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## Development of Protein Farnesyltransferase Variants with Altered Substrate Selectivity

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# **Development of Protein Farnesyltransferase Variants with Altered Substrate Selectivity**

A Capstone Project Submitted in Partial Fulfillment of the  
Requirements of the Renée Crown University Honors Program at  
Syracuse University

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and Renée Crown University Honors  
May 2013

Honors Capstone Project in Biochemistry

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Date: 5/7/2013

## **Abstract**

Post-translational modifications play an essential role in regulating protein structure and function. Enzymes catalyzing these modifications must often recognize and modify multiple substrate proteins from among a plethora of non-substrates with similar structures and amino acid sequences. For example, protein farnesyltransferase (FTase) catalyzes the addition of an isoprenoid group to a cysteine near the C-terminus of a substrate protein and is proposed to modify a pool of substrates numbering more than one hundred. We seek to understand the interactions in the FTase active site that engender substrate selectivity. By mutating two residues within FTase, we have developed FTase variants with expanded substrate selectivity. The alteration in substrate selectivity observed in our variants suggests that FTase selectivity may depend on a small number of “tunable” active site contacts. Our work provides insight into how this multispecific enzyme recognizes its pool of substrates and will also aid in identifying additional FTase substrates.

## **Acknowledgments**

I would like to thank Professor James L. Hougland, the Hougland research group, Professor Heather Coleman, the Renee Crown Honors Program and Syracuse University for all their help and support in making this possible.

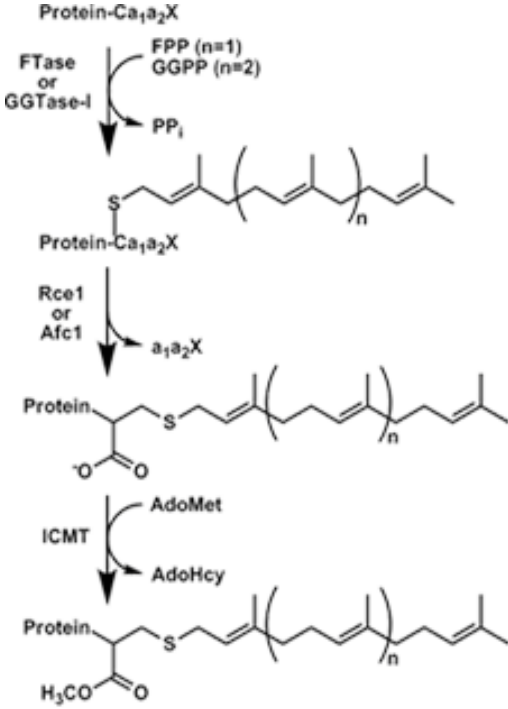
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## Introduction

Protein prenylation is an important posttranslational modification required for the proper localization and function of many eukaryotic proteins (6). Prenylated proteins are involved in vital cell signaling transduction pathways that control cell growth and differentiation, with these proteins linked to diseases including cancer and cardiovascular disease (10). Understanding how protein prenylation affects cellular health requires us to identify which proteins are prenylated and then characterize the impact of prenylation pathway modifications on protein structure and function.

Protein farnesyltransferase (FTase) is a heterodimeric enzyme that catalyzes the addition of a fifteen-carbon prenyl group to the cysteine of the “Ca<sub>1</sub>a<sub>2</sub>X” C-terminal sequence of a substrate protein, the first step in the prenylation pathway (Figure 1). This prenyl group increases the hydrophobicity of the protein and aids in localizing the peptide to cellular membranes (1) FTase readily prenylates a range of Ca<sub>1</sub>a<sub>2</sub>X sequences, but the interactions that lead to selectivity within the Ca<sub>1</sub>a<sub>2</sub>X sequence are still not well defined (2-5, 11). A 2009 study by Houglund and coworkers



**Fig 1.** Prenylation pathway

suggests that the hydrophobicity and steric volume of the  $a_2$  residue serve as determinants of peptide selectivity by

FTase (6).

