, currently under investigation as a treatment for acute myeloid leukemia and breast cancer in which prenylation of H-Ras plays a key role in tumor growth. Tipifarnib prevents the prenylation of the c-terminal motif through binding at the peptide substrate binding site of FTase. When treated with varying amounts of tipifarnib, prenylation was efficiently blocked for both dns-GCMIIM (IC_{50} = 23 +/- 7 nM) and dns-GCAVGP (IC_{50} = 41 +/- 13 nM, Figure 2.4). This suggests prenylation of these non-canonical $C(x)_3X$ sequences occurs through the same binding interaction as those sequences of the type CaaX with the potential to be targeted by the same inhibitors currently being studied for known sequences such as H-Ras.

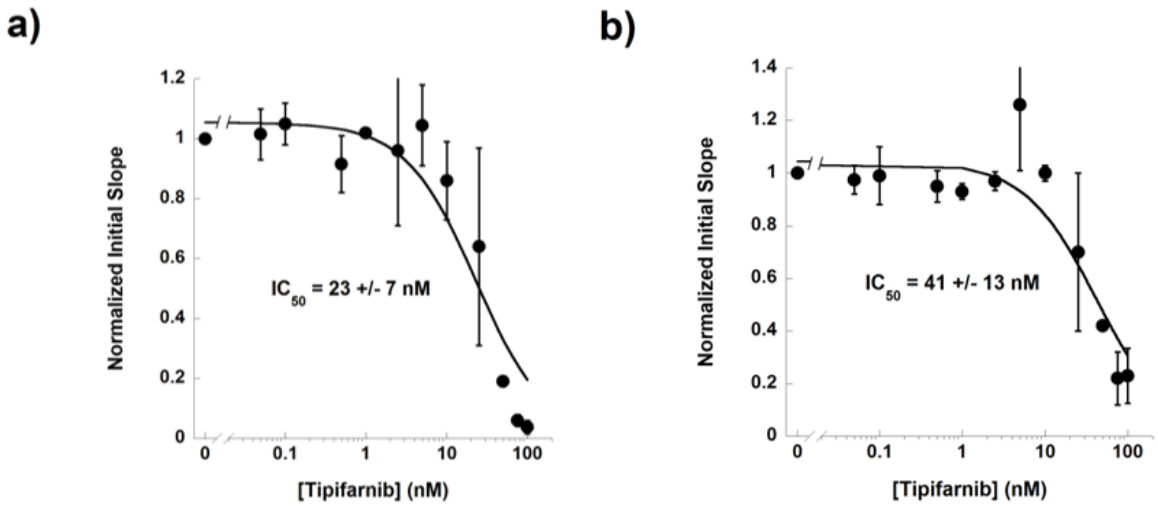


Figure 2.4 Inhibition of FTase-catalyzed farnesylation of dns-GC(x)$_3$X peptides by tipifarnib. a) dns-GCMIIM; b) dns-GCVAGP. Initial slopes were normalized to reactions without tipifarnib and IC_{50} values were calculated as described in Section 2.10. Error bars represent the standard deviation from a minimum of three trials. This figure has been reused with permission from reference 58 (Appendix V).

2.5 Prenylation of His_6-eGFP-GCAVGP reporter protein with product detection through mass spectroscopy and alkyne functionalized FPP labeling

The biological importance of the ability of FTase to prenylate $C(x)_3X$ sequences was examined in a more relevant context of a purified folded protein, eGFP-GCAVGP. We bacterially expressed this protein using an approach validated for detecting modification of eGFP fusion proteins bearing canonical CaaX sequences.[73-74] Farnesylation of eGFP-CAVGP was assessed via LC/MS (Figure 2.5). In a reaction lacking the FPP co-substrate, only the eGFP-CAVGP protein is detected with a mass consistent with the expected unmodified protein weight (28205.1 Da). Upon incubation with both FTase and FPP, a new peak is detected in the chromatogram at longer retention time with a mass of 28408.6 Da. This increase is 205 Da is the consistent with the addition of a farnesyl group, providing evidence of successful farnesylation of a full length protein terminating in a $C(x)_3X$ motif.

To further confirm FTase-catalyzed farnesylation of eGFP-CAVGP under in vitro conditions, we evaluated the ability of purified FTase to modify eGFP-CAVGP using a FPP analogue (C15AlkOPP) bearing an alkyne group to allow for post-prenylation protein labeling (Figure 2.5).[69] Following eGFP-CAVGP incubation with C15AlkOPP in the presence of FTase, the modified protein was derivatized with TAMRA-azide using Cu-catalyzed alkyne-azide cycloaddition.[75-76] A single bold band was seen in TAMRA fluorescence scanning at a size corresponding to 28.4 kDa, the size of farnesylated His_6-eGFP-GCAVGP (Figure 2.5). The negative control gave no band upon fluorescence detection, while Coomassie staining presents one band in each positive and negative control samples indicating that the fluorescence in the prenylation reaction is due to modification with an alkyne farnesyl group.

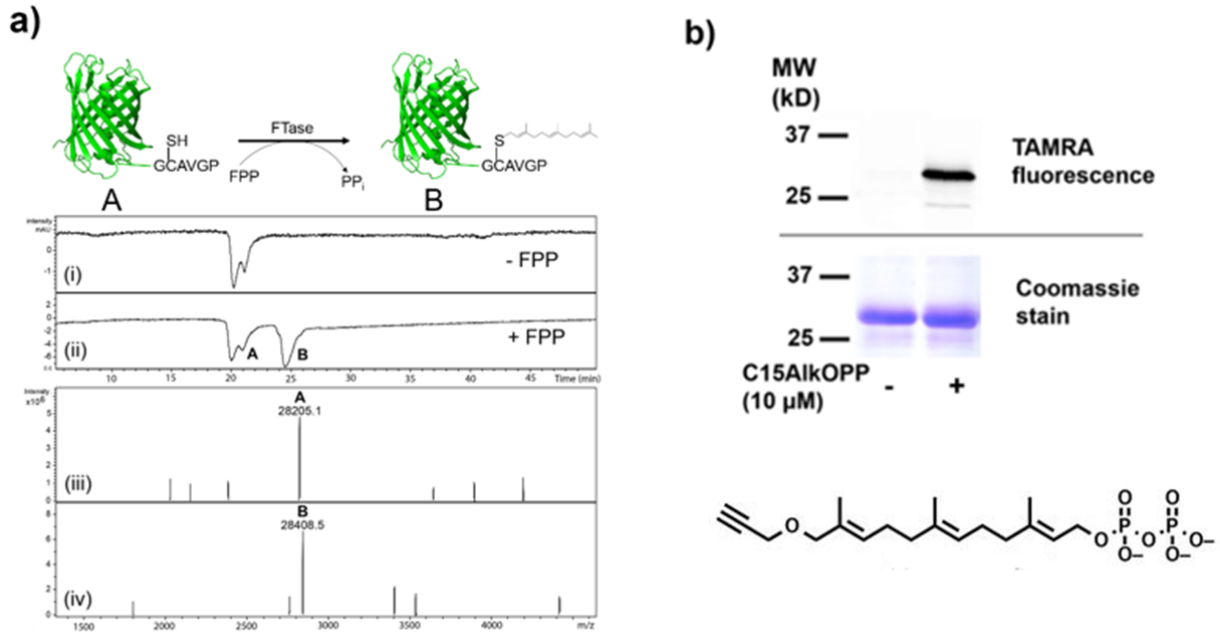


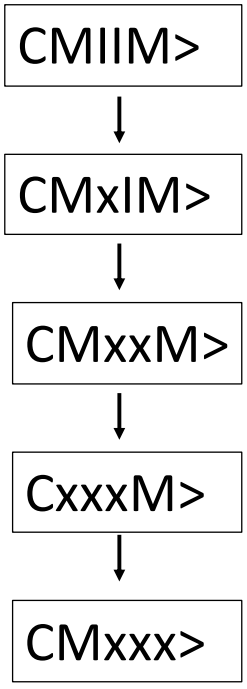
Figure 2.5 A C(x)$_3$X sequence is efficiently farnesylated in the context of a full length protein. a) LC/MS analysis of farnesylation of an eGFP reporter protein terminating in a C(x)$_3$X sequence. LC chromatogram of in vitro farnesylation of eGFP-GCAVGP using purified FTase in the absence (panel i) or presence (panel ii) of FPP, with absorbance detected at 555 nm. Negative absorbances are observed due to background fluorescence from eGFP. Peaks A (panel iii) and B (panel iv) have deconvoluted masses of 28205.1 Da and 28408.6 Da, respectively, that differ by 203.5 Da approximately corresponding to farnesyl modification (theoretical mass of farnesyl group: 205 Da). b) In-gel fluorescence scan (top) and Coomassie staining (bottom) of eGFP-GCAVGP subjected to in vitro prenylation using purified FTase in the presence or absence of C15AlkOPP, shown below. This figure has been reused with permission from reference 58 (Appendix V) with LC/MS and in-gel fluorescence scan performed by Kiall Suazo, University of Minnesota.

2.6 Expansion of the $C(x)_3X$ motif to proteins in the human proteome

To address the relevance of our findings to potential endogenous proteins within humans, we expanded our investigation to include $C(x)_3X$ sequences derived from the human proteome to explore the potential biological impact of this novel FTase substrate class. We began our study into the biological relevance of these finding in the human genome by searching for proteins containing a $C(x)_3X$ C-terminus motif. We approached identifying these sequence candidates through sequence randomization. Using Prosite and our most active sequences from the Ydj1p screening, we allowed for variability throughout the five amino acid motif one position at a time before increasing variability to two, then three positions at random (Scheme 2.1). We were careful to omit any sequence having more than one cysteine in the motif to eliminate classic four amino acid motif (example: CCIIM) or for prenylation by GGTase-II, which recognizes the sewuence motif CC or CxC in Rab proteins and other substrates.[12]

The Prosite scan yielded 965 potential proteins in the human genome which contain a $C(x)_3X$ C-terminus. From our most active sequences in the Ydj1p screening (-CMIIM, -CAVGP, and -CWGEV) six proteins were chosen from over 260 potential targets similar to these sequences for characterization of their $C(x)_3X$ motifs (Table 2.3). This determination was based on the current literature of the proteins, their location within the cell, and their amino acid variability (or similarity) when compared to the Ydj1p sequences. These sequences were tested against the same fluorescence-based assay and product detection via HPLC as used in the yeast screening described above. Of these six sequences tested, half showed a detectable level of activity with FTase (Table 2.3). Those two resembling -CAVGP, dns-GCQTGP and dns-GCSQGP, were found to be most active by exhibiting complete prenylation of the substrate peptide. One other sequence, dns-GCFSKM, also exhibited reactivity with FTase as detected via HPLC. As found previously in our

screening of sequences derived from YDj1p screening, none of these sequences was found to be active with GGTase-I (data not shown). While farnesylation of dns-GCQTGP and dns-GCSQGP was confirmed by HPLC, the fluorescence enhancements for these peptides upon prenylation were not sufficient to support steady state characterization.



Scheme 2.1 An example of introducing variability into $C(x)_3X$ sequences for Prosite Search "x" indicates position in which variability is allowed in the Prosite Search; ">" indicates the sequence must be C-terminal.

Table 2.3 Mammalian FTase reactivity of $C(x)_3X$ sequences derived from the human genome using randomization of yeast screening hits

Peptide	Human protein name and gene identifier	Fluorescence Enhancement assay	HPLC detection of farnesylated peptide
dns-CLLHP	Ras association domain-containing protein 5	INACTIVE	INACTIVE
dns-CSQGP	Sushi, nidogen and EGF-like domain-containing protein 1	**ACTIVE**	**ACTIVE**
dns-CQTGP	Putative glycosylation-dependent cell adhesion molecule 1	**ACTIVE**	**ACTIVE**
dns-CSVKM	Olfactory receptor protein	INACTIVE	INACTIVE
dns-CFSKM	sorting nexin 4	INACTIVE	**ACTIVE**
dns-CDREV	Prostamide/prostaglandin F synthase	INACTIVE	INACTIVE

2.7 Use of fluorescence localization as a proxy for prenylation within HEK293 cells: $C(x)_3X$ sequences can be sufficiently prenylated in a biologically relevant context

To be considered biologically relevant, proteins terminating in $C(x)_3X$ sequences must exhibit sufficient reactivity to be modified by endogenous FTase within an intact human cell. The minimum reactivity is estimated to be in the range of k_{cat}/K_m = 0.5-2 x 10^4 $M^{-1}s^{-1}$ when peptide reactivity is determined in an *in vitro* assay using purified FTase.[77] The reactivity of the dns-GCMIIM peptide with purified FTase (k_{cat}/K_m = 1.9 +/- 0.6 x 10^4 $M^{-1}s^{-1}$, Table 2.2) suggests the CMIIM sequence is sufficiently reactive to support protein farnesylation within mammalian cells. The apparent reactivity observed for this sequence within yeast cells further suggests the ability of this motif to be modified in a cellular setting.

Using a pEGFP-KRas mammalian expression vector (Casey Lab, Duke University), the canonical motif -CVIM was modified to contain the four sequences above through PCR extension and subsequent ligation (Section 2.10).[78] Cloning of negative controls in which the cysteine to be prenylated is replaced with a serine were also performed in the same way. Successful cloning was verified by sequencing (Genscript, Inc.) The use of an eGFP-KRas fusion protein serves as a proxy for directly measuring prenylation through visualization of eGFP fluorescence at the membrane (prenylated) or diffuse throughout the cell (not prenylated, or prenylated but not processed). Following transfection into HEK293 cells, eGFP-KRas-CMIIM displays membrane localized fluorescence consistent with reporter protein farnesylation (Figure 2.6a). Treatment with tipifarnib during transfection leads to diffuse eGFP-KRas-CMIIM fluorescence, as does mutation of the $C(x)_3X$ cysteine to a serine (eGFP-KRas-SMIIM). A reporter protein lacking the last residue of the CMIIM sequence (eGFP-KRas-CMII) also displays membrane localized fluorescence but is unaffected by tipifarnib treatment. This behavior is consistent with the reactivity of the CMII

sequence with GGTase-I (Table 2.2), which would lead to prenylation and membrane localization in the presence of tipifarnib. This result also indicates CMIIM is modified selectively by FTase in cells and is in fact the sequence being accepted by FTase, as proteolysis of CMIIM to CMII by endogenous proteases would result in recuse of prenylation by geranylgeranyltransferase-I. Overall, these studies support FTase-catalyzed lipidation of eGFP-KRas-CMIIM within the cell, indicating that $C(x)_3X$ sequences can be sufficiently reactive to support biologically relevant protein farnesylation. The ability of eGFP-KRas-GCMIIM to membrane localize in HEK293 cells may be due to its being studied in a yeast a-factor-based screening. This a-factor is known to undergo subsequent proteolysis and methylation in yeast which is also required for mammalian prenylated proteins to exhibit membrane localization.[79]

The relative amount of fluorescence localization of eGFP-Kras-CMIIM in the absence and presence of tipifarnib was also quantified. For each sequence and condition, a minimum of n=50 cells were counted and given the assignment of being membrane associated or diffuse. Upon addition of tipifarnib, membrane localization dropped from 84% membrane associated to only 2% membrane associated. A negative control of eGFP-KRas-SMIIM was also scored and found to have zero cells exhibiting membrane association (Figure 2.6b).

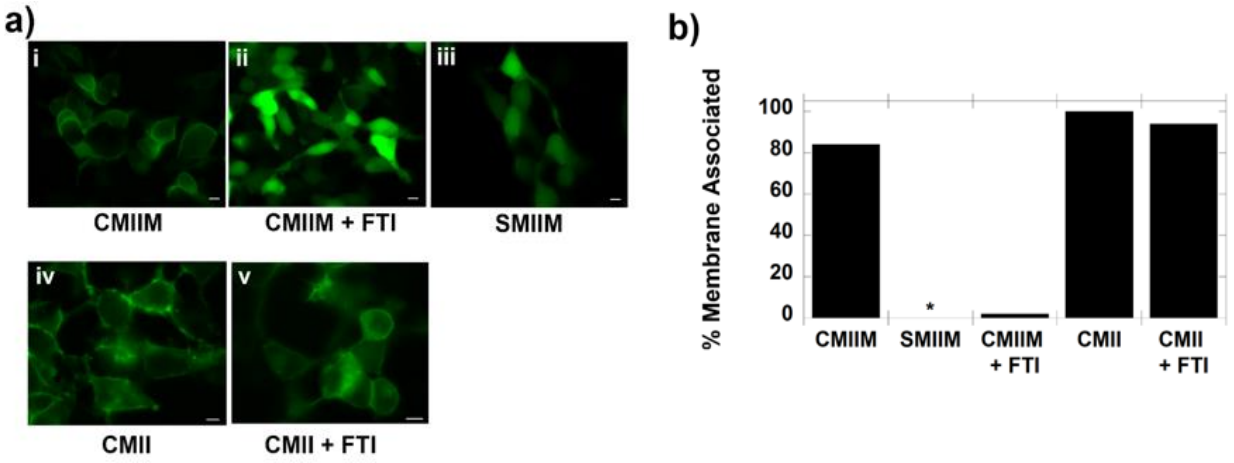


Figure 2.6 eGFP-KRas-CMIIM exhibits localization within a mammalian cell. a) Representative images of HEK293 cells transfected with eGFP-KRas-XMIIM or eGFP-KRas-CMII reporter proteins in the absence or presence of tipifarnib (FTI); scale bar = 20 μm. b) Scoring of fluorescence patterns observed in HEK293 cells after transfection with eGFP-KRas reporter proteins; an asterisk (*) indicates no cells exhibited membrane associated fluorescence. This figure has been reused with permission from reference 58 (Appendix V).

2.8 Metabolic labeling of eGFP-KRas-CMIIM in HEK293 cells

To provide definitive evidence that the eGFPKRas-CMIIM protein is farnesylated within human cells, the protein can be enriched through metabolic labeling with alkyne-modified FPP in HEK293 cells allowing subsequent conjugation with an affinity handle.[80] To directly confirm prenylation of the eGFP-KRas-CMIIM reporter protein by endogenous FTase within a mammalian cell, transfected HEK293 cells were subjected to metabolic labeling using C15AlkOPP FPP analogue. Following transfection with eGFP-KRas-derived fusion proteins, cells were incubated with the C15AlkOPP FPP analogue for alkyne functionalization of the expressed protein. Cell lysates were derivatized with TAMRA-N_3 followed by in-gel imaging of TAMRA fluorescence as described in section 2.5, with eGFP-KRas-CVIM serving as a positive control. A single major band at the expected size of this fusion protein (50 kDa) was observed, with this band absent in untransfected cells or transfected cells not treated with the C15AlkOPP FPP analogue (Figure 2.7a). A similar predominant TAMRA-fluorescent band was observed at the expected size of eGFP-KRas-CMIIM in the presence of C15AlkOPP. This single bold band was not observed when the CMIIM sequence in the reporter was mutated to SMIIM, with the SMIIM sample resembling the negative control. The loss of reporter protein detection with the SMIIM sequence would be expected due to the lack of a cysteine at the farnesylation site.

A quantitative analysis was performed on cell lysates obtained from eGFP-KRas-CMIIM- vs eGFP-KRasSMIIM-transfected cells grown in the presence of C15AlkOPP. Lysates were biotinylated with biotin-N_3 followed by pull-down with avidin resin. Enriched proteins were digested, labeled with a tandem mass tag (TMT) and analyzed via LC-MSMS. A volcano plot generated after performing a two-sample t-test (FDR = 0.05, s0 = 0.5) across three replicates strongly indicates that eGFP-KRasCMIIM is enriched over eGFP-KRas-SMIIM, further

evidencing the in vivo farnesylation of this protein (Figure 2.7b). Parallel studies of eGFP-KRas reporter proteins terminating in other FTase reactive $C(x)_3X$ sequences failed to demonstrate metabolic labeling within transfected cells. This may reflect that the lower reactivity of these sequences (e.g. CAVGP is 10-fold less reactive than CMIIM, Table 2.2) is insufficient to support direct detection of transfected protein prenylation within cells by metabolic labeling or imaging methods.[77] Nevertheless, these imaging, metabolic labeling, and quantitative proteomic studies support FTase-catalyzed lipidation of eGFP-KRas-CMIIM within the cell, indicating that a $C(x)_3X$ sequence can be sufficiently reactive to support biologically relevant protein farnesylation.

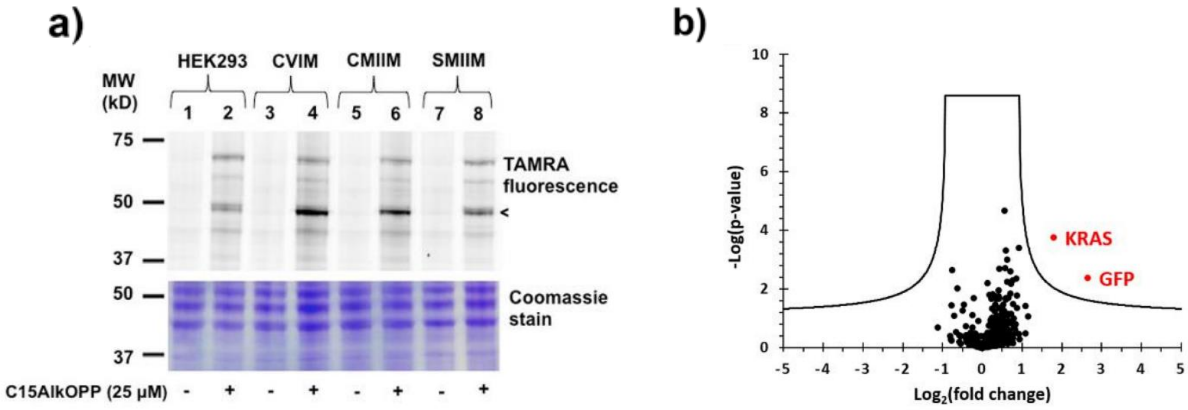


Figure 2.7 eGFP-KRas-CMIIM is efficiently modified by FTase within a mammalian cell. a) In-gel fluorescence scan (top) and Coomassie staining (bottom) of lysates from HEK293 cells transfected with eGFP-KRas reporter proteins and metabolically labeled with C15AlkOPP followed by conjugation of a TAMRA-N3 fluorophore. Cells were either non-transfected (HEK293) or transfected with eGFP-KRas-CVIM, eGFP-KRas-CMIIM, or eGFP-KRas-SMIIM reporter proteins in the absence or presence of C15AlkOPP. b) Volcano plot for TMT-labeled quantitative proteomic analysis of eGFP-KRasCMIIM- vs eGFP-KRas-SMIIM-transfected HEK293 cells treated with C15AlkOPP and enriched via biotin-avidin pull-down. A two sample t-test (FDR = 0.05, s0 = 0.5) from three replicates shows that GFP and KRas are statistically enriched in eGFP-KRas-CMIIM transfected cells. This figure has been reused with permission from reference 58 (Appendix V) with in-gel fluorescence and proteomic analysis performed by Kial Suazo, Univeristy of Minnesota.

2.9 Conclusions

Protein prenylation by FTase and GGTase-I is an important post-translational modification involved in cell signaling and protein membrane localization. For several decades, a C-terminal CaaX sequence has served as the required recognition motif for a protein to be considered a substrate for prenylation. We have identified multiple C-terminal $C(x)_3X$ sequences representing a new class of substrates for FTase. While biochemical, structural, and computation studies have provided the essential foundation for the study of the modification, these $C(x)_3X$ substrates found to undergo prenylation broaden the range of FTase substrate selectivity and expand the list of potential prenylation targets within the human proteome.

The ability of FTase to prenylate $C(x)_3X$ sequences was not anticipated through previous predictive studies. Multiple structural studies of FTase and GGTase-I complexes with peptide substrates supported substrate length selectivity defined by two contact points: 1) the distance between the coordination of the cysteine of the CaaX sequence to the catalytic zinc ion; and 2) multiple hydrogen bonds (both direct and water-mediated) between the C-terminal carboxylate of the CaaX sequence and residues in both subunits of FTase or GGTase I.[48, 81-83] In turn, these contact points were considered in the FlexPepBind computational approach for predicting peptide sequence reactivity with FTase. FlexPepBind only predicts ~50% of the HPLC-verified FTase $C(x)_3X$ sequences as potential FTase substrates. The ability of FTase to accept the longer $C(x)_3X$ motif indicates that its active site is more flexible than the previously proposed more rigid model.

The inability of GGTase-I to accept these $C(x)_3X$ substrates shows that it exhibits distinct length and/or sequence requirements, unlike its close relative FTase. Two enzymes thought to be so alike in their substrate requirements are now presenting an instance where they have distinct differences in substrate specificity. Future studies will work to define the sequence selectivity

within the $C(x)_3X$ sequence and explore the effect of changing the prenyl donor cosubstrate on $C(x)_3X$ substrate reactivity, for comparison to prenyl-donor dependent changes in peptide substrate selectivity seen with CaaX substrates.[60, 84] These studies will provide functional insight into the interactions formed with these longer peptide sequences within the FTase active site. Understanding the active site adjustments required for FTase recognition and modification of $C(x)_3X$ sequences will be aided by structural and computational modeling studies of peptides from this new substrate class in complex with FTase.

The ability of both mammalian and yeast FTase to farnesylate proteins with the longer $C(x)_3X$ motif has the potential to greatly expand the number of potential FTase substrates within the human and yeast proteomes. The human proteome is predicted to contain 1008 proteins terminating in $C(x)_3X$ sequences (as of June 2018, Appendix II). This prediction accounts for canonical prenylation motifs, such as CCxxX or CxC. Allowing cysteine residues in only the first position, 816 of these sequences contain only a single cysteine residue. This representation nearly matches that of CaaX sequences in the human proteome (1205 sequences; 1059 with a single cysteine). Biochemical and computational studies indicate that a large fraction of these CaaX sequences can serve as FTase substrates.[43, 47] Logically then, it is possible that a fraction of these identified $C(x)_3X$ sequences could also be farnesylated. A similar proportion of proteins bearing $C(x)_3X$ and CaaX motifs are observed in the *S. cerevisiae* proteome, with 117 $C(x)_3X$ proteins (88 with a single cysteine, Appendix III) and 120 CaaX proteins (101 with a single cysteine). Based on these numbers, inclusion of $C(x)_3X$ proteins doubles the potential scope of prenylation within the human and yeast proteomes. This idea of proteomic expansion could also have significance in pathogenic organisms that employ either endogenous or host-mediated farnesylation, such as *Plasmodium falciparum, Candida albicans,* and *Legionella pneumophila.*[80, 85-89] Each of these

pathogenic organsims contain a small number of proteins bearing a $C(x)_3X$ motifs that may undergo prenylation by either pathogenic or host protein prenyltransferases (Appendix IV).

Our expansion of the potential substrates recognized by FTase highlights the importance of continuing studies towards identifying additional new and biologically relevant protein substrates and determining their prenylation state within the cell. In particular, identification and isolation of endogenously prenylated $C(x)_3X$ proteins will be essential to understanding the impact of prenylation of non-canonical FTase substrates within the cell. Given the established involvement of prenylation in a range of disease states, our findings also carry the potential to establish a link between farnesylation of unanticipated protein targets and cellular dysfunction that could be exploited for treatment with the available range of potent farnesyltransferase inhibitors.

Our findings further support the "shunt pathway" model for protein prenylation wherein a subset of farnesylated proteins can sidestep subsequent C-terminal processing steps.[31] Such proteins with canonical CaaX motifs include Ydj1p, Rab38, Phkα/β, and Gγ5.[25, 31, 90-91] The majority of $C(x)_3X$ sequences described in this study were identified in yeast using a Ydj1p-based screen that discriminates for shunted sequences. While none of the Ydj1p screen-derived $C(x)_3X$ sequences exist in either the yeast or human proteomes, similar sequences do exist and are reactive for farnesylation as demonstrated by the human sequences examined in this work. The state of these substrates in a cellular context will also provide insight into the ability of the processing enzymes, Rce1 and ICMT to accept these $C(x)_3X$ motifs as substrates. Another avenue of study will be whether the ability for these new substrates to be prenylated, but perhaps not proteolytically processed, gives way for new roles of prenylated proteins outside of membrane localization in mammalian cells. After determination of how many naturally occurring $C(x)_3X$ proteins are prenylated, the determination of what fraction of these $C(x)_3X$ proteins are shunted away from

subsequent processing would provide insight into potentially new roles of prenylation in the cell. Identifying protein prenyltransferase $C(x)_3X$ protein substrates may require application of chemical biology, proteomics-based approaches or development of new analytical tools for detecting endogenously prenylated proteins, especially since traditional assays for detecting *in vivo* prenylation (e.g. fluorescence membrane localization) may not be relevant to shunted proteins regardless of their C-terminal motif.

2.10 Materials and Methods

2.10.1 Miscellaneous Methods: All *in vitro* FTase and GGTase-I assays were performed at 25 °C. All curve fitting was performed with KaleidaGraph (Synergy Software, Reading, PA). Geranylgeranyl diphosphate (GGPP) and farnesyl diphosphate (FPP) were purchased from Isoprenoids.com (Tampa, FL). Peptides were commercially synthesized (Sigma-Genosys, The Woodlands, TX) and exhibited >90% purity, as determined by HPLC or after semi-prep purification via HPLC. Peptides were solubilized in ethanol containing 10% (v/v) DMSO and stored at -20 °C. Peptide concentrations were determined spectrophotometrically using Ellman's reagent. The isoprenoid analogue C15Alk-OPP was prepared as previously described.[73]

2.10.2 Yeast strains and plasmids (These experiments were performed in the Schmidt laboratory, University of Georgia): The yeast strains used in this study were IH1793 (*MAT*α *lys1*), RC757 (*MAT*α *sst2-1 rme his6 met1 can1 cyh2*), SM2331 (*MAT***a** *trp1 leu2 ura3 his4 can1 mfa1 mfa2*), yWS304 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 ydj1::KAN*^{R}), and yWS1632 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 ram1::KAN*^{R}) and have been previously described.[31, 63, 92-93] These strains were routinely propagated at 30 °C (RC757, SM2331) or room temperature (yW304, yWS1632) on either YPD or appropriate selective media when transformed with plasmid pRS315 (*CEN LEU2*), pSM1605 (*2μ URA3 MFA1*), pWS610 (*CEN LEU2 MFA1*), pWS612 (*CEN LEU2 MFA1-CASQ*), pWS942 (*CEN URA3 YDJ1*), pWS1132 (*CEN URA3 YDJ1-SASQ*), pWS1246 (*CEN URA3 YDJ1-CTLM*), and pWS1286 (*CEN URA3 YDJ1-CVIA*) have been previously described.[31, 94-95] pWS705 (*2μ URA3 MFA1-CGGDD*), pWS1170 (*CEN URA3 YDJ1-CVLMM*), and pWS1488 (*CEN URA3 YDJ1-CMIIM*) were constructed by PCR-directed, recombination-mediated plasmid construction.[96] In brief, PCR products designed to amplify the 3´ ends of *MFA1*

and *YDJ1* with encoding for desired $C(x)_3X$ motifs were used to gap repair plasmids pSM1605 (*2μ URA3 MFA1*) and pWS1132 (*CEN URA3 YDJ1-SASQ*), respectively, as previously described.[31, 94] These plasmids and others identified through our screening methods were introduced into strains via a lithium acetate-based transformation procedure.[97]

2.10.3 Oligo design for generation of randomized $C(x)_3X$ sequences by PCR (These experiments were performed in the Schmidt laboratory, University of Georgia): Synthetic oligonucleotides were designed to PCR amplify the CaaX encoding region of *YDJ1* and its 5´untranslated region as encoded in pWS1132. The forward PCR oligo was designed to encode the $C(x)_3X$ sequences and was flanked on the 5´ end by 39 nucleotides homologous to the *YDJ1* gene immediately before the CaaX motif (to facilitate recombination), and 19 nucleotides homologous the UTR immediately after the stop codon (to facilitate PCR priming). The oligo used for generation of CGGDD was designed specifically. The oligo used to generate a library of $C(x)_3X$ sequences was synthesized to encode a cysteine codon followed by 4 random codons and a stop codon. To limit the complexity of the codons synthesized yet allow for all possible amino acids, only C, G and T were used at the wobble position of the $(x)_3$ codons. To prevent formation of premature stop codon yielding a canonical length motif (i.e. Cxxx) that would lead to false positives, only A, C, and G were used at the first position of the X codon; this strategy unfortunately disallowed incorporation of Cys, Phe, Trp, and Tyr codons.

2.10.4 a-factor mating pheromone screen, halo assay, and mating test (These experiments were performed in the Schmidt laboratory, University of Georgia): SM2331 yeast (*MAT***a** t*rp1 leu2 ura3 his4 mfa1 mfa2*) were co-transformed using a previously described PEG lithium-acetate

method with *MluI* and *SphI* digested pWS1024 (*CEN LEU2 mfa1 V_{34}TAG*; amber mutation at a_1 position of CaaX motif) and PCR product engineered to encode 5-mer sequences. The transformation mix was plated on SC-leucine solid media and incubated at 30 °C for 72-96 hours. Estimates of colony numbers were determined, then colonies were replica plated onto a fresh SC-leucine plate as well as a YPD plate containing a thin lawn of RC757 yeast (*MATα sst2-1 rme his6 met1 can1 cyh2*) prepared in the presence of 0.071% TX-100 (final concentration in cell suspension prior to lawn preparation) and plates incubated at 30 °C for 16-20 hours. Colonies displaying a halo were identified, and the corresponding colony on the SC-leucine replicate plate was recovered. Expression of **a**-factor was confirmed by amplifying selected colonies in SC-leucine liquid media and applying 20X concentrated spots of liquid culture onto RC757 lawns in the presence and absence of TX-100. Plasmids were isolated and sequenced from the strongest halo producing strains.

A modified version of the halo assay was used to assess relative strength of **a**-factor production by the various **a**-factor CaaX mutants evaluated. Plasmid transformed *MAT***a** strains were cultured in appropriate selective media (SC-leucine or SC-uracil), spotted onto YPD, and incubated for 24-36 hours at 30 °C. The spots on the plate were replica transferred onto a thin lawn RC757 yeast (no TX-100), and plates were incubated at 30 °C for 16-20 hours.

The quantitative mating test was performed essentially as previously described.[31] In brief, the *MAT***a** strains were independently cultured to saturation in selective media, and the IH1793 *MATα lys1* strain was cultured in YPD. All strains were diluted to A_{600} ~1.0 using fresh culture media. Empirically determined dilutions were spread on SD and SC-lysine solid media; the former media is diploid selective while the latter is selective for *MAT***a** haploid cells and *MAT***a** cells that have undergone mating to form a diploid cell. The total count of colony forming units (CFUs) on

each media type was determined and used to calculate a mating frequency (i.e. diploid CFUs over total CFUs); this value was used to determine the percentage mating of each condition relative to the strain producing wildtype **a**-factor.

2.10.5 Thermotolerance screen (These experiments were performed in the Schmidt laboratory, University of Georgia): yWS304 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 ydj1::KAN*^{R}) yeast was co-transformed using a previously described PEG lithium-acetate method with *Nhe*I digested pWS1132 (*CEN URA3 YDJ1-SASQ*) and PCR product engineered to encode randomized 5-mer sequences. A small portion of the transformation mix was plated on SC-uracil solid media and incubated at 25 °C for 96 hours to assess the total number of plasmids created by the transformation procedure. The remaining transformation mix was plated on YPD solid media and incubated at 40 °C for 96 hours. Colonies recovered by high temperature selection were re-tested for thermotolerance as patches on YPD, and individual plasmids recovered and sequenced. The isolated plasmids were re-introduced into yWS304 to confirm plasmid-linked thermotolerance prior to detailed thermotolerance analysis.

2.10.6 Thermotolerance assay (These experiments were performed in the Schmidt laboratory, University of Georgia): Saturated cultures grown in SC-uracil liquid media at room temperature were serially diluted into YPD and spotted onto YPD solid media (5 µl per spot) as previously described.[31] Plates were incubated at various temperatures (25 °C, 37 °C, or 40 °C) for several days before plate imaging. Each experiment was performed at least twice on separate days, and each strain was evaluated in duplicate within each experiment.

2.10.7 Estimate of $C(x)_3X$ complexity in a-factor and thermotolerance screens (These experiments were performed in the Schmidt laboratory, University of Georgia): The estimated coverage of $C(x)_3X$ sequences evaluated was calculated using the GLUE-IT algorithm based on the number of colonies screened and the number and redundancy of the codons used for amino acid randomization.[64] The number of colonies screened with the **a**-factor reporter was estimated by direct colony counts on all plates evaluated. For the Ydj1p reporter, a known percentage of the transformation mix was plated onto SC-uracil solid media, and the number of colonies observed was used to determine the total number of colony-forming units (CFUs) in the rest of the transformation mixture. The number of false positive colonies (i.e. background colonies containing re-circularized or uncut plasmid) were not counted toward the total number of CFUs; the false positive numbers were determined from transformation mixtures containing the linearized plasmids alone and typically yielded a smaller number of CFUs relative to co-transformed sample (typically <2%).

2.10.8 Immunoblot analysis for protein prenylation in yeast (These experiments were performed in the Schmidt laboratory, University of Georgia): Whole cell lysates of mid-log yeast were prepared as previously described using alkaline hydrolysis and TCA precipitation.[31, 98] Samples and PageRuler size standards (Thermo Scientific, Waltham, MA) were separated by SDS-PAGE (12.5%), transferred onto nitrocellulose, and blots incubated with rabbit anti-Ydj1p primary antibody (courtesy of Dr. Avrom Caplan) and HRP-conjugated donkey anti-rabbit secondary antibody (GE Healthcare, Chicago, IL). Immune complexes were detected by X-ray film after treatment of blot with HyGLO development solution (Denville Scientific, South Plainfield, NJ).

2.10.9 Image analysis for yeast assays and immunoblots (These experiments were performed in the Schmidt laboratory, University of Georgia): Plates and developed films were imaged using a flat-bed scanner (300 dpi; grayscale), and resultant TIFF image files manipulated with Photoshop (i.e. image rotation, image contrast adjustments, cropping) before final figure assembly using PowerPoint. Plates were scanned face down without lids using a black background. Films were scanned using a white background. For all plate scans, contrast settings were adjusted manually using the same settings for all images. For film scans, contrast settings were adjusted using Photoshop's 'Auto Contrast' function.

2.10.10 Activity screening of dns-GC(x)$_3$X peptides by fluorescence-based prenylation assay: Prenylation of dansylated dns-GC(x)$_3$X peptides was assessed by a time-dependent increase in fluorescence (λ_{ex} 340 nm, λ_{em} 520 nm) upon prenylation of the dansylated peptide.[42, 48, 65-67] Assays were performed at 25 °C in a 96-well plate (Corning, Corning, NY); Fluorescence was measured as a function of time in a Synergy H1 multimode plate reader (Biotek, Winooski, VT). Negative controls lacked the FPP or GGPP cosubstrate.

To determine if a given dns-GC(x)$_3$X peptide is considered reactive with FTase or GGTase-I, the observed fluorescence at time zero for each peptide was subtracted from the fluorescence at four time points ($F_t - F_0$; t = 30, 60, 90, and 120 min) to determine the corrected fluorescence at each time point for both the reactions containing the FPP or GGPP prenyl donor and the negative control reactions lacking the prenyl donor cosubstrate. To be considered reactive, a peptide must exhibit at least a 5-fold enhancement of fluorescence in the FTase or GGTase-I reaction compared to the negative control [e.g. Corrected fluorescence (+FPP) / Corrected fluorescence (-FPP) > 5]. Fluorescence data and calculated fluorescence enhancements for reaction with FTase/FPP are

reported in Appendix I. No dns-GC$(x)_3$X peptides exhibited detectable fluorescence enhancement in reaction with GGTase-I/GGPP.

2.10.11 Activity screening prenylation of dns-GC$(x)_3$X peptides via HPLC: Reverse phase HPLC analysis was performed on all dns-GC$(x)_3$X peptides to confirm farnesylation (FPP and FTase) or geranylgeranylation (GGPP and GGTase-I. Reactions were prepared as described above for fluorescence-based activity screening and incubated at room temperature for 14 hours in low-adhesion tubes wrapped in foil. Reactions were halted by addition of an equal volume of 20% acetic acid in isopropanol prior to analysis by HPLC (Zorbax Eclipse XDB-C18 column). Peptides and products were detected by fluorescence (λ_{ex} = 340 nm and λ_{em} = 496 nm). In all cases, HPLC analysis indicates that the peak for the dns-GC$(x)_3$X peptide shifts to a longer retention time upon farnesylation, whereas parallel reactions performed without FPP showed no change in peptide retention time. Representative HPLC traces are included in the Appendix I. Analogous reactions with GGTase-I and GGPP provided no evidence for geranylgeranylation of any dns-GC$(x)_3$X peptides.

2.10.12 Steady-state characterization of dns-GC$(x)_3$X and dns-GCaaX peptides: Steady-state kinetics were determined as previously described for prenylation of dns-GC$(x)_3$X and dns-GCaaX peptides by FTase or GGTase-I by monitoring the time-dependent increase in fluorescence (λ_{ex} 340 nm, λ_{em} 520 nm) upon prenylation of the dansylated peptide in a Synergy HI multimode plate reader (Biotek).[44, 68, 99]

2.10.13 Determination of tipifarnib inhibition of dns-GC(x)$_3$X peptide farnesylation: Assays were performed as described above, with varying concentrations of tipifarnib (0-100 nM) included in the reaction. Initial slopes (fluorescence change per second) were determined for each reaction and normalized to the reaction without tipifarnib. Normalized slope values were plotted against tipifarnib concentration and analyzed using equation 1 to calculate IC_{50} values.

(1) $$\text{Normalized Slope} = 1 - \frac{[\text{tipifarnib}]}{[\text{tipifarnib}] + IC_{50}}$$

2.10.14 ESI-MS analysis of farnesylated dns-GC(x)$_3$X peptides (ESI-MS experiments were performed in the Distefano laboratory, University of Minnesota): *In vitro* reactions with dns-GC(x)$_3$X peptides (5 µM) were prepared in prenylation buffer in the presence of 100 nm FTase and 10 µM FPP and incubated overnight at room temperature. Each reaction mixture was separately loaded onto a pre-conditioned and pre-equilibrated Sep-Pak reverse-phase C18 cartridge (Waters Corp., Milford, MA) and washed with 0.1% TFA in H_2O. A step-gradient elution was carried out with 6-mL volumes of 10% (CH_3CN/H_2O; 0.1% TFA), 35% (CH_3CN/H_2O; 0.1% TFA), and 100% CH_3CN with 0.1% TFA. The 100% CH_3CN fractions containing the modified peptides (confirmed by fluorescence under UV lamp) were evaporated and residues were redissolved in 30% CH_3CN/H_2O with 0.1% TFA. Samples were loaded to an LC column (Agilent Zorbax SB-C18, 5 µm x 150 mm x 0.5 mm) coupled to an ESI-MS/MS ion trap mass analyzer (Agilent 1100 Series LC/MSD). Runs were set to positive ion mode and CID MS/MS fragmentations were triggered at 1.5 V. The theoretical masses of farnesylated peptides and their fragments were calculated using Protein Prospector v 5.19.1 (http://prospector.ucsf.edu).

2.10.15 Construction of pJExpress414 plasmid encoding the His_6-eGFP-GCAVGP reporter protein: A gene encoding the His_6-eGFP-GCAVGP reporter protein was prepared by PCR using the pJExpress414-eGFP-CVIA vector as a template with the GCAVGP C-terminal sequence and *Hind*III restriction site encoded in the 3′ primer.[100] PCR products were purified using the BioBasic Inc.(Amherst, NY) EZ-10 Spin Column PCR Purification Kit following the manufacturer's instructions. Following digestion by *Nhe*I and *Hind*III, the His_6-eGFP-GCAVGP insert was ligated into the pJExpress414 expression plasmid using the Quick Ligase kit (New England Biolabs, Ipswich, MA) per manufacturer instructions. Insert ligation was verified by analytical restriction digest and DNA sequencing (Genewiz, South Plainfield, NJ).

2.10.16 Expression and purification of His_6-eGFP-GCAVGP: Chemically competent BL21 (DE3) *E. coli* (Z-competent, Zymo Research, Irvine, CA) were transformed with pJExpress414_His_6-eGFP-GCAVGP per the manufacturer's protocol. Following transformation and antibiotic selection, a colony from the transformation plate was inoculated into LB media (5 mL) containing 100 µg / mL ampicillin. The culture was incubated with shaking (225 rpm) for 4 hours at 37 °C and subsequently used to inoculate 1 L of prewarmed autoinduction media supplemented with 100 µg / mL ampicillin.[101] Following overnight incubation at 28 °C with shaking, bacteria were harvested, lysed, and His_6-eGFP-GCAVGP was purified as previously described.[74] Protein concentration was measured using absorbance of eGFP at 488 nm (λ_{488} = 55,000 $M^{-1}cm^{-1}$).[102]

2.10.17 Farnesylation reactions with eGFP-GCAVGP (ESI-MS/MS and in-gel fluorescence labeling experiments were performed in the Distefano laboratory, University of Minnesota): Farnesylation of purified eGFP-GCAVGP was performed by incubation of purified eGFP-

GCAVGP (5 µM) with 100 nM FTase, 10 µM FPP or C15AlkOPP, and 5 mM $MgCl_2$ in reaction buffer (50 mM NaHEPPSO, 5 mM TCEP, pH 7.8) in a final volume of 2 mL (FPP, mass spectrometry analysis) or 500 µL (C15AlkOPP, TAMRA labeling and in-gel fluorescence analysis). Substrate protein was incubated in reaction buffer for 20 min prior to reaction initiation by addition of FTase and prenyl donor to reduce disulfide bonds. Reactions were incubated overnight at room temperature wrapped in foil, and then frozen for storage. *In vitro* reaction mixtures were concentrated by lyophilization and injected to an LC column (Agilent [Santa Clara, CA] Zorbax 300SB-C8, 3.5 µm x 100 mm x 0.3 mm) coupled to an ESI-MS/MS ion trap mass analyzer (Agilent 1100 Series LC/MSD. The proteins were eluted with buffer A (0.1% HCO_2H in H_2O) and buffer B (0.1% HCO_2H in CH_3CN) in the following gradient segments of buffer B: 2 mins, 10%; 3 mins, 10-25%; 35 mins, 25-60%; 10 mins, 60-90%. The m/z values from protein fragments were deconvoluted to estimate parent protein masses. For in-gel fluorescence labeling, eGFP-GCAVGP from *in vitro* farnesylation reactions (+/- 10 µM C15AlkOPP) was precipitated out using a protein precipitation kit (ProteoExtract, Calbiochem, San Diego, CA). Protein pellets were redissolved in PBS + 1% SDS and 14 µg of the proteins were aliquoted and subjected to click reaction (25 µM TAMRA-N_3, 1 mM TCEP, 0.1 mM TBTA, and 1 mM $CuSO_4$) for 1 hour at room temperature. An aliquot (3.5 µg) from the click reactions were mixed with Laemmli loading buffer, boiled for 5 min, and loaded into a 15% SDS-PAGE gel. In-gel fluorescence was detected at 542/568 excitation/emission wavelengths on a fluorescence scanner (Typhoon FLA 9500, GE Healthcare). Gels were stained with 1X Coomassie Blue and destained to visualize protein loading.

2.10.18 Identification of human $C(x)_3X$ sequences: To identify human $C(x)_3X$ sequence candidates, we interrogated the Prosite database (http://prosite.expasy.org/scanprosite/) using

highly active sequences from the yeast studies (CAVGP, CMIIM, CWGEV) and allowing sequence variability at one, two, or three positions downstream of the cysteine residue. Sequences containing more than one cysteine were eliminated to avoid canonical four amino acid CaaX-compliant motifs (example: CCIIM) or sequences potentially recognized by GGTase-II (examples: CC or CxC).[18,20,21] The candidate sequences were chosen based on previous studies of the protein bearing the $C(x)_3X$ sequences, their location within the cell (e.g. membrane associated), and the degree of sequence similarity to the parent $C(x)_3X$ sequence from the yeast studies.

2.10.19 Construction of eGFP-KRas-C(x)$_3$X reporter protein plasmids: Gene inserts encoding eGFP-KRas-XMIIM and eGFP-KRas-XMII reporter proteins were prepared by PCR using the pEGFP-KRas vector (Casey Lab, Duke University) as a template with the CMIIM, SMIIM, or CMII C-terminal sequences and *Kpn*I restriction site encoded in the 3′ primers.[78] PCR products were purified using the BioBasic Inc. EZ-10 Spin Column PCR Purification Kit following the manufacturer's instructions. Following digestion by *Nhe*I and *Kpn*I, the eGFP-KRas-XMIIM or eGFP-KRas-XMII insert was ligated into the pEGFP-KRas expression plasmid using the Quick Ligase kit (NEB) per manufacturer instructions. Insert ligation was verified by analytical restriction digest and DNA sequencing (Genewiz).

2.10.20 Cell culture, transfection, and imaging: HEK293 cells (ATCC) was maintained in 75 mL vented tissue culture flasks (Celltreat, Pepperell, MA), and were split upon reaching 80% confluency. Cells were grown in complete DMEM (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin [MediaTech, Manassas, VA]) in 5% CO_2 at 37 °C. Transfections and imaging were performed as previously described,[77] in a single glass well

imaging dish (Corning) and allowed to adhere for 24 hours prior to addition of Turbofect transfection reagent (Thermo Scientific) according to the manufacturer's protocol. Tipifarnib was added to a final concentration of 10 nM to the transfection mixture for those cells undergoing inhibitor studies. Following 36 hours of transfection, live cells were imaged at 63x magnification using a Zeiss (Jena, Germany) Axio Vert.A1 inverted fluorescence microscope with a 470/40 nm excitation filter, a 495 nm beam splitter and a 525/50 nm emission filter.

2.11 References

1. Beck-Sickinger, A. G.; Mörl, K., Posttranslational Modification of Proteins. Expanding Nature's Inventory. By Christopher T. Walsh. *Angewandte Chemie International Edition* **2006,** *45* (7), 1020-1020.

2. Casey, P. J., Lipid modifications of G proteins. *Current opinion in cell biology* **1994,** *6* (2), 219-25.

3. Ganesan, L.; Levental, I., Pharmacological Inhibition of Protein Lipidation. *The Journal of membrane biology* **2015,** *248* (6), 929-41.

4. Hentschel, A.; Zahedi, R. P.; Ahrends, R., Protein lipid modifications--More than just a greasy ballast. *Proteomics* **2016,** *16* (5), 759-82.

5. Mann, M.; Jensen, O. N., Proteomic analysis of post-translational modifications. *Nature biotechnology* **2003,** *21* (3), 255-61.