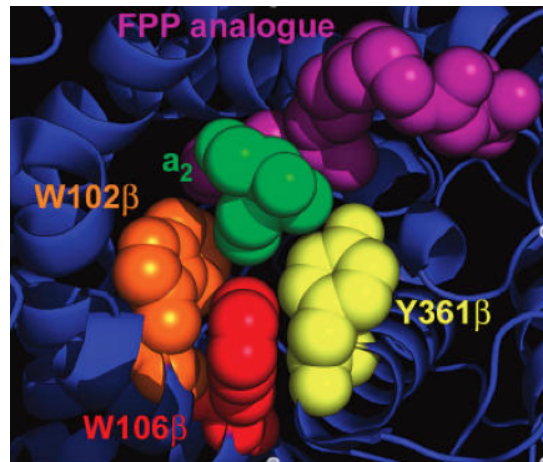
In addition to understanding what properties within the protein substrate are recognized by FTase, we must also identify the amino acids within the FTase active site that transduce these

interactions involved in substrate selectivity. Both structural and

biochemical studies indicate that the

W102 $\beta$  and W106 $\beta$  residues within the active site of FTase play the key roles in recognizing the  $a_2$  residue (Figure 2) (2, 7). Mutation of these two residues has also been demonstrated to alter FTase substrate selectivity, thereby revealing the “tunable” nature of substrate selectivity in FTase (7).

To explore more fully the potential for altering FTase substrate selectivity through mutation of two active site residues, we developed a library of ~400 FTase variants using a previously generated FTase plasmid library wherein the codons for the two active site amino acids, W102 $\beta$  and W106 $\beta$ , have been randomized. A subset of these variants were screened for prenylation activity with three target peptides, CVDS, CVYS, and CVGS, that bear  $Ca_1a_2X$  sequences that are markedly less reactive with FTase than “natural” substrates such as H-Ras (-GCVLS). Using a fluorescence-based assay that probes prenyltransferase activity



**Fig 2.** Interaction of  $a_2$  with the W102 $\beta$  (orange) and W106 $\beta$  (red) residues of wildtype FTase. The lipid tail of a FPP analogue (purple) is also shown here to contact the  $a_2$  residue. Figure from ref 6.

in bacterial cell lysates, we isolated two variant FTases from among the screened variants that exhibited prenylation activity with the CVYS target peptide. These two variants were then expressed, purified, and their enzymatic activity characterized under steady state conditions. Unexpectedly, we also observed and characterized the resistance of these two variants to the highly selective and potent FTase inhibitor, FPTII.

These studies provide a deeper understanding of the interactions that engender  $\alpha_2$  selectivity in FTase and promote the development of FTase variants that will serve as tools for studying prenylation pathway modifications within the cell. In addition, the characterization of FTase substrate recognition derived from my research will also provide insight for the development of better FTase inhibitors, an active area of drug research that is currently hindered in part because the specific protein targets of FTase are unknown (8, 12).



## MATERIALS AND METHODS

*Preparation of W102 $\beta$  and W106 $\beta$  FTase variant library.* A previously generated variant FTase plasmid library with mutations at W102 $\beta$  and W106 $\beta$  in the context of the pET23aPFT vector was utilized to generate the FTase variant libraries described herein (7). Codons for positions 102 $\beta$  and 106 $\beta$  have been randomized using an NNK codon (N = equal mixture of A, T, C, and G, K = G or T). To generate a library of FTase variants, 24 ng (3  $\mu$ L of 8 ng/ $\mu$ L) of the variant FTase plasmid DNA was transformed into each of four 100  $\mu$ L aliquots of Z-competent BL21(DE3) *E. coli* cells (73.4 ng/ $\mu$ L). A plasmid for wild-type FTase (21.4 ng) was also transformed into a 100  $\mu$ L aliquot of Z-competent BL21(DE3) *E. coli* cells. The cells were plated onto LB agar plates containing 100  $\mu$ g/mL ampicillin and grown overnight (~ 16 hours).

Following overnight incubation, 376 single colonies were inoculated into single wells of four 96-well (2.2 mL well volume) growth plates using sterile toothpicks. Each well contained 900  $\mu$ L of filter sterilized (0.22  $\mu$ m) growth media composed of LB media, 1% glucose, 100  $\mu$ g/mL ampicillin and 60  $\mu$ M isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). Each of the four 96-well plates contained a well inoculated with one colony transformed with wild-type FTase to serve as a positive control, a well inoculated with one colony transformed with an empty pET23a vector to serve as a negative control, and 94 wells inoculated with colonies transformed with variants. The four plates were labeled Cultures 1 – 4, sealed with gas-permeable adhesive seals, and incubated with shaking (400 RPM) for 16 hours at 28°C. Following incubation, cultures 2- 4 were sealed and stored

at -80°C. Glycerol stocks for variants in the Culture 1 plate were prepared by aliquoting 50 µL of culture from each well into another growth plate with 450 µL of LB with 100 µg/mL ampicillin in each well. Following incubation with shaking (400 RPM) for 16 hours at 28°C, the cultures in each well was mixed with 500 µL of 20% sterile glycerol. The resulting glycerol stock was stored at -80°C.

*Screening of FTase variants.* Cultures in each well of the Culture 1 plate were lysed using 100 µL of cell lysis reagent per well. The cell lysis reagent consists of 9.6 mL Fast Break cell lysis reagent (Promega), 4.8 µL benzonase (125 U/mL), 1 mL lysozyme (20 mg/mL in purified water) and 384 µL phenylmethanesulfonylfluoride (PMSF, 5 mg/mL in ethanol), with PMSF added last. Following addition of 100 µL of cell lysis reagent per well to the Culture 1 plate, the plate was shaken (380 rpm) for 20 minutes at 28°C and then stored on ice until used in screening assays.

A steady-state kinetics fluorescence assay was used to screen for FTase variants capable of farnesylating selected dansylated peptides. Farnesylation of dansylated peptides was monitored by fluorescence using a POLARstar Galaxy plate reader ( $\lambda_{\text{ex}}$  340 nm,  $\lambda_{\text{em}}$  520 nm) for 4 hours. Farnesylation of the dansylated peptides leads to an increase in fluorescence of the dansyl fluorophore, (9) providing a signal for peptide prenylation. Screening reactions for FTase variant activity with target peptides were as follows, with reactions run at room temperature: 3 µM dansylated peptide, 5 µL cell lysate, 10 µM farnesyl pyrophosphate (FPP), 50 mM HEPPSO pH 7.8, 5 mM tris(2-carboxyethyl)phosphine (TCEP), and 5 mM MgCl<sub>2</sub>.

Recent studies of *in vivo* protein prenylation in the Hougland Lab have identified a set of peptide sequences with point mutations at the a<sub>2</sub> residue that appear to block prenylation by wild type FTase (S. Flynn, manuscript in preparation). From among these sequences, CVDS, CVYS, and CVGS were selected as initial targets for FTase variant selection. In addition to these three target peptides, FTase variant activity reactions were run in parallel with the “natural” substrate CVLS to determine if the FTase variants retain activity with a positive control substrate.

For each screening reaction, a 96 well reaction plate was prepared for each peptide substrate as follows; a typical set of screening reactions involved four 96 well plates. A reaction stock solution was prepared, consisting of 11.9 mL dH<sub>2</sub>O, 2 mL of 10x reaction buffer (500 mM Heppso pH 7.8, 50 mM TCEP), 100 μL 5 mM MgCl<sub>2</sub>, and 4 mL 10 μM FPP. The stock solution (45 μL) was aliquoted into each well of the 96 well reaction plates. Cell lysate (5 μL) from each well of the Culture 1 growth plates was transferred into its corresponding well in each of the four reaction plates (lysate from well A01 was added to well A01 of reaction plates 1,2,3 and 4, lysate from well A02 was added to well A02 of reaction plates 1,2,3 and 4, etc.) Reactions were initiated by the addition of 50 μL of a 6 μM peptide stock into each well. Each reaction plate was initiated by a different peptide (reaction plate 1 with dansyl- GCVLS, reaction plate 2 with dansyl- GCVYS, etc.)

Fluorescence was measured at 12 time points (0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 90 min, 120 min, 150 min, 180 min and 240 min). The

results were combined to determine the time dependence of the fluorescence change for each variant with each peptide. Reactions whose variants who both exhibited a minimum fluorescence change that is at least 70% of the maximum change of wild-type FTase with dansyl-GCVLS (~2450 Au) and reached completion (a plateau in fluorescence) within 2-fold of the time required for wild-type FTase with dansyl-GCVLS (~100 min) were considered active. Two active variant FTases exhibiting prenylation activity with the CVYS target peptide (Wells A05 and F03) were selected from this screen, sequenced, and purified.

*Sequencing of Selected FTase Variants:* The BL21(DE3) *E. coli* cells containing the plasmids for the two selected variants were regrown from the glycerol stock. For each variant, a minimal amount of frozen culture was inoculated into a culture tube containing 5 mL 2xYT media supplemented with 2% glucose and 100 µg/mL ampicillin. The cells were incubated with shaking (275 rpm) for 16 hours at 37°C. Following overnight culture, the plasmid encoding the FTase variant was purified using the protocol for purification of low copy plasmid DNA from the EZ-10 Spin Column Plasmid DNA MiniPrep Kit (Bio Basic Inc). Plasmid concentrations were determined by UV-Vis spectroscopy using the Nanodrop 2000c UV-Vis spectrometer, and the mutations at positions 102 and 106 were determined by DNA sequencing (Genewiz).