6. Martin, D. D.; Beauchamp, E.; Berthiaume, L. G., Post-translational myristoylation: Fat matters in cellular life and death. *Biochimie* **2011,** *93* (1), 18-31.

7. Nadolski, M. J.; Linder, M. E., Protein lipidation. *The FEBS journal* **2007,** *274* (20), 5202-10.

8. Resh, M. D., Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochimica et biophysica acta* **1999,** *1451* (1), 1-16.

9. Resh, M. D., Trafficking and signaling by fatty-acylated and prenylated proteins. *Nature chemical biology* **2006,** *2* (11), 584-90.

10. Resh, M. D., Covalent Lipid Modifications of Proteins. *Current biology : CB* **2013,** *23* (10), R431-5.

11. Benetka, W.; Koranda, M.; Eisenhaber, F., Protein Prenylation: An (Almost) Comprehensive Overview on Discovery History, Enzymology, and Significance in Physiology and Disease. *Monatshefte für Chemie / Chemical Monthly* **2006,** *137* (10), 1241.

12. Casey, P. J.; Seabra, M. C., Protein prenyltransferases. *The Journal of biological chemistry* **1996,** *271* (10), 5289-92.

13. Marshall, C. J., Protein prenylation: a mediator of protein-protein interactions. *Science (New York, N.Y.)* **1993,** *259* (5103), 1865-6.

14. Zhang, F. L.; Casey, P. J., Protein prenylation: molecular mechanisms and functional consequences. *Annual review of biochemistry* **1996,** *65*, 241-69.

15. Guo, Z.; Wu, Y. W.; Das, D.; Delon, C.; Cramer, J.; Yu, S.; Thuns, S.; Lupilova, N.; Waldmann, H.; Brunsveld, L.; Goody, R. S.; Alexandrov, K.; Blankenfeldt, W., Structures of RabGGTase-substrate/product complexes provide insights into the evolution of protein prenylation. *The EMBO journal* **2008,** *27* (18), 2444-56.

16. Khosravi-Far, R.; Clark, G. J.; Abe, K.; Cox, A. D.; McLain, T.; Lutz, R. J.; Sinensky, M.; Der, C. J., Ras (CXXX) and Rab (CC/CXC) prenylation signal sequences are unique and functionally distinct. *The Journal of biological chemistry* **1992,** *267* (34), 24363-8.

17. Shen, F.; Seabra, M. C., Mechanism of digeranylgeranylation of Rab proteins. Formation of a complex between monogeranylgeranyl-Rab and Rab escort protein. *The Journal of biological chemistry* **1996,** *271* (7), 3692-8.

18. Stenmark, H.; Olkkonen, V. M., The Rab GTPase family. *Genome Biology* **2001,** *2* (5), reviews3007.1.

19. Bonni, A.; Brunet, A.; West, A. E.; Datta, S. R.; Takasu, M. A.; Greenberg, M. E., Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and-independent mechanisms. *Science (New York, N.Y.)* **1999,** *286* (5443), 1358-1362.

20. Casey, P. J., Protein lipidation in cell signaling. *Science (New York, N.Y.)* **1995,** *268* (5208), 221.

21. Feig, L. A.; Buchsbaum, R. J., Cell signaling: life or death decisions of ras proteins. *Current Biology* **2002,** *12* (7), R259-R261.

22. Lau, H. Y.; Tang, J.; Casey, P. J.; Wang, M., Isoprenylcysteine carboxylmethyltransferase is critical for malignant transformation and tumor maintenance by all RAS isoforms. *Oncogene* **2017**.

23. Wang, M.; Casey, P. J., Protein prenylation: unique fats make their mark on biology. *Nat Rev Mol Cell Biol* **2016,** *17* (2), 110-22.

24. Gutierrez, L.; Magee, A. I.; Marshall, C. J.; Hancock, J. F., Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *The EMBO journal* **1989,** *8* (4), 1093-8.

25. Leung, K. F.; Baron, R.; Ali, B. R.; Magee, A. I.; Seabra, M. C., Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation. *The Journal of biological chemistry* **2007,** *282* (2), 1487-97.

26. Michaelson, D.; Ali, W.; Chiu, V. K.; Bergo, M.; Silletti, J.; Wright, L.; Young, S. G.; Philips, M., Postprenylation CAAX Processing Is Required for Proper Localization of Ras but Not Rho GTPases. *Molecular Biology of the Cell* **2005,** *16* (4), 1606-16.

27. Ota, I. M.; Clarke, S., Enzymatic methylation of 23-29-kDa bovine retinal rod outer segment membrane proteins. Evidence for methyl ester formation at carboxyl-terminal cysteinyl residues. *Journal of Biological Chemistry* **1989,** *264* (22), 12879-12884.

28. Winter-Vann, A. M.; Casey, P. J., Post-prenylation-processing enzymes as new targets in oncogenesis. *Nature reviews. Cancer* **2005,** *5* (5), 405-12.

29. Roberts, P. J.; Mitin, N.; Keller, P. J.; Chenette, E. J.; Madigan, J. P.; Currin, R. O.; Cox, A. D.; Wilson, O.; Kirschmeier, P.; Der, C. J., Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. *The Journal of biological chemistry* **2008,** *283* (37), 25150-63.

30. Solski, P. A.; Helms, W.; Keely, P. J.; Su, L.; Der, C. J., RhoA Biological Activity Is Dependent on Prenylation but Independent of Specific Isoprenoid Modification(). *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **2002,** *13* (8), 363-373.

31. Hildebrandt, E. R.; Cheng, M.; Zhao, P.; Kim, J. H.; Wells, L.; Schmidt, W. K., A shunt pathway limits the CaaX processing of Hsp40 Ydj1p and regulates Ydj1p-dependent phenotypes. *eLife* **2016,** *5*.

32. Clarke, S.; Vogel, J. P.; Deschenes, R. J.; Stock, J., Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** *85* (13), 4643-7.

33. Farnsworth, C. C.; Wolda, S. L.; Gelb, M. H.; Glomset, J. A., Human lamin B contains a farnesylated cysteine residue. *The Journal of biological chemistry* **1989,** *264* (34), 20422-9.

34. Hancock, J. F.; Magee, A. I.; Childs, J. E.; Marshall, C. J., All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **1989,** *57* (7), 1167-77.

35. Kitten, G. T.; Nigg, E. A., The CaaX motif is required for isoprenylation, carboxyl methylation, and nuclear membrane association of lamin B2. *The Journal of cell biology* **1991,** *113* (1), 13-23.

36. Powers, S.; Michaelis, S.; Broek, D.; Santa Anna, S.; Field, J.; Herskowitz, I.; Wigler, M., RAM, a gene of yeast required for a functional modification of RAS proteins and for production of mating pheromone a-factor. *Cell* **1986,** *47* (3), 413-22.

37. Sefton, B. M.; Buss, J. E., The covalent modification of eukaryotic proteins with lipid. *The Journal of cell biology* **1987,** *104* (6), 1449-53.

38. Vorburger, K.; Kitten, G. T.; Nigg, E. A., Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif. *The EMBO journal* **1989,** *8* (13), 4007-13.

39. Schaber, M. D.; O'Hara, M. B.; Garsky, V. M.; Mosser, S. C.; Bergstrom, J. D.; Moores, S. L.; Marshall, M. S.; Friedman, P. A.; Dixon, R. A.; Gibbs, J. B., Polyisoprenylation of Ras in vitro by a farnesyl-protein transferase. *The Journal of biological chemistry* **1990,** *265* (25), 14701-4.

40. Gao, J.; Liao, J.; Yang, G. Y., CAAX-box protein, prenylation process and carcinogenesis. *American journal of translational research* **2009,** *1* (3), 312-25.

41. Hancock, J. F.; Cadwallader, K.; Paterson, H.; Marshall, C. J., A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *The EMBO journal* **1991,** *10* (13), 4033-9.

42. Hougland, J. L.; Gangopadhyay, S. A.; Fierke, C. A., Expansion of Protein Farnesyltransferase Specificity Using "Tunable" Active Site Interactions: DEVELOPMENT OF BIOENGINEERED PRENYLATION PATHWAYS. *Journal of Biological Chemistry* **2012,** *287* (45), 38090-38100.

43. Hougland, J. L.; Hicks, K. A.; Hartman, H. L.; Kelly, R. A.; Watt, T. J.; Fierke, C. A., Identification of novel peptide substrates for protein farnesyltransferase reveals two substrate classes with distinct sequence selectivities. *Journal of molecular biology* **2010,** *395* (1), 176-90.

44. Hougland, J. L.; Lamphear, C. L.; Scott, S. A.; Gibbs, R. A.; Fierke, C. A., Context-dependent substrate recognition by protein farnesyltransferase. *Biochemistry* **2009,** *48* (8), 1691-701.

45. Lane, K. T.; Beese, L. S., Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *J Lipid Res* **2006,** *47* (4), 681-99.

46. Moores, S. L.; Schaber, M. D.; Mosser, S. D.; Rands, E.; O'Hara, M. B.; Garsky, V. M.; Marshall, M. S.; Pompliano, D. L.; Gibbs, J. B., Sequence dependence of protein isoprenylation. *The Journal of biological chemistry* **1991,** *266* (22), 14603-10.

47. London, N.; Lamphear, C. L.; Hougland, J. L.; Fierke, C. A.; Schueler-Furman, O., Identification of a novel class of farnesylation targets by structure-based modeling of binding specificity. *PLoS computational biology* **2011,** *7* (10), e1002170.

48. Reid, T. S.; Terry, K. L.; Casey, P. J.; Beese, L. S., Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. *Journal of molecular biology* **2004,** *343* (2), 417-33.

49. Al-Quadan, T.; Price, C. T.; London, N.; Schueler-Furman, O.; AbuKwaik, Y., Anchoring of bacterial effectors to host membranes through host-mediated lipidation by prenylation: a common paradigm. *Trends in microbiology* **2011,** *19* (12), 573-9.

50. Cui, G.; Merz, K. M., Jr., Computational studies of the farnesyltransferase ternary complex part II: the conformational activation of farnesyldiphosphate. *Biochemistry* **2007,** *46* (43), 12375-81.

51. Maurer-Stroh, S.; Eisenhaber, F., Refinement and prediction of protein prenylation motifs. *Genome Biology* **2005,** *6* (6), R55.

52. Maurer-Stroh, S.; Koranda, M.; Benetka, W.; Schneider, G.; Sirota, F. L.; Eisenhaber, F., Towards complete sets of farnesylated and geranylgeranylated proteins. *PLoS computational biology* **2007,** *3* (4), e66.

53. Caplan, A. J.; Douglas, M. G., Characterization of YDJ1: a yeast homologue of the bacterial dnaJ protein. *The Journal of cell biology* **1991,** *114* (4), 609-21.

54. Caplan, A. J.; Tsai, J.; Casey, P. J.; Douglas, M. G., Farnesylation of YDJ1p is required for function at elevated growth temperatures in Saccharomyces cerevisiae. *The Journal of biological chemistry* **1992,** *267* (26), 18890-5.

55. Kimura, Y.; Yahara, I.; Lindquist, S., Role of the protein chaperone YDJ1 in establishing Hsp90-mediated signal transduction pathways. *Science (New York, N.Y.)* **1995,** *268* (5215), 1362-5.

56. Lu, Z.; Cyr, D. M., The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding. *The Journal of biological chemistry* **1998,** *273* (10), 5970-8.

57. Qiu, X.-B.; Shao, Y.-M.; Miao, S.; Wang, L., The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and Molecular Life Sciences CMLS* **2006,** *63* (22), 2560-2570.

58. Blanden, M. J.; Suazo, K. F.; Hildebrandt, E. R.; Hardgrove, D. S.; Patel, M.; Saunders, W. P.; Distefano, M. D.; Schmidt, W. K.; Hougland, J. L., Efficient farnesylation of an extended C-terminal C(x)3X sequence motif expands the scope of the prenylated proteome. *The Journal of biological chemistry* **2018,** *293* (8), 2770-2785.

59. DeGraw, A. J.; Palsuledesai, C.; Ochocki, J. D.; Dozier, J. K.; Lenevich, S.; Rashidian, M.; Distefano, M. D., Evaluation of alkyne-modified isoprenoids as chemical reporters of protein prenylation. *Chemical biology & drug design* **2010,** *76* (6), 460-71.

60. Jennings, B. C.; Danowitz, A. M.; Wang, Y. C.; Gibbs, R. A.; Distefano, M. D.; Fierke, C. A., Analogs of farnsyl diphosphate alter CaaX substrate specificity and reactions rates of protein farnesyltransferase. *Bioorganic & medicinal chemistry letters* **2016,** *26* (4), 1333-6.

61. Kho, Y.; Kim, S. C.; Jiang, C.; Barma, D.; Kwon, S. W.; Cheng, J.; Jaunbergs, J.; Weinbaum, C.; Tamanoi, F.; Falck, J.; Zhao, Y., A tagging-via-substrate technology for detection and proteomics of farnesylated proteins. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (34), 12479-12484.

62. Kpetemey, M.; Dasgupta, S.; Rajendiran, S.; Das, S.; Gibbs, L. D.; Shetty, P.; Gryczynski, Z.; Vishwanatha, J. K., MIEN1, a novel interactor of Annexin A2, promotes tumor cell migration by enhancing AnxA2 cell surface expression. *Molecular cancer* **2015,** *14*, 156.

63. Chan, R. K.; Otte, C. A., Isolation and genetic analysis of Saccharomyces cerevisiae mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol Cell Biol* **1982,** *2* (1), 11-20.

64. Firth, A. E.; Patrick, W. M., GLUE-IT and PEDEL-AA: new programmes for analyzing protein diversity in randomized libraries. *Nucleic Acids Research* **2008,** *36* (Web Server issue), W281-5.

65. Cassidy, P. B.; Dolence, J. M.; Poulter, C. D., Continuous fluorescence assay for protein prenyltransferases. *Methods in enzymology* **1995,** *250*, 30-43.

66. Pickett, W. C.; Zhang, F. L.; Silverstrim, C.; Schow, S. R.; Wick, M. M.; Kerwar, S. S., A fluorescence assay for geranylgeranyl transferase type I. *Analytical biochemistry* **1995,** *225* (1), 60-3.

67. Pompliano, D. L.; Gomez, R. P.; Anthony, N. J., Intramolecular fluorescence enhancement: a continuous assay of Ras farnesyl:protein transferase. *Journal of the American Chemical Society* **1992,** *114* (20), 7945-7946.

68. Stirtan, W. G.; Poulter, C. D., Yeast protein geranylgeranyltransferase type-I: steady-state kinetics and substrate binding. *Biochemistry* **1997,** *36* (15), 4552-7.

69. Hosokawa, A.; Wollack, J. W.; Zhang, Z.; Chen, L.; Barany, G.; Distefano, M. D., Evaluation of an Alkyne-containing Analogue of Farnesyl Diphosphate as a Dual Substrate for Protein-prenyltransferases. *International Journal of Peptide Research and Therapeutics* **2007,** *13* (1), 345-354.

70. Subramanian, T.; Pais, J. E.; Liu, S.; Troutman, J. M.; Suzuki, Y.; Leela Subramanian, K.; Fierke, C. A.; Andres, D. A.; Spielmann, H. P., Farnesyl diphosphate analogues with aryl moieties are efficient alternate substrates for protein farnesyltransferase. *Biochemistry* **2012,** *51* (41), 8307-19.

71. Alsina, M.; Fonseca, R.; Wilson, E. F.; Belle, A. N.; Gerbino, E.; Price-Troska, T.; Overton, R. M.; Ahmann, G.; Bruzek, L. M.; Adjei, A. A.; Kaufmann, S. H.; Wright, J. J.; Sullivan,

D.; Djulbegovic, B.; Cantor, A. B.; Greipp, P. R.; Dalton, W. S.; Sebti, S. M., Farnesyltransferase inhibitor tipifarnib is well tolerated, induces stabilization of disease, and inhibits farnesylation and oncogenic/tumor survival pathways in patients with advanced multiple myeloma. *Blood* **2004,** *103* (9), 3271-7.

72. Zhu, K.; Hamilton, A. D.; Sebti, S. M., Farnesyltransferase inhibitors as anticancer agents: current status. *Current opinion in investigational drugs (London, England : 2000)* **2003,** *4* (12), 1428-35.

73. Rashidian, M.; Kumarapperuma, S. C.; Gabrielse, K.; Fegan, A.; Wagner, C. R.; Distefano, M. D., Simultaneous dual protein labeling using a triorthogonal reagent. *J Am Chem Soc* **2013,** *135* (44), 16388-96.

74. Zhang, Y.; Blanden, M. J.; Sudheer, C.; Gangopadhyay, S. A.; Rashidian, M.; Hougland, J. L.; Distefano, M. D., Simultaneous Site-Specific Dual Protein Labeling Using Protein Prenyltransferases. *Bioconjug Chem* **2015,** *26* (12), 2542-53.

75. Palsuledesai, C. C.; Ochocki, J. D.; Kuhns, M. M.; Wang, Y.-C.; Warmka, J. K.; Chernick, D. S.; Wattenberg, E. V.; Li, L.; Arriaga, E. A.; Distefano, M. D., Metabolic Labeling with an Alkyne-modified Isoprenoid Analog Facilitates Imaging and Quantification of the Prenylome in Cells. *ACS Chemical Biology* **2016,** *11* (10), 2820-2828.

76. Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D., Chemoenzymatic Reversible Immobilization and Labeling of Proteins without Prior Purification. *Journal of the American Chemical Society* **2012,** *134* (20), 8455-8467.

77. Flynn, S. C.; Lindgren, D. E.; Hougland, J. L., Quantitative Determination of Cellular Farnesyltransferase Activity: Towards Defining the Minimum Substrate Reactivity for

Biologically Relevant Protein Farnesylation. *Chembiochem : a European journal of chemical biology* **2014,** *15* (15), 2205-2210.

78. Chen, Z.; Otto, J. C.; Bergo, M. O.; Young, S. G.; Casey, P. J., The C-terminal polylysine region and methylation of K-Ras are critical for the interaction between K-Ras and microtubules. *The Journal of biological chemistry* **2000,** *275* (52), 41251-7.

79. Marcus, S.; Caldwell, G.; Miller, D.; Xue, C.; Naider, F.; Becker, J., Significance of C-terminal cysteine modifications to the biological activity of the Saccharomyces cerevisiae a-factor mating pheromone. *Molecular and Cellular Biology* **1991,** *11* (7), 3603-3612.

80. Suazo, K. F.; Schaber, C.; Palsuledesai, C. C.; Odom John, A. R.; Distefano, M. D., Global proteomic analysis of prenylated proteins in Plasmodium falciparum using an alkyne-modified isoprenoid analogue. *Scientific Reports* **2016,** *6*, 38615.

81. Long, S. B.; Casey, P. J.; Beese, L. S., The basis for K-Ras4B binding specificity to protein farnesyltransferase revealed by 2 A resolution ternary complex structures. *Structure (London, England : 1993)* **2000,** *8* (2), 209-22.

82. Strickland, C. L.; Windsor, W. T.; Syto, R.; Wang, L.; Bond, R.; Wu, Z.; Schwartz, J.; Le, H. V.; Beese, L. S.; Weber, P. C., Crystal structure of farnesyl protein transferase complexed with a CaaX peptide and farnesyl diphosphate analogue. *Biochemistry* **1998,** *37* (47), 16601-11.

83. Turek-Etienne, T. C.; Strickland, C. L.; Distefano, M. D., Biochemical and Structural Studies with Prenyl Diphosphate Analogues Provide Insights into Isoprenoid Recognition by Protein Farnesyl Transferase. *Biochemistry* **2003,** *42* (13), 3716-3724.

84. Wang, Y.-C.; Dozier, J. K.; Beese, L. S.; Distefano, M. D., Rapid Analysis of Protein Farnesyltransferase Substrate Specificity Using Peptide Libraries and Isoprenoid Diphosphate Analogues. *ACS Chemical Biology* **2014,** *9* (8), 1726-1735.

85. Hast, M. A.; Nichols, C. B.; Armstrong, S. M.; Kelly, S. M.; Hellinga, H. W.; Alspaugh, J. A.; Beese, L. S., Structures of Cryptococcus neoformans protein farnesyltransferase reveal strategies for developing inhibitors that target fungal pathogens. *The Journal of biological chemistry* **2011,** *286* (40), 35149-62.

86. Ivanov, S. S.; Charron, G.; Hang, H. C.; Roy, C. R., Lipidation by the host prenyltransferase machinery facilitates membrane localization of Legionella pneumophila effector proteins. *The Journal of biological chemistry* **2010,** *285* (45), 34686-98.

87. Piispanen, A. E.; Bonnefoi, O.; Carden, S.; Deveau, A.; Bassilana, M.; Hogan, D. A., Roles of Ras1 membrane localization during Candida albicans hyphal growth and farnesol response. *Eukaryot Cell* **2011,** *10* (11), 1473-84.

88. Selvig, K.; Ballou, E. R.; Nichols, C. B.; Alspaugh, J. A., Restricted substrate specificity for the geranylgeranyltransferase-I enzyme in Cryptococcus neoformans: implications for virulence. *Eukaryot Cell* **2013,** *12* (11), 1462-71.

89. Zverina, E. A.; Lamphear, C. L.; Wright, E. N.; Fierke, C. A., Recent Advances in Protein Prenyltransferases: Substrate Identification, Regulation, and Disease Interventions. *Current opinion in chemical biology* **2012,** *16* (5-6), 544-552.

90. Heilmeyer, L. M.; Serwe, M.; Weber, C.; Metzger, J.; Hoffmann-Posorske, E.; Meyer, H. E., Farnesylcysteine, a constituent of the alpha and beta subunits of rabbit skeletal muscle phosphorylase kinase: localization by conversion to S-ethylcysteine and by tandem mass spectrometry. *Proceedings of the National Academy of Sciences* **1992,** *89* (20), 9554-9558.

91. Kilpatrick, E. L.; Hildebrandt, J. D., Sequence dependence and differential expression of Ggamma5 subunit isoforms of the heterotrimeric G proteins variably processed after prenylation in mammalian cells. *The Journal of biological chemistry* **2007,** *282* (19), 14038-47.

92. Chen, P.; Sapperstein, S. K.; Choi, J. D.; Michaelis, S., Biogenesis of the Saccharomyces cerevisiae mating pheromone a-factor. *The Journal of cell biology* **1997,** *136* (2), 251-69.

93. Michaelis, S.; Herskowitz, I., The a-factor pheromone of Saccharomyces cerevisiae is essential for mating. *Mol Cell Biol* **1988,** *8* (3), 1309-18.

94. Schmidt, W. K.; Tam, A.; Michaelis, S., Reconstitution of the Ste24p-dependent N-terminal proteolytic step in yeast a-factor biogenesis. *The Journal of biological chemistry* **2000,** *275* (9), 6227-33.

95. Sikorski, R. S.; Hieter, P., A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **1989,** *122* (1), 19-27.

96. Oldenburg, K. R.; Vo, K. T.; Michaelis, S.; Paddon, C., Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Research* **1997,** *25* (2), 451-2.

97. Elble, R., A simple and efficient procedure for transformation of yeasts. *BioTechniques* **1992,** *13* (1), 18-20.

98. Kim, S.; Lapham, A. N.; Freedman, C. G.; Reed, T. L.; Schmidt, W. K., Yeast as a tractable genetic system for functional studies of the insulin-degrading enzyme. *The Journal of biological chemistry* **2005,** *280* (30), 27481-90.

99. Gangopadhyay, S. A.; Losito, E. L.; Hougland, J. L., Targeted reengineering of protein geranylgeranyltransferase type I selectivity functionally implicates active-site residues in protein-substrate recognition. *Biochemistry* **2014,** *53* (2), 434-46.

100. Rashidian, M.; Dozier, J. K.; Lenevich, S.; Distefano, M. D., Selective Labeling of Polypeptides Using Protein Farnesyltransferase via Rapid Oxime Ligation. *Chemical communications (Cambridge, England)* **2010,** *46* (47), 8998-9000.

101. Studier, F. W., Protein production by auto-induction in high density shaking cultures. *Protein expression and purification* **2005,** *41* (1), 207-34.

102. Heim, R.; Cubitt, A. B.; Tsien, R. Y., Improved green fluorescence. *Nature* **1995,** *373*, 663.

Chapter 3: Chemoenzymatic protein labeling and isolation from eukaryotic cell lysates using enzymes with reengineered substrate selectivity

3.1 Introduction

Protein immobilization is a valuable tool for studying protein structure, function, and interactions within biological systems.[1-5] Immobilization through bioorthogonal chemistry is particularly useful in its ability to isolate a protein of interest without interfering with native chemical processes in a cell. There are two main steps in the use of bioorthogonal chemistry to isolate proteins. First, the protein is modified by a chemical reporter probe in the cell followed by a chemical reaction between the functionalized protein and a chemically complementary secondary probe which allows for isolation. The most common example of bioorthogonal chemistry for protein modification is the Cu(I)-catalyzed "click" reaction (also known as the Huisgen 1,3-dipolar cycloaddition), but the utility of this approach is limited as the Cu(I) necessary for this reaction is toxic to cells (Figure 3.1a).[6] Oxime and hydrazone reactions have found popularity due to their ability to selectively modify proteins in which aldehyde or ketone additions have been made to the proteins of interest for immobilization (Figure 3.1b-c). This approach, however, is limited in the number of N-termini which can be modified with a ketone or aldehyde functional group.[7-12]

Another route for immobilization of proteins involves the use of enzymatic labeling with chemically modified groups. The use of enzymes for biorthogonal labeling solves several issues such as promiscuous substrate selectivity and eliminates the need to add non-natural functional groups to proteins for recognition by a secondary probe. An example of such an enzymatic reaction used for bioothogonal labeling is prenylation.[13-16] Prenylation is a post-translational modification in which a hydrophobic isoprenoid, either a farnesyl or geranylgeranyl group, is transferred to the

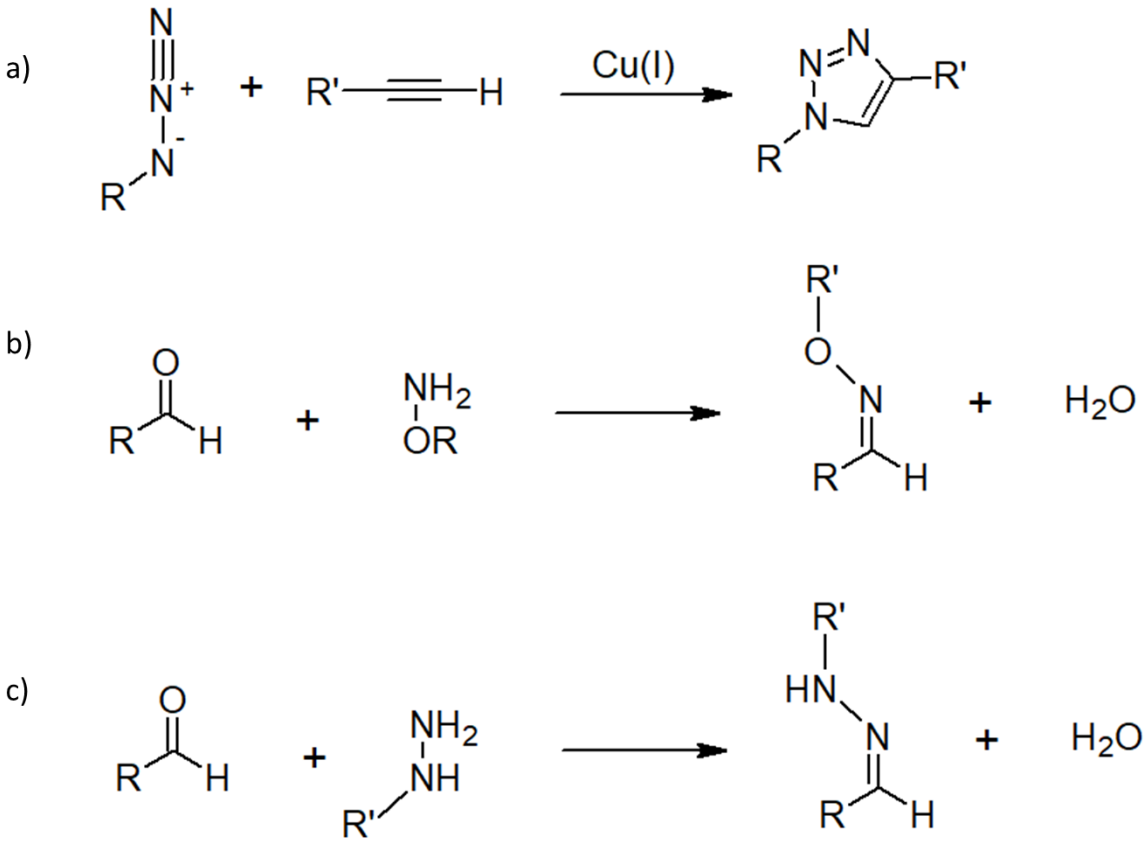
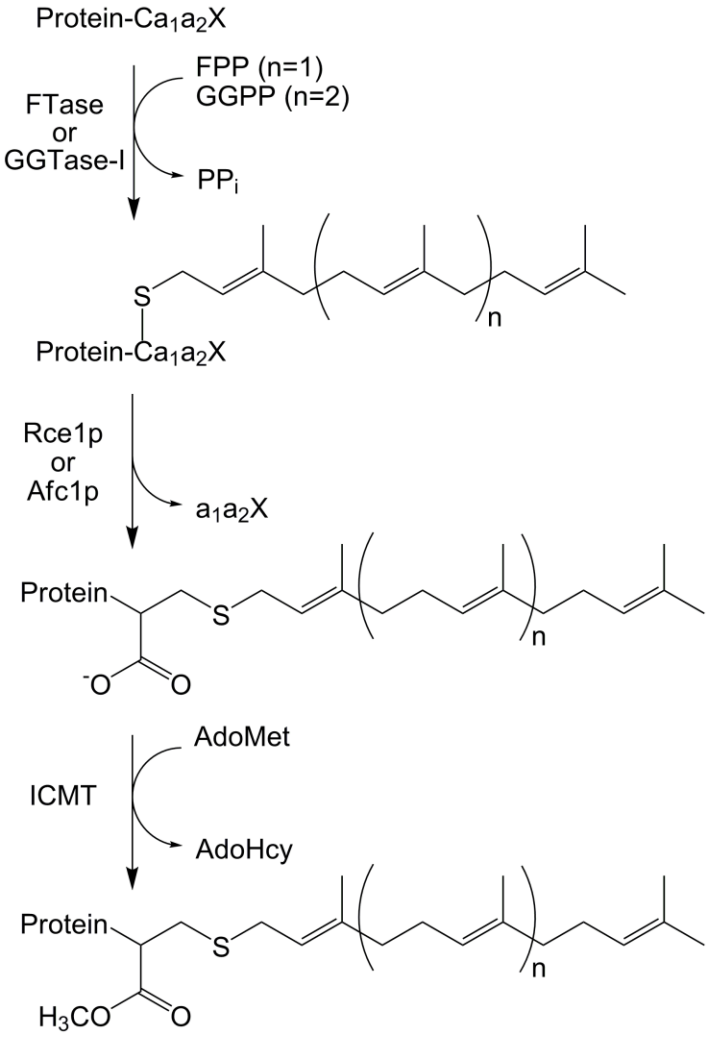


Figure 3.1 A representation of chemical modifications commonly used in biorthogonal labeling of proteins. (a) Cu(I) catalyzed azide-alkyne cycloaddition reaction (b) oxime ligation (c) hydrazone ligation; R and R' represent protein and/or probe used for isolation.

C-terminal cysteine of certain proteins.[17-21] This cysteine is typically part of the CaaX sequence in which 'C' is a cysteine, 'a' is any aliphatic amino acid, and 'X' is an amino acid which determines which type of isoprenoid will be attached to the substrate protein. The prenylation pathway follows a specific set of modifications before the protein is transported to the cell membrane, with the first step comprising cysteine alkylation by the FPP or GGPP prenyl donor (Scheme 3.1). The first step involves the enzymes FTase or GGTase which catalyze the addition of a farnesyl or geranylgeranyl group, via FPP or GGPP, respectively. The CaaX sequence then undergoes a series of other modifications, including proteolysis and methylation, which lead to its final incorporation to the membrane.

Several labs have utilized the specificity of farnesyltransferase (FTase) to label proteins with functionalized FPP.[15, 22-28] These analogues include the addition of ketones and aldehydes to the end of the isoprenoid chain for use in hydrazone reaction which immobilize the protein onto hydrazide beads (Figure 3.2, Scheme 3.2). For example, Rashidian and coworkers in 2012 developed an approach using protein prenyltransferase-catalyzed modification to label and immobilize green fluorescent protein (GFP) in *E. coli* lysates.[15] The GFP to be isolated contained a C-terminal -CVIA recognition sequence to be prenylated by FTase in the prescence of an aldehyde functionalized FPP. Upon prenylation, GFP was captured using hydrazide beads and could be released from the beads using aniline as a catalyst, successfully isolating GFP from the lysate. This technique requires no prior purification of the bacterial lysate and is useful in its ability to release the targeted protein from the hydrazide beads after capture, however, there are limitations to its usefulness. Methods such as these are limited to use in bacteria due to competition between the target prenylation reaction and the endogenous prenylation that occurs in mammalian cells and other eukaryotes.



Scheme 3.1 The prenylation pathway Prenylation consists of lipidation with an isoprenoid group followed by proteolysis and methylation.[18-19, 21] Figure provided by Dr. James L. Hougland.

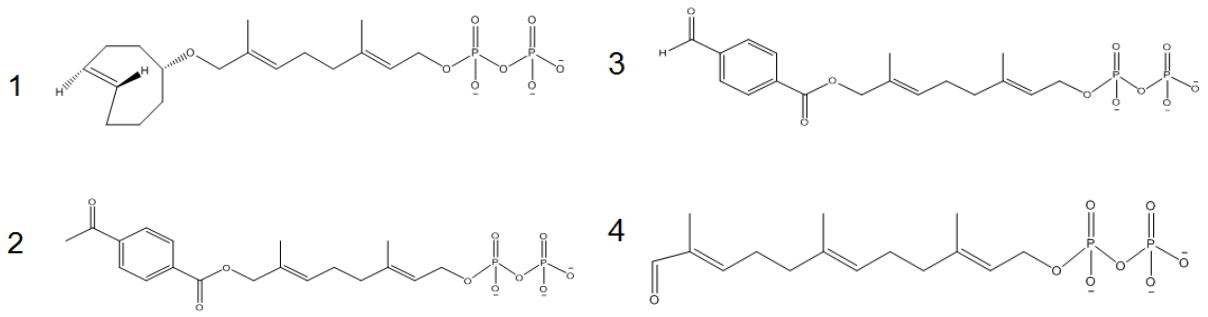
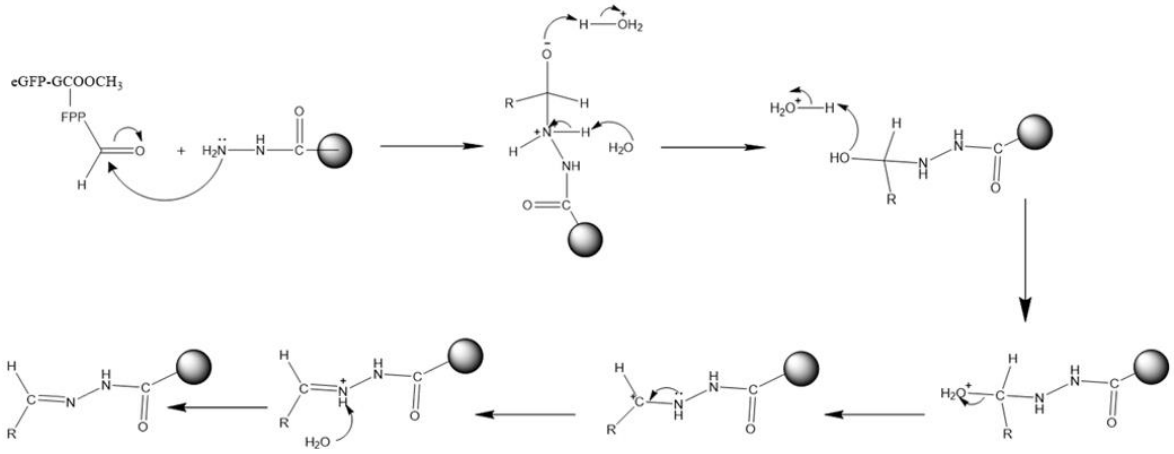


Figure 3.2 Functionalized FPP analogues for use in hydrazone ligation bead pulldown and isolation.[15] (1) Strained alkene (2) Aryl ketone (3) Aryl aldehyde (4) Alkyl aldehyde



Scheme 3.2 Mechanism for hydrazide ligation. Prenylation is highly specific and can be used in a pulldown as a means for isolation when used in conjunction with hydrazone chemistry at the C-terminus of proteins. Labeling with aldehyde functionalized FPP allows for isolation of fluorescent reporter proteins on hydrazide beads through the mechanism shown.

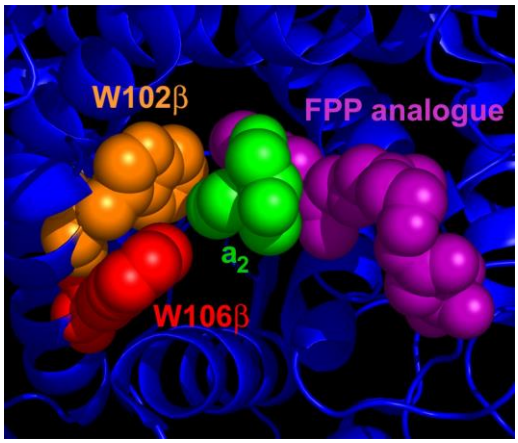
An ideal biorthogonal labeling system for proteins in cells would be one in which competition from endogenous reactions is limited or absent. One way in which this could be achieved is through alteration of enzyme substrate selectivity. For instance, it has been found that FTase can tolerate different mutations to its active site, with these mutations leading to differences in reactivity with natural and non-natural CaaX sequences.[29] In this work by Hougland and coworkers in 2012, a study of the multiple interactions between the active site of FTase and selected peptide substrates were examined to determine the requirements for FTase discrimination against nonsubstrates in the cell. For example, through mutation of tryptophan 102 and 106 on the β strand of FTase, selectivity of Ca_1a_2X sequences can be altered through affecting interactions with the a_2 position of the substrate (Figure 3.3a). One enzyme mutant of interest in the context of bioorthogonal labeling is W102R W106L FTase (referred to as RL FTase) which was found to more selectively prenylate the non-natural CaaX sequence –CVDS than the natural –CVLS sequence derived from H-Ras (Figure 3.3b).

Using this engineered FTase variant with non-natural peptide selectivity in addition with the previously designed FPP derivatives, it is possible to bioorthogonally select and purify proteins of interest in the presence of other competing proteins in both bacterial and mammalian cells. Prenylation by farnesyltransferase can be combined with the immobilization capabilities of hydrazone ligation chemistry to isolate proteins through use of specific CaaX sequences.

This work herein aims to demonstrate the expansion of a previously designed approach to bioorthogonal protein labeling, making it applicable across different systems, specifically in both bacterial and mammalian cells. Using the combined techniques of a selective modification, like prenylation, and an immobilization technique, such as a hydrazide bead pulldown, a new bioorthogonal pulldown technology can be used to isolate proteins of interest that may be difficult

to otherwise obtain. More specifically, isolation of proteins of interest can be achieved through use of site-specific modifications involving engineered FTase variants and functionalized FPP analogues with subsequent immobilization through hydrazone ligation chemistry. Modification of the specific protein of interest with a non-natural CaaX sequence can act as a C-terminal tag to allow for immobilization in the presence of natural, competing proteins in the cell, not recognized by RL FTase.

a)



b)

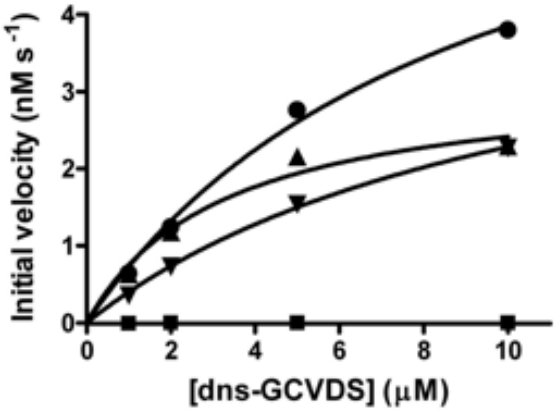


Figure 3.3 The FTase active site and its reactivity. (a) Structure of a peptide substrate bound to FTase with emphasis on interactions with the a_2 binding pocket. The a_2 residue of the peptide substrate (*green*) is surrounded by residues Trp-102β (*orange*), Trp-106β (*red*), and Tyr-361β (not shown) within the active site of FTase. The $a_2$residue also contacts the isoprenoid tail of the FPP analogue inhibitor FPT-II (purple). The figure was derived from PDB ID 1D8D and adapted from Reid *et al.*[30] (b) Initial velocity for reaction with dns-GCVDS is shown as determined by a fluorescence enhancement assay; *squares*, WT FTase; *circles*, W102R/W106L variant; *triangles*, W102L/W106L variant; *inverted triangles*, W106L/W106K variant. The "RL" FTase variant displays preference for noncanonical dns-GCVDS over the endogenous dns-GCVLS. These figures have been reused with permission from reference 29 (Appendix VII).

3.2 RL FTase farnesylation reactivity with dns–GCVDS and dns–GCVLS peptide substrates

As stated in section 3.1, unpurified RL FTase in crude bacterial lysate shows minimal reactivity with a fluorescently labeled dns–GCVLS peptide substrate in comparison to reactivity with dns-GCVDS peptide in reaction with the natural FPP prenyl donor cosubstrate.[29] This preference was found to hold true in reactions in which functionalized FPP analogues (Figure 3.2) were used in place of non-functionalized FPP prenyl donor, with the aryl aldehyde FPP (**2**) showing the greatest reactivity and discrimination between the two peptides (J. Hougland, unpublished data). However, once purified, RL FTase exhibited comparable reactivity with both the dns-GCVLS and dns– GCVDS peptide substrates (Figure 3.4). To determine the origin of this change in relative reactivity between reactions using purified enzyme and enzyme in crude lysates, components used to grow and lyse the bactreria in the original lysate studies were titrated into reactions using purified RL FTase to determine the impact of each component on enzyme substrate selectivity. Components included in these titrations were total bacterial lysate, ampicillin, luria broth (LB) media, and Fast Break lysis reagent (Promega). It was determined that Fast Break (FB) lysis reagent used to lyse cells in the original activity tests decreases reactivity of RL FTase with the dansyl–GCVLS peptide, while maintaining its reactivity with dns-GCVDS, as determined by HPLC analysis and product peak integration (Figure 3.5a). While the specific component of Fast Break (FB) reagent responsible for altering RL FTase selectivity remains unknown, this findings enables optimization of reaction conditions necessary for selective prenylation of –GCVDS tagged proteins *in vitro* using purified components. When WT FTase was tested under the same conditions, it was found that WT FTase reactivity with the dns-GCVLS peptide is not affected as greatly by FB lysis reagent as is the RL FTase (Figure 3.5b).

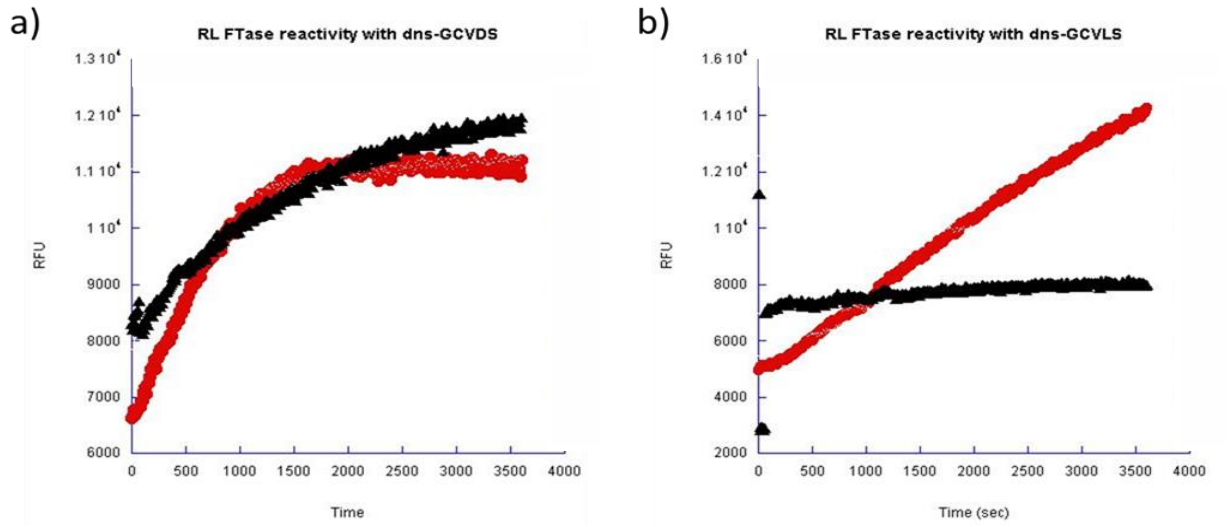
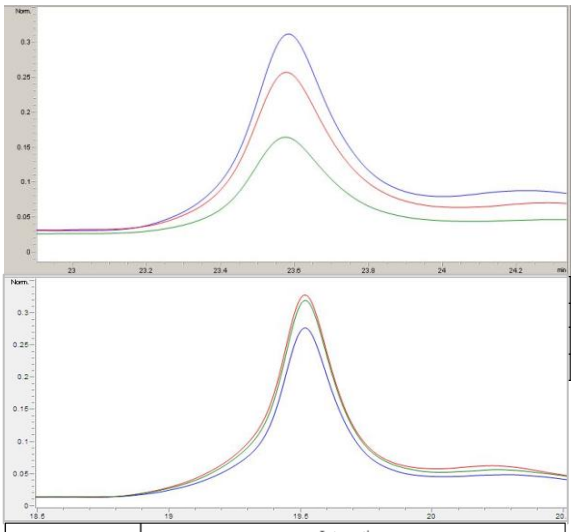


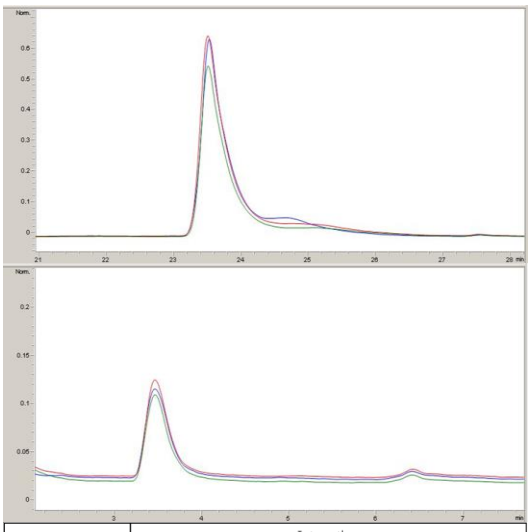
Figure 3.4 RL FTase reactivity with dns-GCVLS changes after purification of enzyme. (a) Reactivity of RL FTase with dns-GCVDS using purified RL FTase (red) or FTase in bacterial cell lysate (black); (b) Reactivity of RL FTase with dns-GCVLS using purified RL FTase (red) or FTase in bacterial cell lysate (black); Reactions conditions include 50 nM RL FTase (or 10 µL cell lysate), 3 µM peptide, 5 mM $MgCl_2$, 50 mM NaHEPPSO (pH 7.5), and 5 mM TCEP.

a)



Reaction	Integration		
	2 µL	5 µL	10 µL
RL FTase with dns-GCVLS	5.93	3.85	3.44
RL FTase with dns-GCVDS	7.41	6.85	6.42

b)



Reaction	Integration		
	2 µL	5 µL	10 µL
WT FTase with dns-GCVLS	14.7	13.4	12.9
WT FTase with dns-GCVDS	1.48	1.45	1.44

Figure 3.5 Fast Break Reagent affects the farnesylation selectivity of the RL FTase variant. a) Effects of Fast Break lysis reagent on the activity of RL FTase with dns-GCVLS or dns-GCVDS and endogenous FPP. Varying amounts of reagent, 2, 5, and 10 uL, were titrated into a reaction of 50 nM RL FTase, 3 µM peptide, and 20 µM FPP. Blue, 2 µL; Red, 5 µL; Green, 10 µL – Product peak shown at 23.5 min and 19.5 min for dns-GCVLS and dns-GCVDS, respectively. b) WT FTase activity with dns-GCVLS is not affected as greatly by addition of Fast Break reagent. In the presence of FB reagent, dns-GCVLS is still nearly all converted to product (product peak shown at 23.5 min) No product peak is detectable for WT FTase with dns-GCVDS (substrate peak shown at 3.5 min).

Characterization of the reactions catalyzed by purified RL FTase with dns-GCVLS and dns-GCVDS using either endogenous FPP or aryl aldehyde FPP (**2**) is aided by steady-state kinetic analysis of these reactions. Steady state characterization can be pursued using a previously developed fluorescence-based assay for FTase-catalyzed peptide prenylation which utilizes the environmentally sensitive dansyl fluorophore.[13, 31-33] However, initial reactions using the FPP analogue (**2**) indicated that this analogue could not be successfully support this assay due to the low fluorescence enhancement obsereved upon peptide modification with this FPP analogue. Consequently, steady state kinetic parameters (k_{cat}/K_m) for reactions with FPP were determined for WT FTase with dns-GCVLS and for RL FTase with dns-GCVDS and dns-GCVLS (Table 1, Figure 3.6). In the presence of Fast Break reagent, steady state kinetic constants for prenylation of the dns-CVLS peptide by the RL FTase variant, as well as dns-CVDS peptide by WT FTase (both with and without Fast Break), could not be determined due to low or no evidence of reactivity. Catalysis of prenylation of the dns-GCVDS peptide by the RL FTase variant with the dns-CVDS peptide is comparable (within ~4-fold) to that for dns-CVLS farnesylation cataltyzed by WT FTase, suggesting that the RL FTase is sufficiently active to allow CVDS prenylation in biological samples.

Table 3.1 Steady-state kinetics for farnesylation of dns-GCVLS and dns-GCVDS catalyzed by WT and RL FTases.

		dns-GCVLS	dns-GCVDS
FTase variant	Fast Break	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat}/K_m ($M^{-1}s^{-1}$)
WT (WW)	+	7.2 +/- 2.4 x10^4	b.d.
WT (WW)	-	7.7 +/- 0.8 x10^4	b.d.
RL	+	b.d.	1.8 +/- 0.3 x10^4
RL	-	4.5 +/- 1.5 x10^3	5.1 +/- 1.0 x10^4

Steady state reactions were performed under the following conditions:100 nM FTase , 10 µM FPP, 0.5-10 µM peptide substrate (dns-GCVLS or dns-GCVDS), 5 mM $MgCl_2$, 50 mM NaHEPPSO, 5 mM TCEP. "b.d." denotes reactivity is below detectable limits.