*Purification of Selected FTase Variants.* For each FTase variant, 1 µL of plasmid (30.7 ng/µL for A05 and 92.7 ng/µL for F03) was transformed into a 100 µL aliquot of BL21-DE3 *E. coli* cells and plated on a LB agar plate containing 100 µg/mL ampicillin. Following overnight incubation, a single colony was

inoculated into 5 mL 2xYT growth media with 100 µg/mL ampicillin and incubated with shaking (225 rpm) at 37°C. After 5 hours incubation, the 5 mL starter culture was inoculated into a 3 L baffled flask containing 1 L of sterile autoinduction media (10 g tryptone, 5 g yeast extract, 872.5 mL of H<sub>2</sub>O (pH adjusted to 7.45 with KOH), 25 mL 1M Na<sub>2</sub>HPO<sub>4</sub>, 25 mL 1M KH<sub>2</sub>PO<sub>4</sub>, 50 mL 1M NH<sub>4</sub>Cl, 5 mL 1M Na<sub>2</sub>SO<sub>4</sub>, 2 mL 1M MgSO<sub>4</sub>, 200 µL trace metals) with 100 µg/mL ampicillin and incubated with shaking (200 rpm) for 24 hours at 28°C.

Following incubation, cells were harvested by centrifugation (6000 rpm, 15 min, 4°C) and the pellet was re-suspended in 50 mL resuspension buffer (50 mM HEPES pH 7.8, 10 µM ZnCl<sub>2</sub>, 2 mM TCEP, 17 µg/mL PMSF). Prior to lysis, benzonase (7.875 µL/63mL), lysozyme (0.2 mg/mL) and PMSF (17 µg/mL) were added to the cell suspension. After a 45 min. incubation period at 37°C, the cells were lysed by sonication (8 x 30 seconds at 30 Watts, with 30 second rests between cycles). Following sonication, cell debris was removed by centrifugation (18000 x g, 45 min, 4°C). The supernatant was treated with <sup>1</sup>/<sub>10</sub> volume of 10% streptomycin sulfate added drop wise in the cold room followed by stirring for 20 min at 4°C. The resulting solution was clarified by centrifugation (18000 x g, 45 min, 4°C), followed by filtration through a 0.45 µM syringe filter.

The filtered supernatant was loaded onto a HiTrap DEAE anion-exchange column (3 x 5mL prepacked columns) washed with HTZ buffer (50 mL 1 M Hepes pH 7.8, 4 mL 2.5 mM ZnCl<sub>2</sub>, 946 mL H<sub>2</sub>O and 0.6 g TCEP) and FTase was eluted by a NaCl gradient (HTZ + 0.5 M NaCl buffer) per preprogrammed gradient (AktaPrime, GE Healthsciences). Fractions containing FTase were

determined by SDS-PAGE analysis, collected, concentrated with 30K concentrators (PALL MacroSep Advance Centrifugal Devices), and diluted to 10 mL with HZ buffer (HTZ buffer without TCEP). The partially purified FTase was likewise purified using a HiTrapQ anion-exchange column (3 x 5mL prepacked columns) using a NaCl gradient, concentrated to  $\sim 10 \mu\text{M}$  as determined by UV absorbance at 280 nm (extinction coefficient  $1.5 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ ) and stored in 25  $\mu\text{L}$  aliquots at  $-80^\circ\text{C}$ .

*Active Site Titration of purified FTase Variants:* The concentration of active enzyme was determined by active site titration of the purified variants. . Increasing concentrations (between 0 nM to 1000 nM) of the dansyl- CVLS and dansyl - CVYS peptides were titrated into 100  $\mu\text{L}$  solutions containing 300 nM enzyme, 5  $\mu\text{M}$  FPTII inhibitor, 1x FTase reaction buffer (50 mM Heppso pH 7.8, 5 mM TCEP) and 5 mM  $\text{MgCl}_2$ . After the addition of peptide, the sample was incubated in the dark for 10 minutes to allow peptide binding to reach completion before a fluorescence ( $\lambda_{\text{ex}}$  280 nm,  $\lambda_{\text{em}}$  496 nm) reading was obtained. Reactions lacking enzyme were run in parallel as negative controls. The concentration of enzyme active sites was calculated from the collected data by finding the intersection of the linear slope lines for pre-saturated and saturated conditions of the FTase-FPTII complex by dansylated peptide.