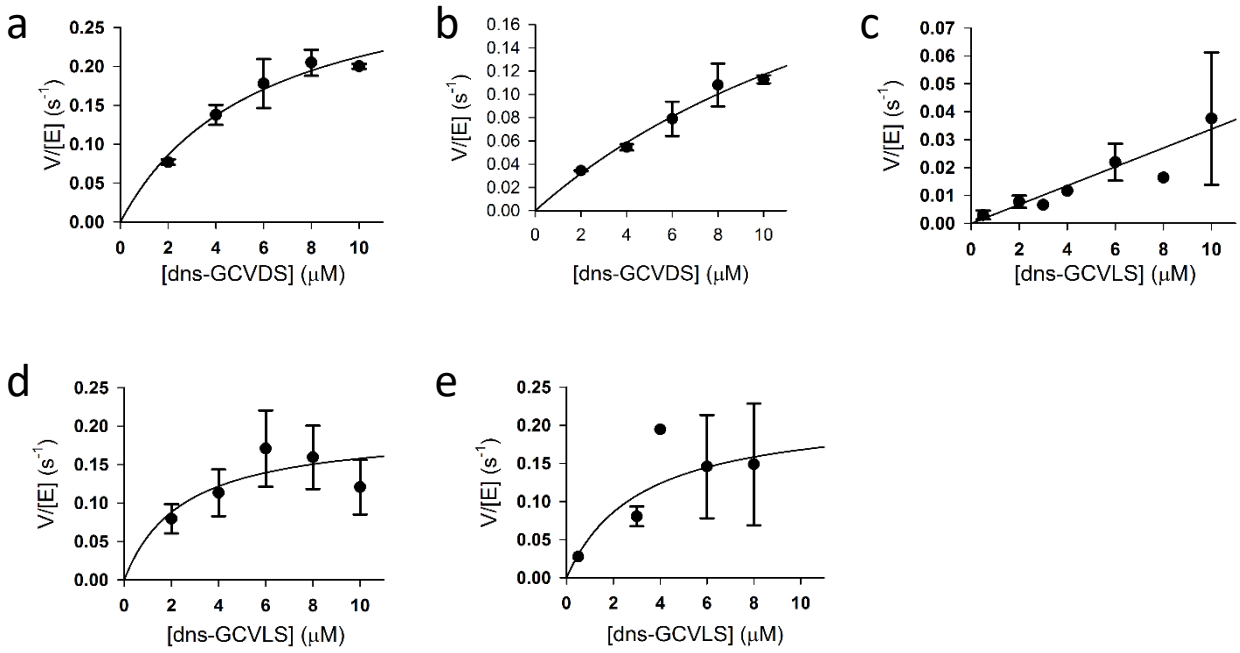


Figure 3.6 Steady state characterization of the reactivity of dns-GCVxS peptides with RL or WT FTase. a-c) Dependence of farnesylation activity on peptide substrate concentration catalyzed by RL FTase with and without Fast Break: a) dns-GCVDS; b) dns-GCVDS with Fast Break; c) dns-GCVLS; . d-e) Dependence of farnesylation activity on peptide substrate concentration catalyzed by WT FTase with and without Fast Break: d) dns-GCVLS; e) dns-GCVLS with Fast Break. The curve represents the best fit to the Michaelis-Menten equation. Reactions were performed and analyzed as described in Section 3.9.8. Error bars represent the standard deviation from a minimum of two replicates.

3.3 WT FTase and RL FTase-catalyzed prenylation of dns-GCVDS and dns-GCVLS peptides using an aryl aldehyde FPP analogue

To determine conditions for prenylation of full length proteins using FPP analogue (**2**) WT FTase and RL FTase were used to explore prenylation of both the GCVDS and GCVLS peptides using either FPP or FPP analogue (**2**). No activity was observed for WT FTase-catalyzed prenylation of the dns–GCVDS peptide when monitored by HPLC, while the RL FTase catalyzed complete modification of the dns–GCVDS peptide under the same conditions using the FPP analogue (**2**) at varying concentrations between 10 - 70 μM (Figure 3.7a, 10 μM FPP analogue (**2**) reaction shown). Under the same reaction conditions in the presence of the FB reagent, RL FTase catalyzed a low level of prenylation of the dns-GCVLS but did not completely modify this substrate (Figure 3.7b). To determine the optimum reaction time to maximize modification of the dns-GCVDS substrate while minimizing dns-GCVLS prenylation, a reaction time course was performed to monitor RL FTase-catalyzed prenylation of both peptide substrates with the FPP analogue (**2**). Prenylation of the dns-GCVLS substrate with FPP analogue (**2**) by the RL FTase is not detectible before approximately 3 hours reaction time as monitored by HPLC, whereas modification of the dns-GCVDS peptide is more rapid with evidence of prenylated product by 1 hour (Table 3.2). This time course allows us to optimize our reaction time, increasing the amount of dns–GCVDS prenylated with FPP analogue (**2**) while minimizing prenylation of dns–GCVLS.

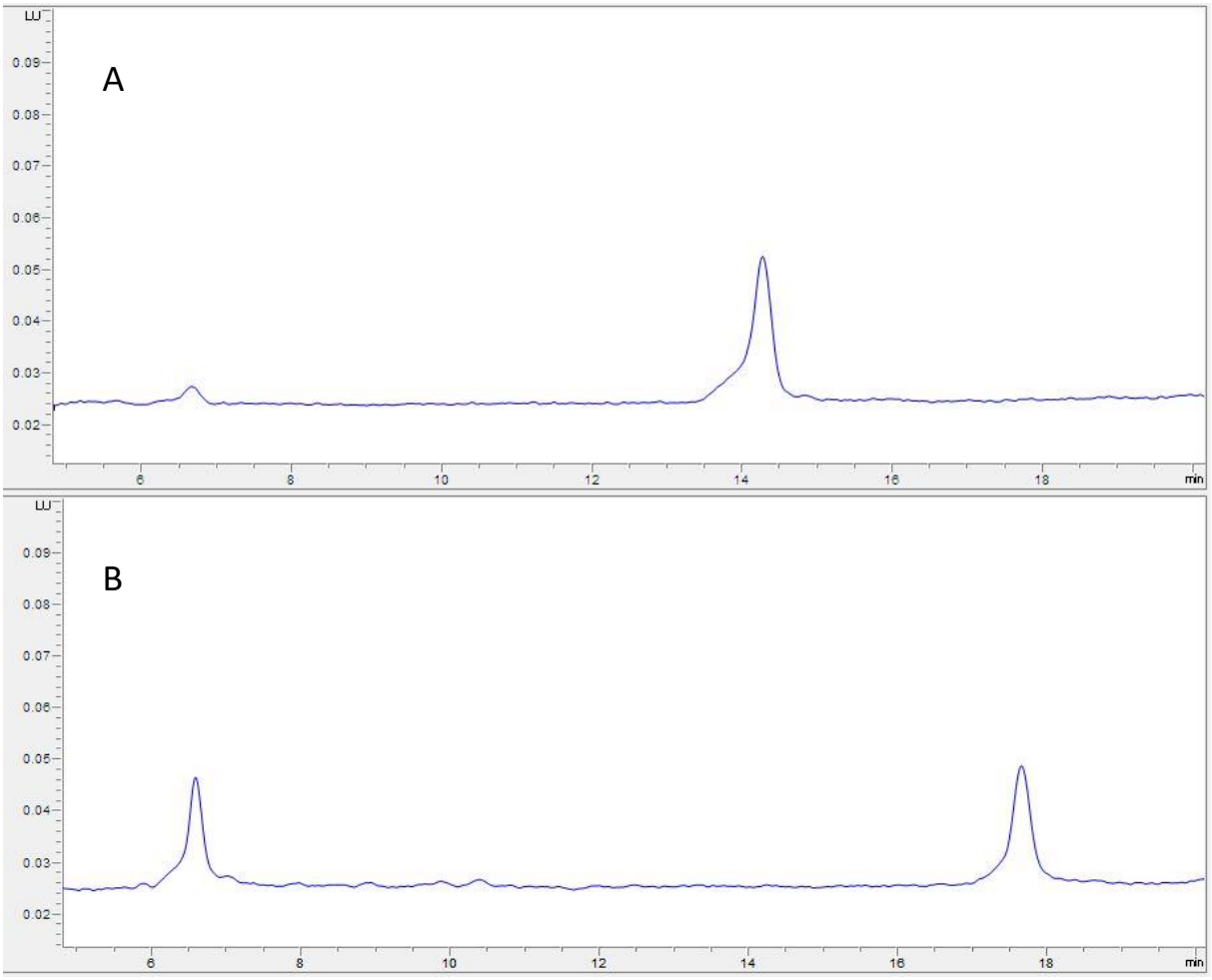


Figure 3.7 RL FTase-catalyzed prenylation of dns-GCVDS and dns-GCVLS in the presence of 10 μM FPP analogue (2). (A) Prenylation reaction with dns-GCVDS; product peak is observed at 14.3 min retention time. (B) Reaction with dns-GCVLS; product preak is observed at 17.6 min retention time with unreacted peptide substrate detected at 6.6 min.

Table 3.2 Comparative extent of reaction for RL FTase-catalyzed prenylation with dns-GCVDS and dns-GCVLS

	Peak Integrations			
Reaction Time (min)	CVLS substrate	CVLS product	CVDS substrate	CVDS product
10	0.724	n.d.	0.415	n.d.
30	0.796	n.d.	0.312	n.d.
60	0.990	n.d.	0.269	0.290
120	0.799	n.d	0.166	0.346
180	1.129	0.061	0.157	0.456
300	0.781	0.109	0.066	0.622

Dns-GCVDS reacts >90% with RL FTase and FPP analogue (**2**) within 5 hours while prenylation of dns-GCVLS is only detectible after 3 hours. "n.d." denotes no detection of product peak. Reaction conditions: 100 nM FTase , 10 µM analogue (**2**), 3 µM peptide substrate (dns-GCVLS or dns-GCVDS), 5 mM $MgCl_2$, 50 mM NaHEPPSO, and 5 mM TCEP.

3.4 Examining RL FTase selectivity between dns–GCVDS and dns– GCVLS peptide substrates in direct competition reactions

While the studies described above provided insight into the substrate specificity of RL FTase in the presence of functionalized FPP and differing peptides, these reactions did not directly examine the ability of RL FTase to selectively catalyze prenylation of the dns-GCVDS substrate in the presence of competing peptides. As these peptides have distinct retention times on HPLC in both their unmodified and prenylated forms, prenylation of both peptides can be monitored simultaneously in reactions containing both peptides in direct competition for prenylation by RL FTase. This competition experiment more closely resembles the reaction that will take place in cell lysates when attempting to isolate a protein of interest.

In the presence of both peptides substrates at equal concentrations (3 μM), RL FTase preferentially catalyzed prenylation of the dns-GCVDS peptide in the presence of FPP analogue (**2**). No prenylated product from reaction with the dns–GCVLS peptide was observed until after 8 hours reaction time (Table 3.3). Modification of the dns–GCVDS peptide was observed within 30 minutes (chromatograms for 30 min and 8 hour reaction time points provided in Figure 3.8). In competition for RL FTase, dns-GCVDS is more active than dns–GCVLS and an optimal incubation time for the prenylation reactions for isolation of target proteins was experimentally determined.

Table 3.3 Competition for farnesylation between dns-GCVDS and dns-GCVLS

	Peak Integrations			
Reaction Time (min)	CVLS substrate	CVLS product	CVDS substrate	CVDS product
30	1.95	n.d.	0.92	0.12
60	2.31	n.d.	1.12	0.14
120	2.01	n.d.	0.80	0.23
180	1.96	n.d	0.66	0.31
240	2.15	n.d.	0.43	0.40
300	2.14	n.d.	2.15	1.13
480	1.45	0.05	0.25	0.62
960	6.67	0.11	n.d	0.61

In competition, dns-GCVDS shows evidence of prenylation by RL FTase and aryl aldehyde FPP analogue as early as 30 min while dns-GCVLS prenylation is not observed until 8 hours. Reaction conditions: 100 nM FTase , 10 µM FPP analogue (**2**), 3 µM peptide substrate of each, dns-GCVLS and dns-GCVDS, 5 mM $MgCl_2$, 50 mM NaHEPPSO, and 5 mM TCEP.

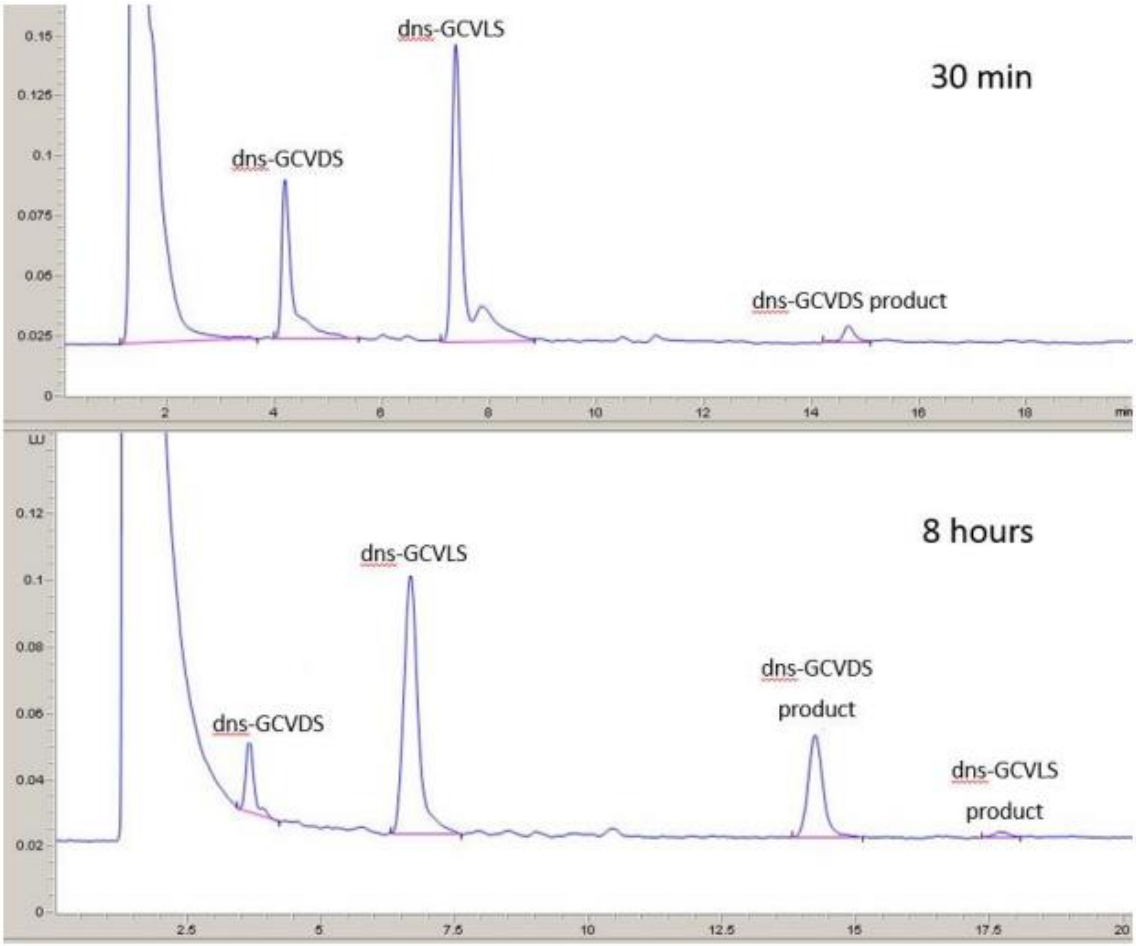


Figure 3.8 HPLC analysis of dns-GCVDS and dns-GCVLS direct competition prenylation reactions with RL FTase. Retention times are as follows: dns-GCVDS unreacted – 4.1 min (30 min), 3.6 min (8 hours); dns-GCVLS unreacted – 7.3 min (30 min), 6.7 (8 hours); dns-GCVDS product – 14.6 min (30 min), 14.2 min (8 hours); dns-GCVLS product – 17.7 min (8 hours) Large peak at ~2 min is due to buffer and is present in buffer-only control injection.

3.5 Design and expression of fluorescent reporter proteins for validating FTase variant protein labeling

To confirm the ability of engineered FTase variants to catatlyze modifications of folded proteins, TagRFP (red fluorescent protein) and eGFP (green fluorescent protein) fluorescent proteins were designed to serve as target and competitor proteins for RL FTase-catalyzed prenylation (Figure 3.9). Fluorescent proteins allow for facile visual monitoring of protein expression and for probe protein detection during and after protein pulldown. Use of both eGFP and TagRFP as target and competitor proteins, respectively, allows for a direct measure of not only the amount of target protein being immobilized (via fluorescence) but as an easy means of measuring the amount of any competitior protein which may be labeled and captured as well.

To serve as probes for FTase activity, these proteins required addition of a C-terminal"CaaX" motif through PCR and subsequent cloning. As stated previously, the engineered RL FTase variant recognizes a -GCVDS target sequence, and a –GCVLS sequence serves as a competitor sequence representing natural FTase substrates. As a negative control, a probe protein with a–GSVDS C-terminal sequence was also designed. In this protein, replacement of the "CaaX" motif cysteine with a serine blocks prenylation by removing the thiol sidechain required for the thioether bond present in prenylated proteins. The reporter protein sequences TagRFP-GCVDS, TagRFP-GCVLS, and TagRFP-GSVDS proteins were cloned into the CDFDuet-1expression vector, which contains a streptomycin resistance gene, while the eGFP-GCVDS protein was cloned into pJExpress-414 vector which carries ampicillin resistance. (Vectors were a gift from Mark DiStefano, University of Minnesota.) Use of these two expression vectors allows for probe protein expression in separate samples alone or as a co-expression within the same cells using simultaneous selection with both ampicillin and streptomycin. Each bacterial expression vector

also contains a 6x-HisTag upstream of each cloning site for future purification of the probe proteins proteins, if needed. The sequences of the final expression vectors were confirmed through DNA sequencing (GenScript, Inc).

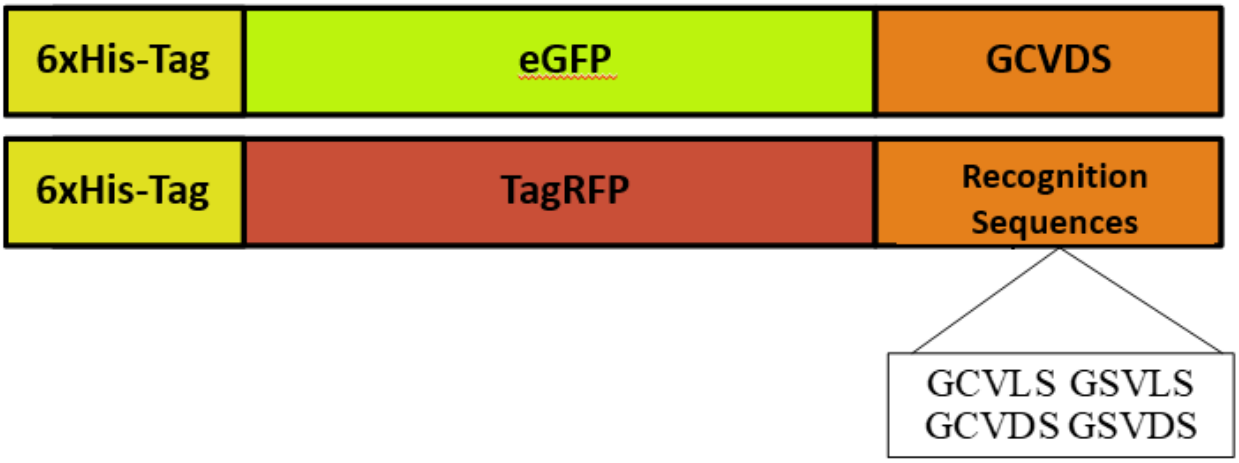
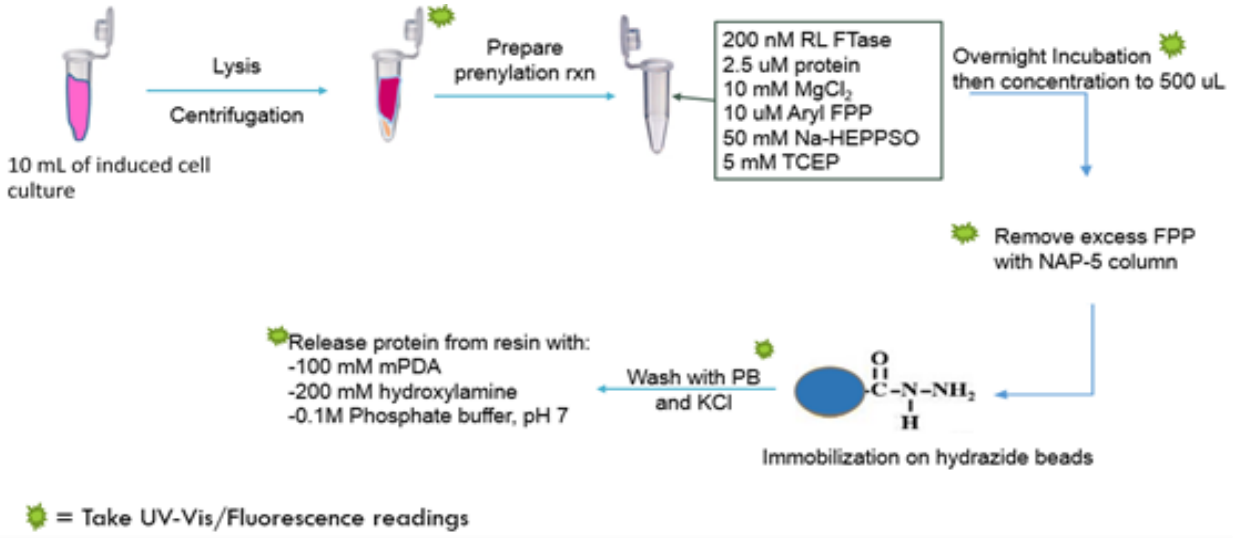


Figure 3.9 Design of protein probes for bioorthogonal labeling using RL FTase and FPP analogues. The target protein was designed as eGFP while competitor and negative control sequences were designed into TagRFP.

3.6 Isolation of –GCVDS tagged protein on hydrazide beads from bacterial lysates via hydrazone ligations

Two approaches were used for isolation of C-terminally tagged -GCVDS proteins from bacterial lysates using RL FTase and FPP analogue (**2**). The first utilizes the pulldown of the aldehyde-functionalized proteins using hydrazide-functionalized agarose beads, as described in section 3.1.[15] A general overview of the immobilization is provided in Scheme 3.3.

During the immobilization and release reactions, measurements of both UV-Vis absorbance (555 nm and 488 nm for TagRFP and eGFP, respectively) and fluorescence (excitation/emission wavelength of 555/585 or 488/515 for TagRFP and eGFP, respectively) were taken at each step during the procedure to track the fluorescent protein, determine potential protein loss, and observe evidence of immobilization/release from the hydrazide beads. Absorbance measurements of protein immobilized to the hydrazide beads were found to be non-reproducible due to settling of the beads and high background arising from light scattering. Initial immobilization attempts were performed using varying initial reaction volumes of 500 µL, 1 mL, or 10 mL prenylation reactions containing 2.5 µM of fluorescent protein in bacterial lysates based on UV-Vis absorbance of the lysate. These reactions are then concentrated to 500 µL, if necessary, before being run through a NAP-5 gravity flow desalting column (GE Healthcare) to remove excess FPP analogue (**2**) via gel filtration to avoid competition of protein immobilization to the hydrazide resin by free aldehyde analogue. It was found that 10 mL reaction volumes produced enough protein to efficiently track immobilization as described below.



Scheme 3.3 A general overview for the immobilization of aldehyde-functionalized prenylated proteins. PB = phosphate buffer, KCl = 50 mM potassium chloride

Hydrazone bond formation between the prenylated protein and resin was catalyzed with the addition of m-phenylenediamine, consistent with literature reports.[34] Protein immobilization by hydrazone formation was followed by washing of the column with phosphate buffer and KCl to remove nonspecifically bound protein, before removal the resin from the column for analysis and subsequent release of the immobilized protein.

While tracking fluorescence during the procedure, a small amount of fluorescence was detected on the hyrdrazide resin after immobilization. To confirm that these small fluorescence readings were evidence of protein immobilization, negative controls were performed through several different immobilization conditions in which WT FTase, endogenous FPP, or –GCVLS tagged protein replaced the use of RL FTase, aryl FPP analogue, or –GCVDS tagged protein, respectively. Under these conditions, no immobilization on hydrazide resin should be observed as indicated by presence of the target protein in the flow-through and PB or KCl washes with no fluorescence detection on the resin. UV-Vis and fluorescence data confirmed that all protein loaded onto the hydrazide resin was present in the flow-through or wash steps in these controls, with no protein being detected on the resin (Table 3.4).

There was evidence of target protein immobilization using this method, as evidenced by a positive fluorescence reading at 555 nm when testing the resin for the presence of TagRFP. However, the technique was found inefficient with only about 50% of the total target protein found to be immobilized and released (Example #1, Table 3.5). Two hypotheses were tested as to why the immobilization was not more efficient. A second pulldown (Example #2, Table 3.5) tested the hypothesis that the amount of hydrazide resin used may not have a sufficient binding capacity to capture a large amount of the target protein. This theory was tested by doubling the amount of hydrazide resin used for immobilization, from 300 µL to 600 µL. A third pulldown (Example #3,

Table 3.4 Control experiments for protein immobilization using hydrazide resin

Pulldown Step	Total Protein		
After lysis	n.d.	n.d.	n.d.
After O/N reaction	40 µg	37.7 µg	38.1 µg
After NAP-5	30.8 µg	26.6 µg	25.6 µg
Conc. To 200 µL	n/a	n/a	n/a
Flow-through after incubation on resin	11.8 µg	13.2 µg	13.0 µg
PB wash	8.1 µg	6.1 µg	6.5 µg
KCl wash	1 µg	1.0 µg	1.0 µg
Hydrazide Resin	0 µg	0 µg	0 µg
Control:	WT FTase	FPP	-CVLS

Controls include (left to right): Use of WT FTase with TagRFP-GCVDS and aryl aldehyde FPP (**2**), RL FTase with FPP and TagRFP-GCVDS, and RL FTase with aryl aldehyde FPP (**2**) and TagRFP-GCV**L**S. Total protein amounts were calculated by first using Beer's Law (ε=Abc) at 555 nm for TagRFP to determine molar concentration. The concentration was then converted to total protein using the volume of the sample and the MW of TagRFP-GCVDS (28 kDa); n.d. = not determined

Table 3.5) tested the hypothesis that the reaction volume was too large for efficient prenylation. The theory was tested by performing the reaction on a smaller scale with the same total protein through the use of 10 - 1 mL reactions in low-adhesion tubes before combining them into the 10 mL total volume after overnight reaction. In all three trials under these differing conditions, immobilization and release of the aldehyde-functionalized TagRFP-GCVDS protein was observed via fluorescence (Table 3.5).

When comparing the three different pulldown conditions, pulldown #2 results were comparable to those previously observed in pulldown #1 with a recovery of 0.04 mg protein. A small improvement in pulldown #3 was observed with recovery of 0.07 mg from an initial 0.31 mg protein but much of the sample was still being lost after immobilization in the flow through.

After evidence of successful isolation and release in pulldown #1 as reflected UV-Vis and fluorescence detection of TagRFP, it was decided to track UV-vis absorbance measurements at time points throughout pulldowns #2 and #3 that would monitor the progress of immobilization and release of the protein (Figure 3.10). A decrease in protein concentration in the supernatant was observed during the first thirty minutes of the two hour immobilization as the protein was isolated to the resin. Release of the protein was observed to occur more slowly over the course of 1-3 hours in both of the pulldown/release reactions monitored.

As a complement and verification for the spectroscopic measurements of protein release described above, the supernatant from the resin release reactions was analyzed by gel electrophoresis followed by Coomassie staining to detect total protein and anti-His_6 Western blotting to specifically detect the target protein containing a His_6 affinity tag. These analyses provided evidence of release of the immobilized protein, but the detection of released target

Table 3.5 Tracking of total protein throughout three pulldowns.

	Pulldown Step	Total Protein		
	After Lysis	n.d	n.d	n.d
	After o/n rxn	0.30 mg	0.69 mg	0.50 mg
	Filtration of sample	0.28 mg	0.53 mg	0.38 mg
	Conc. to 500 uL	0.12 mg	0.36 mg	0.31 mg
Immobilization	After NAP-5	0.12 mg	0.33 mg	0.31 mg
	Flow through	0.06 mg	0.12 mg	0.13 mg
	PB Wash	0.02 mg	0.08 mg	0.05 mg
	KCl Wash	0 mg	0.01 mg	0 mg
	Hydrazide resin	n.a	n.a	n.a
Release	Release Supernatant	0.04 mg	0.04 mg	0.07 mg
	Resin after release	n.a	n.a	n.a
		#1	**#2**	**#3**

Total protein amounts were calculated by first using Beer's Law (ε=Abc) at 555 nm for TagRFP to determine molar concentration. The concentration was then converted to total protein using the volume of the sample and the MW of TagRFP-GCVDS (28 kDa); n.d. = not determined, n.a. = not applicable

protein was inconsistent between trials (Figure 3.11). Although UV-Vis absorbance and fluorescence readings provide evidence for successful release of protein in all three trials, , no bands consistent with the target protein were present in the “release” lane of the Coomassie stained gel or Western blot for pulldown #3. With these inconsistent results, the question arises as to whether the issue of immobilization and release lies in the immobilization of the protein on the resin or in the ability of RL FTase to prenylate a full length protein with enough efficiency to allow subsequent labeling and isolation. Chapter 4 answers the latter question through development of a new technique for detecting protein lipidation. This work aimed and was successful in showing successful and complete prenylation of eGFP-GCVDS by RL FTase under multiple conditions.

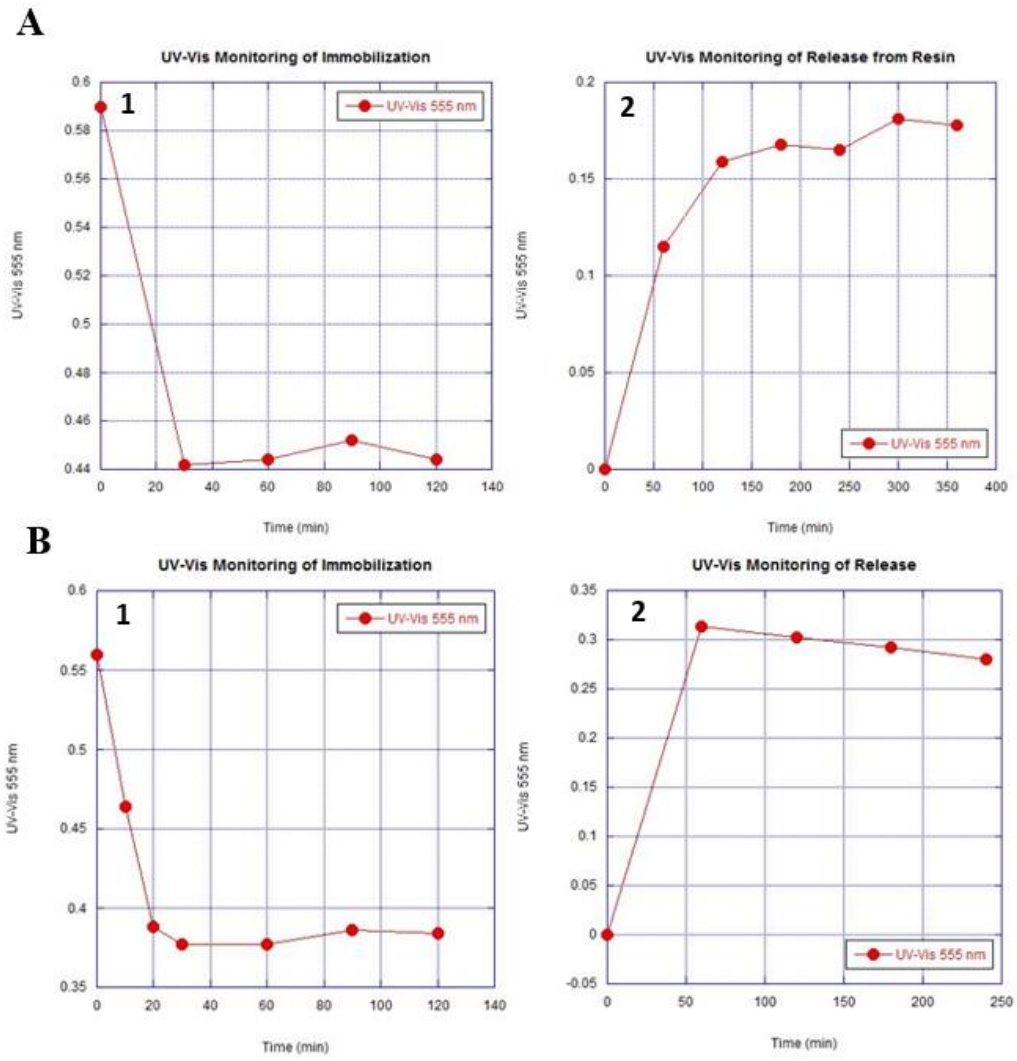


Figure 3.10 Monitoring of isolation and release of His_6-TagRFP-GCVDS via UV-Vis absorbance at 555 nm over time. A. Pulldown #2, B. Pulldown #3; 1) immobilization, 2) release

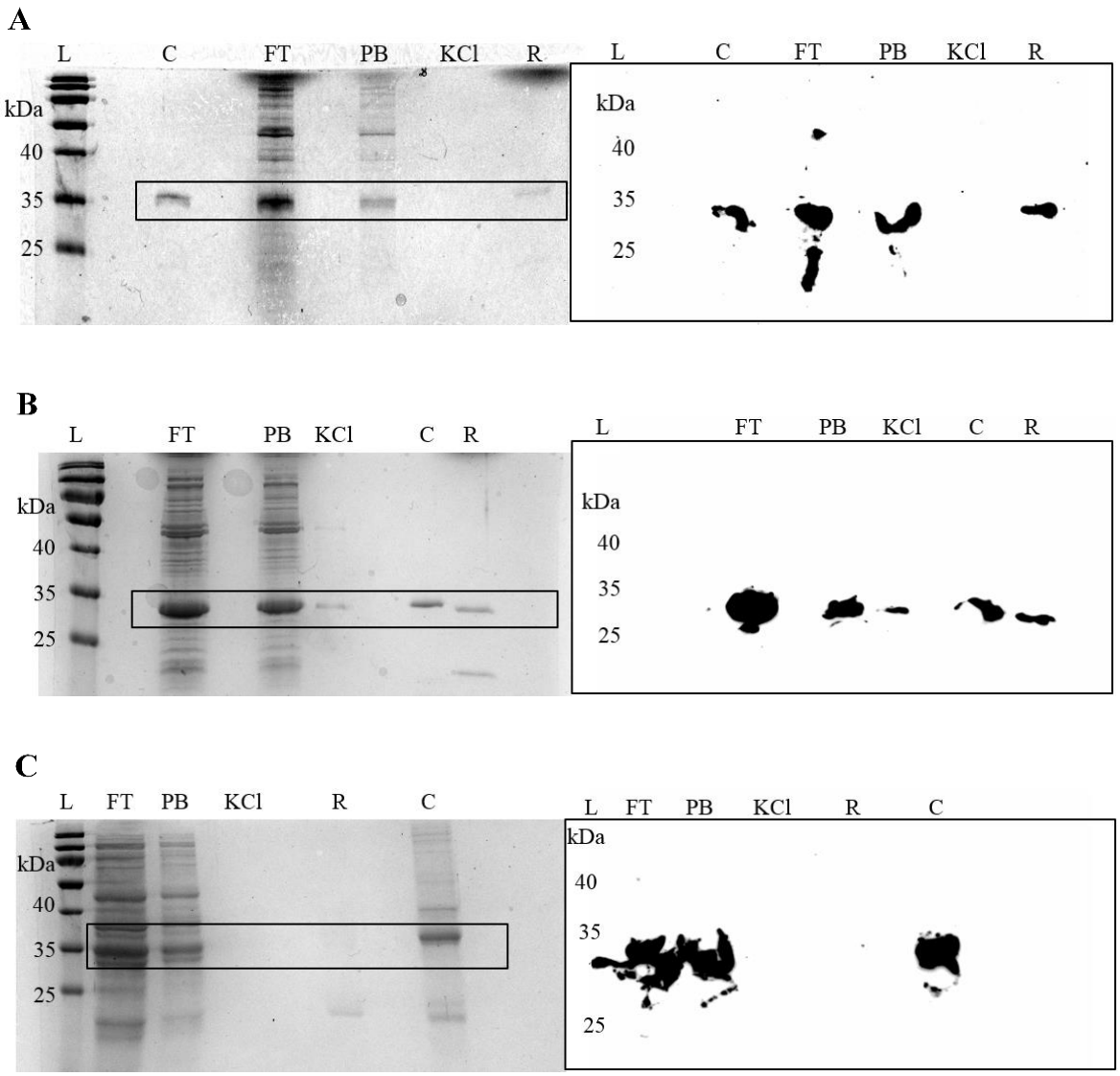
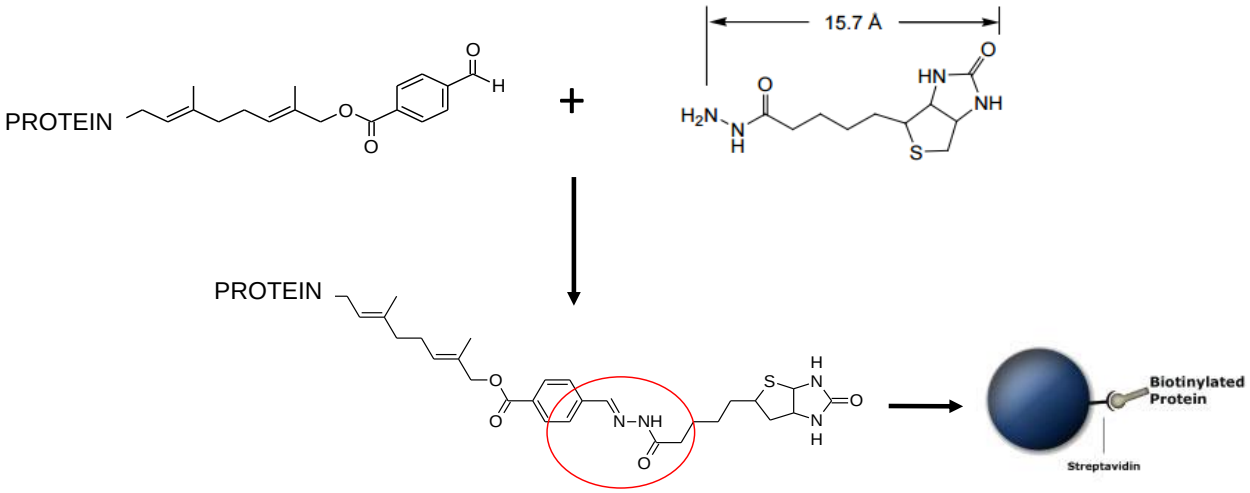


Figure 3.11 Coomasie staining and anti-His Western Blot analysis of hydrazide immobilization of His_6-TagRFP-GCVDS. A) Pulldown #1, B) Pulldown #2, C) Pulldown #3 (see Table 2) Legend: (C) His_6-TagRFP control, (FT) flow-through after immobilization reaction, (PB) PB wash of resin, (KCl) KCl wash of resin, (R) supernatant after release of protein from resin. His_6-TagRFP-GCVDS = 28 kDa

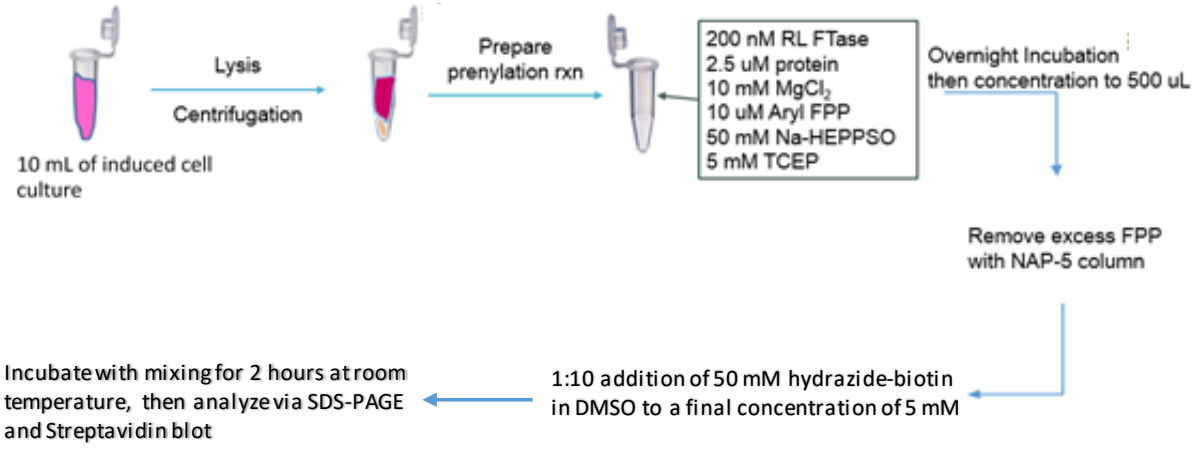
3.7 Streptavidin beads and biotin-hydrazide as an alternative pulldown system

While the use of hydrazide beads to isolate functionalized prenylated protein shows promising results, inconsistency between trials led us to another alternative method for use of biorthogonal selectivity and subsequent pulldown of target proteins. This alternative utilizes biotin-streptavidin interactions through use of a hydrazide-linked biotin and streptavidin beads (Scheme 3.4). The interaction between biotin and streptavidin has one of the highest reported affinities with a K_d of ~10^{-14} M.[35] While this interaction is a popular tool for protein isolation,[36-38] this high affinity leads to issues in retrieving proteins after binding to the streptavidin beads used for isolation. Using hydrazone ligation chemistry, functionalized protein with FPP analogues can be retrieved from streptavidin beads using hydroxylamine cleavage of the hydrazone bond. This will leave the hydrazide-biotin still bound to the streptavidin but release the protein with the FPP analogue "tag" intact. To release the immobilized protein, hydroxylamine is added to the resin to reverse the hydrazone bond via oxime formation with m-PDA being used as a catalyst.

As done previously for pulldowns with hydrazide beads, fluorescent fusion proteins TagRFP-GCVDS or eGFP-GCVDS were used to monitor and track pulldown and release of functionalized protein. Readings of both UV-Vis absorbance (555 nm and 488 nm for TagRFP and eGFP, respectively) and fluorescence (excitation/emission wavelength of 555/585 or 488/515 for TagRFP and eGFP, respectively) were taken at each step during the procedure to track the fluorescent protein, determine potential protein loss, and observe evidence of immobilization/release from the streptavidin beads (Scheme 3.5). Absorbance measurements of protein immobilized to the streptavidin beads were found to be non-reproducible due to settling of the beads and high background arising from light scattering, resulting in only fluorescence measurement being used for monitoring of bead-bound proteins.



Scheme 3.4 Hydrazide-biotin labeling of functionalized protein allows for isolation on streptavidin beads Hydrazide bond is shown in red and streptavidin bead is shown in blue.



Scheme 3.5 Immobilization of target proteins using aryl aldehyde FPP (2) and hydrazide-biotin.

Using bacterial lysates, prenylation with FPP analogue (**2**) was performed as previously described above and as shown in the flow chart above. Biotinylation was performed using 1 mL total volume prenylation reactions with 2.5 µM of fluorescent protein based on UV-Vis absorbance of the lysate. For several trials of the biotin-streptavidin pulldown protocol, samples of TagRFP-GCVDS were taken at each step in the biotinylation process for both fluorescence analysis as before and detection via SDS-PAGE gels using Coomassie stain and streptavidin-HRP blotting (Figures 3.12 and 3.13). In trial #1, TagRFP-GCVDS is present throughout the preparation and biotinylation process evidenced by bands at 28 kDa, visualized by Coomassie stain. Exposure of proteins to streptavidin-HRP after transfer from SDS-PAGE gels to a PVDF membrane and blocking with 3% BSA in TBST produces a band at the correct size in the biotinylation lane only. These two gels together provides evidence for retention of the target protein throughout prenylation with FPP analogue (**2**), NAP-5 desalting column, and biotinylation which resulted in the target protein biotinylated as evidence by lane 3 in the Streptavidin blot.

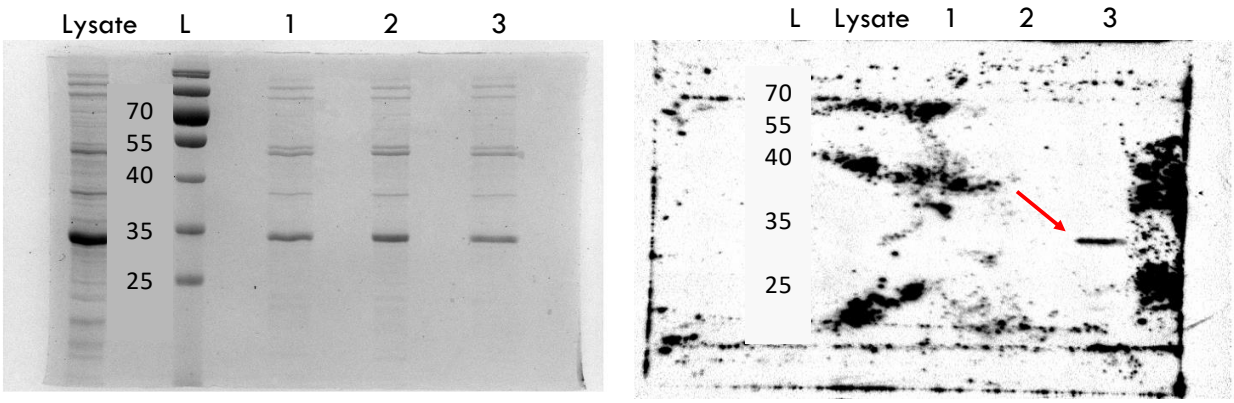


Figure 3.12 Results of TagRFP-GCVDS immobilization using biotin and streptavidin - Trial #1. SDS PAGE and Streptavidin blot analysis of TagRFP-GCVDS Lysate is bacterial lysate prior to prenylation, L) Ladder, 1) after overnight prenylation, 2) after elution on NAP-5 column, 3) after biotinylation. Red arrow denotes band believed to be biotinylated target protein. His_6-TagRFP-GCVDS = 28 kDa. Streptavidin-HRP = 1:10,000 dilution

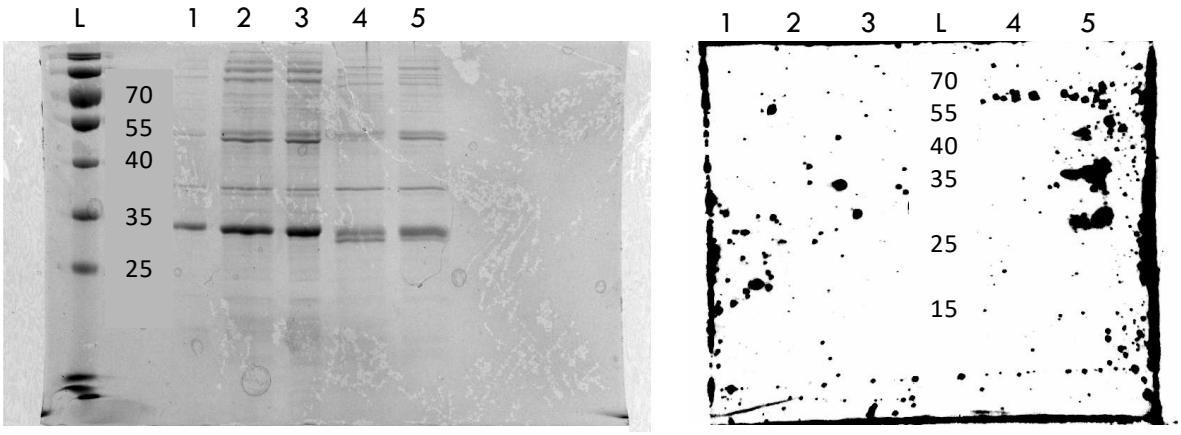


Figure 3.13 Results of TagRFP-GCVDS immobilization using biotin and streptavidin - Trial #2. SDS PAGE and Streptavidin blot analysis of TagRFP-GCVDS Lysate is bacterial lysate prior to prenylation, L) Ladder, 1) bacterial lysate prior to prenylation, 2) after overnight prenylation, 3) after concentration to 500 uL, 4) after elution on NAP-5 column, 5) after biotinylation. It is unclear if either of the two bands in lane 5 are the target protein. His_6-TagRFP-GCVDS = 28 kDa. Streptavidin-HRP = 1:20,000 dilution

In Trial #2, protein loss can be seen after elution from the NAP-5 column which coincides with loss of total protein in the fluorescence data of Table 3.2. Fortunately, sufficient protein is retained to visualize TagRFP-GCVDS before and after biotinylation. Unlike Trial #1, two bands are present in lane 5 of the Streptavidin blot for trial #2, which was performed with half the amount of Streptavidin-HRP as in Trial #1. The close proximity of the bands makes it difficult to determine which band represents an interaction with biotin or that biotinylation occurred in this trial. The appearance of two bands indicated that optimization of streptavidin blot conditions was needed before further analysis of biotinylated products during the streptavidin bead isolation and release of the target protein.

In the two trials above, 3% BSA in TBST was used to block PVDF membranes before exposure to streptavidin-HRP as per the manufacturer's recommendation. BSA is typically preferred over non-fat milk in streptavidin blots as it has been found that using the traditional non-fat milk blocking procedure can result in non-specific binding of streptavidin to the membrane. The two blots above were also incubated with streptavidin-HRP overnight at room temperature as opposed to incubation at 4˚C. To optimize the results of streptavidin-HRP blotting, several changes to these conditions were explored. Blocking solution was changed to 10% non-fat milk (NFM) in TBST with rocking at room temperature for 3 hours. The 1:10,000 dilution of streptavidin-HRP was repeated as in Trial #1 with incubation at 4˚C overnight. As shown in Figure 3.14, optimization of these two conditions yields clean, single bands for both previously run trials at the expected size of TagRFP-GCVDS after adjustment of both the blocking solution and streptavidin-HRP incubation temperature.

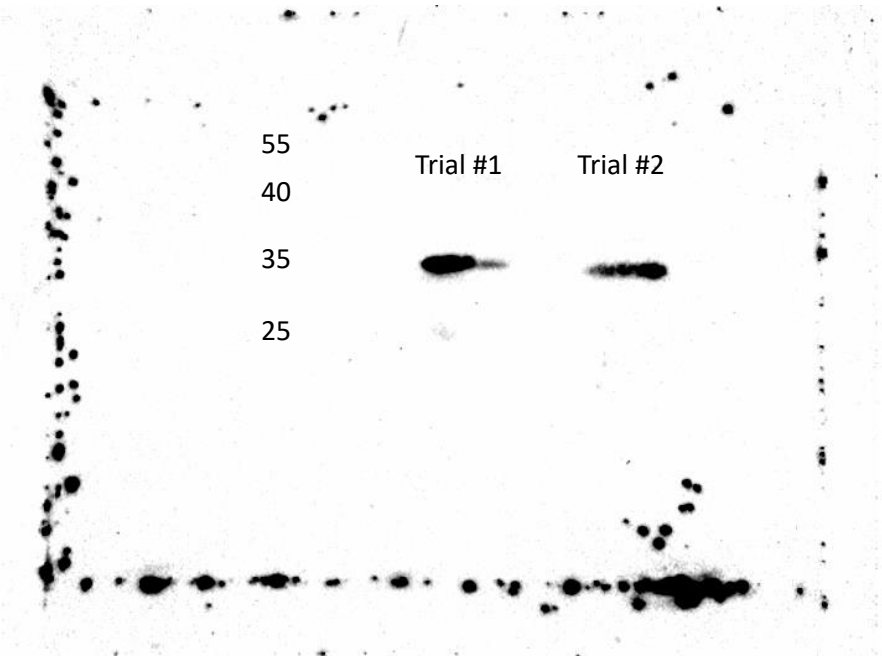


Figure 3.14 Optimization of streptavidin blot detection of TagRFP-CVDS following functionalization with biotin hydrazide. Trials 1 and 2 above were analyzed under new conditions to yield single bands at the appropriate size. His_6-TagRFP-GCVDS = 28 kDa. Streptavidin-HRP = 1:10,000 dilution

3.8 Conclusions

Prenylation presents a promising avenue for chemoselective protein immobilization. The use of a naturally occurring protein modification with the substrate specificity of a mutant enzyme would allow for precise protein immobilization with minimal endogenous protein interference during purification. While this technique has shown promising results, inconsistent success in release of immobilized protein makes this work difficult to bring to fruition at a usable level. With inconsistent results from both methods of protein isolation, the question is still raised as to whether or not these full length proteins are undergoing efficient prenylation with RL FTase and functionalized FPP. In Chapter 4 we describe a new technique for direct detection of full-length, prenylated proteins from both purified and cell lysate samples. This work confirms complete prenylation of our target proteins prior to immobilization and provides evidence that the issue of immobilization is not at the level of prenylation.

While this work describes a new avenue for bioorthogonal labeling of proteins in the presence of competitor proteins, further optimization with consistent results would be needed for this work to be considered at a level which is useful for protein research. Avenues of optimization include increasing the scale of total protein being used, altering the concentrations of m-PDA or hydroxylamine used in both the pulldown and release steps, and controlling for loss of protein during NAP-5 and concentration steps. If optimization is achieved, a target model protein should be chosen for isolation. The target protein should be one in which the C-terminal sequence can be varied to allow for the addition of the –GCVDS motif necessary for the chemoselective pulldown approach.

3.9 Materials and Methods

3.9.1 Purification of RL FTase using FPLC: Chemically competent BL21(DE3) *E. coli* were thawed on ice for 15 minutes. An aliquot of each plasmid containing the RL FTase α and β subunit genes (100 ng) was added to the cells (50 µL). Bacteria were incubated on ice for 30 min before heat shocking at 42ºC for 10 seconds with subsequent incubation on ice for 5 min. An aliquot (50 µL) of cells were then plated on warm LB-Amp plates were incubated overnight at 37°C. A colony was chosen from the plate and inoculated into a 5 mL culture of LB media with ampicillin (final concentration 100 µg/mL). Cultures were incubated and shaken at 225 rpm for 4 hours at 37°C. Cultures were then added to 1L rich media (20 g tryptone, 10 g yeast extract, 5 g NaCl, 1% glucose, and 100 µg/mL ampicillin) and grown at 37°C with shaking (225 rpm) until an OD_{600} of between 0.6-0.8 was reached as monitored using a NanoDrop 2000c spectrophotometer. Cultures were then induced with 0.5 mM IPTG. Cultures were grown 14 hours at 25ºC with shaking (225 rpm). Cells were harvested by centrifugation and resuspended in 50 mL buffer (50 mM HEPES – pH 7.8, 10 mM $ZnCl_2$, 2 mM TCEP). Cell resuspension was incubated at 37°C for 45 min in the presence of 0.625U/mL benzonase, 0.25 mg/mL lysozyme, and 17 µg/mL PMSF before incubation on ice. Lysis of cell culture was done using a Misonix Sonicator (Langford lab, Syracuse University) using 8 rounds of 30 seconds sonication with 30 seconds rest while on ice (pulse method). Lysates were centrifuged at 4°C and supernatant was transferred to a clean flask. At 4°C, precipitation of DNA was performed through addition dropwise of $1/10^{th}$ volume of 10% (w/v) streptomycin sulfate. Precipitation was incubated for 30 min with gentle stirring. Precipitated DNA was then separated from the sample through centrifugation at 4°C before clarification of the final sample though filtration with a 0.45 µm filter.

The prepared sample was purified using a AKTAprime Plus FPLC, first on a 15 mL HiTrap DEAE FF column (GE Healthcare) using 75 mL running buffer (50 mM HEPES – pH 7.8, 10 μM $ZnCl_2$, 2 mM TCEP) to equilibrate the column before addition of sample. A 30 mL wash was then done with running buffer before elution. Elution buffer (50 mM HEPES – pH 7.8, 10 μM $ZnCl_2$, 50 mM NaCl, and 2 mM TCEP) was used to elute the protein over the course of 300 mL elution with fractions containing protein then concentrated using a PALL Macrosep 10 MWCO concentrator to a volume of 2 mL following manufacturer's protocol. Concentrated sample was then desalted using a 10 mL HiTrap desalting column (GE Healthcare) with running buffer following the AKTAprime Plus FPLC desalt application template. Desalted sample (15 mL) was then loaded onto a 15 mL HiTrap Q sepharose column (GE Healthcare) with an equilibration with 75 mL running buffer before loading of the sample. The column was then washed with 30 mL running buffer before elution using 500 mL elution buffer into 7 mL fractions. The resulting product was then concentrated and desalted as described above before a final concentration was done to a volume of 2 mL. This sample was divided into 20 μL aliquots before being flash frozen with liquid nitrogen and storage at -80°C. Enzyme concentration was measured using absorbance at 280 nm using a NanoDrop 2000c spectrophotometer.

3.9.2 PCR mutagenesis of pJExpress-414_eGFP-GCVIA to eGFP-GCVDS: Forward and reverse primers for the isoleucine/alanine mutation to arginine/serine consisted of two point mutations and were synthesized by Integrated DNA Technologies. Primers were dissolved in 100 μL of ultra pure water and their concentrations were determined by UV absorbance of 260 nm using a NanoDrop 2000c spectrophotometer. The PCR reaction (50 μL) contained 10x PFU Turbo buffer (5 μL), 10mM dNTPs (1 μL), reverse primer(125 ng), forward primer (125 ng), and

template plasmid (10 ng). The reaction was mixed and centrifuged before adding PFU Turbo DNA polymerase (1 μL, 2.5U/μL). PCR mutagenesis was performed on a BioRad MyCycler under the following thermal program: Initial denaturation (95˚C, 1 min.); eighteen cycles of denaturation (95˚C, 50s), annealing (60˚C, 50s), and extension (68˚C, 12min); final extension (68˚C, 12min); and a final hold (10˚C, ∞). The reaction was then digested with DpnI (1 μL, 10U/μL) for 1 hour at 37ºC. 18 PCR mutagenesis reactions were transformed into chemically competent E.coli for plasmid isolation using the Z-competent transformation system (Zymo Research). Chemically competent DH5α E. coli were thawed on ice for 15 minutes. An aliquot of the PCR reaction (5 μL) was added to DH5α cells (50 μL) and incubated on ice for 30 min before plating cells on warm LB-Amp plates. Cells were incubated overnight at 37°C. Three colonies were chosen from the LB-Amp plate and inoculated into separate 5 mL culture of LB media with ampicillin (final concentration 100 μg/ml). Cultures were incubated overnight at 37°C with shaking (225 rpm). After incubation, glycerol stocks were made for each culture by adding 500 μL of cultured cells into 500 μL of 20% glycerol in water. These were stored at -80°C. The remaining plasmid DNA was then purified using BIO Basic Inc. EZ-10 Spin Column Plasmid DNA Minipreps Kit following the manufacturer's protocol. Site mutation from the sequence IA to DS was verified using DNA sequencing (Genscript, Inc).

3.9.3 PCR construction of TagRFP to include -GCVDS, -GCVLS, or -GSVDS C-terminal motifs and insertion into expression vectors: One round of PCR was performed for the extension of TagRFP in the TagRFP-N vector (Evrogen) with either –GCVDS, -GCVLS, or –GSVDS C-terminal motifs in the 3' primers along with HindIII restriction sites for CDF-Duet-1 cloning. The 5' primer included addition of the EcoRI restriction site for CDF-Duet-1 cloning For this PCR,

forward and reverse primers were designed by Dr. James Hougland and synthesized by Integrated DNA Technologies. Primers were dissolved in 100 μL of ultra pure water and their concentrations were measured using a NanoDrop 2000c spectrophotometer at a UV absorbance of 260 nm. The PCR reaction (50 μL) contained Standard OneTaq buffer (10 μL), 10mM dNTPs (1 μL), reverse primer (125 ng), forward primer (125 ng), and template plasmid (10 ng). The reaction was mixed and centrifuged before adding OneTaq DNA polymerase (0.25 μL, 5U/μL). PCR was performed on BioRad MyCycler under the following conditions: Initial denaturation (94°C, 1 min.); thirty cycles of denaturation (94°C, 30s), annealing (56°C, 1min.), and extension (68°C, 2min); final extension (68°C, 5min); and a final hold (10°C, ∞). After PCR, products were purified using BIO Basic Inc. EZ-10 Spin Column PCR Purification Kit following the manufacturer's instructions. A double digestion (20 μL) was then performed. The reaction consisted of 10x NEB buffer 4 (2 μL), 100x BSA (0.2 μL), plasmid DNA or PCR product that will act as insert (3 μg), and restriction enzymes depending on the vector being cloned into: EcoRI (1 μL, 20 U/μL) and HindIII (1 μL, 20U/μL) for ligation into CDF-Duet-1. The reactions were mixed and centrifuged prior to adding enzymes and the reactions were incubated for 2 hours at 37ºC. Digested DNA was then purified using 0.8% agarose gel (1x TAE buffer, 120 V). DNA was excised from the gel and purified using BIO Basic Inc. EZ-10 Spin Column DNA Gel Extraction Kit following the manufacturer's instructions. Ligation reactions (40 μL) contained: 2x quick ligase (20 μL), vector DNA (200 ng), insert DNA 20 (85.7 ng), and quick ligase (1 μL). Controls contained the same reaction components excluding the enzyme. The reaction was incubated at room temperature for 5 minutes then placed on ice before transformation. Chemically competent DH5α E. coli cells were thawed on ice for 15 minutes. An aliquot of the ligation reaction (5 μL) was added to the cells (50 μL) and kept on ice for 30 min. before plating. Cells were plated on prewarmed LB-Strep plates for CDF-

Duet-1 vector ligations. Plates were then incubated overnight at 37ºC. Three colonies were chosen from each plate and inoculated into separate 5 mL cultures of LB media with streptomycin (final concentration 100 μg/mL). Cultures were incubated and shaken at 225 rpm overnight at 37°C. After incubation, glycerol stocks were made for each culture by adding 500 μL of cultured cells into 500 μL of 20% glycerol in water. These were stored at -80°C. The remaining plasmid DNA was then purified using BIO Basic Inc. EZ-10 Spin Column Plasmid DNA Minipreps Kit following the manufacturer's protocol. Successful ligation was confirmed through an analytical digest (500 ng scale) using EcoRI and HindIII restriction enzymes (0.5 μL each) and the appearance of bands matching the size of the insert on 0.8% agarose gel (1x TAE buffer, 120V).

3.9.4 Expression of eGFP and TagRFP proteins in BL21(DE3) cells: Vectors described above were transformed into competent *E.coli* for plasmid isolation using the Z-competent transformation system (Zymo Research). Chemically competent BL21(DE3) 21 *E. coli* were thawed on ice for 15 minutes. An aliquot of each plasmid (100 ng) was added to the cells (50 μL). These were incubated on ice for 30 min before heat shocking at 42ºC for 10 seconds with subsequent incubation on ice for 5 min. SOC media (950 μL) was added to the cells and incubated at 37ºC for 1 hour with shaking (225 rpm). An aliquot (50 μL) of cells were then plated on warm LB-Strep plates (50 mg/mL streptomycin sulfate). Cells were incubated overnight at 37°C. A colony was chosen from each plate and inoculated into separate 5 mL cultures of LB media with streptomycin (final concentration 100 μg/mL for both). Cultures were incubated and shaken at 225 rpm overnight at 37°C. An aliquot of each culture (100 μL) was inoculated into 10 mL of fresh LB media containing streptomycin (final concentration 100 μg/mL) and incubated at 37ºC with shaking (225 rpm) until an OD_{600} of between 0.6-0.8 was reached as monitored using a NanoDrop

2000c spectrophotometer. Cultures were then induced with 0.5 mM IPTG (10 μL/mL of culture using a 500 mM IPTG stock solution). Cultures were then grown 14 hours at 28ºC with shaking (225 rpm). Aliquots of 1 mL cultures were then harvested and stored at -80˚C. SDS-PAGE and Coomasie staining confirmed the expression of target proteins in cells.

3.9.5 Titration of FB lysis reagent, ampicillin, and LB media into RL FTase reactions: Prior to reactions, dns-GCVDS or dns–GCVLS peptide (20 μM) was incubated in the presence of 1x reaction buffer (50 mM NaHEPPSO – pH 7.8. 5 mM TCEP) and 5 mM $MgCl_2$ (50 μL total) for 20 minutes. Additionally 0, 2, 5, 10, or 20 μL of either FB lysis reagent (1/10th diluted stock), amipicillin, or LB media were titrated into these reactions. After incubation, reactions were initiated with a reaction mixture (50 μL containing 100 nM RL FTase, 40 μM FPP, 5 mM $MgCl_2$, and 1x reaction buffer and incubated at RT for 14 hours before adding an equal volume of 20% acetic acid in isopropanol to stop the reaction. Reactions (100 μL) were analyzed via HPLC (Zorbax EclipseXDB-C18 column) using a linear gradient of 30:70 TFA in water (0.05%):acetonitrile (HPLC grade) to 0:100 TFA in water(0.05%):acetonitrile with a flow rate of 1 mL/min over 30 min. The column was then equilibrated for 2 min back to the original 30:70 ratio. Peptides and products were detected by fluorescence with excitation/emission of 340/496 nm.

3.9.6 Monitoring of RL FTase reactions with aryl aldehyde FPP analogue via HPLC: Prior to reactions, dns-GCVDS or dns–GCVLS peptide (6 μM) was incubated in the presence of 1x reaction buffer (50 mM NaHEPPSO – pH 7.8. 5 mM TCEP), 1/10th diluted FB lysis reagent (10 μL) and 5 mM $MgCl_2$ (50 μL total) for 20 minutes in 0.65 mL low-adhesion eppendorf tubes. For titration of the aryl aldehyde analogue, reactions were initiated with a reaction mix (50 μL)

containing 100 nM RL FTase or WT FTase, 5 mM $MgCl_2$, 1x reaction buffer, and 10, 30, 70, or 100 μM aryl aldehyde analogue. Reactions were incubated at RT for 14 hours before adding an equal volume of 20% acetic acid in isopropanol. Reactions were analyzed via HPLC as outlined in section 3.9.5. For timed reactions between RL FTase, aryl aldehyde analogue, and either dns-GCVLS or dns-GCVDS, reactions conditions were as described above with final concentrations of 50 nM RL FTase, 10 μM aryl aldehyde analogue, 5 μM dns-GCVLS or dns-GCVDS. Time points were taken at 10 and 30 min, and 1, 2, 3, and 5 hours.

3.9.7 Competing reactions between dns-GCVDS and dns-GCVLS with RL FTase and aryl aldehyde analogue: Reactions conditions were as described in section 3.9.6 with a final concentration of 10 μM aryl aldehyde analogue, 50 nM RL FTase and 3 μM each of dns-GCVDS and dns-GCVLS. Reactions were run at RT and stopped using 20% acetic acid in isopropanol at time points of 30 min, 1 2, 3, 4, 5, 8, and 16 hours. After all time points had been taken, analysis using HPLC was done as described previously.

3.9.8 Reactions for Steady-state analysis: Reactions consisted of a peptide mix of 0.5-3.0 μM dns-GCVDS or dns-GCVLS, reaction buffer (50 mM HEPPSO, pH 7.8 and 5 mM TCEP) and 10 mM $MgCl_2$ incubated for 20 min at RT to reduce disulfide bonds. To this mix was added an enzyme mix consisting of 100 nM RL FTase or 20 nM WT FTase, 10 μM FPP, and 10 mM $MgCl_2$ in reaction buffer at 25 °C in a low-adhesion Eppendorf tube. Fluorescence at 340/520 nm was measured as a function of time, over 2 hours, in a BioTek H1 Synergy plate reader from which the initial linear velocity (slope) as well as the reaction end point were determined. The total change in fluorescence (amplitude, amp) was divided by the initial concentration of peptide for each

reaction to yield a conversion factor (conv), changing relative fluorescence units to concentration of product. Velocity (μM product/sec) was determined through the equation V = slope/conv. The steady-state kinetics were determined from a fit of the Michaelis−Menten equation to the initial velocity divided by enzyme concentration (V/E) in the presence of saturating FPP.

3.9.9 Pulldown of TagRFP-GCVDS on hydrazide beads (10 mL scale): Several 1 mL aliquots (3 – 1.5 mL samples) of Bl21(DE3) cells containing TagRFP-GCVDS was thawed on ice. These aliquots were pelleted at 10,000xg for 2 min and all three pellets were resuspended in supernatant together as a 1 mL total sample. To 900 μL of this cell suspension, 100 μL of Fast Break lysis reagent was added and the mixture was moderately shaken for 15 min. The resulting lysate was centrifuged at 4°C and 1000xg for 5 min. The UV-Vis absorbance of the supernatant was measured on a NanoDrop 2000c spectrophotometer at 555 nm. An extinction coefficient (ε) of 100,000 was used for TagRFP. Fluorescence of 200 μL of this sample (excitation/emission of 555/585) was taken in a 96 well plate on a BioTek H1 Synergy plate reader. Prenylation was initiated in a 10 mL glass culture tube containing 200 nM RL FTase, 10 μM aryl aldehyde analogue, 5 mM $MgCl_2$, reaction buffer (50 mM NaHEPPSO – pH 7.8. 5 mM TCEP), and sufficient lysate supernatant to reach a final concentration of 1-2 μM TagRFP-GCVDS. Supernatant containing the protein was incubated with reaction buffer for 20 min prior to initiation of the reaction to reduce disulfide bonds. The reaction was incubated for overnight at room temperature at a final volume of 10 mL.

The 10 mL sample was then concentrated to 500 μL, first using a 10K PALL 15 mL Macrosep concentrator (to 2 mL), followed by a PALL 1 mL Nanosep concentrator. Removal of excess FPP analogue (**2**) was done through use of a 0.5 mL illustra NAP-5 column (GE Healthcare). NAP-5 columns were equilibrated with 50 mM Tris-HCl (10 mL, pH 7.5) before

application of the 0.5 mL sample. Elution followed with 50 mM Tris-HCl (1.0 mL, pH 7.5) into a low-adhesion eppendorf tube. Concentration of the protein was calculated using UV-Vis absorbance of the eluent, fluorescence readings were also taken. The eluent was then added to the hydrazide beads. An aliquot (600 μL) of Ultralink hydrazide resin slurry (Thermo Scientific) was added to a gravity column and allowed to settle for 15 min. Liquid present from the slurry was then drained from the column and the resin was washed with 0.1M phosphate buffer (3 x 500 μL, pH 7). The bottom of the column was capped and more 0.1M phosphate buffer was added to the resin (300 μL) in addition to the 1 mL prepared sample. Immobilization was initiated with m-phenylenediamine (100 mM) to a final volume of 2 mL. The column was gently rocked at 25°C for 2 hours. The resin was then drained before being washed with 0.3M phosphate buffer, pH 7.3 (3 x 300 μL), followed by 1M KCl in 50 mM Tris-Cl, pH 7.5 (3 x 300 μL). Fluorescence was read on a BioTek Synergy H1 Microplate Reader using a 96-well transparent-bottom plate containing a 200 μL sample of the hydrazide resin. Blank control readings consisted of unreacted hydrazide beads washed with 1M KCl. UV-Vis absorbance and fluorescence of the flow through and phosphate buffer and KCl washes were also read.

3.9.10 Release of immobilized protein from hydrazide resin using m-phenylenediamine: To immobilized resin (300 μL) was added a final concentration of 100 mM m-PDA and 200 mM hydroxylamine HCl. Phosphate buffer (0.3M, pH 7) was added to a final reaction volume of 1 mL in a low adhesion Eppendorf tube. The tube was incubated for 6 hours at RT with gentle shaking. The sample was spun down at 5000xg for 5 min and supernatant was analyzed by UV-Vis at 555 nm and by fluorescence of 200 μL of the sample (excitation/emission of 555/585) taken in a 96 well plate on a BioTek H1 Synergy plate reader.

3.9.11 SDS PAGE and anti-His Western blot analysis of proteins: For SDS PAGE and Coomasie blue staining: Each sample (15 μL) was denatured by boiling at 95ºC in 3x SDS sample buffer for 3 min. before loading each sample onto a 12% SDS-PAGE gel. The gel was run at 150v for 1 hour.

The gel was then visualized through Coomasie blue staining. The gel was rinsed in milli-Q water and covered in ~75 mL of destain solution (stock: 2L water, 1.6L MeOH, 0.4L acetic acid). Gels in this solution were then microwaved for 18 seconds. Destain solution was poured off and to the gels ~75 mL of Coomasie blue stain (stock: 0.25g Coomasie blue dye, 500 mL MeoH, 75 mL acetic acid) was added. The gel was microwaved for 30 seconds and then rocked in the stain for 5 min. Coomasie blue stain was poured off and ~75 mL destain solution was added. The gel was rocked for 15 min before destain was poured off and fresh destain was added and rocked for another 15 min. After this incubation, destain was poured off and milli-Q water was added and the gel was rocked overnight. Gels were imaged on BioRad XRS+ gel imager.

For anti-His Western blot analysis: Samples were run on a 12% SDS-PAGE gel until loading dye was removed, about 1 hour. Gels were transferred to PVDF membranes using the TransBlot Turbo RTA Transfer kit, following manufacturer's instructions. Protein was transferred to the membrane using a BioRad TurboBlot with 3x 10 min transfers at 25 volts. Blocking was immediately performed using 30 mL of 10% nonfat milk in TBST. Membranes were rocked in blocking solution a minimum of 2 hours at room temperature. Blocking solution was then poured off the membranes and 10 mL of 1:60 HRP conjugated anti-His monoclonal antibody His-probe (H-3) (Santa Cruz Biotechnology, Inc.) in TBST was added. The membrane was rocked overnight

prior to washing of membrane with 30 mL of TBST six times before incubation with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaging.

3.9.12 Biotinylation of TagRFP-GCVDS after functionalization with aryl aldehyde analogue:

A 1 mL aliquot of Bl21(DE3) cells containing TagRFP-GCVDS was thawed on ice. To 900 μL of these cells, 100 μL of Fast Break lysis reagent was added and the mixture was moderately shaken for 15 min. The resulting lysate was centrifuged at 4°C and 1000xg for 5 min. The UV-Vis absorbance of the supernatant was measured on a NanoDrop 2000c spectrophotometer at 555 nm. This absorbance was converted to concentration in μM using Beer's law, $A = \varepsilon bc$, where A is the absorption, b is the path length of 1 cm, and c is the molar concentration. The extinction coefficient (ε) of 100,000 was used for TagRFP. Fluorescence of 200 μL of sample (excitation/emission of 555/585) was taken in a 96 well plate on a BioTek H1 Synergy plate reader. Prenylation was initiated in a 0.65 mL low-adhesion eppendorf tube containing 200 nM RL FTase, 10 μM aryl aldehyde analogue, 5 mM $MgCl_2$, reaction buffer (50 mM NaHEPPSO – pH 7.8. 5 mM TCEP), and enough lysate supernatant to reach a final concentration of 2.5 μM TagRFP-GCVDS. Supernatant containing the protein was incubated with 1x reaction buffer for 20 min prior to initiation of the reaction to reduce disulfide bonds. The reaction was incubated for overnight at room temperature at a final volume of 1 mL.

The reaction was then concentrated to a volume of 500 μL before addition of the sample to NAP-5 column. Removal of excess FPP analogue (**2**) was done through use of a 0.5 mL illustra NAP-5 column (GE Healthcare). NAP-5 columns were equilibrated with PBS (10 mL, pH 7.5) before application of the 0.5 mL sample. Elution followed with PBS (1.0 mL, pH 7.5) into a low-adhesion eppendorf tube. Concentration of the protein was calculated using UV-Vis absorbance

of the eluent, fluorescence readings were also taken. 50 mM hydrazide-biotin in DMSO was then added to the eluent in a 1:10 ratio, giving a final concentration of 5 mM hydrazide-biotin. The sample was mixed for two hours at room temperature gently before being analyzed via SDS-PAGE gel and Streptavidin blots.

3.9.13 Analysis via streptavidin HRP blot: Samples were run on a 12% SDS-PAGE gel until loading dye was removed, about 1 hour. Gels were transferred to PVDF membranes using the TransBlot Turbo RTA Transfer kit, following manufacturer's instructions. Protein was transferred to the membrane using a BioRad TurboBlot with 3x 10 min transfers at 25 volts. Blocking was immediately performed using 30 mL of 3% BSA in TBST or 10% nonfat milk in TBST. Membranes were rocked in blocking solution a minimum of 2 hours at room temperature. Blocking solution was then poured off the membranes and a 1:10,000 dilution of Pierce High Sensitivity Streptavidin HRP in TBST was added (10 mL). The membrane was rocked overnight.

Following incubation, membranes were washed 6x in TBST, 5 min of rocking for each. Membranes were then treated with West Pico Chemi substrate kit following manufacturer's instructions before imaging on a BioRad Imager with 500 sec exposure.

3.10 References

1. Luk, Y. Y.; Tingey, M. L.; Dickson, K. A.; Raines, R. T.; Abbott, N. L., Imaging the binding ability of proteins immobilized on surfaces with different orientations by using liquid crystals. *J Am Chem Soc* **2004,** *126* (29), 9024-32.

2. Hernandez, K.; Fernandez-Lafuente, R., Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. *Enzyme and microbial technology* **2011,** *48* (2), 107-22.

3. Barbosa, O.; Torres, R.; Ortiz, C.; Berenguer-Murcia, A.; Rodrigues, R. C.; Fernandez-Lafuente, R., Heterofunctional supports in enzyme immobilization: from traditional immobilization protocols to opportunities in tuning enzyme properties. *Biomacromolecules* **2013,** *14* (8), 2433-62.

4. Brena, B.; Gonzalez-Pombo, P.; Batista-Viera, F., Immobilization of enzymes: a literature survey. *Methods in molecular biology (Clifton, N.J.)* **2013,** *1051*, 15-31.

5. Steen Redeker, E.; Ta, D. T.; Cortens, D.; Billen, B.; Guedens, W.; Adriaensens, P., Protein Engineering For Directed Immobilization. *Bioconjugate Chemistry* **2013,** *24* (11), 1761-1777.

6. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie (International ed. in English)* **2001,** *40* (11), 2004-2021.

7. P., T. Z.; Wade, R.; Erhan, B.; J., S. C.; P., P. D.; K., D. J.; D., D. M.; Bruce, D.; D., M. H., Chemoselective Immobilization of Proteins by Microcontact Printing and Bio-orthogonal Click Reactions. *Chembiochem : a European journal of chemical biology* **2013,** *14* (18), 2464-2471.

8. Long, Y.; Hongyan, S.; Yao-Wen, W.; Gemma, T.; Herbert, W.; S., G. R., A Highly Efficient Strategy for Modification of Proteins at the C Terminus. *Angewandte Chemie International Edition* **2010,** *49* (49), 9417-9421.

9. Blanden, A. R.; Mukherjee, K.; Dilek, O.; Loew, M.; Bane, S. L., 4-aminophenylalanine as a biocompatible nucleophilic catalyst for hydrazone ligations at low temperature and neutral pH. *Bioconjug Chem* **2011,** *22* (10), 1954-61.

10. Rashidian, M.; Dozier, J. K.; Lenevich, S.; Distefano, M. D., Selective labeling of polypeptides using protein farnesyltransferase via rapid oxime ligation. *Chem Commun (Camb)* **2010,** *46* (47), 8998-9000.

11. Yeo, J. E.; Wickramaratne, S.; Khatwani, S.; Wang, Y.-C.; Vervacke, J.; Distefano, M. D.; Tretyakova, N. Y., Synthesis of Site-Specific DNA–Protein Conjugates and Their Effects on DNA Replication. *ACS Chemical Biology* **2014,** *9* (8), 1860-1868.

12. Duckworth, B. P.; Xu, J.; Taton, T. A.; Guo, A.; Distefano, M. D., Site-specific, covalent attachment of proteins to a solid surface. *Bioconjug Chem* **2006,** *17* (4), 967-74.

13. Blanden, M. J.; Suazo, K. F.; Hildebrandt, E. R.; Hardgrove, D. S.; Patel, M.; Saunders, W. P.; Distefano, M. D.; Schmidt, W. K.; Hougland, J. L., Efficient farnesylation of an extended C-terminal C(x)3X sequence motif expands the scope of the prenylated proteome. *The Journal of biological chemistry* **2018,** *293* (8), 2770-2785.

14. DeGraw, A. J.; Palsuledesai, C.; Ochocki, J. D.; Dozier, J. K.; Lenevich, S.; Rashidian, M.; Distefano, M. D., Evaluation of alkyne-modified isoprenoids as chemical reporters of protein prenylation. *Chemical biology & drug design* **2010,** *76* (6), 460-71.

15. Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D., Chemoenzymatic Reversible Immobilization and Labeling of Proteins without Prior Purification. *Journal of the American Chemical Society* **2012,** *134* (20), 8455-8467.

16. Suazo, K. F.; Schaber, C.; Palsuledesai, C. C.; Odom John, A. R.; Distefano, M. D., Global proteomic analysis of prenylated proteins in Plasmodium falciparum using an alkyne-modified isoprenoid analogue. *Scientific Reports* **2016,** *6*, 38615.

17. Casey, P. J.; Seabra, M. C., Protein prenyltransferases. *The Journal of biological chemistry* **1996,** *271* (10), 5289-92.

18. Marshall, C. J., Protein prenylation: a mediator of protein-protein interactions. *Science (New York, N.Y.)* **1993,** *259* (5103), 1865-6.

19. Zhang, F. L.; Casey, P. J., Protein prenylation: molecular mechanisms and functional consequences. *Annual review of biochemistry* **1996,** *65*, 241-69.

20. Resh, M. D., Trafficking and signaling by fatty-acylated and prenylated proteins. *Nature chemical biology* **2006,** *2* (11), 584-90.

21. Benetka, W.; Koranda, M.; Eisenhaber, F., Protein Prenylation: An (Almost) Comprehensive Overview on Discovery History, Enzymology, and Significance in Physiology and Disease. *Monatshefte für Chemie / Chemical Monthly* **2006,** *137* (10), 1241.

22. Duckworth, B. P.; Chen, Y.; Wollack, J. W.; Sham, Y.; Mueller, J. D.; Taton, T. A.; Distefano, M. D., A universal method for the preparation of covalent protein-DNA conjugates for use in creating protein nanostructures. *Angewandte Chemie (International ed. in English)* **2007,** *46* (46), 8819-22.

23. Xu, J.; Degraw, A. J.; Duckworth, B. P.; Lenevich, S.; Tann, C. M.; Jenson, E. C.; Gruber, S. J.; Barany, G.; Distefano, M. D., Synthesis and reactivity of 6,7-dihydrogeranylazides: reagents

for primary azide incorporation into peptides and subsequent staudinger ligation. *Chemical biology & drug design* **2006,** *68* (2), 85-96.

24. P., D. B.; Zhiyuan, Z.; Ayako, H.; D., D. M., Selective Labeling of Proteins by Using Protein Farnesyltransferase. *Chembiochem : a European journal of chemical biology* **2007,** *8* (1), 98-105.

25. Weinrich, D.; Lin, P. C.; Jonkheijm, P.; Nguyen, U. T.; Schroder, H.; Niemeyer, C. M.; Alexandrov, K.; Goody, R.; Waldmann, H., Oriented immobilization of farnesylated proteins by the thiol-ene reaction. *Angewandte Chemie (International ed. in English)* **2010,** *49* (7), 1252-7.

26. Palsuledesai, C. C.; Distefano, M. D., Protein prenylation: enzymes, therapeutics, and biotechnology applications. *ACS Chem Biol* **2015,** *10* (1), 51-62.

27. Khatwani, S. L.; Kang, J. S.; Mullen, D. G.; Hast, M. A.; Beese, L. S.; Distefano, M. D.; Taton, T. A., Covalent protein–oligonucleotide conjugates by copper-free click reaction. *Bioorganic & Medicinal Chemistry* **2012,** *20* (14), 4532-4539.

28. Gauchet, C.; Labadie, G. R.; Poulter, C. D., Regio- and chemoselective covalent immobilization of proteins through unnatural amino acids. *J Am Chem Soc* **2006,** *128* (29), 9274-5.

29. Hougland, J. L.; Gangopadhyay, S. A.; Fierke, C. A., Expansion of protein farnesyltransferase specificity using "tunable" active site interactions: development of bioengineered prenylation pathways. *The Journal of biological chemistry* **2012,** *287* (45), 38090-100.

30. Reid, T. S.; Terry, K. L.; Casey, P. J.; Beese, L. S., Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. *Journal of molecular biology* **2004,** *343* (2), 417-33.

31. Dozier, J. K.; Distefano, M. D., An enzyme-coupled continuous fluorescence assay for farnesyl diphosphate synthases. *Analytical biochemistry* **2012,** *421* (1), 158-163.

32. Gangopadhyay, S. A.; Losito, E. L.; Hougland, J. L., Targeted reengineering of protein geranylgeranyltransferase type I selectivity functionally implicates active-site residues in protein-substrate recognition. *Biochemistry* **2014,** *53* (2), 434-46.

33. Hougland, J. L.; Lamphear, C. L.; Scott, S. A.; Gibbs, R. A.; Fierke, C. A., Context-dependent substrate recognition by protein farnesyltransferase. *Biochemistry* **2009,** *48* (8), 1691-701.

34. Rashidian, M.; Mahmoodi, M. M.; Shah, R.; Dozier, J. K.; Wagner, C. R.; Distefano, M. D., A Highly Efficient Catalyst for Oxime Ligation and Hydrazone–Oxime Exchange Suitable for Bioconjugation. *Bioconjugate Chemistry* **2013,** *24* (3), 333-342.

35. Green, N. M., Avidin. *Advances in protein chemistry* **1975,** *29*, 85-133.

36. Hirsch, J. D.; Eslamizar, L.; Filanoski, B. J.; Malekzadeh, N.; Haugland, R. P.; Beechem, J. M.; Haugland, R. P., Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection, and isolation. *Analytical biochemistry* **2002,** *308* (2), 343-57.

37. Gretch, D. R.; Suter, M.; Stinski, M. F., The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpesvirus hydrophobic proteins or glycoproteins. *Analytical biochemistry* **1987,** *163* (1), 270-7.

38. Maertens, B.; Spriestersbach, A.; Kubicek, J.; Schafer, F., Strep-Tagged Protein Purification. *Methods in enzymology* **2015,** *559*, 53-69.

Chapter 4: Development of a quantitative method to analyze prenylation activity: Protein-Lipidation Quantitation

A portion of this chapter has been previously published in reference 80. Reprinted with permission (Appendix VIII) from Shala-Lawrence, A.; Blanden, M. J.; Krylova, S. M.; Gangopadhyay, S. A.; Beloborodov, S. S.; Hougland, J. L.; Krylov, S. N., Simultaneous Analysis of a Non-Lipidated Protein and Its Lipidated Counterpart: Enabling Quantitative Investigation of Protein Lipidation's Impact on Cellular Regulation. Analytical Chemistry 2017, 89 (24), 13502-13507. Copyright 2017, American Chemical Society

Shala-Lawrence, A. and Blanden, M. J. contributed equally to the work.

Co-author contributions include: ASL, SMK, SSB, and SNK performed capillary electrophoresis and prepared electropherograms. SAG prepared mammalian cell lysates and conducted p53 experiments.

4.1 Introduction

Capillary electrophoresis (CE) is a separation technique first introduced in the 1960s in which target molecules are separated by travel through electrolytic solutions under the influence of an electric field.[1-5] As the name suggests, CE is performed in small capillaries allowing separation of samples based on ionic mobility and non-covalent interactions. CE can also be used to focus, or separate and concentrate, analytes of a mixture by means of pH and conductivity gradients.[6-7]

While there are many ways in which to separate analytes in CE, the overall scheme is the same (Figure 4.1). After the sample to be analyzed is introduced into the capillary tube, analytes in the sample are moved by force of an electric field to a destination vial which is read by a detector. Ions within the sample move through the capillary at different rates in the same direction as they are affected by the electroosmotic flow caused by the electric field in the mobile phase. Upon reaching the detector, samples can be analyzed via UV-Vis absorbance or fluorescence and may also be coupled with mass spectroscopy.[2, 5, 8-12] Separation and detection is rather quick, on the order of 3-30 minutes.[5]

The most commonly studied analytes by CE are nucleic acids, peptides, and small molecules. CE has a wide variety of uses including forensic DNA fingerprinting, pharmaceutical analysis, and testing for genetic abnormalities.[13-21] One example of the analytical power of CE is in its use as a means of detection in infectious disease. This is done most commonly through detection of nucleic acids and recognition of sequences from specific proteins produced by the infectious agent.[22] This is achieved through PCR-based techniques which amplify conserved or mutated gene sequences belonging to the microorganism prior to detection of that sequence using CE.

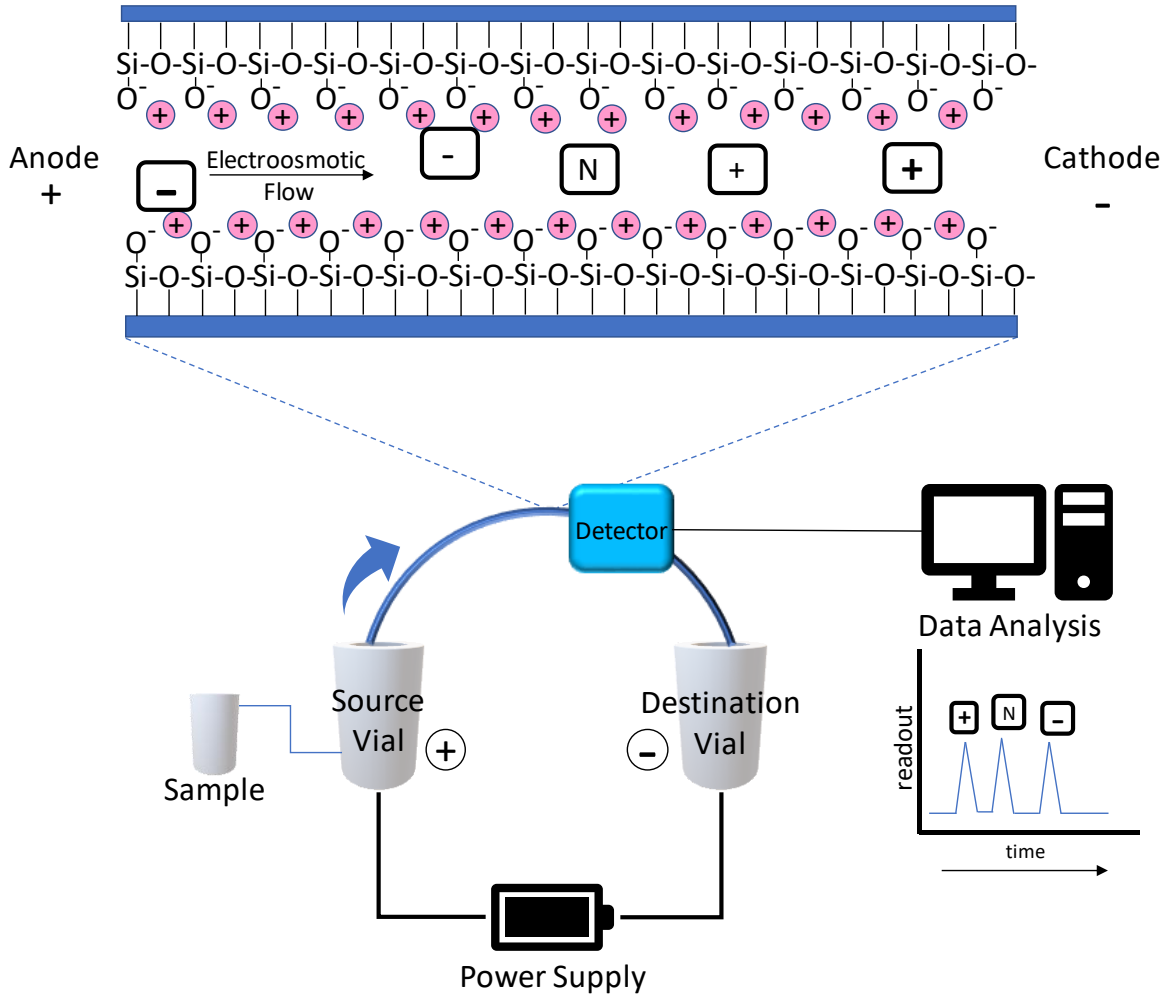


Figure 4.1 A general diagram of a capillary electrophoresis system. After sample injection, analytes flow from the anode to the cathode through an electric field before reaching a detector. Readout can be fluorescence or UV-Vis, for example. Separation of charged and neutral analytes are shown as boxes labeled +, -, and N for positive, negative and neutral, respectively. Circles shown in pink represent the positively charged cations present in the buffer solution.

In 2012, Sobral and coworkers developed a CE method called High Throughput Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA-CE).[22] This method uses several loci containing variable number tandem repeats (VNTR) in *Staphylococcus aureus* which are amplified through PCR and genotyped using CE to provide a quick identification of *S. aureus* in food sources that could cause food poisoning.[23-24] MLVA-CE was able to successfully identify and separate all 16 *S. aureus* VNTR DNA sequences from multiple sources. This one example highlights the ability to specialize CE for detection of a variety targets under a different means of separation.

Despite the diversity of this technique, there are several limitations. Separation in these capillaries is extremely sensitive to changes in pH and ionic strength of the mobile phase, sometimes causing inconsistent results. The size of the molecules is also a factor, with large molecules exhibiting poor separation. The work herein aims to develop a new CE method entitled Protein-Lipidation Quantitation (PLQ), which eliminates the limitation of analyzing large biomolecules through the detection and quantification of the modification state of a full length, lipidated protein compared to its unmodified counterpart. This method builds upon one variant of CE termed Micellar Electrokinetic Capillary Electrophoresis (MEKC).[25] To design this new technique, the reality in the challenges of studying protein lipidation need to be understood and met with solutions as described below.

Posttranslational modification of proteins, such as phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation, play important roles in cellular function and biological regulation.[26-27] Among this wide range of protein modifications, protein lipidation plays a unique and essential role in regulating protein localization and trafficking within the cell by directly mediating protein-membrane interactions (see Chapter 1). There are several distinct lipidation modifications, such as N-terminal myristoylation, palmitoylation,

prenylation of cysteine sidechain thiols, and attachment of glycophosphoinositol (GPI) groups.[28-43] However, the significance of each of the diverse lipid modifications, as well as the mechanism by which lipidation controls function and activity of the proteins, remain topics of intense study.

One central challenge in studying protein lipidation has been the difficulty in measuring the distribution and relative populations of precursor and lipidated forms of proteins. The lack of a quantitative method that could simultaneously measure the amounts of both protein forms has complicated quantitative analysis of the regulation, functional impact, and biological requirement for protein lipidation. To date, analytical methods used to study protein lipidation have focused primarily on identifying lipidated proteins.[44-46] In terms of protein prenylation, the earliest studies utilized metabolic labeling with radioactive prenyl donors or biosynthetic precursors, separating the prenylated product from the excess lipid donor by gel electrophoresis followed by detection by autoradiography.[34, 47-50] Subsequent studies have employed chemically modified FPP and GGPP analogues containing groups allowing for protein detection by bioorthogonal chemical tagging or affinity detection.[51-60] Proteins labeled with these analogues can be detected using either gel electrophoresis and/or mass spectrometry following affinity-based pulldown protein isolation.[51, 61-62] Prenylation of specific proteins can be detected within the cell using fluorescent fusion proteins and microscopic imaging to detect the lipidated product based on protein localization to cellular membranes.[56, 63-66] Each of the above methods has only limited quantitative capabilities for measuring protein prenylation and none of them can efficiently quantitate the unmodified substrate. Some prenylated proteins such as H-Ras exhibit a mobility shift on gel electrophoresis following prenylation pathway modifications,[67-68] although this behavior is considered highly ambiguous amongst naturally occurring prenylated proteins. In these cases, simultaneous detection of both the precursor and modified forms of the prenylated protein can be achieved by

separation with SDS-PAGE and detection by Western blot analysis.[69-70] Known limitations of Western blot analysis make this method only semi-quantitative.

A quantitative understanding of the role played by protein lipidation in cells requires a method that measures both unmodified substrate (S) and modified product (P); an approach which the aforementioned techniques fall short of. In designing a suitable method, we determined three requirements that should be met by the approach. The first is its ability to detect both S and P with a known relation between their signal/concentration ratios. This allows for direct quantitation. The second is its ability to discriminate between signals for S and P clearly from each other. Third, for biological application, it should be able to examine crude cell lysates as to avoid bias of S or P from use of sample purification. The methods listed above find difficulty in satisfying the ability to identify unmodified substrate and those that do, cannot effectively separate the two species.

In exploring analytical methods that satisfy all three requirements for efficiently separating the prenylated and non-prenylated forms of a protein, we can draw inspiration from the proposed role of protein lipidation modifications in increasing protein association with cellular membranes. Micellar Electro-Kinetic Chromatography (MEKC) utilizes detergent or lipid micelles to provide molecular separation during capillary electrophoresis (Figure 4.2).[71-73] In MEKC, a detergent such as SDS is used to form micelles in the running buffer with which the analyte can interact. Micelles with or without interacting analyte travel quite slowly through the capillary tube as compared to free analyte or buffer. This is due to an electrophoretic mobility caused by the drag associated with the viscosity and charge of the micelle as it moves through the electric field within capillary. This electrophoretic mobility is counter to the electroosmotic flow (EOF) of the capillary resulting in micelle-associated analytes exhibiting a slower overall velocity towards the cathode.

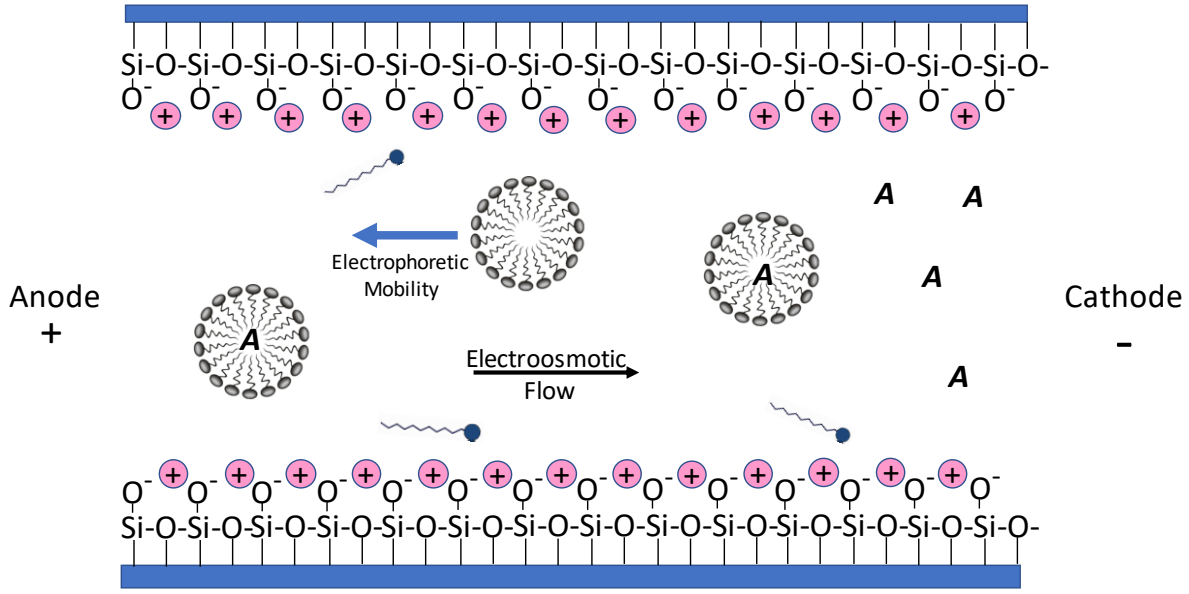


Figure 4.2 Micellar Electrokinetic Chromatography. Analytes (A) are distributed based on their hydrophobicity, with those associated with micelles reaching the detector later than unassociated analytes. Circles shown in pink represent the positively charged cations present in the buffer solution.