*Steady-State Kinetics of Variant Enzymes.* To measure the effects of the mutations on the catalytic efficiency ( $k_{\text{cat}}/K_{\text{M}}$ ) of FTase, the velocity for peptide farnesylation at multiple peptide concentrations was determined using the previously reported fluorescence-based assay (9). Increasing concentrations of

dansyl- CVLS and dansyl - CVYS peptide (0.2  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$ , 2  $\mu\text{M}$ , 5  $\mu\text{M}$  and 10  $\mu\text{M}$ ) were titrated into 100  $\mu\text{L}$  solutions containing 20 nM enzyme, 10  $\mu\text{M}$  FPP, 1x FTase reaction buffer and 5 mM  $\text{MgCl}_2$ . Immediately after reaction initiation by the addition of the enzyme stock, fluorescence was measured as a function of time for 4 hours.  $K_M$  was determined from Lineweaver-Burk plots; a linear fit to the dependence of the inverse substrate concentration on inverse initial velocity (eq 1). Initial velocity ( $V$ ,  $\mu\text{M/s}$ ) was determined by dividing the linear velocity of the reaction in fluorescence units per second by an amplitude conversion derived from an average of the total fluorescence change observed upon reaction completion divided by the initial peptide substrate concentration.  $k_{\text{cat}}$  was derived from equation 2, where  $[\text{E}_0]$  = enzyme concentration.

$$1/V = (K_M/V_{\text{max}})(1/[s]) + 1/V_{\text{max}} \quad (1)$$

$$V_{\text{max}} = k_{\text{cat}}[\text{E}_0] \quad (2)$$

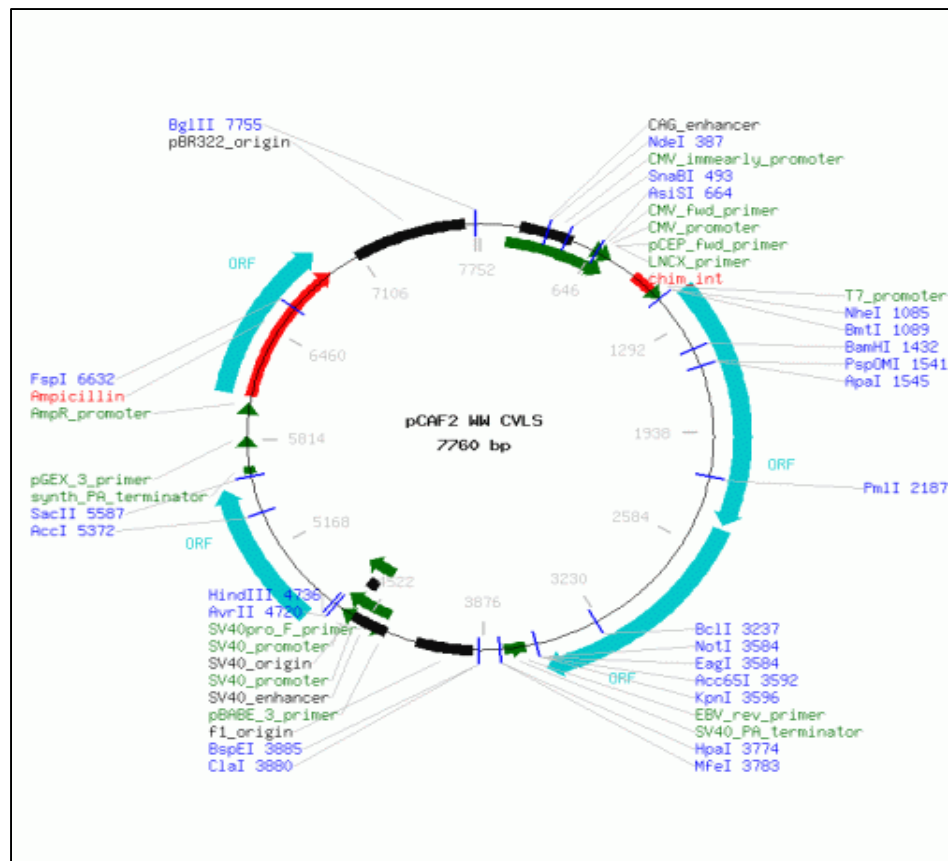
*Testing of FPTII Inhibitor Resistance.* To determine the effects of the mutations on the affinity of FPTII inhibitor with FTase, increasing concentrations of FPTII inhibitor (0  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , 1.0  $\mu\text{M}$  and 10  $\mu\text{M}$ ) were titrated into solutions with 20 nM enzyme, 2  $\mu\text{M}$  peptide, 1x FTase reaction buffer, 5 mM  $\text{MgCl}_2$  and 10  $\mu\text{M}$  FPP. The velocity for peptide farnesylation in the presence of FPTII concentrations was determined using the previously reported fluorescence-based assay (9). The dissociation constant  $K_i$  of FPTII was determined from a fit of the Michaelis-Menten equation adjusted for inhibition (eq 3) to the dependence of initial velocity on FPTII concentration.