This technique has been used to separate peptides and small molecules based on hydrophobicity-dependent partitioning into the detergent micelles,[74-76] and has been used to separate short farnesylated peptides from the non-lipidated precursor.[77-78] An example from Berezovski and coworkers in 2002 demonstrated separation of unmodified and farnesylated small peptides labeled with 2',7'-difluorofluorescein (dff) for fluorescence detection. The samples were analyzed using CE in the presence and absence of SDS micelles, with addition of SDS as used in MEKC providing the best resolution between substrate and modified product (Figure 4.3). This technique was applied further in using the ability of MEKC to measure the rate of accumulating product to determine kinetic parameters of the farnesylation of the pentapeptides.[78]

As with any analytical technique, MEKC has experimental limitations. It has generally been considered not applicable for full-length protein separation due to the inability of a protein to fit within the hydrophobic micelle interior without denaturation.[79-80] This work was motivated by our insight that the unique chemical characteristics of protein lipidation modifications may allow us to leverage MEKC as a method to separate and identify lipidated proteins. To examine the applicability of MEKC for analysis of protein lipidation, we used fluorescent reporter proteins (eGFP and TagRFP) which have been designed to become substrates of protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase-I) through cloning at their C-terminus (See Chapter 3). The use of fluorescent proteins allows simultaneous detection of the lipidated and non-lipidated forms of the reporter proteins. We found that MEKC utilizing SDS micelles can rapidly and efficiently separate a farnesylated protein from the non-farnesylated precursor. The efficiency of this separation is dependent on protein charge, with eGFP separating more efficiently than the more negatively charged TagRFP.

We foresee that PLQ will facilitate transformation of protein-lipidation studies towards quantitative analysis of *in vivo* protein modifications and may prove extendable to the analysis of a wide range of different protein lipidation modifications. PLQ has the advantage of both positively and negatively charged proteins reaching the detector due to the presence of a strong electroosmotic flow (EOF). It also allows for separation of two analytes with similar charge-to-size ratios through addition of a pseudo-stationary phase (micelles) to the running buffer. Specifically, addition of suspended molecules to the buffer can introduce a shift in an analyte's electrophoretic mobility through preferential analyte binding. PLQ was inspired by an insight that an ionic surfactant could serve as a pseudo-stationary phase for separation of P and S through binding of the lipid moiety of P. While this approach as been achieved using small lipidated peptides, our fine tuning of the stationary and mobile phases using differing concentrations of SDS achieves enhancement of full-length protein separation.

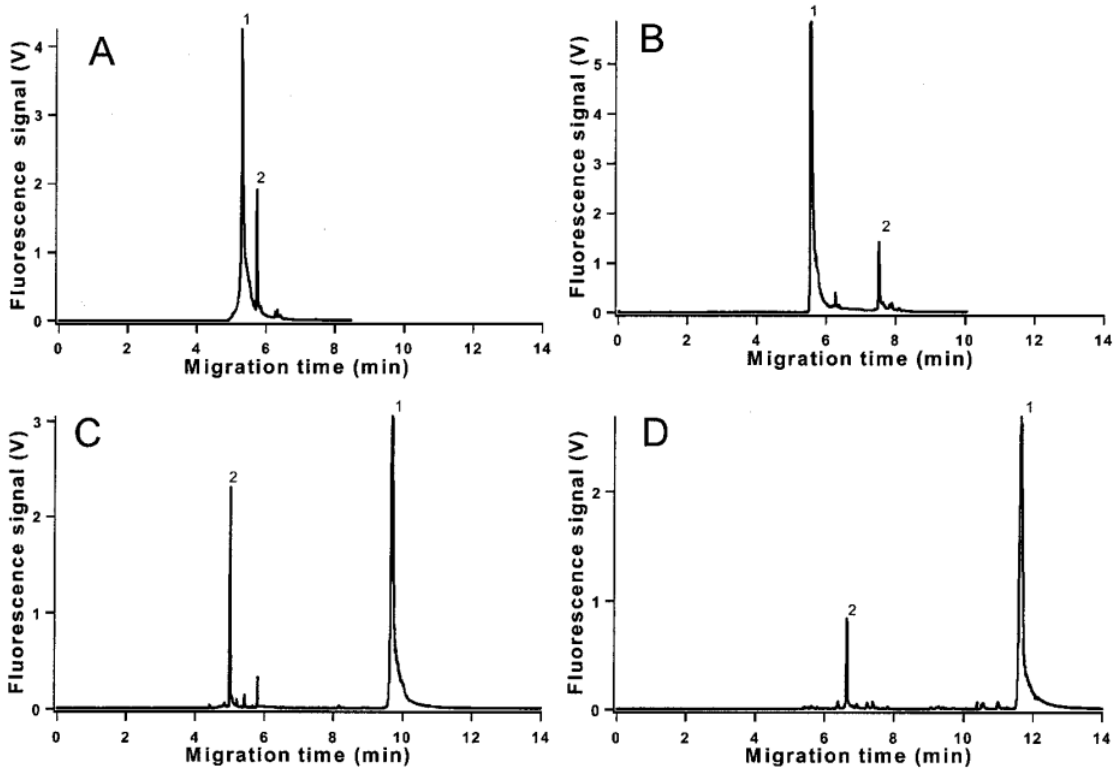


Figure 4.3 CE separation of a farnesylated peptide (peak 1) from the parent unmodified peptide (peak 2). The reaction mixture contained 0.8 M dff-GCVIA, 20 M FPP and 46 nM PFTase after 48 h incubation at 30˚C in the enzymatic buffer. Running buffers: (A) 25 mM borax, pH 9.1; (B) 50 mM borax, 20 mM β-cyclodextrin, pH 9.5; (C) 50 mM TES, 50 mM SDS, pH 7.3; (D) 25 mM borax, 25 mM SDS, pH 9.3. This figure has been reused with permission from reference 78 (Appendix IX).

4.2 PLQ separation of lipid modified fluorescent proteins from their unmodified counterparts

To validate PLQ as a potential method for spearation, we chose purified enhanced green fluorescent protein (eGFP) fused with a GCVDS peptide to permit modification by protein farnesyltransferase (FTase) using a farnesyl diphosphate (FPP) lipid donor (see section 4.7 and Chapter 3 for design and cloning of this protein). The use of a fluorescent protein grants the ability to detect substrate and produce via a readout of fluorescence at the detector. The GCVDS motif was chosen as part of an ongoing study into a bioorthogonal substrate-enzyme relationship in which a mutant FTase lipidates a noncanonical prenylation motif (see Chapter 3).[81]

In order to achieve sufficient separation of substrate and product in PLQ, an appropriate surfactant, along with its ideal concentration, must first be determined. This was achieved through testing of different surfactants at varying concentrations for their ability to separate a mixture of the purified, unmodified eGFP-GCVDS and its farnesylated product. Nonionic surfactants were tested and did not introduce noticeable mobility shift. We began tests of anionic surfactants with sodium dodecyl sulfate (SDS) because it has been previously used in CE as a pseudo-stationary phase for small-molecule separation.[82] The concentration of SDS was kept well below that typically used for protein denaturation (~70 mM SDS, with sample heating to 100 °C and addition of reducing agents). To determine optimum SDS conditions, the mixture of farnesylated and non-farnesylated eGFP were analyzed at SDS concentrations ranging from 0 to 25 mM at pH 9.2 (Figure 4.4). The maximum resolution was achieved with SDS concentration in a range of 15 to 25 mM, with the electropherograms being exhibiting reproducibility in this range. As expected, the farnesylated product migrated slower in agreement with our model in which a negatively charged surfactant should slow down migration of P during PLQ analysis (Figure 4.5).

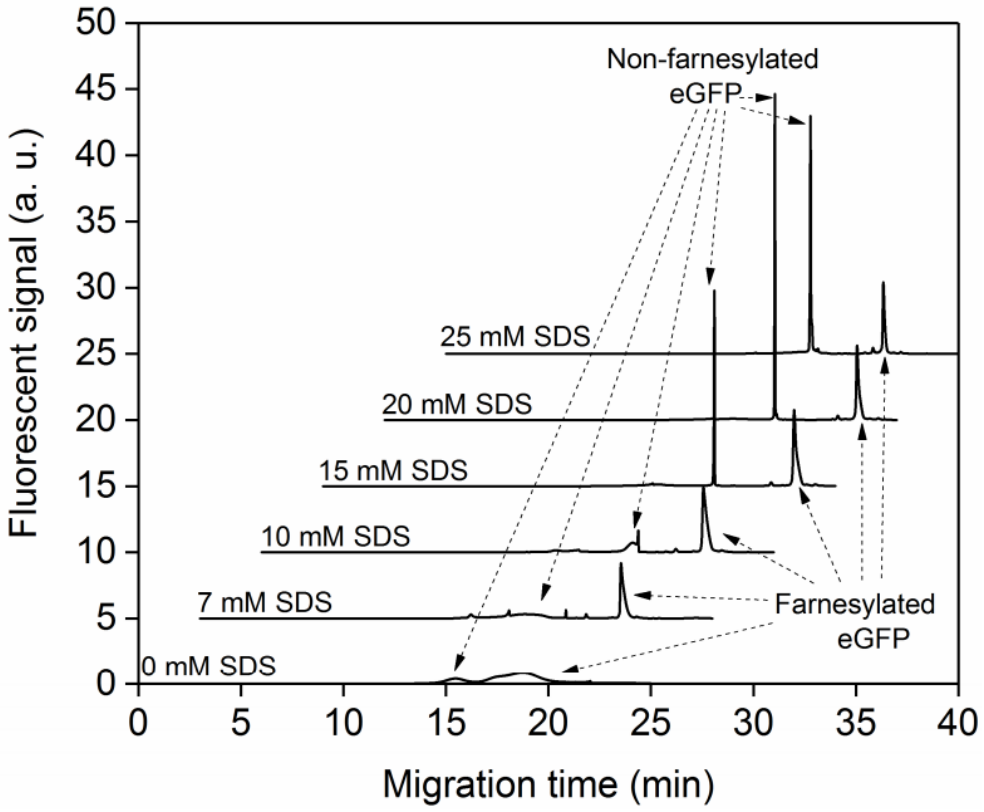


Figure 4.4 Influence of changing SDS concentration on separation of the farnesylated and non-farnesylated eGFP. All of the experiments were conducted in a buffer containing 25 mM borax at different concentrations of SDS (indicated at left) and pH of 9.2. For clarity of presentation, 3-min time offsets and 5-unit signal offsets units are applied. This figure has been reused with permission from reference 80 (Appendix VIII) with CE analysis performed by Krylov and coworkers, York Univeristy.

The composition of the run buffer was then optimized with regards to SDS concentration, pH, and ionic strength to achieve maximum resolution between peaks of S and P. This optimized buffer (20 mM SDS, 25 mM sodium tetraborate, pH 9.2) facilitated baseline separation of farnesylated and nonfarnesylated eGFP-GCVDS (Figure 4.6a). A test of the ability of PLQ to separate S and P efficiently at a biological level required analysis of crude bacterial cell lysate containing both eGFP-GCVDS and its farnesylated product. We observed high-quality separation undistinguishable from that of purified S and P (Figure 4.6a). We found that farnesylation did not affect relative fluorescence intensity of eGFP-GCVDS, and the presence of 20 mM SDS (optimal concentration) did not change fluorescence from eGFP-GCVDS above normal experiment-to-experiment variation. This important observation defines PLQ as quantifiable with accurate signal/concentration ratios for both S and P, not influenced by the surfactant or buffer. This allows simple quantitation of S and P concentrations in the sample based on areas of the corresponding peaks, with absolute quantitation possible through use of an internal standard.

To determine if PLQ-based separation was farnesylation specific or applicable to a wider variety of lipid moieties, we next explored a different lipid donor by replacing FPP with geranylgeranyl diphosphate (GGPP). The substrate of lipidation was eGFP fused with the GCVLL peptide sequence with the enzyme geranylgeranyltransferase type I (GGTase-I) used to geranylgeranylate eGFP-GCVLL. Separation was achieved for nongeranylgeranylated and geranylgeranylated eGFP-GCVLL without reoptimization of the conditions used for farnesylation (Figure 4.6b).

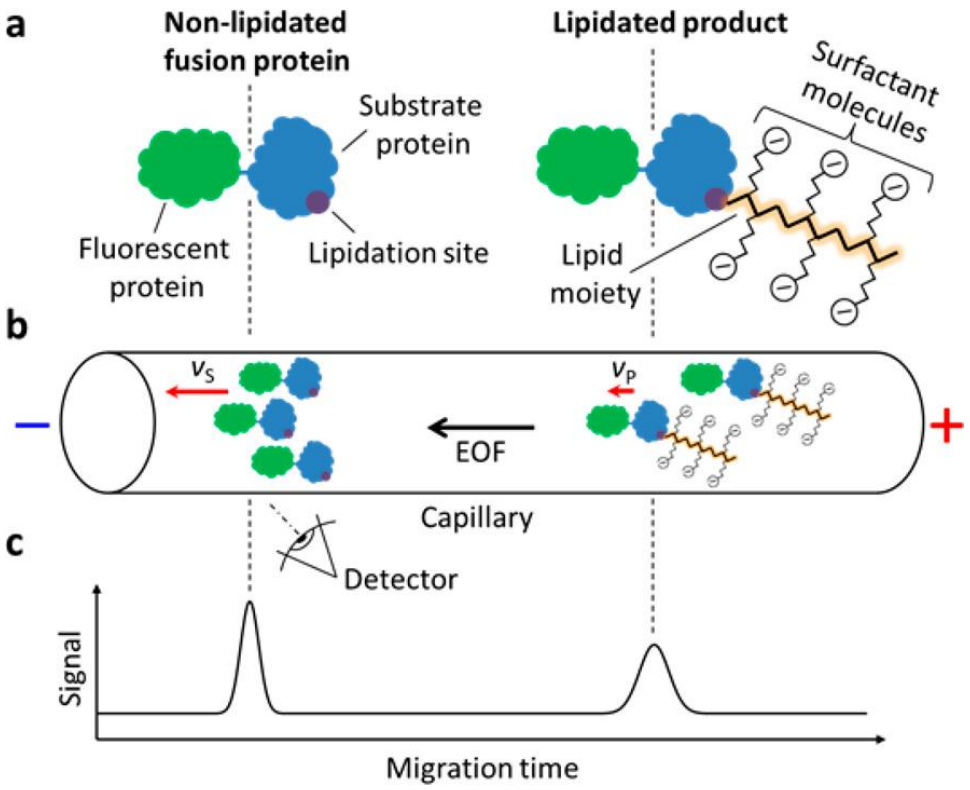


Figure 4.5 Conceptual illustration of PLQ. (a) Nonlipidated fusion protein (S) and its lipidated product (P) with an anionic (for example) surfactant bound to a lipid moiety of the latter. (b) S and P in a capillary filled with the surfactant in the run buffer (not shown), an electric field and EOF. Both S and P are propelled toward the cathode by EOF, however, the charge added to P by the lipid-bound surfactant makes its resulting velocity (v_P) different (smaller in our example) than that of S (v_S). (c) Schematic dependence of fluorescence signal on time for the separated S and P. Areas of peaks are proportional to the respective concentrations. This figure has been reused with permission from reference 80 (Appendix VIII).

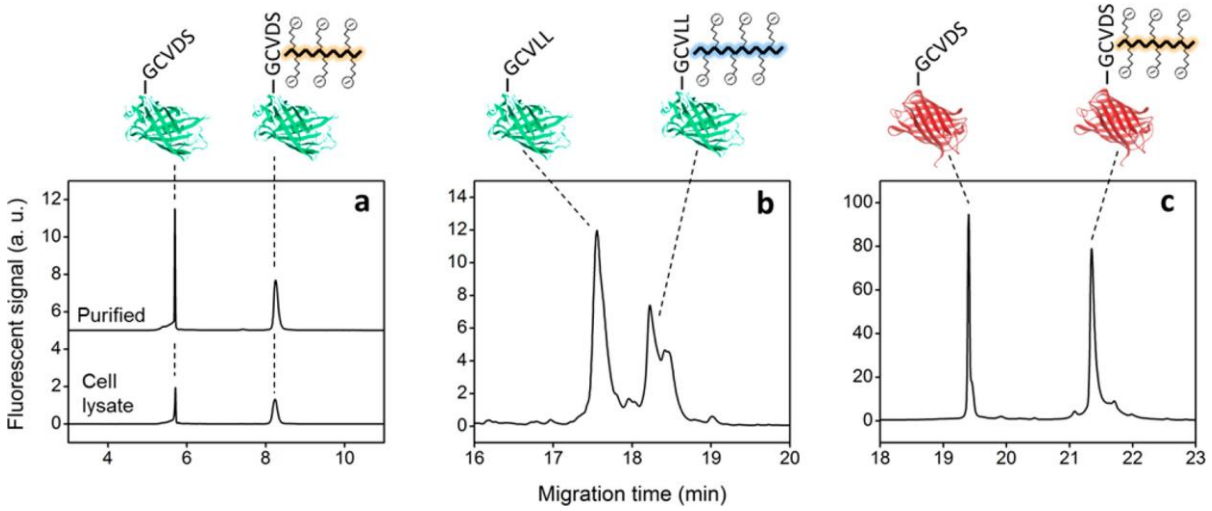


Figure 4.6 PLQ separation of various prenylation constructs. (a) S, eGFP-GCVDS; P; farnesylated eGFP-GCVDS. Top trace, purified S and P in 50 mM NaHEPPSO pH 7.8 and 5 mM TCEP; bottom trace, S and P present in the crude bacterial cell lysate. The run buffer was 25 mM Borax, 20 mM SDS at pH 9.2. (b) S, eGFP-GCVLL; P, geranylgeranylated eGFP-GCVLL. Purified S and P were in 50 mM NaHEPPSO pH 7.8 and 5 mM TCEP; run buffer was 25 mM Borax, 20 mM SDS at pH 9.2 (c) S, TagRFP-GCVDS; P, farnesylated TagRFP-GCVDS. Purified S and P were in 50 mM Na-HEPPSO pH 7.8 and 5 mM TCEP; run buffer was 25 mM sodium tetraborate, 20 mM SDS at pH 10.0. This figure has been reused with permission from reference 80 (Appendix VIII) with CE analysis performed by Krylov and coworkers, York Univeristy.

To determine if PLQ is applicable to different full length proteins, eGFP was replaced with red fluorescent protein (TagRFP) while maintaining the GCVDS farnesylation motif. With differing isoelectric points of 5.8 and 7.1 for eGFP and TagRFP, respectively (determined using ExPASy Bioinformatics Resource Portal), a reoptimization of pH was needed for maximum resolution between the S and P forms of TagRFP-GCVDS and was achieved at pH 10.0 (Figure 4.6c). Interestingly, the need to optimize pH based on different protein suggests that while the lipid moiety directly interacts with SDS micelles during separation as expected, there is some indirect interaction of the fluorescent protein itself with the micelle. Through adjustment of the buffer pH we can minimize this interaction and measure separation based solely on the presence or absence of lipid modification.

4.3 PLQ separation of unmodified and farnesylated eGFP-HRas from mammalian cell lysate

To investigate the biologically relevant use of PLQ, the short C-terminal peptide appended to eGFP in previous samples was replaced with full length human HRas, a well-studied protein which undergoes farnesylation.[83-85] In cells, eGFP-HRas is readily prenylated by endogenous FTase, providing us with a product but potentially no unmodified substrate from which to separate it from. In order to generate an unmodified form of eGFP-HRas with which to analyze separation between S and P, the FTase inhibitor tipifarnib was used to block prenylation in one sample of HEK293 cells.[86] The cell lysate prepared from cells treated with tipifarnib produced two prominent peaks less than 1 minute apart (Figure 4.7, lower curve). The cell lysate prepared from cells grown without the FTase inhibitor presented a new peak corresponding to the farnesylated protein and the decreased 2 peaks associated with the substrate (Figure 4.7, upper curve). Separation of S from P for this larger size fusion protein (54 kDa) required reoptimization of conditions, with the optimum separation achieved at a higher ionic strength of the SDS-containing run buffer: 50 mM instead of 25 mM sodium tetraborate. Quantitative features of PLQ allowed us to determine the level of lipidated substrate in the presence of inhibitor being below the limit of detection while in the absence of inhibitor it grew to 58%, which demonstrates the ability of PLQ to accurately determine the fraction of a given protein within a cell that undergoes lipid modification.

PLQ is sensitive to a wide range of protein concentrations making it widely applicable in research. The commercial instrument used in this study supports a limit of detection below 10 pM and a linear dynamic range of at least 3 orders of magnitude. Importantly, all quantitative characteristics are retained in the analysis of crude cell lysates since the cell debris is fully separated from the fluorescent analytes and does not affect detection. The quantitative characteristics are also independent of the substrate protein nature because they are defined by the

fluorescent properties of GFP or RFP which do not change significantly upon fusion to a substrate protein and are not greatly affected by substrate lipidation. We confirmed that PLQ satisfied the requirements necessary to produce a quantitative analysis of protein modification while also showing that PLQ is robust to changes in lipid donor, fluorescent protein, and lipid acceptor with minimal reoptimization. These findings emphasize the practicality of PLQ approach and suggest that PLQ implementation with a fluorescent fusion protein and SDS-mediated CE as a stationary-phase-free column separation method is a robust approach for investigating protein lipidation in biologically relevant contexts.

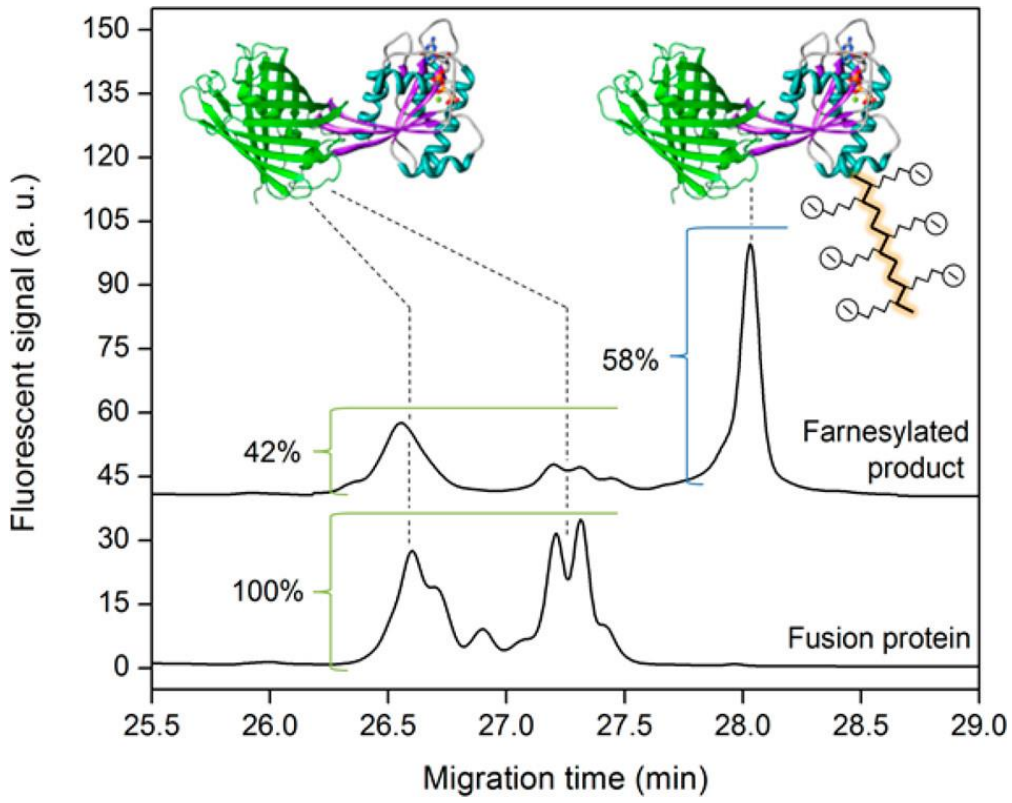


Figure 4.7 PLQ analysis of endogenous farnesylation of eGFP-HRas fusion protein. The lower curve corresponds to sampling cell lysate prepared from HEK293 cells grown in the presence of a FTase inhibitor and shows peaks generated by the nonfarnesylated eGFP-HRas. The upper trace corresponds to sampling cell lysate prepared from HEK293 cells grown without inhibition of FTase and shows the peak of farnesylated eGFP-HRas at the right and peaks of nonfarnesylated eGFP-HRas at the left. The run buffer was 50 mM sodium tetraborate, 20 mM SDS at pH 9.2. Percentile shows concentrations of nonfarnesylated and farnesylated eGFP-HRas calculated from the areas of corresponding peaks. This figure has been reused with permission from reference 80 (Appendix VIII) with mammalian lysates prepared by Soumyashree Gangopadhyay, Syracuse University, and CE analysis performed by Krylov and coworkers, York Univeristy.

4.4 PLQ analysis of the impact of p53 mutation on cellular prenylation activity

The most commonly mutated gene in cancer is p53, a tumor suppressor gene which codes for a protein responsible for regulation of the cell growth cycle.[87-90] The majority of p53 mutations are found to be single amino acid missense mutations affecting the DNA binding domain of the protein.[91] It was recently reported that the presence of mutated in the MDA-231 breast cancer cell line is associated with prenylation-dependent changes in cell morphology and growth.[90] Prenylation is suggested in this study to be impacted by the mutated p53 in the breast cancer cells. Our ability to separate nonprenylated and prenylated proteins using PLQ provides an ideal approach to directly probe the impact of the presence of mutated p53 on cellular protein prenylation and to test PLQ as a valuable tool in discerning prenylation's role in disease states.

To examine the influence of a p53 mutation on prenylation activity, we used the MDA-231shp53 cell line which has been engineered to express a doxycycline (Dox)-inducible short hairpin RNA (shRNA) silencing of the endogenous mutant p53 gene.[90] MDA231shp53 cells were transfected with an RFP-tagged substrate for GGTase-I in the presence and absence of Dox (Figure 4.8a). Crude lysates from these cells were analyzed by PLQ using conditions optimized for TagRFP-containing fusion protein substrates. In the presence of Dox (when mutated p53 was silenced), the peak of the geranylgeranylated form of the TagRFP substrate was below the limit of detection, confirming that less than 5% of the substrate was prenylated (Figure 4.8b). In contrast, in the absence of Dox (when mutated p53 was expressed), the peak corresponding to the geranylgeranylated product of TagRFP indicated that more than 95% of the substrate was prenylated. Control experiments using HEK293 cells indicate that Dox treatment alone does not influence protein geranylgeranylation in the absence of the mutated p53 gene (Figure 4.9). This study demonstrates that silencing of mutant p53 expression exhibits a functional effect on cellular

GGTase-I activity similar to that of direct inhibition of cellular FTase activity by FTase inhibitor treatment with Tipifarnib. These findings support the proposed model wherein p53 mutations lead to altered cellular prenylation activity and suggests that altered protein prenylation may play a direct role in cellular changes involved in development of cancer cells.

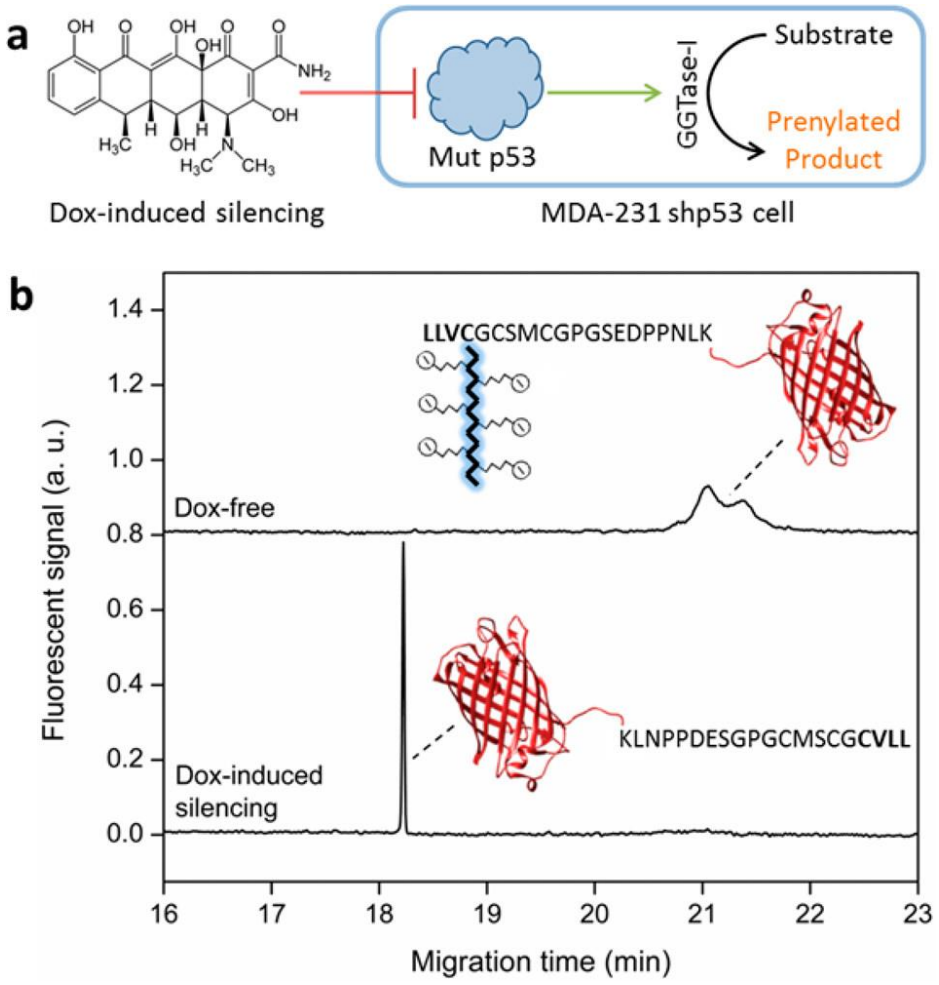


Figure 4.8 PLQ analysis of the impact of p53 mutation on cellular prenylation activity. (a) Schematic representation of cellular model for testing the impact of mutant p53 expression on protein prenylation. (b) PLQ analysis of cell lysates prepared from cells grown in the absence (top trace) and presence (bottom trace) of Dox. This figure has been reused with permission from reference 80 (Appendix VIII) with samples prepared by Soumyashree Gangopadhyay, Syracuse University, and CE analysis performed by Krylov and coworkers, York Univeristy.

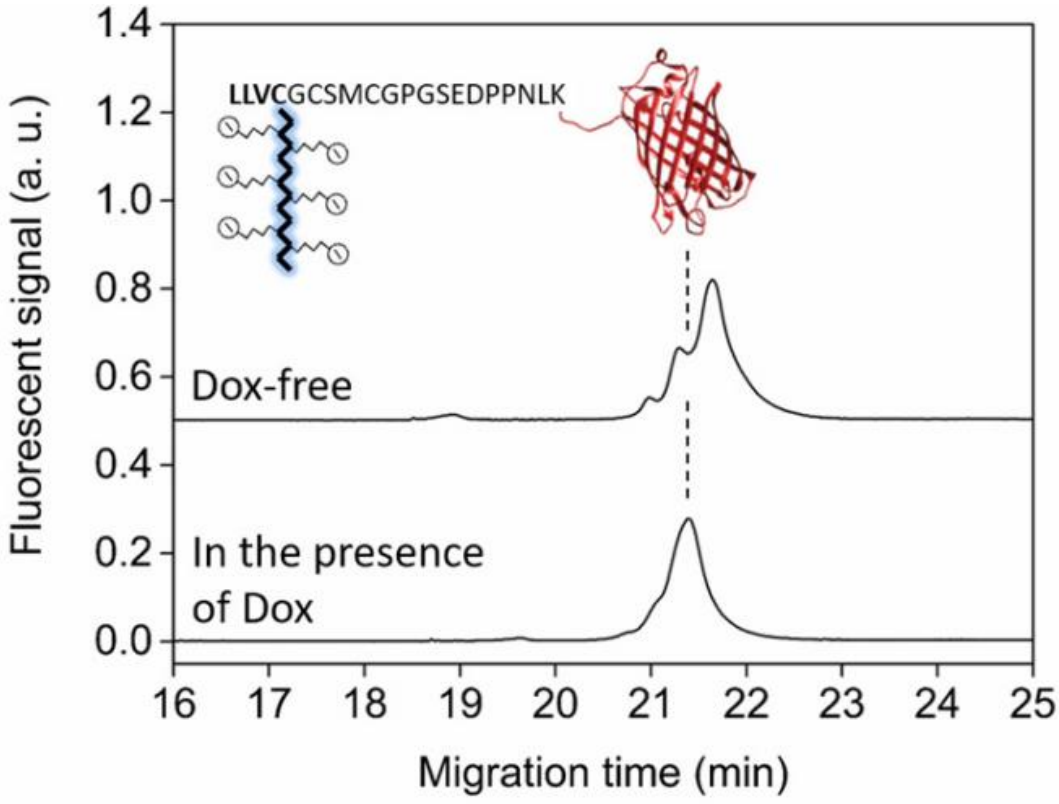


Figure 4.9 Control experiments with HEK293 cells in the absence of mutated p53 gene. The upper trace shows signal of geranylgeranylated product of TagRFP-link(HRas)-CVLL expressed in HEK293 cells in the absence of doxycycline. The bottom trace shows the same peak of geranylgeranylated product expressed in HEK293 cells in the presence of doxycycline, which indicates that doxycycline treatment alone does not influence protein geranylgeranylation in the absence of the mutated p53 gene. This figure has been reused with permission from reference 80 (Appendix VIII) with samples prepared by Soumyashree Gangopadhyay, Syracuse University and CE analysis performed by Krylov and coworkers, York University.

4.5 Conclusions

Protein-Lipidation Quantitation is the first successful approach for simultaneous analysis of substrate and product levels for full length proteins capable of undergoing lipidation. We have been able to demonstrate its practical execution with multiple fluorescent fusion proteins as substrates in an SDS-mediated CE as a stationary-phase-free column separation method. This method is applicable not only to purified sample but also in crude cell lysates without any sample purification, emphasizing its value in providing a snapshot of the extent of lipidation of target proteins in a cell. PLQ provides a powerful complement to While fluorescence microscopy is valuable for determining the ability for a protein to be lipidated, PLQ offers a complementary picture of the extent of lipidation in a quantitative manner. Used together, the change in protein cellular localization upon lipidation can be combined with an accurate assessment in the extent of protein lipidation potentially at both the single-cell and population scales. The combined use of PLQ and microscopy will uniquely enable researchers to identify and study proteins in which the role of lipidation is not to enhance plasma localization, for example, Ydj1p heat shock protein recently reported on by Hildebrant and co-workers.[92] With this shunt pathway for prenylated proteins, as described in Chapter 2, membrane-localized fluorescence analysis will not provide a complete picture of the prenylated proteome. In the growing field of protein lipidation, the quantitative capabilities of PLQ will allow researchers to formulate and address quantitative questions that were once too difficult to answer. PLQ thus promises to become a powerful tool in studies of roles of protein lipidation in cellular regulation and biological function. This work also adds lipidation to a set of postrtranslational modifications of proteins (PEGylation, glycosylation, carbonylation, phosphorylation) for which CE analyses have been developed.[93-96]

4.6 Materials and Methods

4.6.1 Miscellaneous: Fused silica capillary (75 μm inner diameter, 360 μm outer diameter) was purchased from Molex (Phoenix, AZ). All chemicals were obtained from SigmaAldrich (Oakville, ON, Canada) unless otherwise stated. Disodium Tetrborate (borax) was purchased from EMD (Toronto, ON, Canada). All solutions were made using deionized Mili-Q water filtered through 0.22 μm Millipore filter (Millipore, Nepean, ON, Canada).

4.6.2 Design of pJExpress414 plasmid containing His_6-eGFP-GCVDS or His_6-eGFP-GCVLL: For the eGFP-GCVDS reporter protein, site directed mutagenesis of the plasmid pJExpress_eGFP-GCVIA[59] was used to prepare an eGFP reporter protein with a C-terminal -GCVDS sequence. PCR reactions contained final concentrations of 1× Pfu Turbo buffer, 10 mM dNTPs, reverse primer (125 ng), forward primer (125 ng), template plasmid (10 ng), and Pfu Turbo DNA polymerase (1 μL, 2.5 U/μL). PCR mutagenesis was performed under the following thermal program: Initial denaturation (95°C, 1 min.); eighteen cycles of denaturation (95°C, 50 s), annealing (60°C, 50 s), and extension (68°C, 12 min); final extension (68°C, 12 min); and a final hold (10°C, ∞). The reaction was then digested with DpnI (1 μL, 10 U/μL) for 1 h at 37°C. PCR mutagenesis reactions were transformed into chemically competent E.coli for plasmid isolation using the Z-competent transformation system (Zymo Research). An aliquot of the PCR reaction (5 μL) was added to DH5α cells (50 μL) and incubated on ice for 30 min before plating cells on warm LB-Amp plates. Cells were incubated overnight at 37°C. Colonies were chosen from the LB-Amp plate and inoculated into separate 5 mL culture of LB media with ampicillin (final concentration 100 μg/mL). Cultures were incubated overnight at 37°C with shaking. The plasmid DNA was then purified using BIO Basic Inc. EZ-10 Spin Column Plasmid DNA Minipreps Kit following the

manufacturer's protocol. Mutations were verified by commercial DNA sequencing (Genewiz). The gene for the His_6-eGFP-GCVLL reporter protein was prepared by above protocol using appropriately designed mutagenic primers. Mutations were verified by commercial DNA sequencing (Genewiz).

4.6.3 pCDF-Duet-1 plasmid containing the His_6-TagRFP-GCVDS reporter protein: A gene encoding the His_6-TagRFP-GCDVS reporter protein was prepared by PCR using the TagRFP-N vector[97] (Evrogen) as template with the –GCVDS C-terminal sequence and HindIII restriction site present in the 3' primer. The PCR reaction (50 μL) contained final concentrations of 1x Standard OneTaq buffer, 10 mM dNTPs, reverse primer (125 ng), forward primer (125 ng), template plasmid (10 ng), and OneTaq DNA polymerase (0.25 μL, 5 U/μL). PCR was performed S3 under the following conditions: Initial denaturation (94°C, 1 min); thirty cycles of denaturation (94°C, 30 s), annealing (56°C, 1 min), and extension (68°C, 2 min); final extension (68°C, 5 min); and a final hold (10°C, ∞). PCR products were purified using BIO Basic Inc. EZ-10 Spin Column PCR Purification Kit following the manufacturer's instructions. Following digestion by EcoRI and HindIII, the His_6-TagRFP-GCDVS insert was ligated into the pCDF-Duet-1 expression plasmid. Insert ligation was verified by analytical restriction digest and DNA sequencing (Genewiz).

4.6.4 Expression and purification of RL FTase and GGTase-I: Wild-type (WT) rat GGTase-I and W102R W106L rat FTase variants were expressed in BL21(DE3) Escherichia coli (E. coli) employing previously described pET23a-based GGTase-I and variant FTase vectors, with expression and purification using previously reported protocols with the following changes to protein expression.[98-100] Initial cultures were added to 1 L rich media (20 g tryptone, 10 g yeast

extract, 5 g NaCl, 1% glucose, 0.5 mM IPTG and 100 µg/mL ampicillin) and grown at 28°C with shaking for 18 hours. Upon purification, enzyme concentration was measured by active-site titration using dansylated peptides, as described previously.[101]

4.6.5 Expression and purification of His_6-eGFP-GCVxx and His_6-TagRFP-GCVDS: proteins: Chemically competent BL21 (DE3) E. coli were transformed with reporter protein expression vectors (pJExpress414-eGFP-GCVDS, pJExpress414-GCVLL, or CDF-Duet-1_TagRFP-GCVDS). Following transformation and antibiotic selection, a colony from the transformation plate was inoculated into LB media (5 mL) containing either 100 µg/mL ampicillin (for pJExpress414 vectors) or 100 µg/mL streptomycin (for CDF-Duet-1 vector). Cultures were incubated and shaken at 225 rpm for 4 h at 37°C. Each culture was then transferred to 1 liter of prewarmed auto-induction media (10 g tryptone, 5 g yeast extract, 20 mL 50× 5052 media [25% glycerol, 10% lactose, 2.5% glucose], 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 200 µL trace metals [50 µM $FeCl_2$, 20 µM $CaCl_2$, 10 µM $MnCl_2$, 10 µM $ZnCl_2$], 100 µg/mL ampicillin or streptomycin.7 Expression cultures were incubated for 19 h at 37˚C with shaking. Cells were harvested by centrifugation and resuspended in 50 mL resuspension buffer (20 mM NaH_2PO_4, 500 mM NaCl, and 10 mM imidazole). Bacterial cell suspensions were lysed by sonication, clarified by centrifugation, and purified by affinity chromatography using a Ni-NTA HisTrap column (GE S4 Healthcare). Fractions containing the fluorescent protein were combined and concentrated using a centrifugal concentrator. Concentrated samples were buffer exchanged to 50 mM Tris buffer (pH 7.5), divided into 20 µL aliquots, and flash frozen with liquid nitrogen for storage at −80°C. Protein concentrations were determined using molar absorptivities

of eGFP at 488 nm of ε488 = 55,000 $M^{-1}cm^{-1}$ and of TagRFP at 555 nm of ε555 = 100,000 M^{-1} cm^{-1} , respectively.[59, 97]

4.6.6 Farnesylation of purified His_6-eGFP-GCVDS and His_6-TagRFP-GCVDS proteins: Farnesylation of purified fluorescent proteins was performed by incubation of the appropriate substrate protein (His_6-eGFP-GCVDS or His_6-TagRFP-GCVDS, 2.5 µM) with 200 nM FTase, 10 µM of Farnesyl pyrophosphate (FPP), and 5 mM $MgCl_2$ in reaction buffer (50 mM NaHEPPSO, 5 mM TCEP, pH 7.8) in a final volume of 0.5 mL. Substrate proteins were incubated in reaction buffer for 20 min prior to reaction initiation by addition of FTase and FPP to reduce disulfide bonds. Reactions were covered with foil and incubated overnight at room temperature.

4.6.7 Geranylgeranylation of purified His_6-eGFP-GCVLL: Geranylgeranylation of purified His_6-eGFP-GCVLL fluorescent proteins was performed $MgCl_2$ in reaction buffer (50 mM NaHEPPSO, 5 mM TCEP, pH 7.8) in a final volume of 0.5 mL. The substrate protein was incubated in reaction buffer for 20 min prior to reaction initiation by addition of GGTase-I and Geranylgeranyl pyrophosphate (GGPP) to reduce disulfide bonds. Reactions were covered with foil and incubated overnight at room temperature.

4.6.8 Farnesylation of His_6-eGFP-GCVDS in bacterial lysates: A frozen 1 mL aliquot of BL21 (DE3) cells expressing His_6-eGFP-GCVDS was thawed on ice. Bacterial cells in suspension (900 mL) were lysed by addition of 100 mL of Fast Break lysis reagent (Promega) followed by moderate shaking for 15 min at room temperature. The resulting lysate was clarified by centrifugation (1,000 × g, 5 min). The concentration of the His_6-eGFPGCVDS in the clarified lysate was determined by

absorbance of eGFP at 488 nm of ε488 = 55,000 $M^{-1}cm^{-1}$). Farnesylation of His_6-eGFP-GCVDS in crude lysate was performed by incubation of sufficient lysate supernatant to reach a final concentration of 2.5 μM protein with 200 nM RL FTase, 10 μM FPP, 5 mM $MgCl_2$, reaction buffer (50 mM NaHEPPSO, 5 mM TCEP, pH 7.8) in a final volume of 0.5 mL. Supernatant containing the protein was incubated with 1× reaction buffer for 20 min to reduce disulfide bonds prior to initiation of the reaction. Reactions were incubated for overnight at room temperature.

4.6.9 Preparation of HEK293 cells expressing eGFP-HRas (There experiments were performed Soumyashree Gangopadhyay, Syracuse University): The mammalian cell line HEK293 (ATCC) was maintained in 75 mL vented tissue culture flasks (Celltreat), and were split upon reaching 80% confluency. The cells were grown in complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (MediaTech)) in a humidified atmosphere with 5% CO_2 at 37°C. For expression of the eGFP-HRas reporter protein, 9×10^4 cells were placed in 2 mL of complete DMEM per well of a tissue culture treated 6-well plate (Corning) (total of 5 wells). The cells were incubated 18-20 hours prior to transfection. The DNA-transfection reagent complex was prepared by incubating 4 μg of the reporter protein expression plasmid pEGFP-HRas (Casey laboratory, Duke University) and 9 μL of the Turbofect transfection reagent (Thermo Scientific) in a total volume of 500 μL supplement free DMEM for 15 minutes at room temperature.[102] The cells were then transfected with the prepared DNA-transfection reagent complex by drop wise addition into the wells of a 6-well tissue culture plate. A parallel set of wells was treated with the FTase inhibitor tipifarnib at a concentration of 500 nM. Following transfection for 24 h, live cells were imaged using a Zeiss Axio Vert.A1 inverted fluorescence microscope with a 470/40 nm excitation filter, a 495 nm beam

splitter and a 525/50 nm emission filter to verify fluorescent protein expression and analyze fluorescence localization behavior in presence and absence of tipifarnib. The cells were then scraped and resuspended in phosphate buffered saline (PBS) followed by centrifugation to harvest the cell pellet. The cell pellets were stored at –80°C.

4.6.10 Creation of pCAF mammalian expression plasmid containing the TagRFP-link(HRas)- CVLL reporter protein: A gene encoding the Tag RFP-link(HRas)-CVLL reporter protein was prepared by PCR mutagenesis by mutating the terminal serine residue of the previously reported TagRFPlink(HRas)-CVLS reporter protein to a leucine residue to obtain the pCAF1-TagRFP-link(HRas)-CVLL vector.[63] In the Tag RFP-link(HRas)-CVLL reporter protein, the 15 amino acids of HRas upstream of the farnesylation site within Hras and a C-terminal -GCVLL sequence are fused to TagRFP (-KLNPPDESGPGCMSCGCVLL). Primers were dissolved in ultra-pure water and concentrations were measured by UV absorbance at 260 nm (1 OD = 33 µg/mL). The PCR mutagenesis reaction (50 µL) contained 10 x Pfu reaction buffer (5 µL), pCAF1-TagRFPlink(HRas)-CVLS template plasmid (10 ng), forward primer (125 ng), reverse primer (125 ng), 10 mM dNTPs, and Pfu Turbo DNA polymerase (1 µL, 2.5 U/µL). PCR mutagenesis was performed using the following temperature program: Initial denaturation (95°C, 1 min); 18 cycles of denaturation (95°C, 50 sec); annealing (60°C, 50 sec) and extension (68°C, 12 min) followed by final extension (68°C, 12 min). Following PCR, reactions were treated with DpnI (1 µL, 10 units/µL) and incubated at 37°C for 1 hour. Z-competent DH5α E. coli cells (Zymo Research) were thawed on ice for 10 minutes and then transformed with 5 µL of the PCR reaction following which the cells were plated on pre-warmed LB-Amp plates and incubated overnight at 37°C. Two colonies were picked from each plate and inoculated into LB media (5 mL) containing

100 µg/mL ampicillin and incubated with shaking (225 rpm) overnight at 37°C. Following overnight growth, a 10% glycerol stock was prepared stored at -80°C. Plasmid DNA was purified from the remaining saturated culture using EZ-10 spin column DNA purification kit (BioBasic) per the manufacturer's protocol. The mutations were confirmed by DNA sequencing (Genscript).

4.6.11 Preparation of MDA-231shp53 cells expressing TagRFP-link(HRas)-CVLL in the presence and absence of doxycycline (There experiments were performed Soumyashree Gangopadhyay, Syracuse University): The mammalian cell line MDA-231shp53 (CUNY-Hunter College, New York) was maintained in 75 mL vented tissue culture flasks (Celltreat), and were split upon reaching 80% confluency.[90] The cells were grown in complete DMEM in a humidified atmosphere with 5% CO_2 at 37°C. For doxycycline treatment, doxycycline (6 µg/mL) was added to the 75 mL culture flask and the cells were incubated as described above. The cells were grown in the presence of doxycycline for at least 8 days to ensure suppression of mutant p53 expression. For expression of the reporter protein, 1×10^5 cells were placed in 2 mL of complete DMEM per well (5 total wells) of a tissue culture treated 6-well plate (Corning). For the doxycycline treated cells, 6 µg/mL doxycycline was added to each well prior to transfection. Both doxycycline treated and untreated cells were incubated 18- 20 h prior to transfection. The DNA-transfection reagent complex was prepared by incubating 1 µg of the pCAF1-TagRFP-link(Hras)-CVLL reporter protein plasmid and 2 µL of the XtremeGENE HP transfection reagent (Roche) in a total volume of 100 µL supplement free DMEM for 30 min at room temperature. The cells were then transfected with the prepared DNA-transfection reagent complex by drop wise addition into the wells of a 6-well tissue culture plate. Upon 24 h transfection, live cells were imaged using a Zeiss Axio Vert.A1 inverted fluorescence microscope with a 545/525 nm excitation filter, a 565

nm beam splitter, and a 605/70 nm emission filter to verify fluorescent protein expression. The cells were then scraped and resuspended in PBS, followed by centrifugation to harvest the cell pellet. The cell pellets were stored at −80°C.

4.6.12 Control experiments with HEK293 cells expressing TagRFP-link(HRas)-CVLL (There experiments were performed Soumyashree Gangopadhyay, Syracuse University): The mammalian cell line HEK293 (ATCC) was maintained in 75 mL vented tissue culture flasks (Celltreat), and were split upon reaching 80% confluency. The cells were grown in complete DMEM penicillin-streptomycin (MediaTech)) an in a humidified atmosphere with 5% CO_2 at 37°C. For expression of the reporter protein, 1×10^5 cells were placed in 2 mL of complete DMEM per well of a tissue culture treated 6-well plate (Corning) (5 total wells). For the doxycycline treated cells, 6 µg/mL doxycycline was added to each well prior to transfection. Both doxycycline treated and untreated cells were incubated 18-20 h prior to transfection. The DNA-transfection reagent complex was prepared by incubating 4 µg of the pCAF1-TagRFP-link(Hras)-CVLL reporter protein plasmid and 9 µL of the Turbofect transfection reagent (Thermo Scientific) in a total volume of 500 µL supplement free DMEM for 15 min at room temperature. The cells were then transfected with the prepared DNA-transfection reagent complex by drop wise addition into the wells of a 6-well tissue culture plate. Upon 24 h transfection, live cells were imaged using a Zeiss Axio Vert.A1 inverted fluorescence microscope with a 545/525 nm excitation filter, a 565 nm beam splitter and a 605/70 nm emission filter to verify fluorescent protein expression. The cells were then scraped and resuspended in PBS followed by centrifugation to harvest the cell pellet. The cell pellets were stored at −80°C.

4.6.13 Preparation and analysis of proteins by capillary electrophoresis (Capillary electrophoresis experiments were performed in the Krylov laboratory, York University):

Prior to the CE experiments, HEK293 and MDA-231 shp53 cells were centrifuged for 2 min at ~13000 × g (Eppendorf 5417R centrifuge with F45−30−11 rotor (Fisher scientific, PA, USA)) at 4 °C and the supernatant was collected. The obtained supernatant as well as purified proteins were diluted with the sample buffer consisting of 50 mM HEPES sodium salt (NaHEPPSO), 10 mM $MgCl_2$, 5 mM tris(2-carboxyethyl)- phosphine hydrochloride (TCEP), and 10 mg/mL bovine serum albumin (BSA) at pH 7.8. The BSA was added to reduce adsorption of the proteins onto capillary surface.

CE experiments were carried out with MDQ-PACE instrument (Sciex, formerly BeckmanCoulter, Caledon, ON, Canada) using laser-induced fluorescence (LIF) detection with excitation at 488 nm and emission at 520 and 610 nm for the detection of eGFP and TagRFP derivatives, respectively. Fused silica capillaries with total length of 84 cm were preconditioned by sequential washing with 100 mM HCl, 100 mM NaOH, Milli-Q water and run buffer each for 2 min at 30 psi (206.84 kPa). In a beginning of each experiment capillaries were rinsed with each of the following solutions: 100 mM HCl, 100 mM NaOH Milli-Q water for 2 min at 30 psi (206.84 kPa) and with run buffer for 2 min at 40 psi (275.79 kPa). The samples were injected into the capillary by 1 psi (6.89 kPa) pressure pulse of 28 s. Electrophoretic separation was carried out with a positive electrode at the injection end of the capillary, with electric field strength of 297 V/cm.

4.7 References

1. Otieno, A. C.; Mwongela, S. M., Capillary electrophoresis-based methods for the determination of lipids--a review. *Analytica chimica acta* **2008,** *624* (2), 163-74.

2. Harstad, R. K.; Johnson, A. C.; Weisenberger, M. M.; Bowser, M. T., Capillary Electrophoresis. *Analytical Chemistry* **2016,** *88* (1), 299-319.

3. Grossman, P. D.; Colburn, J. C., *Capillary Electrophoresis: Theory and Practice*. Elsevier Science: 2012.

4. Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T., Electrokinetic separations with micellar solutions and open-tubular capillaries. *Analytical Chemistry* **1984,** *56* (1), 111-113.

5. Lian, D.-S.; Zhao, S.-J., Capillary electrophoresis based on nucleic acid detection for diagnosing human infectious disease. In *Clinical Chemistry and Laboratory Medicine (CCLM)*, 2016; Vol. 54, p 707.

6. Britz-McKibbin, P.; Chen, D. D. Y., Selective Focusing of Catecholamines and Weakly Acidic Compounds by Capillary Electrophoresis Using a Dynamic pH Junction. *Analytical Chemistry* **2000,** *72* (6), 1242-1252.

7. Wu, X. Z.; Zhang, L. H.; Onoda, K., Isoelectric focusing sample injection for capillary electrophoresis of proteins. *Electrophoresis* **2005,** *26* (3), 563-70.

8. Froim, D.; Hopkins, C. E.; Belenky, A.; Cohen, A. S., Method for phosphorothioate antisense DNA sequencing by capillary electrophoresis with UV detection. *Nucleic Acids Research* **1997,** *25* (21), 4219-4223.

9. Forteschi, M.; Zinellu, A.; Assaretti, S.; Mangoni, A. A.; Pintus, G.; Carru, C.; Sotgia, S., An isotope dilution capillary electrophoresis/tandem mass spectrometry (CE-MS/MS) method for

the simultaneous measurement of choline, betaine, and dimethylglycine concentrations in human plasma. *Analytical and Bioanalytical Chemistry* **2016,** *408* (26), 7505-7512.

10. Dominguez-Vega, E.; De Vijlder, T.; Romijn, E. P.; Somsen, G. W., Capillary electrophoresis-tandem mass spectrometry as a highly selective tool for the compositional and site-specific assessment of multiple peptide-deamidation. *Analytica chimica acta* **2017,** *982*, 122-130.

11. Swinney, K.; Bornhop, D. J., Detection in capillary electrophoresis. *Electrophoresis* **2000,** *21* (7), 1239-50.

12. Won, S. G.; Sung, H. H.; Boram, C.; Yeol, J. G., Recent developments in CE-based detection methods for food-borne pathogens. *Electrophoresis* **2010,** *31* (13), 2137-2153.

13. Murphy, K. M.; Levis, M.; Hafez, M. J.; Geiger, T.; Cooper, L. C.; Smith, B. D.; Small, D.; Berg, K. D., Detection of FLT3 Internal Tandem Duplication and D835 Mutations by a Multiplex Polymerase Chain Reaction and Capillary Electrophoresis Assay. *The Journal of Molecular Diagnostics* **2003,** *5* (2), 96-102.

14. Ren, J., High-throughput single-strand conformation polymorphism analysis by capillary electrophoresis. *Journal of Chromatography B: Biomedical Sciences and Applications* **2000,** *741* (2), 115-128.

15. Le, H.; Fung, D.; Trent, R. J., Applications of capillary electrophoresis in DNA mutation analysis of genetic disorders. *Molecular Pathology* **1997,** *50* (5), 261-265.

16. Wei, S.; Song, G.; Lin, J.-M., Separation and determination of norepinephrine, epinephrine and isoprinaline enantiomers by capillary electrophoresis in pharmaceutical formulation and human serum. *Journal of Chromatography A* **2005,** *1098* (1), 166-171.

17. Hermann, W.; Matthias, D.; Annette, K., Strategies for capillary electrophoresis: Method development and validation for pharmaceutical and biological applications. *Electrophoresis* **1998,** *19* (16-17), 2695-2752.

18. Ahmad, A., Recent developments in chiral capillary electrophoresis and applications of this technique to pharmaceutical and biomedical analysis. *Electrophoresis* **2001,** *22* (15), 3107-3130.

19. Cheng, J.; Waters, L. C.; Fortina, P.; Hvichia, G.; Jacobson, S. C.; Ramsey, J. M.; Kricka, L. J.; Wilding, P., Degenerate Oligonucleotide Primed–Polymerase Chain Reaction and Capillary Electrophoretic Analysis of Human DNA on Microchip-Based Devices. *Analytical biochemistry* **1998,** *257* (2), 101-106.

20. Woolley, A. T.; Mathies, R. A., Ultra-High-Speed DNA Sequencing Using Capillary Electrophoresis Chips. *Analytical Chemistry* **1995,** *67* (20), 3676-3680.

21. M., B. J.; Eric, B.; Federica, C.; R., M. B., Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. *Electrophoresis* **2004,** *25* (10-11), 1397-1412.

22. Sobral, D.; Schwarz, S.; Bergonier, D.; Brisabois, A.; Feßler, A. T.; Gilbert, F. B.; Kadlec, K.; Lebeau, B.; Loisy-Hamon, F.; Treilles, M.; Pourcel, C.; Vergnaud, G., High Throughput Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA) of Staphylococcus aureus from Human, Animal and Food Sources. *PloS one* **2012,** *7* (5), e33967.

23. Pourcel, C.; Hormigos, K.; Onteniente, L.; Sakwinska, O.; Deurenberg, R. H.; Vergnaud, G., Improved multiple-locus variable-number tandem-repeat assay for Staphylococcus aureus genotyping, providing a highly informative technique together with strong phylogenetic value. *Journal of clinical microbiology* **2009,** *47* (10), 3121-8.

24. te Witt, R.; van Belkum, A.; MacKay, W. G.; Wallace, P. S.; van Leeuwen, W. B., External quality assessment of the molecular diagnostics and genotyping of meticillin-resistant Staphylococcus aureus. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **2010,** *29* (3), 295-300.

25. El Deeb, S.; AbuIriban, M.; Gust, R., *MEKC as a Powerful Growing Analytical Technique*. 2011; Vol. 32, p 166-83.

26. Walsh, C. T., *Posttranslational Modification of Proteins: Expanding Nature's Inventory*. Roberts & Company Publishers: 2005.

27. Walsh, C. T.; Garneau-Tsodikova, S.; Gatto, G. J., Jr., Protein posttranslational modifications: the chemistry of proteome diversifications. *Angewandte Chemie (International ed. in English)* **2005,** *44* (45), 7342-72.

28. Martin, D. D.; Beauchamp, E.; Berthiaume, L. G., Post-translational myristoylation: Fat matters in cellular life and death. *Biochimie* **2011,** *93* (1), 18-31.

29. Resh, M. D., Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochimica et biophysica acta* **1999,** *1451* (1), 1-16.

30. Hancock, J. F.; Paterson, H.; Marshall, C. J., A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* **1990,** *63* (1), 133-9.

31. Lin, D. T. S.; Davis, N. G.; Conibear, E., Targeting the Ras palmitoylation/depalmitoylation cycle in cancer. *Biochemical Society transactions* **2017,** *45* (4), 913-921.

32. Nadolski, M. J.; Linder, M. E., Protein lipidation. *The FEBS journal* **2007,** *274* (20), 5202-10.

33. Rocks, O.; Peyker, A.; Kahms, M.; Verveer, P. J.; Koerner, C.; Lumbierres, M.; Kuhlmann, J.; Waldmann, H.; Wittinghofer, A.; Bastiaens, P. I., An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science (New York, N.Y.)* **2005,** *307* (5716), 1746-52.

34. Benetka, W.; Koranda, M.; Eisenhaber, F., Protein Prenylation: An (Almost) Comprehensive Overview on Discovery History, Enzymology, and Significance in Physiology and Disease. *Monatshefte für Chemie / Chemical Monthly* **2006,** *137* (10), 1241.

35. Casey, P. J.; Seabra, M. C., Protein prenyltransferases. *The Journal of biological chemistry* **1996,** *271* (10), 5289-92.

36. Cox, A. D.; Der, C. J., Protein prenylation: more than just glue? *Current opinion in cell biology* **1992,** *4* (6), 1008-16.

37. Gao, J.; Liao, J.; Yang, G. Y., CAAX-box protein, prenylation process and carcinogenesis. *American journal of translational research* **2009,** *1* (3), 312-25.

38. Inglese, J.; Koch, W. J.; Caron, M. G.; Lefkowitz, R. J., Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature* **1992,** *359* (6391), 147-50.

39. Marshall, C. J., Protein prenylation: a mediator of protein-protein interactions. *Science (New York, N.Y.)* **1993,** *259* (5103), 1865-6.

40. Wang, M.; Casey, P. J., Protein prenylation: unique fats make their mark on biology. *Nat Rev Mol Cell Biol* **2016,** *17* (2), 110-22.

41. Birgit, E.; Sebastian, M. S.; Maria, N.; Georg, S.; Frank, E., Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. *BioEssays* **2003,** *25* (4), 367-385.

42. Nosjean, O.; Briolay, A.; Roux, B., Mammalian GPI proteins: sorting, membrane residence and functions. *Biochimica et biophysica acta* **1997,** *1331* (2), 153-86.

43. Paulick, M. G.; Bertozzi, C. R., The Glycosylphosphatidylinositol Anchor: A Complex Membrane-Anchoring Structure for Proteins. *Biochemistry* **2008,** *47* (27), 6991-7000.

44. Maurer-Stroh, S.; Koranda, M.; Benetka, W.; Schneider, G.; Sirota, F. L.; Eisenhaber, F., Towards complete sets of farnesylated and geranylgeranylated proteins. *PLoS computational biology* **2007,** *3* (4), e66.

45. Reid, T. S.; Terry, K. L.; Casey, P. J.; Beese, L. S., Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. *Journal of molecular biology* **2004,** *343* (2), 417-33.

46. Hougland, J. L.; Hicks, K. A.; Hartman, H. L.; Kelly, R. A.; Watt, T. J.; Fierke, C. A., Identification of novel peptide substrates for protein farnesyltransferase reveals two substrate classes with distinct sequence selectivities. *Journal of molecular biology* **2010,** *395* (1), 176-90.

47. Caplin, B. E.; Hettich, L. A.; Marshall, M. S., Substrate characterization of the Saccharomyces cerevisiae protein farnesyltransferase and type-I protein geranylgeranyltransferase. *Biochimica et biophysica acta* **1994,** *1205* (1), 39-48.

48. Hancock, J. F., Reticulocyte lysate assay for in vitro translation and posttranslational modification of Ras proteins. *Methods in enzymology* **1995,** *255*, 60-5.

49. Wilson, A. L.; Maltese, W. A., [8] Coupled translation/ prenylation of rab proteins in Vitro. In *Methods in enzymology*, Academic Press: 1995; Vol. 250, pp 79-91.

50. Gibbs, B. S.; Zahn, T. J.; Mu, Y.; Sebolt-Leopold, J. S.; Gibbs, R. A., Novel farnesol and geranylgeraniol analogues: A potential new class of anticancer agents directed against protein prenylation. *Journal of medicinal chemistry* **1999,** *42* (19), 3800-8.

51. Nguyen, U. T.; Guo, Z.; Delon, C.; Wu, Y.; Deraeve, C.; Franzel, B.; Bon, R. S.; Blankenfeldt, W.; Goody, R. S.; Waldmann, H.; Wolters, D.; Alexandrov, K., Analysis of the

eukaryotic prenylome by isoprenoid affinity tagging. *Nature chemical biology* **2009,** *5* (4), 227-35.

52. Kho, Y.; Kim, S. C.; Jiang, C.; Barma, D.; Kwon, S. W.; Cheng, J.; Jaunbergs, J.; Weinbaum, C.; Tamanoi, F.; Falck, J.; Zhao, Y., A tagging-via-substrate technology for detection and proteomics of farnesylated proteins. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (34), 12479-12484.

53. Berry, A. F.; Heal, W. P.; Tarafder, A. K.; Tolmachova, T.; Baron, R. A.; Seabra, M. C.; Tate, E. W., Rapid multilabel detection of geranylgeranylated proteins by using bioorthogonal ligation chemistry. *Chembiochem : a European journal of chemical biology* **2010,** *11* (6), 771-3.

54. Wang, Y.-C.; Dozier, J. K.; Beese, L. S.; Distefano, M. D., Rapid Analysis of Protein Farnesyltransferase Substrate Specificity Using Peptide Libraries and Isoprenoid Diphosphate Analogues. *ACS Chemical Biology* **2014,** *9* (8), 1726-1735.

55. Zhang, Y.; Blanden, M. J.; Sudheer, C.; Gangopadhyay, S. A.; Rashidian, M.; Hougland, J. L.; Distefano, M. D., Simultaneous Site-Specific Dual Protein Labeling Using Protein Prenyltransferases. *Bioconjug Chem* **2015,** *26* (12), 2542-53.

56. Blanden, M. J.; Suazo, K. F.; Hildebrandt, E. R.; Hardgrove, D. S.; Patel, M.; Saunders, W. P.; Distefano, M. D.; Schmidt, W. K.; Hougland, J. L., Efficient farnesylation of an extended C-terminal C(x)3X sequence motif expands the scope of the prenylated proteome. *The Journal of biological chemistry* **2018,** *293* (8), 2770-2785.

57. Hosokawa, A.; Wollack, J. W.; Zhang, Z.; Chen, L.; Barany, G.; Distefano, M. D., Evaluation of an Alkyne-containing Analogue of Farnesyl Diphosphate as a Dual Substrate for Protein-prenyltransferases. *International Journal of Peptide Research and Therapeutics* **2007,** *13* (1), 345-354.

58. P., D. B.; Zhiyuan, Z.; Ayako, H.; D., D. M., Selective Labeling of Proteins by Using Protein Farnesyltransferase. *Chembiochem : a European journal of chemical biology* **2007,** *8* (1), 98-105.

59. Rashidian, M.; Dozier, J. K.; Lenevich, S.; Distefano, M. D., Selective labeling of polypeptides using protein farnesyltransferase via rapid oxime ligation. *Chem Commun (Camb)* **2010,** *46* (47), 8998-9000.

60. Turek-Etienne, T. C.; Strickland, C. L.; Distefano, M. D., Biochemical and Structural Studies with Prenyl Diphosphate Analogues Provide Insights into Isoprenoid Recognition by Protein Farnesyl Transferase. *Biochemistry* **2003,** *42* (13), 3716-3724.

61. Mahmoodi, M. M.; Rashidian, M.; Dozier, J. K.; Distefano, M. D., Chemoenzymatic site-specific reversible immobilization and labeling of proteins from crude cellular extract without prior purification using oxime and hydrazine ligation. *Current protocols in chemical biology* **2013,** *5* (2), 89-109.

62. Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D., Chemoenzymatic Reversible Immobilization and Labeling of Proteins without Prior Purification. *Journal of the American Chemical Society* **2012,** *134* (20), 8455-8467.

63. Flynn, S. C.; Lindgren, D. E.; Hougland, J. L., Quantitative Determination of Cellular Farnesyltransferase Activity: Towards Defining the Minimum Substrate Reactivity for Biologically Relevant Protein Farnesylation. *Chembiochem : a European journal of chemical biology* **2014,** *15* (15), 2205-2210.

64. Ochocki, J. D.; Igbavboa, U.; Wood, W. G.; Arriaga, E. A.; Wattenberg, E. V.; Distefano, M. D., Evaluation of prenylated peptides for use in cellular imaging and biochemical analysis. *Methods in molecular biology (Clifton, N.J.)* **2014,** *1088*, 213-23.

65. Thissen, J. A.; Gross, J. M.; Subramanian, K.; Meyer, T.; Casey, P. J., Prenylation-dependent association of Ki-Ras with microtubules. Evidence for a role in subcellular trafficking. *The Journal of biological chemistry* **1997,** *272* (48), 30362-70.

66. Lamphear, C. L.; Fierke, C. A., Molecular recognition of FTase and GGTase-I in vivo. *The FASEB Journal* **2010,** *24* (1 Supplement), 904.4.

67. Fiordalisi, J. J.; Johnson, R. L., 2nd; Weinbaum, C. A.; Sakabe, K.; Chen, Z.; Casey, P. J.; Cox, A. D., High affinity for farnesyltransferase and alternative prenylation contribute individually to K-Ras4B resistance to farnesyltransferase inhibitors. *The Journal of biological chemistry* **2003,** *278* (43), 41718-27.

68. Berndt, N.; Sebti, S. M., Measurement of protein farnesylation and geranylgeranylation in vitro, in cultured cells and in biopsies, and the effects of prenyl transferase inhibitors. *Nature protocols* **2011,** *6* (11), 1775-91.

69. Ntantie, E.; Gonyo, P.; Lorimer, E. L.; Hauser, A. D.; Schuld, N.; McAllister, D.; Kalyanaraman, B.; Dwinell, M. B.; Auchampach, J. A.; Williams, C. L., An Adenosine-Mediated Signaling Pathway Suppresses Prenylation of the GTPase Rap1B and Promotes Cell Scattering. *Science Signaling* **2013,** *6* (277), ra39.

70. Troutman, J. M.; Roberts, M. J.; Andres, D. A.; Spielmann, H. P., Tools To Analyze Protein Farnesylation in Cells. *Bioconjugate Chemistry* **2005,** *16* (5), 1209-1217.

71. Hancu, G.; Simon, B.; Rusu, A.; Mircia, E.; Gyéresi, Á., Principles of Micellar Electrokinetic Capillary Chromatography Applied in Pharmaceutical Analysis. *Advanced Pharmaceutical Bulletin* **2013,** *3* (1), 1-8.

72. Gong, F.; Yang, H.; Sun, W.; Cao, J.; Liu, W., Development and validation of a micellar electrokinetic capillary chromatography method for the determination of goserelin and related substances. *Electrophoresis* **2016,** *37* (4), 623-9.

73. Koji, O.; Shigeru, T., Micellar electrokinetic chromatography. *Molecular Biotechnology* **1998,** *9* (3), 253-271.

74. Stanley, B.; Mehr, K. A.; Kellock, T.; Van Hamme, J. D.; Donkor, K. K., Separation and determination of closely related lantibiotics by micellar electrokinetic chromatography. *Journal of separation science* **2009,** *32* (17), 2993-3000.

75. Poitevin, M.; Tranquart, F.; Cherkaoui, S., Use of capillary electrophoresis as a versatile tool to measure interaction constants between a KDR-binding PEGylated lipopeptide and pegylated phospholipid micelles. *Electrophoresis* **2015,** *36* (2), 326-34.

76. Le, Z.; Krylov, S. N.; Hu, S.; Dovichi, N. J., Methyl-beta-cyclodextrin modified micellar electrokinetic capillary chromatography with laser-induced fluorescence for separation and detection of phospholipids. *Journal of chromatography. A* **2000,** *894* (1-2), 129-34.

77. Ochocki, J. D.; Igbavboa, U.; Wood, W. G.; Wattenberg, E. V.; Distefano, M. D., Evaluation of prenylated peptides for use in cellular imaging and biochemical analysis. *Methods in molecular biology (Clifton, N.J.)* **2014,** *1088*, 213-223.

78. Berezovski, M.; Li, W. P.; Poulter, C. D.; Krylov, S. N., Measuring the activity of farnesyltransferase by capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis* **2002,** *23* (19), 3398-403.

79. Seddon, A. M.; Curnow, P.; Booth, P. J., Membrane proteins, lipids and detergents: not just a soap opera. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2004,** *1666* (1), 105-117.

80. Shala-Lawrence, A.; Blanden, M. J.; Krylova, S. M.; Gangopadhyay, S. A.; Beloborodov, S. S.; Hougland, J. L.; Krylov, S. N., Simultaneous Analysis of a Non-Lipidated Protein and Its Lipidated Counterpart: Enabling Quantitative Investigation of Protein Lipidation's Impact on Cellular Regulation. *Analytical Chemistry* **2017,** *89* (24), 13502-13507.

81. Hougland, J. L.; Gangopadhyay, S. A.; Fierke, C. A., Expansion of protein farnesyltransferase specificity using "tunable" active site interactions: development of bioengineered prenylation pathways. *The Journal of biological chemistry* **2012,** *287* (45), 38090-100.

82. Cavazza, A.; Corradini, C.; Lauria, A.; Nicoletti, I.; Stancanelli, R., Rapid analysis of essential and branched-chain amino acids in nutraceutical products by micellar electrokinetic capillary chromatography. *Journal of agricultural and food chemistry* **2000,** *48* (8), 3324-9.

83. Bos, J. L., ras oncogenes in human cancer: a review. *Cancer research* **1989,** *49* (17), 4682-9.

84. Campbell, P. M.; Der, C. J., Oncogenic Ras and its role in tumor cell invasion and metastasis. *Seminars in cancer biology* **2004,** *14* (2), 105-14.

85. Clarke, S.; Vogel, J. P.; Deschenes, R. J.; Stock, J., Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** *85* (13), 4643-7.

86. Alsina, M.; Fonseca, R.; Wilson, E. F.; Belle, A. N.; Gerbino, E.; Price-Troska, T.; Overton, R. M.; Ahmann, G.; Bruzek, L. M.; Adjei, A. A.; Kaufmann, S. H.; Wright, J. J.; Sullivan, D.; Djulbegovic, B.; Cantor, A. B.; Greipp, P. R.; Dalton, W. S.; Sebti, S. M., Farnesyltransferase inhibitor tipifarnib is well tolerated, induces stabilization of disease, and inhibits farnesylation and

oncogenic/tumor survival pathways in patients with advanced multiple myeloma. *Blood* **2004,** *103* (9), 3271-7.

87. Freed-Pastor, W. A.; Prives, C., Mutant p53: one name, many proteins. *Genes & development* **2012,** *26* (12), 1268-86.

88. Labuschagne, C. F.; Zani, F.; Vousden, K. H., Control of metabolism by p53 - Cancer and beyond. *Biochimica et biophysica acta* **2018**.

89. Joerger, A. C.; Fersht, A. R., The p53 Pathway: Origins, Inactivation in Cancer, and Emerging Therapeutic Approaches. *Annual review of biochemistry* **2016,** *85* (1), 375-404.

90. Freed-Pastor, W. A.; Mizuno, H.; Zhao, X.; Langerod, A.; Moon, S. H.; Rodriguez-Barrueco, R.; Barsotti, A.; Chicas, A.; Li, W.; Polotskaia, A.; Bissell, M. J.; Osborne, T. F.; Tian, B.; Lowe, S. W.; Silva, J. M.; Borresen-Dale, A. L.; Levine, A. J.; Bargonetti, J.; Prives, C., Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. *Cell* **2012,** *148* (1-2), 244-58.

91. Blanden, A. R.; Yu, X.; Loh, S. N.; Levine, A. J.; Carpizo, D. R., Reactivating mutant p53 using small molecules as zinc metallochaperones: awakening a sleeping giant in cancer. *Drug discovery today* **2015,** *20* (11), 1391-7.

92. Hildebrandt, E. R.; Cheng, M.; Zhao, P.; Kim, J. H.; Wells, L.; Schmidt, W. K., A shunt pathway limits the CaaX processing of Hsp40 Ydj1p and regulates Ydj1p-dependent phenotypes. *eLife* **2016,** *5*.

93. Cao, P.; Stults, J. T., Mapping the phosphorylation sites of proteins using on-line immobilized metal affinity chromatography/capillary electrophoresis/electrospray ionization multiple stage tandem mass spectrometry. *Rapid communications in mass spectrometry : RCM* **2000,** *14* (17), 1600-6.

94. Feng, J.; Arriaga, E. A., Quantification of carbonylated proteins in rat skeletal muscle mitochondria using capillary sieving electrophoresis with laser-induced fluorescence detection. *Electrophoresis* **2008,** *29* (2), 475-82.

95. Amon, S.; Zamfir, A. D.; Rizzi, A., Glycosylation analysis of glycoproteins and proteoglycans using capillary electrophoresis-mass spectrometry strategies. *Electrophoresis* **2008,** *29* (12), 2485-507.

96. Na, D. H.; Park, M. O.; Choi, S. Y.; Kim, Y. S.; Lee, S. S.; Yoo, S. D.; Lee, H. S.; Lee, K. C., Identification of the modifying sites of mono-PEGylated salmon calcitonins by capillary electrophoresis and MALDI-TOF mass spectrometry. *Journal of chromatography. B, Biomedical sciences and applications* **2001,** *754* (1), 259-63.

97. Merzlyak, E. M.; Goedhart, J.; Shcherbo, D.; Bulina, M. E.; Shcheglov, A. S.; Fradkov, A. F.; Gaintzeva, A.; Lukyanov, K. A.; Lukyanov, S.; Gadella, T. W. J.; Chudakov, D. M., Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nature Methods* **2007,** *4*, 555.

98. Hougland, J. L.; Gangopadhyay, S. A.; Fierke, C. A., Expansion of Protein Farnesyltransferase Specificity Using "Tunable" Active Site Interactions: DEVELOPMENT OF BIOENGINEERED PRENYLATION PATHWAYS. *Journal of Biological Chemistry* **2012,** *287* (45), 38090-38100.

99. Gangopadhyay, S. A.; Losito, E. L.; Hougland, J. L., Targeted reengineering of protein geranylgeranyltransferase type I selectivity functionally implicates active-site residues in protein-substrate recognition. *Biochemistry* **2014,** *53* (2), 434-46.

100. Hartman, H. L.; Bowers, K. E.; Fierke, C. A., Lysine beta311 of protein geranylgeranyltransferase type I partially replaces magnesium. *The Journal of biological chemistry* **2004,** *279* (29), 30546-53.

101. Hartman, H. L.; Hicks, K. A.; Fierke, C. A., Peptide specificity of protein prenyltransferases is determined mainly by reactivity rather than binding affinity. *Biochemistry* **2005,** *44* (46), 15314-24.

102. Chen, Z.; Otto, J. C.; Bergo, M. O.; Young, S. G.; Casey, P. J., The C-terminal polylysine region and methylation of K-Ras are critical for the interaction between K-Ras and microtubules. *The Journal of biological chemistry* **2000,** *275* (52), 41251-7.

Chapter 5: Conclusions and Future Work

5.1 Summary

Prenylation is an important post-translational lipidation involved in cell signaling and growth through changes in protein structure, localization, and function.[1-8] For over 3 decades, the four amino acid, C-terminal "CaaX" box motif has been thought of as the required recognition motif for prenylation through study of yeast mating factors and lamins.[1, 3, 6, 8-15] Study of what makes a protein a target for prenylation by FTase and GGTase-I has focused mainly on amino acid selectivity within these four amino acids. Through biochemical, crystallographic, and computational work, general rules for this selectivity and the proposed contacts within the enzyme's binding site have greatly increased our understanding of prenylation but have restricted investigation to the CaaX box motif.[5, 11, 16-29]

Recently, yeast genetic screening involving the Hsp40 co-chaperone Ydj1p revealed that yeast FTase could efficiently prenylate $C(x)_3X$ sequences.[30] This finding led us to use fluorescent-based biochemical and cell-based studies at both the short peptide and full-length protein level to determine that mammalian FTase can in fact recognize and prenylate a five amino acid recognition motif. A search of the human genome using Prosite results in 1008 (as of June 2018) proteins ending in a $C(x)_3X$ sequence with the potential to undergo prenylation, nearly doubling the number of proteins that can be explored not only in humans, but also species such as yeast.. With previous studies and current computational prediction models restricted to four amino acids, innovative approaches to determining the role of this novel selectivity will need to be addressed for such as large scale of potential proteins.

This finding increases our understanding of the impact prenylation has within cells and raises many new questions concerning the role of these $C(x)_3X$ proteins. With FTase and GGTase-

I showing a stark difference in their acceptance of these longer sequences, a question is raised as to why these "sister" enzymes present such a dissimilarity in their flexibility for substrates. Alongside the question of how FTase recognizes these sequences, is the consideration of the biological role of $C(x)_3X$ proteins. The potential for a shunt pathway in humans, as seen with yeast, would define a new cytosolic role for prenylation in mammalian cells.[31]

The new method protein-lipidation quantitation (PLQ) may prove useful in studying lipidation at a quantitative level for these new $C(x)_3X$ sequences in cells.[32] The ability of PLQ to quantify both unmodified substrate and product rapidly in small quantities gives an opportunity to examine $C(x)_3X$ motifs in relation to endogenous FTase in a biological setting. PLQ sample preparation is minimal due to its ability to examine crude cell lysate and would allow for quicker sample analysis than other traditional methods. While a powerful tool, there is limitation in the cloning of these human proteins to eGFP or TagRFP to allow for detection by PLQ - how best to address this restraint remains unclear.

5.2 Future Directions

5.2.1 Investigation of FTase vs GGTase-I in $C(x)_3X$ substrate selectivity

Perhaps the most surprising finding after determining the ability of FTase to prenylate a $C(x)_3X$ sequence is the inability of GGTase-I to accept any of the sequences tested.[30] These two enzymes recognize the same classic CaaX motif with variations in the amino acids they accept and the type of isoprenoid group to be attached, a chain of 15 carbons for FPP vs 20 carbons for GGPP.[3, 33-34] FTase or GGTase-I present both distinctions and similarities in amino acid preference in the "X" position of the prenylation motif with FTase accepting X = M, Q, S, T, and A and GGTase-I most efficiently prenylating sequences of X = I or L.[1] The difference in activities for these two enzymes lies in their active site with the active site of FTase consisting of amino acids W102, W106, and Y361 as compared to T49, F53, and L320 for GGTase-I (See Chapter 1). These closely related enzymes have the ability to cross-talk in cells, with one enzyme taking up the role of prenylating certain sequences when the other is inhibited.[35] An example of this is seen with the protein KRas, which has shown to be geranylgeranylated in the presence of FTase inhibitors. Despite the evidence in their similarities and its larger active site, not one $C(x)_3X$ sequence tested was found to be accepted by GGTase-I.

To begin understanding this difference between the enzymes, one of the next goals in this research is to determine the crystal structure of FTase interacting with $C(x)_3X$ motifs. The ability to prenylate these large sequences suggests a flexibility within the FTase active site not currently predicted through computational studies or found in GGTase-I. Understanding the contact points and spacing of amino acids within the FTase binding site will provide insight into why GGTase-I in much stricter in its substrate selectivity. To obtain these crystal structures, a high-yielding "single-band" purification of FTase is necessary and work has begun on optimizing that process.

5.2.2 Identification of endogenous $C(x)_3X$ proteins and the potential of a human shunt pathway

Considering activity of both Cxxx and $C(x)_3X$ sequences in yeast and humans, this newly discovered activity of non-canonical sequences nearly doubles the number of proteins that can be potentially prenylated within cells even when removing sequences containing other cysteines within the motif. Through our Prosite search it was found that 1008 human proteins (accessed June 2018) follow a C-terminal $C(x)_3X$ sequence with 816 of those sequences containing no other cysteine other than that required for prenylation. (See Appendix II) This is comparable to the 1205 potential Cxxx proteins in the human genome. The same can be said for yeast in which 120 proteins contain a canonical Cxxx motif. It was found that 117 proteins containing C-terminal $C(x)_3X$ sequences are also possible targets of prenylation with 88 of those containing no other cysteine within the sequence. (See Appendix III) Many of these yeast proteins remain uncharacterized. These numbers underscore the importance of exploring and characterizing more $C(x)_3X$ proteins, especially in the human genome, to determine their prenylation state within the cell.

The original $C(x)_3X$ sequences identified for analysis from yeast used a screening that discriminates for proteins that are prenylated but not proteolyzed. This raises the question as to whether all $C(x)_3X$ sequences that serve as prenyltransferase substrates are unable or resistant to undergo subsequent processing. This possibility makes analysis of $C(x)_3X$ sequences through fluorescence microscopy difficult as membrane localization can no longer be used as a proxy for prenylation. Regardless of the challenges this creates, implications of this newly discovered $C(x)_3X$ sequence reactivity opens a door to the potential of alternate uses of a farnesyl group beyond serving as a membrane anchor in prenylation, with the possible existence and role of a "shunt pathway" in mammalian cells.[31]

An ultimate goal in examining these human $C(x)_3X$ proteins is to find one which undergoes endogenous prenylation. Due to the number of potential proteins to test, this objective presents a "needle in a haystack" obstacle. While the current use of HPLC to identify potential proteins is overall quick, production of the peptides required to test all 1008 sequences is costly and time consuming. In addition to this initial hurdle, the next steps of cloning, transfection, imaging, and metabolic labeling of the proteins whose sequences produce a product peak on HPLC is even more of a financial and time-intensive obstacle than the initial screening.

A reasonable way to tackle the number of potential sequences is to use an approach similar to how we originally chose our six human proteins for an initial screening (see Chapter 2).[30] While it does not solve the concern of how many sequences there are to be tested, it may provide the best option for finding a protein sooner. The first option is to reduce the potential complexity of proteins being examined by choosing proteins which are produced from genes that do not produce $C(x)_3X$ C-termini upon splicing. Protein splice variants are produced during regulation of gene expression and allow for a single gene to produce several functional proteins. For example, the protein Serine/threonine-protein kinase Chk2 ends in "CAAVL" and contains six splice variants (Appendix II).[36] Choosing this protein for study may prove problematic at the cellular level where gene regulation will affect which and how many variants of this one protein we are examining.

Another way to take the first step in eliminating and/or finding potential $C(x)_3X$ human proteins which undergo prenylation is through a "sub-family" approach. Upon examination of the protein list generated from Prosite, some C-terminal sequences are conserved across a related family of proteins. For example, there are nine distinct cAMP-responsive element modulator proteins all ending in the sequence "CHKVE"(Appendix II).[37] This conservation may be important

to the proteins' function and can be used as a guided approach to selecting the first series of $C(x)_3X$ sequences to be studied.

For yeast and other species, the smaller proteome makes for an easier target to finding target proteins. Future work could include pathogenic organisms that employ either endogenous or host-mediated farnesylation, such as *Plasmodium falciparum, Candida albicans,* and *Legionella pneumophila* which have two, ten, and fourteen proteins ending in $C(x)_3X$, respectively (Appendix IV).[23, 38-43]

5.2.3 Expansion of PLQ to include other classes of lipidation

As was discussed earlier in section 5.1, PLQ could prove useful in determining the ability of $C(x)_3X$ proteins to undergo prenylation in cells without using fluorescence membrane localization as the only readout. Besides this directly related work, there is much that can be explored further with PLQ including the examination of how other lipid modifications fare with this new method.

Palmitoylation, myristoylation, and GPI anchors are also important in protein trafficking for cell signaling. The strength of PLQ as a new research method will lie in its ability to be used for multiple lipidation states. Proteins which undergo prenylation are also commonly palmitoylated upstream of the C-terminus While the role of this palmitoylation has been discerned as significant for membrane localization, the extent of palmitoylation needed for efficient localization and its details are still investigated.[11, 44-47] PLQ could be useful for these types of investigations after determining the protocol modifications, if any, necessary to separate these other types of lipidation.

5.3 References

1. Benetka, W.; Koranda, M.; Eisenhaber, F., Protein Prenylation: An (Almost) Comprehensive Overview on Discovery History, Enzymology, and Significance in Physiology and Disease. *Monatshefte für Chemie / Chemical Monthly* **2006,** *137* (10), 1241.

2. Casey, P. J., Lipid modifications of G proteins. *Current opinion in cell biology* **1994,** *6* (2), 219-25.

3. Casey, P. J.; Seabra, M. C., Protein prenyltransferases. *The Journal of biological chemistry* **1996,** *271* (10), 5289-92.

4. Cox, A. D.; Der, C. J., Protein prenylation: more than just glue? *Current opinion in cell biology* **1992,** *4* (6), 1008-16.

5. Gao, J.; Liao, J.; Yang, G. Y., CAAX-box protein, prenylation process and carcinogenesis. *American journal of translational research* **2009,** *1* (3), 312-25.

6. Marshall, C. J., Protein prenylation: a mediator of protein-protein interactions. *Science (New York, N.Y.)* **1993,** *259* (5103), 1865-6.

7. Palsuledesai, C. C.; Distefano, M. D., Protein prenylation: enzymes, therapeutics, and biotechnology applications. *ACS Chem Biol* **2015,** *10* (1), 51-62.

8. Zhang, F. L.; Casey, P. J., Protein prenylation: molecular mechanisms and functional consequences. *Annual review of biochemistry* **1996,** *65*, 241-69.

9. Clarke, S.; Vogel, J. P.; Deschenes, R. J.; Stock, J., Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** *85* (13), 4643-7.

10. Farnsworth, C. C.; Wolda, S. L.; Gelb, M. H.; Glomset, J. A., Human lamin B contains a farnesylated cysteine residue. *The Journal of biological chemistry* **1989,** *264* (34), 20422-9.

11. Hancock, J. F.; Magee, A. I.; Childs, J. E.; Marshall, C. J., All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **1989,** *57* (7), 1167-77.

12. Kitten, G. T.; Nigg, E. A., The CaaX motif is required for isoprenylation, carboxyl methylation, and nuclear membrane association of lamin B2. *The Journal of cell biology* **1991,** *113* (1), 13-23.

13. Powers, S.; Michaelis, S.; Broek, D.; Santa Anna, S.; Field, J.; Herskowitz, I.; Wigler, M., RAM, a gene of yeast required for a functional modification of RAS proteins and for production of mating pheromone a-factor. *Cell* **1986,** *47* (3), 413-22.

14. Sefton, B. M.; Buss, J. E., The covalent modification of eukaryotic proteins with lipid. *The Journal of cell biology* **1987,** *104* (6), 1449-53.

15. Vorburger, K.; Kitten, G. T.; Nigg, E. A., Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif. *The EMBO journal* **1989,** *8* (13), 4007-13.

16. Gangopadhyay, S. A.; Losito, E. L.; Hougland, J. L., Targeted reengineering of protein geranylgeranyltransferase type I selectivity functionally implicates active-site residues in protein-substrate recognition. *Biochemistry* **2014,** *53* (2), 434-46.

17. Gutierrez, L.; Magee, A. I.; Marshall, C. J.; Hancock, J. F., Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *The EMBO journal* **1989,** *8* (4), 1093-8.

18. Hancock, J. F.; Cadwallader, K.; Paterson, H.; Marshall, C. J., A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *The EMBO journal* **1991,** *10* (13), 4033-9.

19. Hougland, J. L.; Gangopadhyay, S. A.; Fierke, C. A., Expansion of protein farnesyltransferase specificity using "tunable" active site interactions: development of bioengineered prenylation pathways. *The Journal of biological chemistry* **2012,** *287* (45), 38090-100.

20. Hougland, J. L.; Lamphear, C. L.; Scott, S. A.; Gibbs, R. A.; Fierke, C. A., Context-dependent substrate recognition by protein farnesyltransferase. *Biochemistry* **2009,** *48* (8), 1691-701.

21. Moores, S. L.; Schaber, M. D.; Mosser, S. D.; Rands, E.; O'Hara, M. B.; Garsky, V. M.; Marshall, M. S.; Pompliano, D. L.; Gibbs, J. B., Sequence dependence of protein isoprenylation. *The Journal of biological chemistry* **1991,** *266* (22), 14603-10.

22. Ota, I. M.; Clarke, S., Enzymatic methylation of 23-29-kDa bovine retinal rod outer segment membrane proteins. Evidence for methyl ester formation at carboxyl-terminal cysteinyl residues. *Journal of Biological Chemistry* **1989,** *264* (22), 12879-12884.

23. Al-Quadan, T.; Price, C. T.; London, N.; Schueler-Furman, O.; AbuKwaik, Y., Anchoring of bacterial effectors to host membranes through host-mediated lipidation by prenylation: a common paradigm. *Trends in microbiology* **2011,** *19* (12), 573-9.

24. Cui, G.; Merz, K. M., Jr., Computational studies of the farnesyltransferase ternary complex part II: the conformational activation of farnesyldiphosphate. *Biochemistry* **2007,** *46* (43), 12375-81.

25. Maurer-Stroh, S.; Eisenhaber, F., Refinement and prediction of protein prenylation motifs. *Genome Biology* **2005,** *6* (6), R55.

26. Maurer-Stroh, S.; Koranda, M.; Benetka, W.; Schneider, G.; Sirota, F. L.; Eisenhaber, F., Towards complete sets of farnesylated and geranylgeranylated proteins. *PLoS computational biology* **2007,** *3* (4), e66.

27. Lane, K. T.; Beese, L. S., Thematic review series: Lipid Posttranslational Modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *Journal of Lipid Research* **2006,** *47* (4), 681-699.

28. London, N.; Lamphear, C. L.; Hougland, J. L.; Fierke, C. A.; Schueler-Furman, O., Identification of a novel class of farnesylation targets by structure-based modeling of binding specificity. *PLoS computational biology* **2011,** *7* (10), e1002170.

29. Reid, T. S.; Terry, K. L.; Casey, P. J.; Beese, L. S., Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. *Journal of molecular biology* **2004,** *343* (2), 417-33.

30. Blanden, M. J.; Suazo, K. F.; Hildebrandt, E. R.; Hardgrove, D. S.; Patel, M.; Saunders, W. P.; Distefano, M. D.; Schmidt, W. K.; Hougland, J. L., Efficient farnesylation of an extended C-terminal C(x)3X sequence motif expands the scope of the prenylated proteome. *The Journal of biological chemistry* **2018,** *293* (8), 2770-2785.

31. Hildebrandt, E. R.; Cheng, M.; Zhao, P.; Kim, J. H.; Wells, L.; Schmidt, W. K., A shunt pathway limits the CaaX processing of Hsp40 Ydj1p and regulates Ydj1p-dependent phenotypes. *eLife* **2016,** *5*.

32. Shala-Lawrence, A.; Blanden, M. J.; Krylova, S. M.; Gangopadhyay, S. A.; Beloborodov, S. S.; Hougland, J. L.; Krylov, S. N., Simultaneous Analysis of a Non-Lipidated Protein and Its

Lipidated Counterpart: Enabling Quantitative Investigation of Protein Lipidation's Impact on Cellular Regulation. *Analytical Chemistry* **2017,** *89* (24), 13502-13507.

33. Hartman, H. L.; Hicks, K. A.; Fierke, C. A., Peptide specificity of protein prenyltransferases is determined mainly by reactivity rather than binding affinity. *Biochemistry* **2005,** *44* (46), 15314-24.

34. Yokoyama, K.; Goodwin, G. W.; Ghomashchi, F.; Glomset, J.; Gelb, M. H., Protein prenyltransferases. *Biochemical Society transactions* **1992,** *20* (2), 489-94.

35. Whyte, D. B.; Kirschmeier, P.; Hockenberry, T. N.; Nunez-Oliva, I.; James, L.; Catino, J. J.; Bishop, W. R.; Pai, J.-K., K- and N-Ras Are Geranylgeranylated in Cells Treated with Farnesyl Protein Transferase Inhibitors. *Journal of Biological Chemistry* **1997,** *272* (22), 14459-14464.

36. Zannini, L.; Delia, D.; Buscemi, G., CHK2 kinase in the DNA damage response and beyond. *Journal of Molecular Cell Biology* **2014,** *6* (6), 442-457.

37. Rauen, T.; Hedrich, C. M.; Tenbrock, K.; Tsokos, G. C., cAMP responsive element modulator: a critical regulator of cytokine production. *Trends in molecular medicine* **2013,** *19* (4), 262-269.

38. Ivanov, S. S.; Charron, G.; Hang, H. C.; Roy, C. R., Lipidation by the host prenyltransferase machinery facilitates membrane localization of Legionella pneumophila effector proteins. *The Journal of biological chemistry* **2010,** *285* (45), 34686-98.

39. Hast, M. A.; Nichols, C. B.; Armstrong, S. M.; Kelly, S. M.; Hellinga, H. W.; Alspaugh, J. A.; Beese, L. S., Structures of Cryptococcus neoformans protein farnesyltransferase reveal strategies for developing inhibitors that target fungal pathogens. *The Journal of biological chemistry* **2011,** *286* (40), 35149-62.

40. Selvig, K.; Ballou, E. R.; Nichols, C. B.; Alspaugh, J. A., Restricted Substrate Specificity for the Geranylgeranyltransferase-I Enzyme in Cryptococcus neoformans: Implications for Virulence. *Eukaryotic Cell* **2013,** *12* (11), 1462-1471.

41. Piispanen, A. E.; Bonnefoi, O.; Carden, S.; Deveau, A.; Bassilana, M.; Hogan, D. A., Roles of Ras1 Membrane Localization during Candida albicans Hyphal Growth and Farnesol Response. *Eukaryotic Cell* **2011,** *10* (11), 1473-1484.

42. Zverina, E. A.; Lamphear, C. L.; Wright, E. N.; Fierke, C. A., Recent Advances in Protein Prenyltransferases: Substrate Identification, Regulation, and Disease Interventions. *Current opinion in chemical biology* **2012,** *16* (5-6), 544-552.

43. Suazo, K. F.; Schaber, C.; Palsuledesai, C. C.; Odom John, A. R.; Distefano, M. D., Global proteomic analysis of prenylated proteins in Plasmodium falciparum using an alkyne-modified isoprenoid analogue. *Scientific Reports* **2016,** *6*, 38615.

44. Lin, D. T. S.; Davis, N. G.; Conibear, E., Targeting the Ras palmitoylation/depalmitoylation cycle in cancer. *Biochemical Society transactions* **2017,** *45* (4), 913-921.

45. Goodwin, J. S.; Drake, K. R.; Rogers, C.; Wright, L.; Lippincott-Schwartz, J.; Philips, M. R.; Kenworthy, A. K., Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway. *The Journal of cell biology* **2005,** *170* (2), 261-72.

46. Rocks, O.; Peyker, A.; Kahms, M.; Verveer, P. J.; Koerner, C.; Lumbierres, M.; Kuhlmann, J.; Waldmann, H.; Wittinghofer, A.; Bastiaens, P. I., An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science (New York, N.Y.)* **2005,** *307* (5716), 1746-52.

47. Hancock, J. F.; Paterson, H.; Marshall, C. J., A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* **1990,** *63* (1), 133-9.

Table S1 Fluorescence screening data for dns-GC(x)$_3$X peptides.

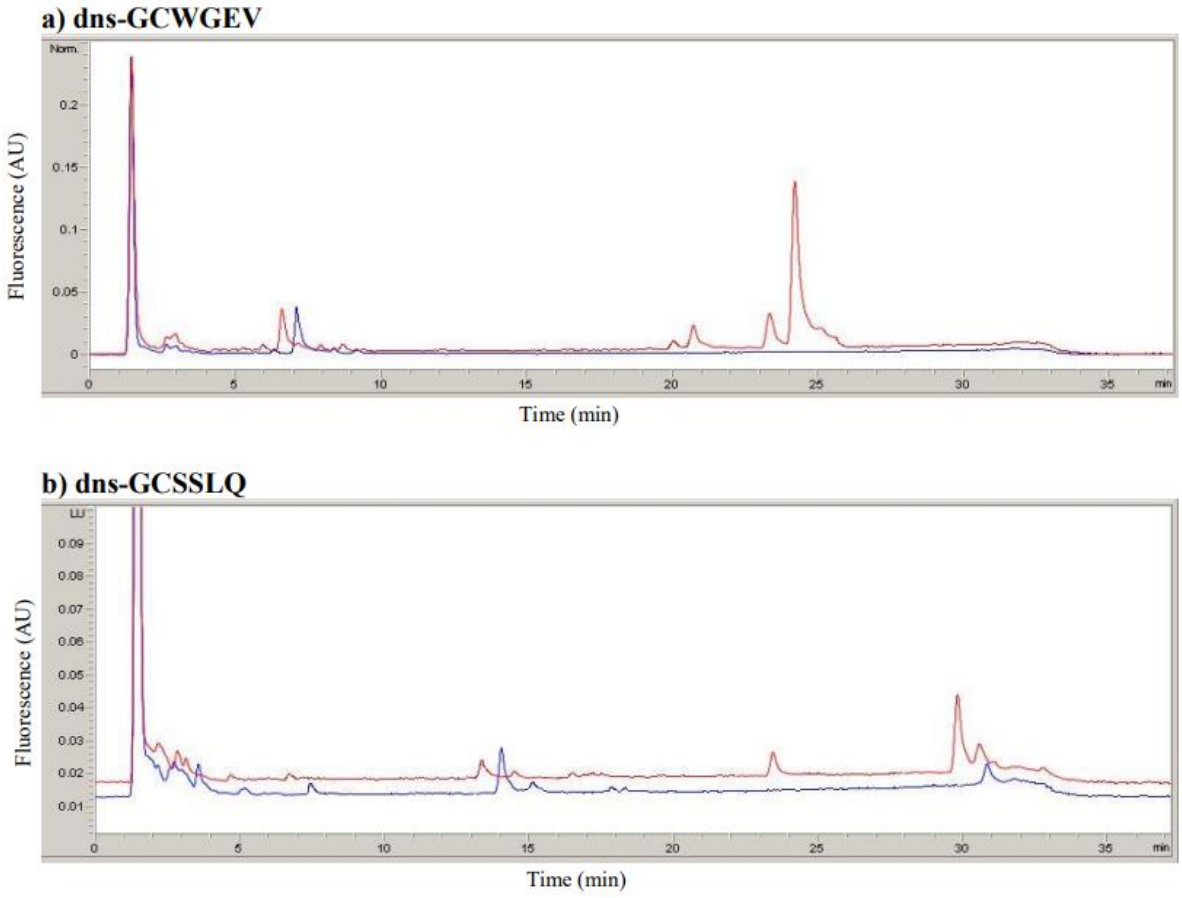
Peptide sequence	Peptide Fluorescence[a] (reactions containing FPP)					Peptide Fluorescence (negative control reactions without FPP)					Corrected Fluorescence (+FPP) / Corrected Fluorescence (-FPP)			
	Time (min)					Time (min)					Time (min)			
	0	30	60	90	120	0	30	60	90	120	30	60	90	120
Dns-GCGGDD	866	1233	1211	1154	1137	429	562	546	497	496	2.8	2.9	4.2	4.0
Dns-GCMIIM	3768	9515	10766	10658	10549	2181	2272	2216	2128	2075	63.2	199.9	130.0	64.0
Dns-GCAVGP	455	1732	2580	3324	3765	535	613	641	614	578	16.4	20.0	36.3	77.0
Dns-GCAYVL	3885	5469	5641	5574	5460	1367	1561	1590	1508	1476	8.2	7.9	12.0	14.4
Dns-GCFFYI	3367	2957	3835	1972	1708	1028	1000	1182	1060	1139	14.6	3.0	43.6	14.9
Dns-GCFNSL	2513	2973	3018	2933	2960	2241	2634	2523	2481	2416	1.2	1.8	1.8	2.6
Dns-GCIPVQ	710	935	969	876	922	610	775	765	757	696	1.4	1.7	1.1	2.5
Dns-GCLPIV	1230	1334	1356	1275	1272	1070	1526	1484	1442	1406	0.2	0.3	0.1	0.1
Dns-GCQGFL	2254	2601	2654	2579	2550	1899	2371	2260	2159	2114	0.7	1.1	1.3	1.4
Dns-GCSIQG	2855	3516	3642	3825	4063	1310	1486	1405	1383	1329	3.8	8.3	13.3	63.6
Dns-GCSRLQ	1815	2291	2185	2204	2163	1558	1907	1816	1788	1732	1.4	1.4	1.7	2.0
Dns-GCSSLQ	1198	1584	1564	1545	1492	1756	2081	2066	1995	1921	1.2	1.2	1.5	1.8
Dns-GCVSFG	1232	1571	1572	1595	1680	884	1085	1104	1037	1100	1.7	1.5	2.4	2.1
Dns-GCWAGG	1141	1384	1429	1467	1484	1007	1316	1294	1263	1166	0.8	1.0	1.3	2.2
Dns-GCWGEV	860	1293	1378	1426	1539	956	1214	1165	1144	1115	1.7	2.5	3.0	4.3
Dns-GCWGGA	2026	2286	2306	2216	2182	1642	2136	2032	1956	1855	0.5	0.7	0.6	0.7

[a] Values for peptide fluorescence are provided as raw fluorescence units (RFU).