$$V = V_0 * \left(1 - \frac{[I]}{K_i + [I]}\right) \quad (3)$$

$K_M$  values for FPP with the selected variants were also measured using the fluorescence-based assay. Increasing concentrations of FPP (0.1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$ , 2  $\mu\text{M}$ , 5  $\mu\text{M}$  and 10  $\mu\text{M}$ ) were titrated into solutions with 15 nM enzyme, 2  $\mu\text{M}$  dansyl-CVLS, 1x FTase reaction buffer and 5 mM  $\text{MgCl}_2$ . Fluorescence ( $\lambda_{\text{ex}}$  340 nm,  $\lambda_{\text{em}}$  520 nm) was monitored for four hours, and initial velocities were measured as described above.  $K_M$  of FPP was determined from a fit of the Michaelis-Menten equation (eq 4) to the dependence of initial velocity on the FPP concentration.

$$V = V_{\text{max}} \left( \frac{[\text{FPP}]}{K_M + [\text{FPP}]} \right) \quad (4)$$





**Fig. 3:** pCAF2 WW CVLS Vector

*Mutagenesis of pCAF2 vectors.* The shuttle vector pCAF was previously modified to allow for co-expression of FTase and a florescent fusion protein (7). In the vector pCAF2 WW CVLS (Figure 3), wild-type FTase was cloned into open reading frame 1 (ORF1) and TagRFP-GCVLS into open reading frame 2 (ORF2). ORF1 is under the control of the CMV promoter while ORF2 is under the control of the SV40 promoter. FTase variants were first introduced into the pCAF2 WW CVLS plasmid by PCR site-directed mutagenesis. Mutations were confirmed by DNA sequencing (GENEWIZ) using the CMV forward primer. The TagRFP-CVYS mutation was then introduced into the pCAF variant CVLS template and confirmed by sequencing using the SV40 reverse primer.