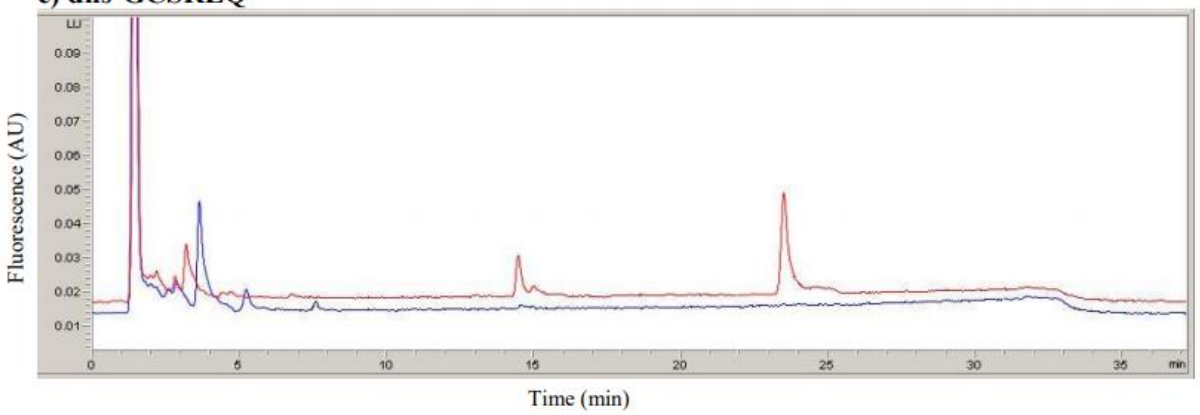
Table S2 Summary of fragments and assignments from ESI-MS analysis of dns-GCMIIM and dns-GCAVGP.

Ion	dns-GCMIIM		dns-GCAVGP	
	Calculated *m/z*	Observed *m/z*	Calculated *m/z*	Observed *m/z*
$[M+H]^+$	1104.4	1104.4	940.5	940.4
$MH - H_2O$	1086.4	1086.7	922.5	922.5
b6	955.5	955.2	825.4	825.4
b5	842.4	842.3	768.4	768.4
b4	729.3	729.4	669.3	668.9
a6	927.5	-	797.4	797.3
a4	701.3	-	641.3	641.0
b4* (b4 – thiofarnesyl)	491.1	490.9	431.1	431.1
b3* (b3 – thiofarnesyl)	360.1	360.2	360.1	360.1

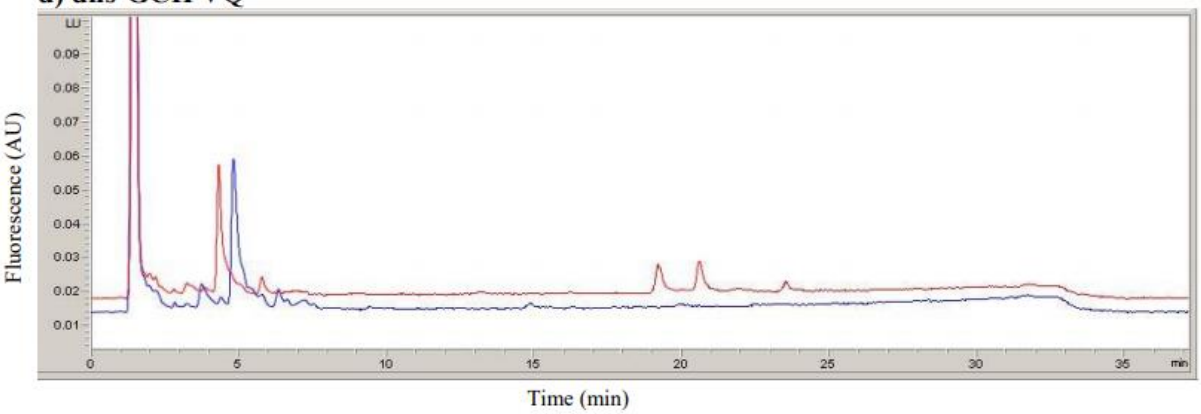
Figure S2 HPLC analysis of dns-GC(x)$_3$X peptide farnesylation by FTase. Farnesylation reactions were performed in the presence (+, red trace) and absence (-, blue trace) of 10 µM FPP. All reactions contained 3 µM peptide substrate and 100 nM FTase as described in Experimental Procedures. The peak observed at short retention time is due to the reaction buffer, with an identical retention time and intensity in the +FPP and -FPP samples. a) dns-GCWGEV; b) dns-GCSSLQ; c) dns-GCSRLQ; d) dns-GCIPVQ; e) dns-GCGGDD; f) dns-GCAYVL; g) dns-GCFFYI; h) dns-GCFNSL; i) dns-GCLPIV; j) dns-GCQGFL; k) dns-GCSIQG; l) dns-GCVSFG; m) dns-GCWAGG; n) dns-GCWGGA; o) dns-GCLLHP; p) dns-GCSQGP; q) dns-GCQTGP; r) dns-GCSVKM; s) dns-GCFSKM; t) dns-GCDREV



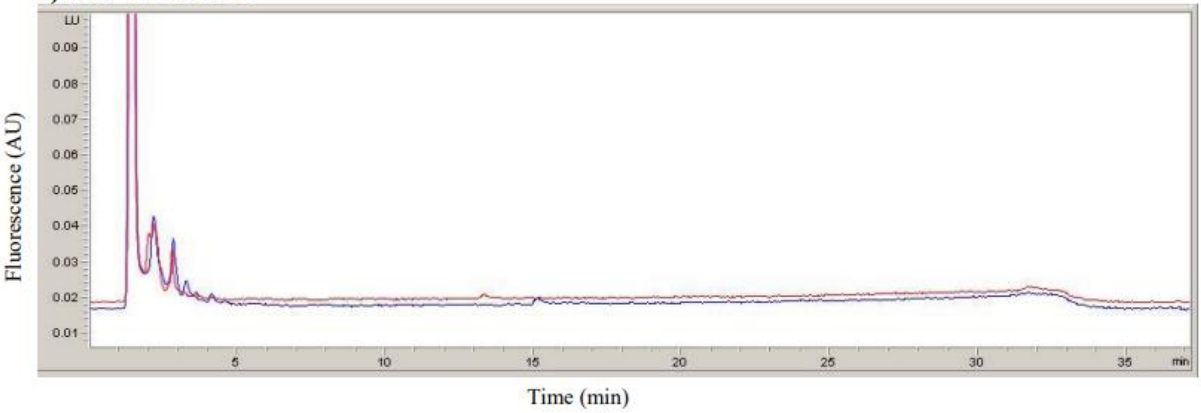
c) dns-GCSRLQ



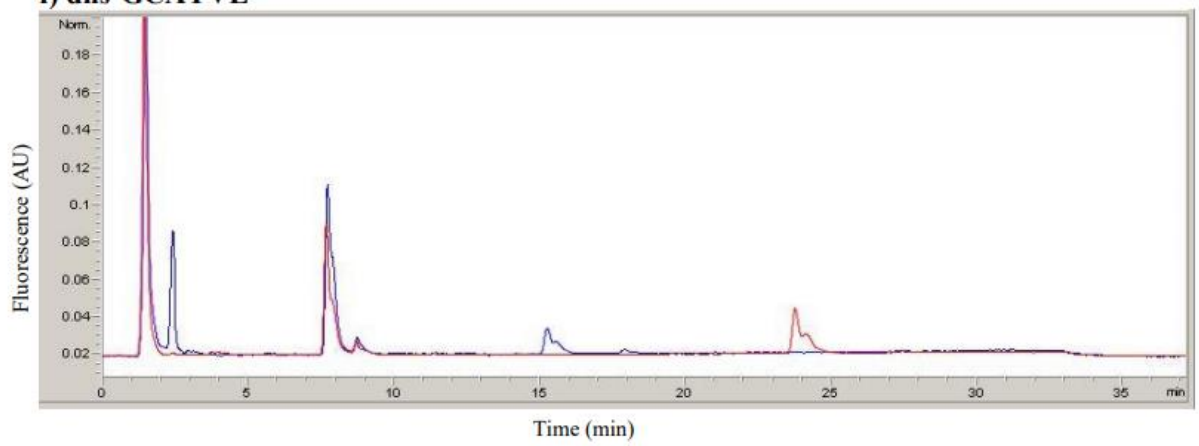
d) dns-GCIPVQ



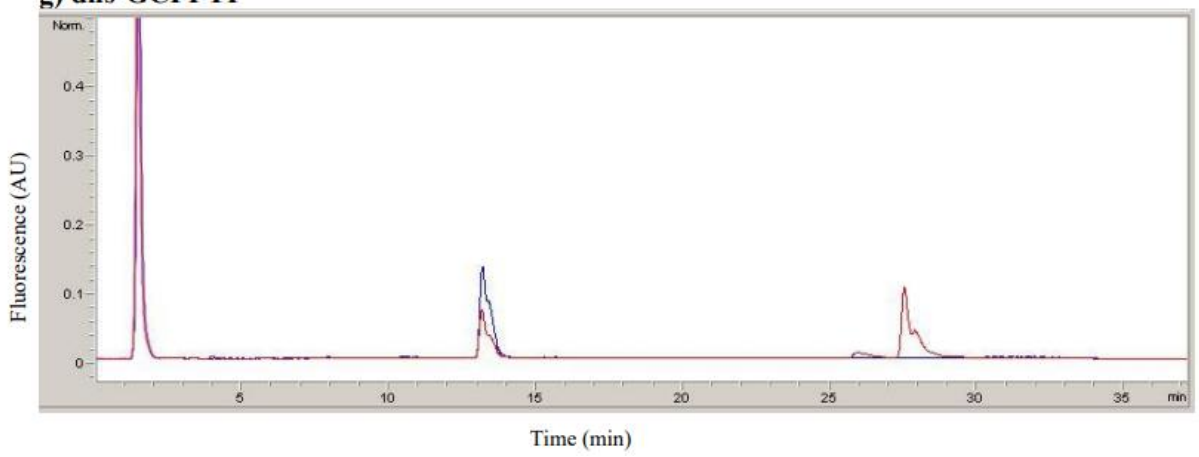
e) dns-GCGGDD



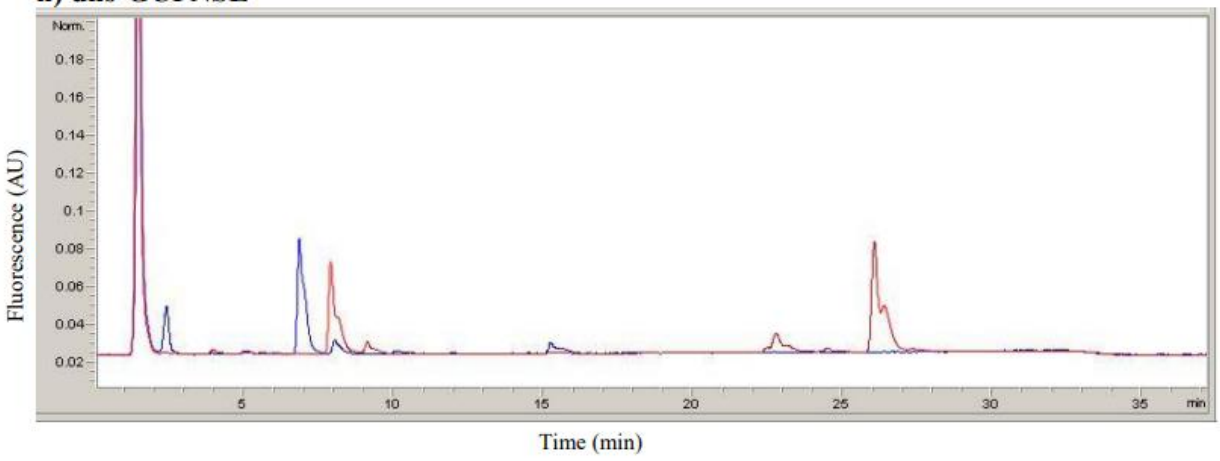
f) dns-GCAYVL



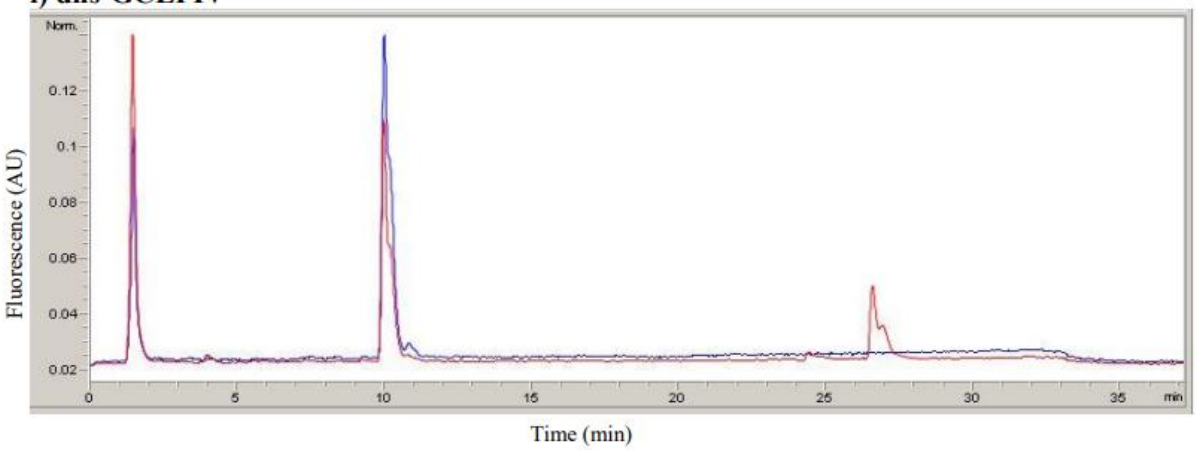
g) dns-GCFFYI



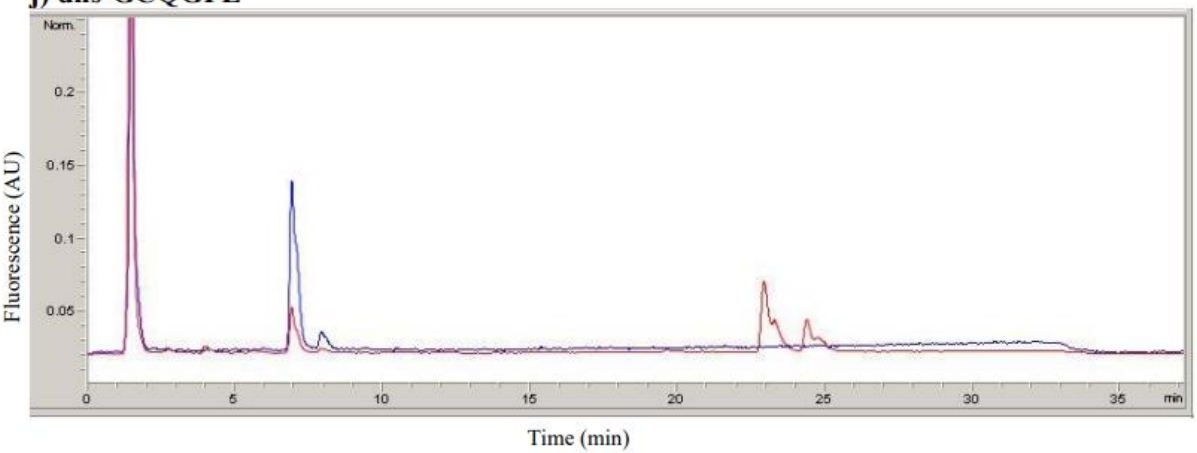
h) dns-GCFNSL



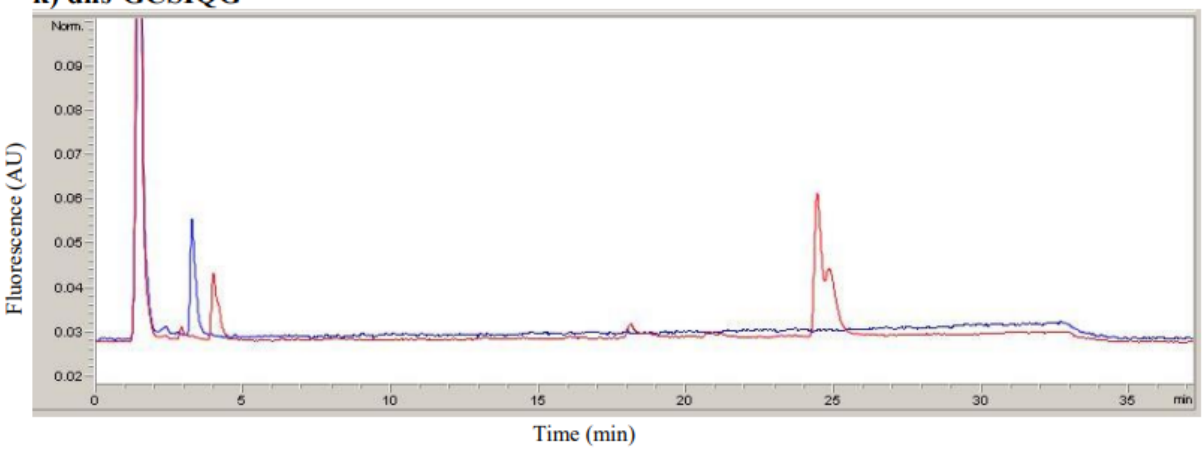
i) dns-GCLPIV



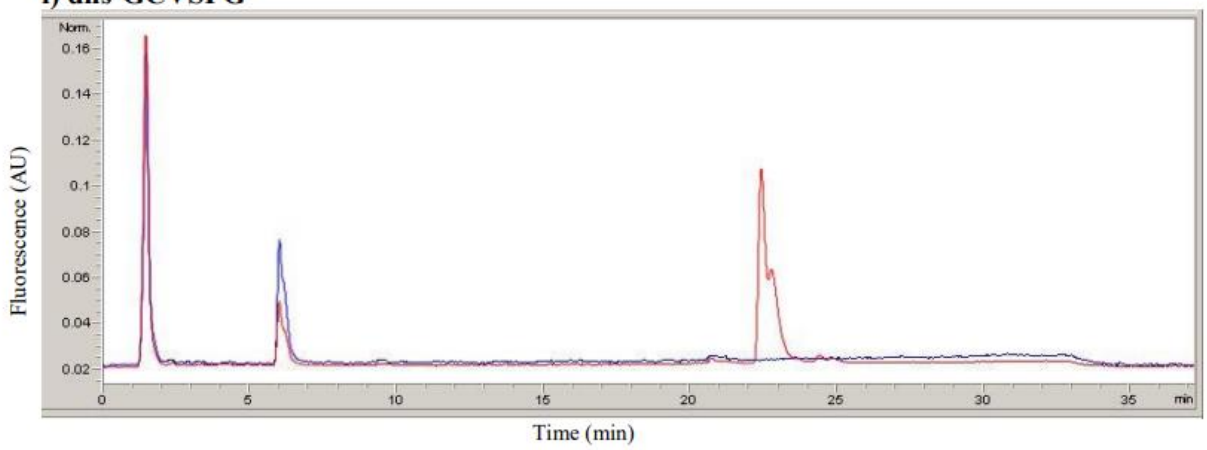
j) dns-GCQGFL



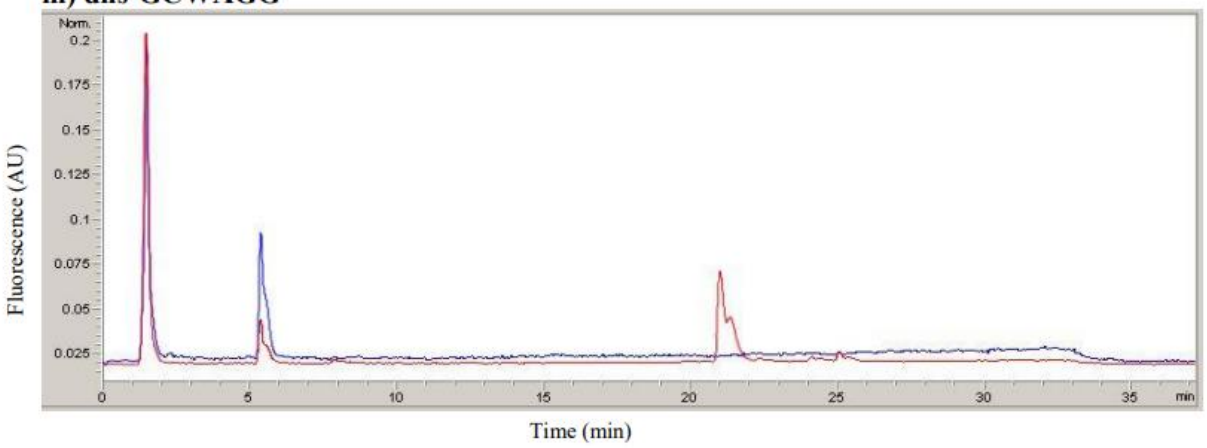
k) dns-GCSIQG



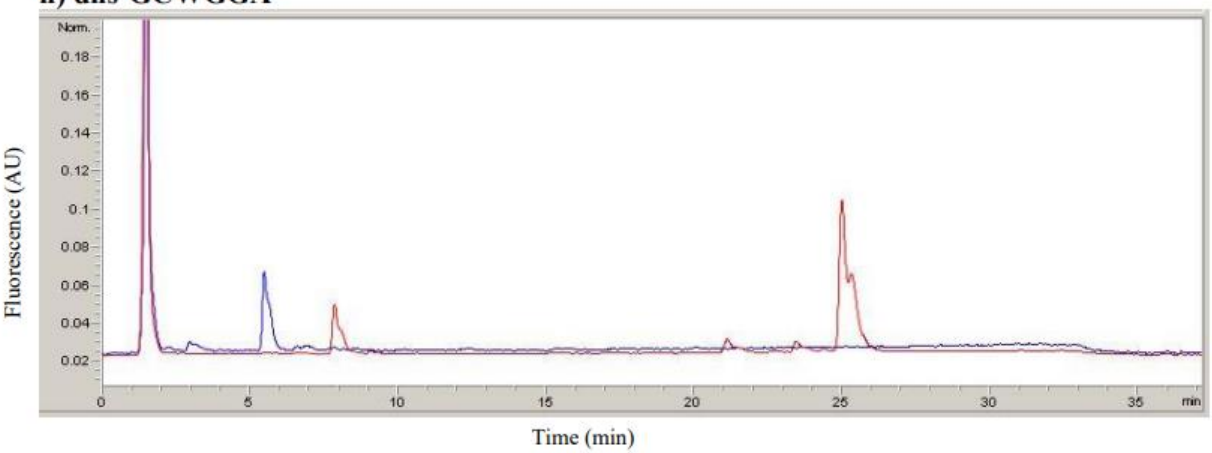
l) dns-GCVSFG



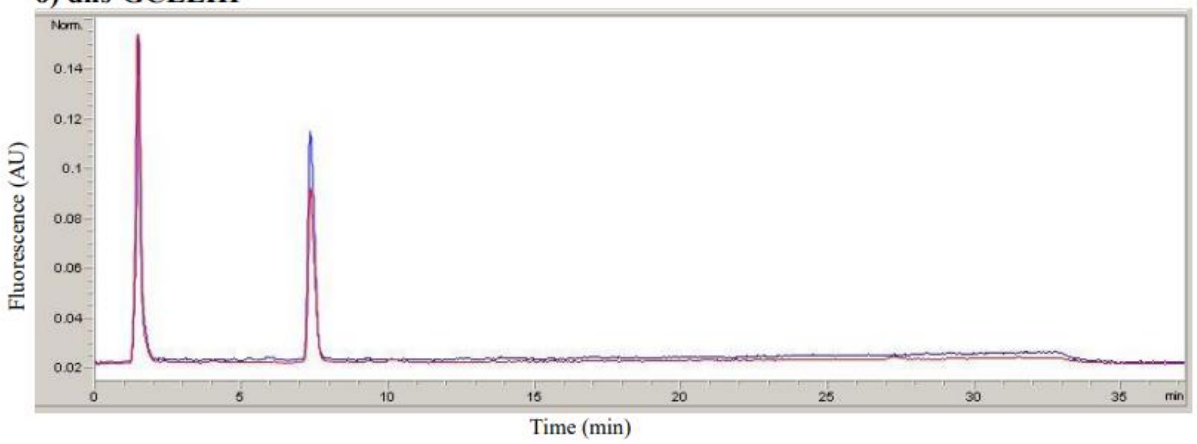
m) dns-GCWAGG



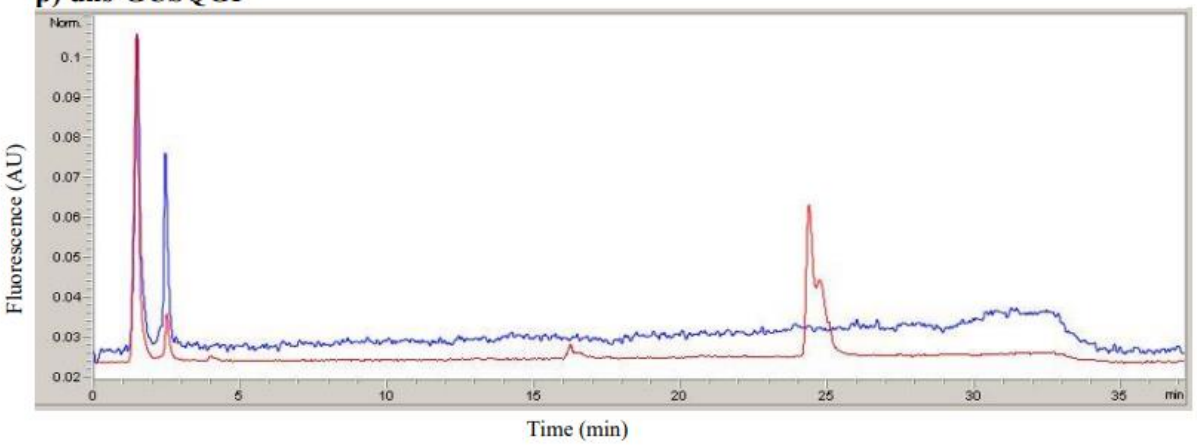
n) dns-GCWGGA



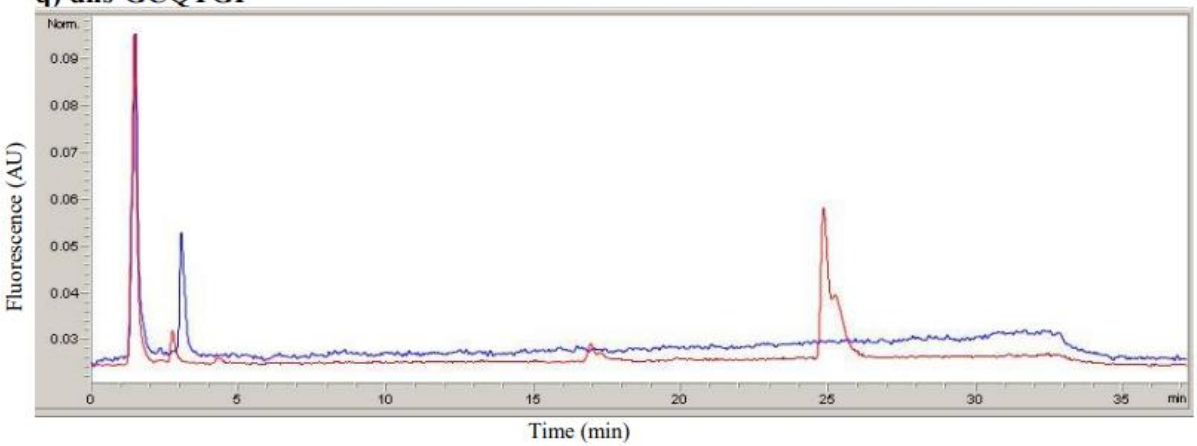
o) dns-GCLLHP



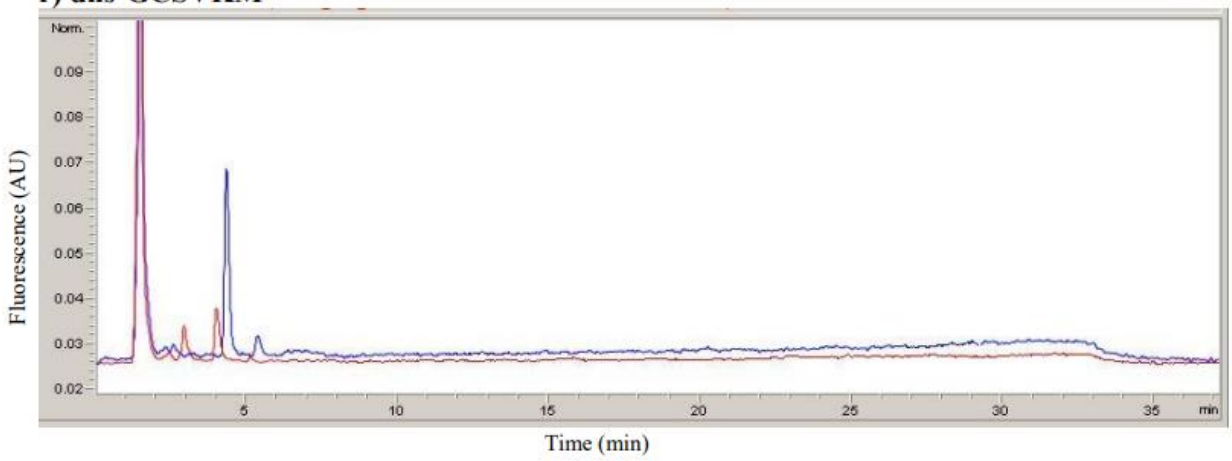
p) dns-GCSQGP



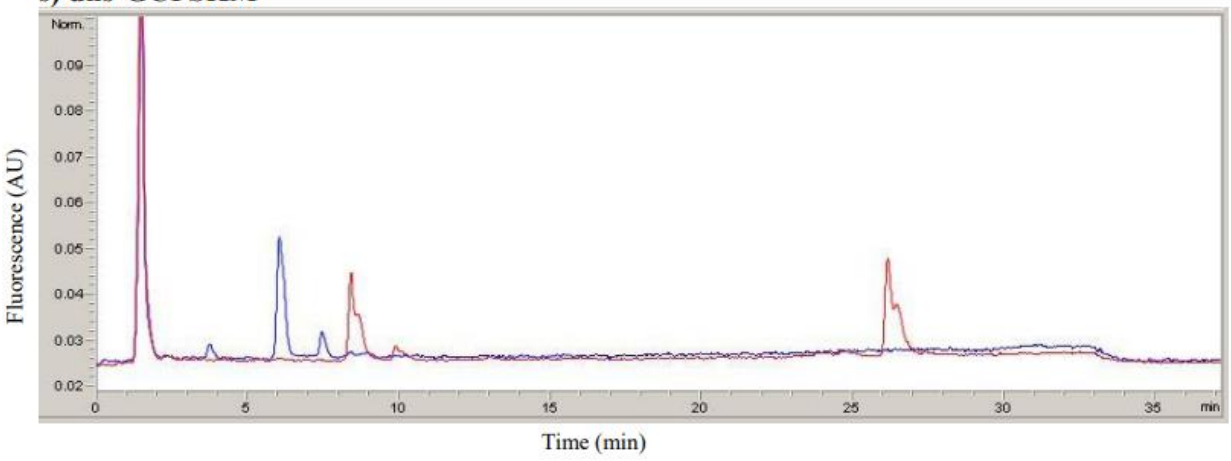
q) dns-GCQTGP



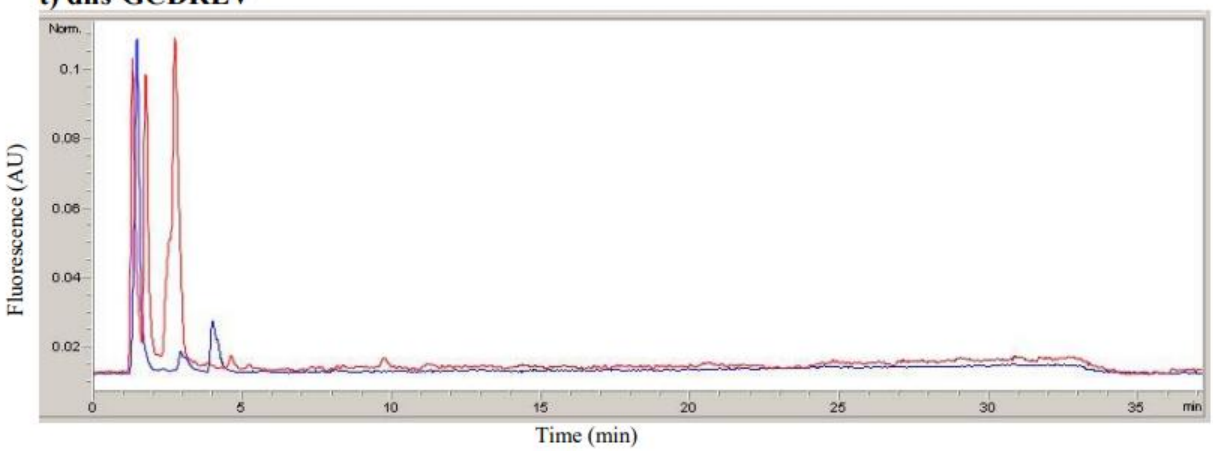
r) dns-GCSVKM



s) dns-GCFSKM



t) dns-GCDREV



C-terminus	Protein Name	Size (aa)	Number of splice variants
Cxxxx			
CREHT	5-hydroxytryptamine receptor 1E	365	0
CLWNT	5-hydroxytryptamine receptor 3D	454	3
CLWNT	5-hydroxytryptamine receptor 3E	456	4
CGFNL	Putative ankyrin repeat domain-containing protein 30B-like	251	0
CDAEV	DNA dC->dU-editing enzyme APOBEC-3H	200	0
CDSVD	Alpha/beta hydrolase domain-containing protein 4	342	0
CDTVD	1-acylglycerol-3-phosphate O-acyltransferase ABHD5	349	0
CKRNN	Acetyl-coenzyme A transporter 1	549	0
CSTAN	Neuronal acetylcholine receptor subunit alpha-7	102	0
CLTIQ	Acetyl-coenzyme A synthetase, cytoplasmic	701	1
CSRYW	Adrenocorticotropic hormone receptor	297	0
CQEEP	Adenylate cyclase type 10	372	0
CILLL	Alcohol dehydrogenase 6	375	0
CFPWG	Afadin	1612	0
CNGTI	Adhesion G-protein coupled receptor F1	218	0
CFEVE	Type-1 angiotensin II receptor	359	0
CTFLP	Aryl hydrocarbon receptor repressor	701	2
CRFVT	Aldehyde dehydrogenase family 8 member A1	160	0
CMEDK	ALK and LTK ligand 2	91	0
CGGND	Annexin A11	505	1
CGGDD	Annexin A2	339	1
CGGDD	Annexin A3	323	0
CGGDD	Annexin A4	319	1
CGEDD	Annexin A5	320	0
CGGED	Annexin A6	673	1
CSIEA	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	147	0
CPKLS	Apolipoprotein D (Apo-D)	189	0
CKNGN	Rho guanine nucleotide exchange factor 28	1651	0
CSPRA	Rho guanine nucleotide exchange factor 10-like protein	1067	0
CVHDS	cAMP-regulated phosphoprotein 21	108	0
CSLLL	Arylsulfatase B	413	0
CTLVL	Acid sphingomyelinase-like phosphodiesterase 3b	455	0
CLAPR	Protein asteroid homolog 1	520	0
CLVVR	Putative Polycomb group protein ASXL1	1541	1 (see below)
CVLSR	Putative Polycomb group protein ASXL1	479	SPLICE OF ABOVE
CLVVR	Putative Polycomb group protein ASXL2	1435	1
CLVVR	Putative Polycomb group protein ASXL3	2248	0
CTVNI	Phospholipid-transporting ATPase ID	387	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CKLAS	Probable phospholipid-transporting ATPase IIB	1147	1
CTRKI	A disintegrin and metalloproteinase with thrombospondin motifs 18	1221	0
CPGRQ	A disintegrin and metalloproteinase with thrombospondin motifs 4	846	0
CGGDD	Putative annexin A2-like protein	339	0
CLYKN	5-azacytidine-induced protein 2	392	1 (see below)
CSLFA	5-azacytidine-induced protein 2	243	SPLICE OF ABOVE
CMTSQ	Beta-1,4 N-acetylgalactosaminyltransferase 1	533	1
CTTDE	Transcription regulator protein BACH1	736	0
CDAFL	Barrier-to-autointegration factor	89	0
CKKDH	B-cell scaffold protein with ankyrin repeats	785	3
CPRPL	Breast carcinoma-amplified sequence 4	203	0
CISVF	B-cell CLL/lymphoma 9-like protein	1395	0
CTEKP	Class E basic helix-loop-helix protein 22	381	0
CHEKP	Class E basic helix-loop-helix protein 23	225	0
CSRNP	BTB/POZ domain-containing protein 1	385	0
CYLVK	Coiled-coil domain-containing protein 144A	691	0
CYLVK	Coiled-coil domain-containing protein 144A	1154	0
CQYLD	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	309	0
CRRVI	Monofunctional C1-tetrahydrofolate synthase, mitochondrial	275	0
CLVAR	Putative inactive cytochrome P450 2G1	146	0
CSLQQ	Uncharacterized protein C1orf109	113	0
CITTL	Voltage-dependent L-type calcium channel subunit alpha-1D	2161	2
CVHAL	Voltage-dependent L-type calcium channel subunit alpha-1F	1977	5
CPRLV	Cadherin-18	574	0
CLLVY	Cadherin-23	530	0
CAGDN	Calbindin	261	1
CPKLK	Calpain-13	423	0
CFSVL	Calpain-2 catalytic subunit	700	1
CVWII	F-actin-capping protein subunit alpha-3	299	0
CMSLL	Carboxypeptidase O	374	0
CLHWP	Putative uncharacterized protein PQLC2L	118	0
CENDG	Coiled-coil domain-containing protein 178	867	2
CVHWD	Coiled-coil domain-containing protein 191	514	0
CMTWN	C-C motif chemokine 4-like	47	0
CMTWN	C-C motif chemokine 4-like	52	0
CSPDD	Coiled-coil domain-containing protein 85B	202	0
CRIQR	Coiled-coil domain-containing protein 34	373	0
CRTSV	Endosialin	757	1
CTSEI	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1	300	0
CREPG	Cyclin-dependent kinase 10	272	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CGAHL	Cyclin-dependent kinase 10	154	0
CSSDA	UPF0461 protein C5orf24	155	0
CRDKR	Centrosomal protein of 126 kDa	1117	0
CNNQY	Centromere protein I	756	0
CSMDD	Ceramide synthase 6	384	1
CVEIT	Cilia- and flagella-associated protein 46	247	0
CWFHK	Uncharacterized protein C7orf69	122	0
CKKAI	Putative uncharacterized protein C8orf49	230	0
CTEPE	Chromodomain-helicase-DNA-binding protein 7	1138	0
CAAVL	Serine/threonine-protein kinase Chk2	543	6
CEAGP	Chordin	86	0
CTSGH	Uncharacterized protein encoded by LINC01561	128	0
CRRAY	Uncharacterized protein C11orf53	236	0
CEFPF	C-type lectin domain family 11 member A	323	0
CFRDE	C-type lectin domain family 4 member M	399	3
CDSDL	Claudin-23	292	0
CHWKS	Clavesin-1	167	0
CHSIA	Uncharacterized protein C15orf41	281	1
CMRRT	Collagen alpha-1(IV) chain	1669	0
CMKNL	Collagen alpha-2(IV) chain	1712	0
CVKYS	Collagen alpha-4(IV) chain	1690	0
CMKRT	Collagen alpha-5(IV) chain	1685	1
CMKSL	Collagen alpha-6(IV) chain	1691	1
CRYFT	Acetylcholinesterase collagenic tail peptide	455	4
CPSAS	Uncharacterized protein C16orf95	158	0
CFIPV	Cytochrome P450 2C8	490	1
CFIPV	Cytochrome P450 2C9	490	0
CFIPV	Cytochrome P450 2C18	490	1
CFIPV	Cytochrome P450 2C19	490	0
CAVPR	Cytochrome P450 2D6	497	1
CAVPR	Putative cytochrome P450 2D7	515	0
CLRPR	Cytochrome P450 2F1	491	0
CAERR	Vitamin D 25-hydroxylase	501	0
CGTSS	Uncharacterized protein C17orf53	647	1
CTTGF	Complement component receptor 1-like protein	181	0
CTTGF	Complement component receptor 1-like protein	444	0
CHKSD	Cyclic AMP-responsive element-binding protein 1	327	1
CHKVE	cAMP-responsive element modulator	285	0
CHKVE	cAMP-responsive element modulator	102	0
CHKVE	cAMP-responsive element modulator	112	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CHKVE	cAMP-responsive element modulator	248	0
CHKVE	cAMP-responsive element modulator	299	0
CHKVE	cAMP-responsive element modulator	236	0
CHKVE	cAMP-responsive element modulator	348	0
CHKVE	cAMP-responsive element modulator	120	0
CHKVE	cAMP-responsive element modulator	108	0
CLSGL	Corticotropin-releasing factor-binding protein	322	0
CIDVR	COP9 signalosome complex subunit 9	252	0
CYNNY	Versican core protein	3370	0
CPRLA	Putative uncharacterized protein encoded by LINC00313	77	0
CVLHE	Cell cycle regulator of non-homologous end joining	69	0
CRRKK	Beta-defensin 103	67	0
CRQRI	Beta-defensin 105	78	0
CSPKA	DAN domain family member 5	189	0
CRIWR	Cynein assembly factor with WDR repeat domains 1	415	0
CFPFS	DDB1- and CUL4-associated factor 4-like protein 1	396	0
CFSYG	DDB1- and CUL4-associated factor 4-like protein 2	395	0
CYSYS	DDB1- and CUL4-associated factor 4	495	3
CWLLR	Glutamate decarboxylase 1	224	0
CTRVD	Neutrophil defensin 4	97	0
CLPEK	DEP domain-containing protein 4	294	0
CTVDV	Microprocessor complex subunit DGCR8	773	1
CTRPA	Homeobox protein DLX-5	191	0
CQDGY	Dentin matrix acidic phosphoprotein 1	513	1
CLSLL	Dipeptidase 1	411	0
CHRHL	Dipeptidase 2	258	0
CAVKS	DNA polymerase alpha catalytic subunit	1462	0
CFSLP	Dipeptidyl peptidase 4	766	0
CKEFS	Down syndrome cell adhesion molecule	1571	0
CSSSK	Protein ripply3	190	1
CSRLI	Desmoglein-3	999	0
CLEQQ	Probable E3 ubiquitin-protein ligase DTX2	622	1
CQLDD	Dynein heavy chain 12, axonemal	3092	2
CALDY	Dynein heavy chain 1, axonemal	4330	1
CQLDN	Dynein heavy chain 3, axonemal	4116	0
CQLSE	Dynein heavy chain 6, axonemal	4158	1 (see below)
CGLSS	Dynein heavy chain 6, axonemal	592	SPLICE OF ABOVE
CQLNS	Dynein heavy chain 7, axonemal	4024	1
CEKDD	Dihydrofolate reductase 2, mitochondrial	187	0
CMAAG	Emopamil-binding protein-like	97	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CTEEE	EGF-like repeat and discoidin I-like domain-containing protein 3	480	1
CKTLM	EF-hand domain-containing family member B	833	1
CKKDS	Epidermal growth factor-like protein 7	273	0
CTYGS	Epidermal growth factor receptor	628	0
CFVPQ	Elafin, Elastase-specific inhibitor	117	0
CVTVM	ETS domain-containing protein Elk-4	405	0
CVLTL	ELMO domain-containing protein 3	185	0
CTQQL	Endogenous retrovirus group K member 25 Env polyprotein	661	0
CVYLH	Ephrin type-A receptor 7	449	0
CLTSG	Ephrin type-B receptor 4	414	0
CPKAF	Receptor tyrosine-protein kinase erbB-3	331	0
CPLPL	Exocyst complex component 1-like	172	0
CFKFI	Exostosin-like 3	919	0
CKRRG	Eyes absent homolog 4	452	0
CFWWI	Protein FAM196B	535	0
CVPSS	Protein FAM32A	107	0
CQAQD	Protein FAM57B	274	1
CQTLI	Protein FAM92A	271	0
CPGKF	Fatty-acid amide hydrolase 2	532	0
CPAGS	Fanconi anemia core complex-associated protein 20	170	0
CSRLF	F-box only protein 3	471	0
CRSGE	F-box/LRR-repeat protein 5	691	1
CPPRS	F-box/WD repeat-containing protein 5	377	0
CSLNG	Forkhead-associated domain-containing protein 1	1453	0
CAKKL	Four and a half LIM domains protein 1	280	0
CAKKL	Four and a half LIM domains protein 1	309	0
CAKKL	Four and a half LIM domains protein 1	296	0
CKALA	Protein flightless-1 homolog	1269	2
CLMLW	Fibronectin type III domain-containing protein 5	153	0
CSRKL	FRAS1-related extracellular matrix protein 1	2179	2
CIWEF	Fascin-3	498	0
CLVIE	F-box/LRR-repeat protein 16	479	1
CHSDT	Frizzled-6	706	1
CLWDG	Glucose-6-phosphatase 2	102	0
CLAFL	Putative uncharacterized protein GAFA-1	74	0
CGSRK	Glutamine amidotransferase-like class 1 domain-containing protein 1	220	1
CLWSH	N-acetylgalactosamine-6-sulfatase	522	0
CEPHF	Glycerophosphodiester phosphodiesterase 1	331	0
CGSSN	Gem-associated protein 2	44	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CLHKE	GTP cyclohydrolase 1 feedback regulatory protein	84	1
CYIFD	Gamma-glutamyl hydrolase	318	0
CTYFF	Glycosyltransferase 1 domain-containing protein 1	152	0
CQTGP	Putative glycosylation-dependent cell adhesion molecule 1	47	0
CESAF	Probable G-protein coupled receptor 150	434	0
CENNA	Probable G-protein coupled receptor 171	319	0
CVTLA	G-protein coupled receptor 35	309	1
CPKGK	G-rich sequence factor 1	480	1
CDYGL	Glutathione S-transferase omega-1	241	2
CPEPT	Solute carrier family 2, facilitated glucose transporter member 5	244	0
CHLYE	Hepatitis A virus cellular receptor 2	142	0
CSDDT	Pseudouridine-5'-phosphatase	208	0
CSEGK	Hepatocyte growth factor	210	0
CKEMV	Homeodomain-only protein	94	0
CKEMV	Homeodomain-only protein	112	0
CYFYQ	Hornerin	2850	0
CDMQK	Hippocalcin-like protein 4	191	0
CYAEN	Hyaluronan and proteoglycan link protein 2	340	0
CYRQH	Hyaluronan and proteoglycan link protein 3	360	0
CVVHA	Hermansky-Pudlak syndrome 3 protein	890	0
CPKGD	Homeobox protein Hox-D9	352	0
CMLGQ	Glucokinase	465	2
CKAKM	Interleukin-12 receptor subunit beta-1	662	0
CSHHL	Interleukin-15 receptor subunit alpha	267	4
CVEIP	Interleukin-22 receptor subunit alpha-2	263	1
CRSVS	Insulin growth factor-like family member 1	110	0
CQHIP	Interleukin-19	207	0
CPTSI	Interleukin-31 receptor subunit alpha	662	0
CPTSI	Interleukin-31 receptor subunit alpha	681	0
CLAKK	Importin subunit alpha-8	516	0
CMYRA	Integrator complex subunit 14	86	0
CTGGQ	Interferon regulatory factor 7	164	0
CVQGI	Iron-sulfur cluster assembly 2 homolog, mitochondrial	60	0
CATST	Intestine-specific homeobox	245	0
CLKQH	Izumo sperm-egg fusion protein 4	232	1
CSKDR	Dyslexia-associated protein KIAA0319	1072	3
CTMKS	UPF0258 protein KIAA1024	916	0
CGNGP	Kynurenine--oxoglutarate transaminase 1	250	0
CGKDS	Potassium channel subfamily K member 17	332	0
CQKRQ	Lysine-specific demethylase 4C	1056	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CREQK	Killer cell immunoglobulin-like receptor 3DS1	382	0
CSRHN	Kinesin-like protein KIF19	998	0
CLGSN	Kinesin-like protein KIF6	814	2
CWQRR	Kinesin-like protein KIFC2	838	0
CSPAN	Thymidine kinase, cytosolic	234	0
CFGLS	Kinesin light chain 4	315	0
CVSDS	Kelch domain-containing protein 8A	350	0
CVYNV	Kelch-like protein 23	558	0
CLTKM	Kelch-like protein 40	621	0
CPVRQ	Kinase non-catalytic C-lobe domain-containing protein 1	1141	0
CHFYH	Kinetochore scaffold 1	2342	1
CVSLL	Keratin-associated protein 10-4	401	0
CKKER	KRAB domain-containing protein 4	171	1
CRTKY	Keratin, type II cuticular Hb4	600	0
CLSVD	Ribosomal protein S6 kinase beta-2	154	0
CTVLF	Putative uncharacterized protein KTN1-AS1	53	0
CASWQ	Laminin subunit gamma-3	1575	0
CHSVH	Putative L-type amino acid transporter 1-like protein IMAA	180	0
CVAFD	Galectin-1	135	0
CPPPE	Late cornified envelope-like proline-rich protein 1	98	0
CDLTV	Leptin receptor	1165	0
CLGHY	Legumain	433	1
CEAVL	Lipase member M	423	1
CGGRH	Hormone-sensitive lipase	1076	1
CGLSR	Leucine-rich repeat protein 1	146	0
CTDSS	Low-density lipoprotein receptor-related protein 5	1615	0
CTDSS	Low-density lipoprotein receptor-related protein 6	1613	0
CIYFV	Leucine-rich repeat-containing protein 9	1111	0
CTAKE	Latent-transforming growth factor beta-binding protein 2	1821	0
CAEEN	Leucine zipper protein 1	1076	0
CDNFK	DNA helicase MCM9	391	0
CAFLS	MDS1 and EVI1 complex locus protein	169	0
CLSAA	Mediator of RNA polymerase II transcription subunit 15	788	1
CLSAA	Mediator of RNA polymerase II transcription subunit 15	677	0
CLARR	MICAL-like protein 2	680	0
CIMNW	Misshapen-like kinase 1	1332	4
CTKHY	Mitochondrial Rho GTPase 1	247	0
CAESV	Mitochondrial Rho GTPase 2	213	0
CVNRT	MMS19 nucleotide excision repair protein homolog	318	0
CLTFI	2-acylglycerol O-acyltransferase 3	341	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CWLRD	Mitochondrial-processing peptidase subunit beta	489	0
CFALE	Mitochondrial carrier homolog 1	389	2
CLSDA	Myotubularin-related protein 5	1868	2
CISDA	Myotubularin-related protein 13	1849	0
CQMSL	Mucin-17	4361	0
CGTTA	Musculin (Activated B-cell factor 1)	206	0
CPLGA	Matrix remodeling-associated protein 8	450	0
CTPAL	Myomegalin	2346	1
CSWGK	Myozenin-3	87	0
CHHGY	NEDD4-binding protein 2-like 1	243	0
CFLSS	N-alpha-acetyltransferase 16, NatA auxiliary subunit	429	0
CLYLA	Putative uncharacterized protein encoded by LINC00301	95	0
CALQA	Nuclear cap-binding protein subunit 1	790	0
CLSEP	Neurochondrin	729	2
CGSQG	Nck-associated protein 5-like	1330	1
CPVSV	Sodium/potassium/calcium exchanger 1	1099	2
CPVSI	Sodium/potassium/calcium exchanger 2	661	1
CREDD	Sodium/potassium/calcium exchanger 4	622	3
CSIFI	Nuclear receptor coactivator 7	102	0
CLVFE	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	120	0
CYRIF	Serine/threonine-protein kinase Nek4	781	0
CTASS	Serine/threonine-protein kinase Nek7	302	0
CRPSL	Serine/threonine-protein kinase Nek9	979	0
CLQNN	Protein kinase C-binding protein NELL1	810	1
CLQEL	Protein kinase C-binding protein NELL2	816	3
CSFPL	Neuferricin	264	1
CSPWH	Neurofascin	619	0
CIEQK	Nidogen-1	1247	1
CLNDR	Substance-P receptor	311	0
CKHKL	NKG2-A/NKG2-B type II integral membrane protein	233	1
CKHKL	NKG2-C type II integral membrane protein	231	0
CTVEM	NLR family CARD domain-containing protein 3	1065	2
CHPRR	Glutamate receptor ionotropic, NMDA 2A	1281	0
CRKMK	Polynucleotide 5'-hydroxyl-kinase NOL9	702	0
CFQLP	Nuclear pore-associated protein 1	1156	0
CRAAA	Neuropeptides B/W receptor type 1 (G-protein coupled receptor 7)	328	0
CSEER	Nephronectin	565	4
CRQIN	Neuronal pentraxin-1	432	0
CVVHS	Neurexin-3	1392	0
CSKLN	NUAK family SNF1-like kinase 1	661	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CSKLT	NUAK family SNF1-like kinase 2	628	0
CLLFS	Nuclear mitotic apparatus protein 1	1776	0
CILVR	Nuclear mitotic apparatus protein 1	1763	0
CPLET	NXPE family member 3	559	0
CPLSS	Olfactory receptor 10H3	316	0
CPPSS	Olfactory receptor 10H4	316	0
CDRSI	Olfactory receptor 2AT4	320	0
CQWKI	Olfactory receptor 51A2	313	0
CQRKI	Olfactory receptor 51A4	313	0
CFWKD	Olfactory receptor 51G1	321	0
CGNIP	Olfactory receptor 52R1	315	0
CLAVK	Olfactory receptor 5AL1	328	0
CRRGS	Putative uncharacterized protein OBSCN-AS1	158	1 (see below)
CGTQA	Putative uncharacterized protein OBSCN-AS1	113	SPLICE OF ABOVE
CVLEH	Odorant-binding protein 2a	170	1
CVPEH	Odorant-binding protein 2b	170	0
CPVLS	Obscurin-like protein 1	1543	0
CGEKG	Mu-type opioid receptor	128	0
CSGKM	Olfactory receptor 2L3	312	0
CSVKM	Olfactory receptor 2L8	312	0
CSKLN	Olfactory receptor 4A8	315	0
CKIAV	Olfactory receptor 5M10	315	0
CNIFV	Olfactory receptor 8K3	312	0
CPDIY	Oxysterol-binding protein-related protein 2	480	1
CPDIF	Oxysterol-binding protein 1	807	0
CPNIF	Oxysterol-binding protein 2	916	5
CTWAK	Protein OSCP1	223	0
CGGTA	Protein odd-skipped-related 2	276	0
CIAGL	3-oxoacyl-[acyl-carrier-protein] synthase, mitochondrial	459	1
CLFRK	Proline-rich protein 23D1	279	0
CLFRK	Proline-rich protein 23D2	279	0
CDEHP	Pannexin-3	392	0
CLKRQ	Monocyte to macrophage differentiation factor 2	193	0
CLKTL	Partitioning defective 6 homolog beta	115	0
CNEDL	Prostate and testis expressed protein 1	126	1
CNFKL	Prostate and testis expressed protein 3	98	0
CVVFS	PAX3- and PAX7-binding protein 1	815	0
CTFQG	Proprotein convertase subtilisin/kexin type 5	913	0
CLLAG	Proprotein convertase subtilisin/kexin type 6	969	1
CSWPL	Programmed cell death protein 1	288	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CDREV	Prostamide/prostaglandin F synthase	198	3
CSALG	PHD finger protein 12	704	0
CKKSK	PHD finger protein 7	381	1
CVSVS	Phosphatidylinositol 4-phosphate 5-kinase type-1 gamma	700	0
CSFIW	GPI ethanolamine phosphate transferase	463	0
CSFIW	GPI ethanolamine phosphate transferase 2	341	0
CTHSP	phospholipase A2 inhibitor & Ly6/PLAUR domain-containing protein	204	2
CQYNS	Pituitary homeobox 1	314	0
CWIIK	Pleckstrin homology domain-containing family S member 1	465	0
CIRLK	Prokineticin receptor 1 (PK-R1) (G-protein coupled receptor 73)	393	0
CIRLK	Prokineticin receptor 2 (G-protein coupled receptor 73-like 1)	384	0
CVWQG	Phospholipase D4	506	0
CYSEA	Plexin-D1	1925	0
CDFFL	Polyamine-modulated factor 1-binding protein 1	1022	0
CGQLE	Peptidyl-prolyl cis-trans isomerase A	165	1
CGEYV	Peptidyl-prolyl cis-trans isomerase E	301	0
CGQLS	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	207	0
CVAHS	Protein phosphatase 1 regulatory subunit 32	425	0
CYRSV	Pre-mRNA-processing factor 40 homolog B	195	0
CFMPN	Melanoma antigen preferentially expressed in tumors	509	0
CRKDE	Probable histone-lysine N-methyltransferase PRDM7	407	0
CREDE	Histone-lysine N-methyltransferase PRDM9	894	0
CQIDE	Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2	984	0
CLAQK	Prokineticin-2	129	1
CASLS	Proline-rich protein 5-like	368	2
CMTEQ	Prostaglandin-H2 D-isomerase	190	0
CNRTG	Receptor-type tyrosine-protein phosphatase eta	539	0
CDIDI	Securin	202	0
CLISS	Regulator of G-protein signaling 7-binding protein	257	0
CLLHP	Ras association domain-containing protein 5	336	0
CESLV	Ras association domain-containing protein 10	507	0
CPSQA	Putative uncharacterized protein encoded by RBM12B-AS1	102	0
CFIPP	RNA-binding protein 34	225	0
CGPQR	ATP-dependent DNA helicase Q5	991	1
CEELG	ATP-dependent DNA helicase Q5	410	SPLICE OF ABOVE
CKFKN	Lithostathine-1-alpha	166	0
CKFKN	Lithostathine-1-beta	166	0
CKFTD	Regenerating islet-derived protein 3-alpha	175	0
CKFKD	Regenerating islet-derived protein 3-gamma	175	1
CKYRP	Regenerating islet-derived protein 4	158	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CRGAE	N-acylglucosamine 2-epimerase	427	0
CRVQP	Resistin	108	1
CLLRG	All-trans-retinol 13,14-reductase	481	0
CSDDA	RNA exonuclease 4	422	1
CQVLH	Replication factor C subunit 4	303	0
CLKKN	Rab11 family-interacting protein 1	142	0
CRNNI	Regulator of G-protein signaling 1	169	0
CRIQQ	Blood group Rh(D) polypeptide	493	0
CGERS	egulating synaptic membrane exocytosis protein 4	269	1
CVLVA	60S ribosomal protein L27a	148	0
CAHKS	60S ribosomal protein L28	169	0
CVGSS	Zinc finger protein Rlf	1914	0
CPTLK	RING finger protein 214	703	1
CEQAV	E3 ubiquitin-protein ligase RNF43	783	2
CHPLW	RING finger and transmembrane domain-containing protein 2	321	0
CGSKL	Probable ribonuclease 11	199	0
CKIPY	60 kDa SS-A/Ro ribonucleoprotein	534	0
CGVES	Reactive oxygen species modulator 1	59	0
CLFLS	Regulation of nuclear pre-mRNA domain-containing protein 2	152	0
CLLMR	Ras-related GTP-binding protein B	374	0
CFTSE	Receptor-transporting protein 4	246	0
CVYYW	Protein RUBCNL-like	635	0
CLGAQ	Mitochondrial RNA pseudouridine synthase RPUSD4	377	1
CPDRP	Protein S100-A2	98	0
CQPPE	Multidrug and toxin extrusion protein 1	586	0
CNVTS	Orphan Na- and Cl-dependent neurotransmitter transporter NTT5	736	0
CLGPK	SUMO-activating enzyme subunit 1	346	1 (see below)
CPLPS	SUMO-activating enzyme subunit 1	299	SPLICE OF ABOVE
CARRN	Scaffold attachment factor B2	115	0
CQLLN	Protein transport protein Sec24D	1032	1
CFPNS	Semaphorin-5B	1151	2
CRLGV	Semaphorin-6B	677	0
CPVPV	tRNA-splicing endonuclease subunit Sen54	177	0
CKLTV	Sentrin-specific protease 3	574	0
CRLMD	Sentrin-specific protease 5	755	1
CPTAE	Neuronal-specific septin-3	358	0
CSTGN	Sperm-associated antigen 11B	103	1
CGNTH	Beta-sarcoglycan	318	1
CLKAP	SH2 domain-containing protein 1A	128	1
CGPVP	Protein shisa-like-2A	190	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CDGAT	Alpha-2,8-sialyltransferase 8B	375	0
CSPSV	Small integral membrane protein 28	152	0
CPVGN	NAD-dependent protein deacetylase sirtuin-7	183	0
CLHKK	SLIT and NTRK-like protein 2	733	0
CRRLT	Schlafen family member 12	578	0
CNSEL	Secreted Ly-6/uPAR-related protein 1	103	0
CSSMS	Mothers against decapentaplegic homolog 2	467	1
CSSVS	Mothers against decapentaplegic homolog 3	425	3
CFFLR	Schlafen family member 12-like	588	1
CSQGP	Sushi, nidogen and EGF-like domain-containing protein 1	1324	0
CFSKM	Sorting nexin-4	450	1
CRYVF	Sterol O-acyltransferase 1	550	2
CVMSV	Sorcin	198	1
CRVHG	Spermatogenesis-associated protein 2-like protein	152	0
CQEAV	Serpin A9	201	0
CPLDV	Spermatogenesis-associated protein 7	599	2
CLMEN	Snurportin-1	360	0
CLLHH	Spermatogenesis-associated protein 3	192	0
CHIPN	Serine palmitoyltransferase 3	175	0
CGQAD	Steroid receptor-associated and regulated protein	169	0
CYGGL	Succinate-semialdehyde dehydrogenase, mitochondrial	535	1
CVDYL	Suppressor of tumorigenicity 7 protein-like	555	0
CVDYL	Suppressor of tumorigenicity 7 protein-like	538	0
CLDQE	StAR-related lipid transfer protein 13	687	0
CRYPD	Metalloreductase STEAP2	454	0
CRYAF	Serine/threonine-protein kinase 4	462	0
CKQPG	Surfeit locus protein 2	256	0
CASYL	Nesprin-2	285	0
CVVHI	TP53-target gene 3 protein	102	0
CVQTS	Thymosin beta-15A	45	0
CVQTS	Thymosin beta-15B	45	0
CRVPG	TANK-binding kinase 1-binding protein 1	222	0
CVLDL	F-box-like/WD repeat-containing protein TBL1Y	522	0
CSLMQ	Transcription elongation factor A protein 3	154	0
CKAYS	Tectonic-2	697	1
CLGSP	Transcription elongation factor, mitochondrial	171	0
CPSQQ	Testis-expressed protein 45	505	0
CVQRE	Acetyl-CoA acetyltransferase, cytosolic	397	1
CHVRR	Acetyl-CoA acetyltransferase, mitochondrial	162	0
CWPDP	Threonine synthase-like 2	384	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CMLLN	Transketolase-like protein 1	596	2
CSDPI	Transmembrane protein 108	487	0
CKTVI	Transmembrane protein 220	160	1
CLKGD	Transmembrane protein 241	123	0
CIEEI	Transmembrane protein 17	198	0
CRSGV	Tropomodulin-1	359	1
CLGPP	Tumor necrosis factor receptor superfamily member 25	253	0
CPQNR	DNA topoisomerase 3-alpha	1001	2
CLKLA	Trafficking protein particle complex subunit 12	735	0
CVKDR	Trafficking protein particle complex subunit 13	309	0
CHPDT	Tryptophan 5-hydroxylase 1	466	0
CAPFP	Putative protein TPRXL	258	0
CAERM	Triggering receptor expressed on myeloid cells 1	225	0
CPPSS	E3 ubiquitin-protein ligase TRIM22	498	1
CPPSS	Tripartite motif-containing protein 34	488	0
CHGSK	Tripartite motif-containing protein 77	450	0
CSPSS	Tripartite motif-containing protein 5	493	0
CEEGG	Transient receptor potential cation channel subfamily M member 1	300	0
CLPGT	Taste receptor type 1 member 1	480	0
CAGLG	Tuberin	239	0
CYRDF	Testis-specific gene 10 protein	698	0
CMGWW	Translocator protein 2	70	0
CLKKL	Tetratricopeptide repeat protein 13	860	1
CKLAF	Putative tetratricopeptide repeat protein 41	1053	0
CRPTS	Tubby-related protein 3	170	0
CAFMK	T cell receptor alpha variable 38-1	116	0
CAYRS	T cell receptor alpha variable 38-2/delta variable 8	116	0
CAMRE	T cell receptor alpha variable 14/delta variable 4	116	0
CALSE	T cell receptor alpha variable 19	116	0
CAVRD	T cell receptor alpha variable 3	114	0
CLVGD	T cell receptor alpha variable 4	109	0
CIVRV	T cell receptor alpha variable 26-1	109	0
CILRD	T cell receptor alpha variable 26-2	109	0
CASSL	T cell receptor beta variable 13	124	0
CASSQ	T cell receptor beta variable 14	115	0
CASSQ	T cell receptor beta variable 16	115	0
CASSP	T cell receptor beta variable 18	115	0
CASSI	T cell receptor beta variable 19	14	0
CASSE	T cell receptor beta variable 2	115	0
CASSQ	T cell receptor beta variable 3-1	114	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CASSQ	T cell receptor beta variable 4-1	114	0
CASSQ	T cell receptor beta variable 4-2	114	0
CASSQ	T cell receptor beta variable 4-3	114	0
CASSL	T cell receptor beta variable 5-1	114	0
CASSL	T cell receptor beta variable 5-4	114	0
CASSL	T cell receptor beta variable 5-5	114	0
CASSL	T cell receptor beta variable 5-6	114	0
CASSL	T cell receptor beta variable 5-8	114	0
CASSE	T cell receptor beta variable 6-1	114	0
CASSY	T cell receptor beta variable 6-2	114	0
CASSY	T cell receptor beta variable 6-3	114	0
CASSD	T cell receptor beta variable 6-4	114	0
CASSY	T cell receptor beta variable 6-5	114	0
CASSY	T cell receptor beta variable 6-6	114	0
CASSY	T cell receptor beta variable 6-8	113	0
CASSY	T cell receptor beta variable 6-9	114	0
CASSL	T cell receptor beta variable 7-2	115	0
CASSL	T cell receptor beta variable 7-4	115	0
CASSL	T cell receptor beta variable 7-6	115	0
CASSL	T cell receptor beta variable 7-7	115	0
CASSL	T cell receptor beta variable 7-8	115	0
CASSL	T cell receptor beta variable 7-9	115	0
CASSV	T cell receptor beta variable 9	114	0
CASSE	T cell receptor beta variable 10-1	114	0
CASSE	T cell receptor beta variable 10-2	114	0
CAISE	T cell receptor beta variable 10-3	114	0
CASSL	T cell receptor beta variable 11-1	115	0
CASSL	T cell receptor beta variable 11-2	115	0
CASSL	T cell receptor beta variable 11-3	115	0
CASSL	T cell receptor beta variable 12-4	115	0
CASGL	T cell receptor beta variable 12-5	115	0
CSKVN	Thioredoxin domain-containing protein 16	825	0
CGRPS	TYMS opposite strand protein	123	0
CPSVG	tRNA wybutosine-synthesizing protein 2 homolog	448	0
CLRIF	NEDD8-conjugating enzyme UBE2F	120	0
CTEDK	(E3-independent) E2 ubiquitin-conjugating enzyme	1292	0
CMHTN	Ubiquitin carboxyl-terminal hydrolase 15	981	2
CKSEE	Ubiquitin carboxyl-terminal hydrolase 30	517	0
CSSAV	Basic helix-loop-helix domain-containing protein USF3	2245	0
CGTDD	Transcription cofactor vestigial-like protein 4	41	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CSNTF	Protein virilizer homolog	1147	0
CDIQK	Visinin-like protein 1	191	0
CGMPE	Synaptic vesicular amine transporter	209	0
CSLSW	Pantetheinase	513	0
CIIMT	Vascular non-inflammatory molecule 2	195	0
CREMK	Visual system homeobox 1	280	0
CHVRG	WD repeat-containing protein 26	216	0
CMSIL	WAP four-disulfide core domain protein 10A	79	0
CMSIL	Protein WFDC10B	73	1
CKKYH	WAP four-disulfide core domain protein 6	131	0
CYNTG	Whirlin	345	0
CYRIA	WW domain-containing oxidoreductase	311	0
CSQAS	Xaa-Pro aminopeptidase 3	507	2
CSRPT	Transmembrane protein LOC653160	218	0
CPVIT	Putative uncharacterized protein FLJ45035	140	0
CVAAL	YjeF N-terminal domain-containing protein 3	299	1
CSDNV	Putative uncharacterized protein FLJ43343	128	0
CNFLT	Putative uncharacterized protein FLJ46792	126	0
CADSF	Putative uncharacterized protein FLJ45275, mitochondrial	168	0
CTAAG	Putative uncharacterized protein FLJ42384	140	0
CSLWR	Putative uncharacterized protein FLJ40606	135	0
CSVFL	Putative uncharacterized protein FLJ39060	123	0
CGKAF	Putative zinc finger protein 355P	428	0
CGEAK	Zinc finger and BTB domain-containing protein 40	1239	0
CRRVQ	Zinc finger CCHC domain-containing protein 24	241	0
CHMAL	Palmitoyltransferase ZDHHC17	216	0
CRRKR	Zinc finger MYND domain-containing protein 11	602	2
CPTDL	RING finger and CHY zinc finger domain-containing protein 1	75	0
CEHFS	Zinc finger protein 407	1660	0
CIDTP	Zinc finger protein 433	673	1
CAGEK	Zinc finger protein 492	531	0
CQATM	Zinc finger protein 680	123	0
CFTNK	Putative protein ZNF720	178	0
CAKAF	Zinc finger protein 729	1252	0
CGWAR	Zinc finger protein 839	811	2
CVIIP	Zinc finger protein 24	193	0
CGKDF	Putative zinc finger protein 56	161	0
CAGEK	Zinc finger protein 98	572	0
CGRHS	Tight junction protein ZO-2	993	0
CQMPF	Protein zyg-11 homolog A	759	1

C-terminus	Protein Name	Size (aa)	Number of splice variants
CWPRS	Zinc finger ZZ-type and EF-hand domain-containing protein 1	811	0
CCxxx			
CCLGM	AP-3 complex subunit mu-2	273	0
CCSRK	Biliverdin reductase A	296	0
CCVLV	Calpain-8	703	0
CCSVK	COBW domain-containing protein 1	113	0
CCNWA	Acidic mammalian chitinase	476	2
CCTWN	Chitotriosidase-1	466	2
CCCIF	Chondroitin sulfate glucuronyltransferase	666	0
CCSEN	utative uncharacterized protein encoded by LINC00469	141	0
CCSGC	Cysteine-rich C-terminal protein 1	99	0
CCVIS	Cysteine-rich tail protein 1	144	0
CCCPR	Cysteine and tyrosine-rich protein 1	64	0
CCLQQ	Beta-defensin 110	67	0
CCKKP	Beta-defensin 4A	64	0
CCGHS	Protein FAM107A	135	0
CCVIL	F-box/LRR-repeat protein 2	423	1
CCPFP	L-fucose kinase	1084	1
CCIIL	F-box/LRR-repeat protein 20	436	1
CCTKF	Neuronal membrane glycoprotein M6-b	265	2
CCVVQ	Type I inositol 1,4,5-trisphosphate 5-phosphatase	412	0
CCSYA	Immunoglobulin lambda variable 2-23	113	0
CCCKK	Mucolipin-3	553	1
CCVSD	Metastasis-associated protein MTA3	594	0
CCACV	Protein myomaker	221	0
CCWPS	Sialidase-4	484	2
CCSEA	Neuropilin-2	931	2
CCVVM	Paralemmin-2	379	1
CCAVM	Paralemmin-3	673	0
CCSIM	Paralemmin-1	387	1
CCVVN	Nuclear pore membrane glycoprotein 210	967	0
CCLLM	Protein phosphatase 1 regulatory subunit 16A	528	0
CCRIS	Protein phosphatase 1 regulatory inhibitor subunit 16B	567	1
CCSSG	Ras-related protein Rab-21	225	0
CCISL	Ras-related protein Rab-25	213	0
CCNFN	Ras-related protein Rab-30	203	0
CCSFM	Ras-related protein Rab-37	223	3
CCQNI	Ras-related protein Rab-11A	216	0

C-terminus	Protein Name	Size (aa)	Number of splice variants
CCQNL	Ras-related protein Rab-11B	218	0
CCHLT	Resistin-like beta	111	0
CCKVL	Rho-related GTP-binding protein RhoB	196	0
CCSII	Rho-related GTP-binding protein RhoJ	214	1
CCLIT	Rho-related GTP-binding protein RhoQ	205	0
CCVAH	Zinc finger protein SNAI2	268	0
CCPGP	Zinc finger protein SNAI3	292	0
CCHVI	SNF-related serine/threonine-protein kinase	765	0
CCSSK	Syntaxin-19	294	0
CCIHF	Putative tripartite motif-containing protein 49B	452	0
CCIHF	Putative tripartite motif-containing protein 49C	452	0
CCVHL	Tripartite motif-containing protein 49D	452	0
CCIHF	Tripartite motif-containing protein 49	452	0
CCSHF	Tripartite motif-containing protein 51	452	1
CCVIL	Ubiquitin-like protein 3	117	0
CCYQN	Vascular non-inflammatory molecule 3	207	0
CCAIM	Synaptobrevin homolog YKT6	198	1
CCYVA	Zinc finger protein 64 homolog, isoforms 3 and 4	415	0
CxCxx			
CGCLG	Artemin	220	2
CACFL	Barrier-to-autointegration factor-like protein	90	1
CSCLR	BSD domain-containing protein 1	474	0
CSCLR	BSD domain-containing protein 1	353	0
CSCLR	BSD domain-containing protein 1	99	0
CKCWS	Cytidine and dCMP deaminase domain-containing protein 1	99	0
CVCDD	Uncharacterized protein C20orf24 (Rab5-interacting protein)	87	0
CSCGG	Ephrin type-B receptor 2	1055	0
CLCNT	Fc receptor-like protein 3	742	0
CTCIF	Interleukin-17B	180	0
CQCKI	Keratin-associated protein 5-10	202	0
CQCKI	Keratin-associated protein 5-11	156	0
CQCKI	Keratin-associated protein 5-1	278	0
CQCKI	Keratin-associated protein 5-2	177	0
CQCKI	Keratin-associated protein 5-3	238	0
CQCKI	Keratin-associated protein 5-4	288	0
CQCKI	Keratin-associated protein 5-5	237	0
CQCKI	Keratin-associated protein 5-6	129	0
CQCKI	Keratin-associated protein 5-7	165	0

C-terminus	Protein Name	Size (aa)	Number of splice variants
CQCKI	Keratin-associated protein 5-8	187	0
CHCVH	Putative L-type amino acid transporter 1-like protein MLAS	180	0
CSCCA	Metallothionein 1H-like protein 1	61	0
CTDEE	Mitogen-activated protein kinase kinase kinase 4	1608	1
CSPVG	Membrane-spanning 4-domains subfamily A member 6A	141	0
CKIEF	Microtubule-associated protein 1A	2803	1
CKIEL	Microtubule-associated protein 1B	2468	0
CKVEF	Microtubule-associated protein 1S	1059	1
CLFHA	Methionine adenosyltransferase 2 subunit beta	259	0
CEFPI	Mannose-binding protein C	248	0
CFTLP	Metallo-beta-lactamase domain-containing protein 2	199	0
CRTSD	Muscleblind-like protein 1	342	0
CLCRL	Malectin	292	0
CSCCA	Metallothionein-1A	61	0
CRCCA	Metallothionein-1B	61	0
CSCCA	Metallothionein-1E	61	0
CSCCD	Metallothionein-1F	61	0
CSCCA	Metallothionein-1G	62	1
CSCCA	Metallothionein-1H	61	0
CSCCA	Metallothionein-1L	61	0
CSCCA	Metallothionein-1M	61	0
CSCCA	Metallothionein-1X	61	0
CSCCA	Metallothionein-2	61	0
CSCCQ	Metallothionein-3	68	0
CSCCP	Metallothionein-4	62	0
CKCSC	Noggin	232	0
CNCCR	Neuropeptide FF receptor 2 (G-protein coupled receptor 74)	132	0
CGCGG	Persephin	156	0
CGCCS	Rho GTPase-activating protein 19	517	0
CPCQL	Protein shisa-5	118	0
CPCLK	Syntaxin-11	287	0
CRCGP	SURP and G-patch domain-containing protein 1	222	0
CPCPC	Transmembrane protein 138	177	0
CVCKL	Transmembrane protein 39A	146	0
CDCVD	Twisted gastrulation protein homolog 1	75	0
CHCSS	Putative uncharacterized protein LOC151760	199	0
CxxCx			
CFSCN	Putative ATP-binding cassette sub-family C member 13	274	1

C-terminus	Protein Name	Size (aa)	Number of splice variants
CVGCS	Bromodomain-containing protein 4	794	0
CGRCP	Coiled-coil domain-containing protein 43	154	0
CWPCW	Protein C19orf12	107	0
CAKCI	Putative uncharacterized protein encoded by ERC2-IT1	136	0
CLYCV	Gamma-tubulin complex component 3	824	0
CVLCR	Probable G-protein coupled receptor 141	305	0
CTGCL	Guanylate cyclase activator 2B	112	0
CQNCH	RanBP-type and C3HC4-type zinc finger-containing protein 1	510	1
CLLCN	LON peptidase N-terminal domain and RING finger protein 3	610	0
CKDCL	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A	741	0
CQGCL	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B	792	2
CEQCC	Membrane-spanning 4-domains subfamily A member 5	200	0
CSICC	PACRG-like protein	248	2
CALCA	Slit homolog 1 protein	1534	0
CLACS	Slit homolog 3 protein	1523	2
CGPCR	Transcription factor 7	383	0
CGPCR	Transcription factor 7	268	0
CFGCT	Putative tripartite motif-containing protein 64B	449	0
CFGCT	Tripartite motif-containing protein 64C	450	0
CFGCT	Tripartite motif-containing protein 64	449	0
CLACV	Tubulin polyglutamylase TTLL13P	459	0
CYYCH	Zinc finger MYM-type protein 2	462	0
CxxxC			
CNVLC	ADAMTS-like protein 1	683	0
CRSRC	Calpain-10	544	0
CRSRC	Calpain-10	513	0
CSVGC	Coiled-coil domain-containing protein 47	480	0
CYDIC	Uncharacterized protein C12orf74	190	0
CKNFC	CCR4-NOT transcription complex subunit 4	767	0
CEPSC	Cartilage acidic protein 1	661	0
CRDRC	Zinc finger protein DPF3	195	0
CPHPC	Peptidyl-prolyl cis-trans isomerase FKBP1B	80	0
CRRTC	Immunoglobulin-like and fibronectin type III domain-containing protein 1	605	0
CRRTC	Keratin-associated protein 11-1	163	0
CRSTC	Keratin-associated protein 13-2	175	0
CYSSC	Keratin-associated protein 21-1	79	0
CEPTC	Keratin-associated protein 1-3	177	0
CEPTC	Keratin-associated protein 1-4	121	0

Appendix II: Table of Human $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)	Number of splice variants
CEPTC	Keratin-associated protein 1-5	174	0
CEPRC	Keratin-associated protein 3-1	98	0
CGSSC	Keratin-associated protein 4-1	146	0
CAMNC	Protein lin-54 homolog	749	3
CQRDC	Arachidonate 5-lipoxygenase	533	0
CLDKC	Volume-regulated anion channel subunit LRRC8B	803	0
CTFFC	RNA-binding protein MEX3B	158	0
CQAWC	Male-specific lethal 3 homolog	416	0
CDFFC	NACHT, LRR and PYD domains-containing protein 7	980	2
CLFLC	Olfactory receptor 2AJ1	328	0
CRLRC	39S ribosomal protein L4, mitochondrial	263	0
CQISC	Sodium-dependent noradrenaline transporter	628	0
CETGC	Structural maintenance of chromosomes flexible hinge domain-containing protein 1	762	0
CQIRC	Taste receptor type 2 member 50	299	0

Appendix III: Table of Yeast $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)
Cxxxx		
CHYEF	Aberrant microtubules protein 1	123
CIFTY	Actin-like protein ARP8	881
CKKSR	Protein ASG7	209
CKKSR	Protein ASG7	209
CISEE	Metal homeostasis factor ATX2	313
CKPTY	Barrierpepsin	587
CKKMT	Putative uncharacterized protein BUD26	95
CASTD	Pre-mRNA-splicing factor BUD31	157
CVVFT	Calnexin homolog	502
CRRHM	Anaphase-promoting complex subunit CDC23	626
CHMQE	Anaphase-promoting complex subunit CDC27	758
CKQKR	S-phase entry cyclin-6	380
CHFTQ	GTP-binding protein CIN4	191
CLEDH	Cobalt uptake protein COT1	439
CRKRE	Probable S-adenosylmethionine-dependent methyltransferase CRG1	291
CAKPF	Exosome complex component CSL4	292
CPITA	Protein ECM13	257
CALVA	Putative glucokinase-2	500
CHLPV	Probable folylpolyglutamate synthase	430
CLYEL	Galactokinase	528
CLYEQ	Protein GAL3	520
CKAAE	E3 ubiquitin-protein ligase HEL1	551
CALVA	Glucokinase-1	500
CVLDA	Dihydroxy-acid dehydratase, mitochondrial	585
CDGKV	J protein JJJ2	584
CDGKV	J protein JJJ2	583
CGTPK	Protein LDB17	491
CLSFM	Protein LIN1	340
CDTGH	M1-1 protoxin	316
CKDNS	Mitochondrial intermembrane space cysteine motif-containing protein MIX23	196
CKNTV	E3 ubiquitin-protein ligase linker protein MMS1	1407
CGLIL	MIOREX complex component 8	314
CLHMP	N-alpha-acetyltransferase 40	285
CTYKI	Nucleoporin NUP145	1317
CFYKE	Nucleoporin NUP170	1502
CQAFM	Nucleoporin NUP85	744
CIDLL	Ornithine aminotransferase	424

Appendix III: Table of Yeast $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)
CADIF	Oxysterol-binding protein homolog 1	1188
CGDIF	Oxysterol-binding protein homolog 2	1283
CRSLW	Peroxisomal coenzyme A diphosphatase 1, peroxisomal	340
CSSKD	Peroxisomal membrane protein PEX25	394
CRIIV	Peroxisomal membrane protein PEX34	144
CKKKL	Transcriptional regulatory protein PHO23	330
CGQQN	Orotidine 5'-phosphate decarboxylase	267
CFERD	25S rRNA	490
CLREQ	DNA repair and recombination protein RDH54	924
CLREQ	DNA repair and recombination protein RDH54	924
CLREQ	DNA repair and recombination protein RDH54	924
CLREQ	DNA repair and recombination protein RDH54	924
CLREQ	DNA repair and recombination protein RDH54	958
CPITQ	DNA-directed RNA polymerases I and III subunit RPAC1	335
CNEYI	Chromatin structure-remodeling complex subunit RSC7	435
CIFGK	37S ribosomal protein SWS2, mitochondrial	143
CRFGG	Protein SWT21	357
CRFGG	Protein SWT21	357
CRFGG	Protein SWT21	357
CRFGG	Protein SWT21	357
CRFGG	Protein SWT21	357
CRFGG	Protein SWT21	357
CLPDE	Uncharacterized oxidoreductase TDA5	326
CLSLS	Vacuolar basic amino acid transporter 4	768
CEAIL	Vacuolar protein sorting-associated protein 13	3144
CWRGT	Putative uncharacterized protein YBL006W-A	49
CAGGM	Uncharacterized protein YBR056W-A	66
CLLNM	Putative uncharacterized protein YBR226C	136
CAIYP	Uncharacterized protein YCR108C	63
CKDFI	Uncharacterized protein YDR124W	324
CFEDG	Uncharacterized protein YDR209C	137
CSTAT	Putative uncharacterized protein YDR445C	135
CFNGE	Putative uncharacterized protein YDR521W	111
CNVYI	Uncharacterized protein YER078W-A	54
CGRTQ	Putative uncharacterized membrane protein YFL021C-A	284
CTIFS	Uncharacterized protein YGR146C-A	53
CGNYF	Uncharacterized protein YGR122W	402
CTSDD	Uncharacterized protein YGL015C	130
CLYSI	Uncharacterized protein YGL193C	103
CKNIT	Uncharacterized protein YHR125W	101
CTDAP	Uncharacterized oxidoreductase YJR096W	282

Appendix III: Table of Yeast $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)
CLMFS	Putative uncharacterized protein YJL169W	122
CRDLA	Uncharacterized protein YLR163W-A	37
CLKAR	Uncharacterized protein YLR255C	117
CSGTS	Putative uncharacterized protein YLR399W-A	34
CEFIL	Uncharacterized protein YMR134W	237
CYYIL	Putative uncharacterized membrane protein YML031C-A	111
CIPFK	Uncharacterized protein YOR376W-A	51
CIRGA	Uncharacterized membrane protein YOR376W	122
CVLII	Putative uncharacterized protein YPL238C	129
CLGNL	Protein ZPS1	249
CCxxx		
CCFFN	Transcriptional regulatory protein EDS1	919
CCFFN	Transcriptional regulatory protein EDS1	919
CCFFN	Transcriptional regulatory protein EDS1	919
CCFFN	Transcriptional regulatory protein EDS1	919
CCFFN	Transcriptional regulatory protein EDS1	919
CCFFN	Transcriptional regulatory protein EDS1	919
CCTLM	Guanine nucleotide-binding protein subunit gamma	110
CCSGK	Copper metallothionein 1-1	61
CCSGK	Copper metallothionein 1-2	61
CCIIC	Ras-like protein 1	309
CCIIS	Ras-like protein 2	322
CCIIL	GTP-binding protein RHO2	192
CCTVM	Flavoprotein-like protein YCP4	247
CCDVF	Cysteine-rich and transmembrane domain-containing protein YDR034W-B	51
CCISK	Putative uncharacterized protein YHR180C-B	34
CCIIM	Synaptobrevin homolog YKT6	200
CxCxx		
CFCCC	Casein kinase I homolog 3	524
CQCNK	SEH-associated protein 4	1038
CHCTT	Uncharacterized protein YJL077W-A	28
CVCTQ	Putative uncharacterized membrane protein YPR170C	111
CxxCx		
CWWCR	Aquaporin-like protein 2	149
CWWCR	Aquaporin-like protein 2	149
CWWCR	Aquaporin-like protein 2	149
CWWCR	Aquaporin-like protein 2	149
CWWCR	Aquaporin-like protein 2	149
CHLCV	Uncharacterized protein YMR122C	124
CVFCV	Uncharacterized protein YPR064W	139

Appendix III: Table of Yeast $C(x)_3X$ Sequences

C-terminus	Protein Name	Size (aa)
CxxxC		
CFRRC	Putative uncharacterized protein YNL097W-A	51
CRRNC	Uncharacterized protein YPL277C	487

Appendix IV: Table of $C(x)_3X$ Sequences in Select Pathogenic Organisms

C-terminus	Protein Name	Size (aa)
	Plasmodium falciparum	
CFFIM	25 kDa ookinete surface antigen	217
CQIEG	Putative phosphatidylethanolamine-binding protein	190
	Candida albicans	
CYVLV	Candidapepsin-9	544
CNMIH	Hypha-specific G1 cyclin-related protein 1	785
CLGQN	Adenylate kinase	249
CQGQI	Mediator of RNA polymerase II transcription subunit 8	239
CNDHQ	Mitogen-activated protein kinase MKC1	509
CTRIE	Protein OS-9 homolog	258
CTATD	Pre-mRNA-splicing factor SLU7	350
CCVIV	Ras-like protein 1	290
CCVIV	Ras-like protein 1	288
CCVIV	Ras-like protein 1	290
	Legionella pneumophila	
CLSSL	ATP-dependent dethiobiotin synthetase BioD	212
CLSSL	ATP-dependent dethiobiotin synthetase BioD	212
CLSSL	ATP-dependent dethiobiotin synthetase BioD	212
CLSSL	ATP-dependent dethiobiotin synthetase BioD	212
CDSHR	30S ribosomal protein S18	75
CDSHR	30S ribosomal protein S18	75
CDSHR	30S ribosomal protein S18	75
CDSHR	30S ribosomal protein S18	75
CGVIK	Superoxide dismutase [Cu-Zn]	162
CGVIK	Superoxide dismutase [Cu-Zn]	162
CIRKG	Thiopurine S-methyltransferase	221
CIRKG	Thiopurine S-methyltransferase	221
CIRKG	Thiopurine S-methyltransferase	221
CPQCQ	Ferric uptake regulation protein	136

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Research Experience

2011-Present **Graduate Assistant (Chemistry)** Syracuse University
Supervisor: Dr. James Hougland
Summary – Discovery and characterization of lipidation for non-canonical protein sequences in vitro and in mammalian cells. Investigated protein isolation techniques using bioorthogonal chemistry of engineered enzymes in bacterial and mammalian cells. Developed new method for studying protein modifications from cells.

Summer 2010 **Intern (Bioinformatics and Integrative Genomics)**
Harvard- MIT Health Sciences and Technology (Cambridge, MA)
Supervisors: Dr. Susanne Churchill and Dr. Robert Plenge
Summary – Evaluated Rheumatoid Arthritis biomarkers in human patient samples. Techniques used include isolation of white blood cells from patient blood samples, qPCR, DNA isolation from white blood cells, and statistically evaluating antibody levels and single nucleotide polymorphisms as risk factors for developing rheumatoid arthritis.

Teaching Experience

2018-Present **Assistant Professor of Chemistry (tenure track)** Utica College
Summary – Responsibilities include teaching four lectures or laboratories each semester in both biochemistry and general chemistry sequences. Courses taught include Biochemistry I/II lecture, Biochemistry lab, General Chemistry I lecture, and General Chemistry I/II lab. Research with undergraduate students in biochemistry.

2017-2018 **One Year Assistant Professor of Chemistry** Utica College
Summary – Responsibilities include teaching four lectures or laboratories each semester in both biochemistry and general chemistry sequences. Courses taught include Biochemistry I/II lecture, Biochemistry lab, General Chemistry I lecture, and General Chemistry I/II lab.

Summer 2016 **Instructor (Organic Chemistry Lab I)** Syracuse University

Summary – Taught lecture, designed lab protocols, report templates, quizzes, and exams. Held studentled discussions sections for review of lecture material. Supervised teaching assistants in running lab experiments and grading reports

Summer 2014 **Research Experience for Undergrate Students (REU) Research Mentor** Syracuse University

Supervisor: Dr. James Hougland

Summary –Advised summer student on experimental design, biochemical lab techniques, and scientific communication skills. Techniques included mammalian tissue culture, protein purification via FPLC, in vitro reactions from cell lysate, fluorescent plate reader methods, bacterial cloning, and protein gel analysis. This research culminated in a poster and oral presentation to the Syracuse University community.

2013-present **Teaching Assistant (Chemistry)** Syracuse University

Classes – General Chemistry I, Organic Chemistry I, Organic Chemistry Lab I/II, Prep & Analysis of Proteins and Nucleic Acids (Lab)

Summary: Lecture course responsibilities included teaching of multiple weekly recitation classes, office hours, proctoring and grading of exams, and one-on-one mentorship. Laboratory course responsibilities included preparation and ordering of materials, maintenance of equipment, testing the validity of experiments prior to class, assisting and guiding students during laboratory, office hours, holding lecture-style study sessions, and grading of lab reports, quizzes, and exams.

2013-2014 **Undergraduate Student Research Mentor** Syracuse University

Supervisor: Dr. James Hougland

Summary – Training and guidance of a sophomore undergradate student in biochemistry lab techniques, experiments, and scientific writing/presentations. Techniques mentored include protein purification via FPLC, bacterial cloning, DNA and protein gel analysis, PCR, HPLC analysis and basic lab maintenance and solution making

2009-2011 **Undergraduate Teaching Assistant (Organic Chemistry I/II Lecture and Lab)**

Bay Path University

Supervisor: Dr. Hsiang-Ching Kung

Summary – Taught weekly lecture recitations and supervised laboratory sections. Prepared laboratory materials and graded lab quizzes. Formatted, typed, and graded lecture exams.

2008-2010 **Student Tutor** The Bashevkin Center for Academic Excellence - Bay Path University

Classes: General Chemistry I/II, Organic Chemistry I/II, Statistics, Calculus I

Summary – Nominated by course instructor. Individual tutoring sessions with undergraduate students several days per week. Duties include review of lecture material, exam preparation, guidance in study methods, and homework help.

Awards and Honors:

Best Graduate Student Poster Presentation – 1st place, FASEB Science Research Conference 2017
William D. Johnson Award for Outstanding Graduate Teaching Assistant 2017
ACS Division of Biological Chemistry Travel Award 2016
Syracuse University Graduate Student Organization Travel Award 2015/2016
GAANN Fellowship 2011-2012
Syracuse University STEM Fellowship 2012 – 2014

Publications:

Zhang, Yi, ***Blanden, Melanie J.****, Sudheer, Ch., Gangopadhyay, Soumyashree A., Rashidian, Mohammad, Hougland, James L., and Distefano, Mark D. Simultaneous Site-Specific Dual Protein Labeling Using Protein Prenyltransferases Bioconjugate Chem.*, 2015, 26 (12), 2542–2553

Shala-Lawrence, Agnesa*, **Blanden, Melanie J.*,** Krylova, Svetlana M., Gangopadhyay, Soumyashree A., Beloborodov, Stanislav S., Hougland, James L., and Krylov, Sergey N. *Simultaneous Analysis of a Non-Lipidated Protein and Its Lipidated Counterpart: Enabling Quantitative Investigation of Protein Lipidation's Impact on Cellular Regulation* Anal Chem., 2017, 89 (24), 13502-13507

Blanden, Melanie J., Suazo, Kiall F., Hildebrandt, Emily R., Hardgrove, Daniel S., Patel, M., Saunders, William P., Distefano, Mark D., Schmidt, Walter K., and Hougland, James L. *Efficient farnesylation of "forbidden" C-terminal C(x)3X sequences expands the scope of the prenylated proteome* J. Biol. Chem., 2018, 293 (8), 2770-2785

Oral Presentations:

Blanden, Melanie J., Suazo, Kiall F., Hildebrandt, Emily R., Hardgrove, Daniel S., Patel, M., Saunders, William P., Distefano, Mark D., Schmidt, Walter K., and Hougland, James L. *Efficient farnesylation of "forbidden" C-terminal C(x)3X sequences expands the scope of the prenylated proteome* FASEB Science Research Conference: Protein Lipidation: Enzymology, Signaling and Therapeutics, Saxtons River, VT, July 2017 (Elevator Pitch Presentation)

Blanden, Melanie J., Schmidt, Walter K., Hougland, James L. *Redefining the potential prenylome: Prenylation of non-canonical C-terminal sequences in peptides and proteins* 252nd American Chemical Society National Meeting & Exposition, Biological Division: Young Investigators. Philadelphia, PA 2016

Blanden, Melanie J., Schmidt, Walter K., Hougland, James L. *Redefining the potential prenylome: Prenylation of non-canonical C-terminal sequences in peptides and proteins* Northeast Regional Meeting of the American Chemical Society, Biological Division: Biochemistry, Binghamton, NY, 2016

Poster Presentations:

Blanden, Melanie J., Suazo, Kiall F., Hildebrandt, Emily R., Hardgrove, Daniel S., Patel, M., Saunders, William P., Distefano, Mark D., Schmidt, Walter K., and Hougland, James L. *Efficient farnesylation of "forbidden" C-terminal C(x)3X sequences expands the scope of the prenylated proteome* FASEB Science Research Conference: Protein Lipidation: Enzymology, Signaling and Therapeutics, Saxtons River, VT, July 2017

Blanden, Melanie J., Shala-Lawrence, Agnesa, Krylova, Svetlana M., Gangopadhyay, Soumyashree A., Beloborodov, Stanislav S., Hougland, James L., and Krylov, Sergey N. *Simultaneous Analysis of a Non-Lipidated Protein and Its Lipidated Counterpart: Enabling Quantitative Investigation of Protein Lipidation's Impact on Cellular Regulation* FASEB Science Research Conference: Protein Lipidation: Enzymology, Signaling and Therapeutics, Saxtons River, VT, July 2017

Blanden, Melanie J., Shala, A., Zhang, Y., Krylov, S., Distefano, M., and Hougland, James L. *Chemoenzymatic protein labeling and isolation from eukaryotic cell lysates using prenyltransferases with reengineered substrate selectivity* 251st American Chemical Society National Meeting & Exposition, Biological Division: Current Topics in Biochemistry. San Diego, CA 2016

Blanden, Melanie J. and Hougland, James, L. *Investigation into the Degradation Pathway of Prenylated Proteins* Syracuse University Life Sciences Research Showcase, Syracuse, NY, 2013.

Blanden, Melanie J. and Hougland, James, L. *Investigation into the Degradation Pathway of Prenylated Proteins* Northeast Regional Meeting of the American Chemical Society, Rochester, NY, 2012.

Leadership & Service:

2014-present **Nursery Childcare Volunteer** Imperfect Church (Camillus, NY)
Care of children ages 0-3 years during church services.

Supervisor of Welcome Team Imperfect Church (Camillus, NY)
Welcoming first-time attendees to our church and providing them with information about our values and Sunday service. Introduce the visitors to other church members, leaders, and pastor, as well as answer questions the guest may have. Organizing a guest information packet and receiving their demographic information for future outreach. Scheduling volunteers to work at the desk.

2012-present **Board of Directors (Vice-President), Volunteer, Foster, Coordinator**
CNY Cat Coalition (Syracuse, NY)
a) Adoption Center volunteer: Care, cleaning and enrichment for adoptable cats at local PetSmart adoption centers. Liaison to the public for adoption information, meet-and-greets of pet with potential adopters.

b) Adoption Center Coordinator: Responsible for organization and direction of fosters for cage assignments of adoptable pets, handled issues with the public concerning the adoption center, organization of the adoption materials, supplies, and volunteer schedule of the adoption center.
c) Board of Directors: Monthly meetings to discuss concerns and issues within the organization. Design of new volunteer positions, donation events, and writing of grants for animal rescue nonprofits

2009-2011 **Resident Assistant** – Bay Path College

Responsibilities included maintenance of dormitory, creating and holding social and community service events, mentoring students, and mediation of concerns and issues between students

Appendix X: Curriculum Vitae