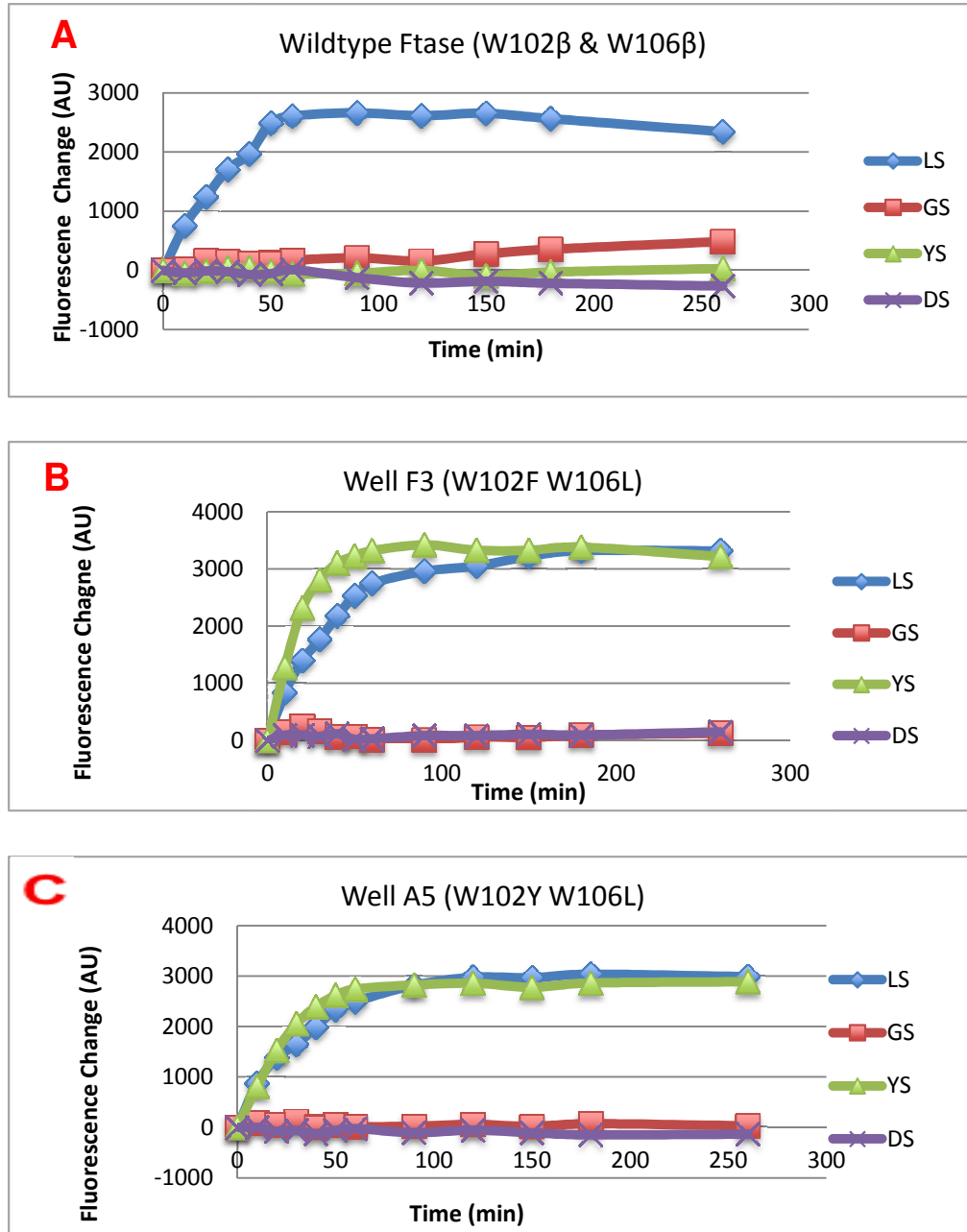
*Cell culture, transfection, and imaging.* HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 100 units/mL penicillin-streptomycin

and L-glutamine (2mM) and 10% fetal bovine serum (Complete Media). Cells were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37<sup>0</sup>C. HEK 293T cells (4 x 10<sup>4</sup> cells) were plated in a 24 -well tissue culture treated plate (2.0 cm<sup>2</sup> well size) and incubated for 24 and 48 hours. Cells were then transfected with 3μg plasmid DNA using TurboFect (Fermenta) according to manufacturer's protocols. Following transfection for 48 hours, the cells were imaged using a Zeiss Axio Vert A1 fluorescence microscope, with a minimum of 50 cells were characterized and reported.

## **RESULTS**

*Selection of W102β and W106β mutants with increased reactivity with dansyl – GCVYS.* In the screening of novel FTase variants, 376 *E. coli* colonies derived from the FTase plasmid library with double mutations at W102β and W106β were selected to be assayed for farnesylation activity with peptide targets. Of the 94 variants screened, 31% (29/94 variants) exhibited high prenylation activity with dansyl–GCVLS while 43% (40/94 variants) exhibited high prenylation activity with dansyl –GCVYS. A variants is defined as exhibiting “high prenylation activity” if the prenylation reaction with the target peptide satisfies two criteria: 1) reaction completion within 2-fold of the time required for wildtype - FTase to reach completion with its native substrate dansyl-GCVLS (~100min); and 2) a minimum fluorescence change at least 70% of the maximum fluorescence change observed for wildtype-FTase with dansyl-GCVLS (~2450 Au). The large pool of variants that were able to prenylate the non-native substrate CVYS suggests that substrate selectivity in FTase is easily altered and depends fundamentally on a

small number of “tunable” active site contacts, consistent with previous studies (7).



**Fig 4.** Prenylation activity as observed by fluorescence (AU) with dansyl-labeled peptides -GCVLS, -GCVYS, -GCVDS, and -GCVGS with (A) wildtype FTase, (B) W102Y W106L, and (C) W102F W106L

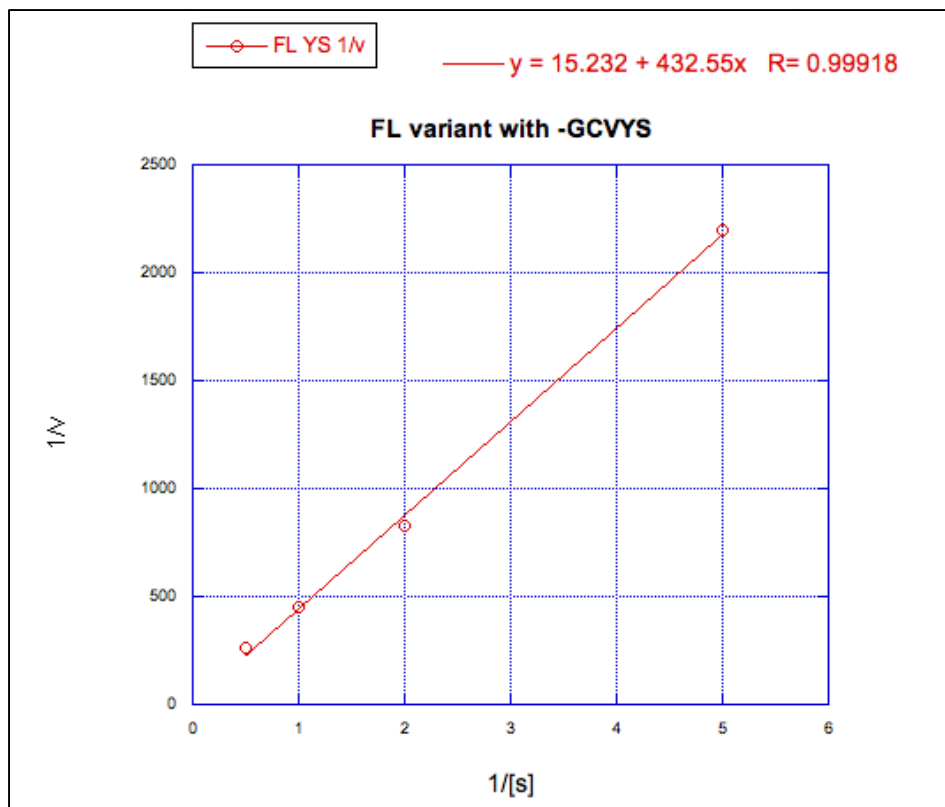
*Selected FTase variants* Two variant FTases that exhibited high prenylation activity with the CVYS target peptide as well as the native CVLS peptide (Figure 4) were isolated; these variants were sequenced and the mutations at W102 $\beta$  and W106 $\beta$  are reported in Table 1. The  $k_{cat}/K_M$  values for these variants for reaction with both the dansyl- CVLS and dansyl-CVYS peptides were calculated from Lineweaver-Burk plots (Figure 5 and Table 2). Both the W102F W102L and W102Y W106L mutants exhibit a ~100-fold increase in dansyl-GCVYS activity when compared with wildtype-FTase. The mutants also simultaneously maintain approximately the same level of activity (within 2-fold) with dansyl-GCVLS as wildtype-FTase. These findings are consistent with our earlier observations that the FTase active site is flexible and that substrate selectivity can be easily modulated.

**Table 1.** Residues at position 102 $\beta$  and position 106 $\beta$  of wildtype and selected variant FTases.

Well	Residue at position 102	Residue at position 106
WW FTase	W	W
A5	Y	L
F3	F	L

**Table 2.** The  $K_{cat}/K_M$  values of wildtype FTase and the two variants (YL, FL) with the peptides -GCCVLS and -GCVYS.

Enzyme	$K_{cat}/K_M$ ( $M^{-1}s^{-1}$ ) LS	$K_{cat}/K_M$ ( $M^{-1}s^{-1}$ ) YS
WW FTase	$1.7 \times 10^5 \pm 40$	$1.21 \times 10^3 \pm 200$
YL Variant	$9.8 \times 10^4$	$1.2 \times 10^5$
FL Variant	$9.6 \times 10^4$	$1.3 \times 10^5$



**Fig 5.** A Lineweaver-Burk plot of the FL variant with dansyl-GCVYS. The plot fits a linear model corresponding to the inverse of the Michaelis-Menten equation from which the  $K_{cat}/K_M$  value can be calculated. This reaction was run with 20 nM enzyme.

*Variant Resistance to FPTII Inhibitor.* During the active site titrations of the purified WT FTase and FTase variants, wildtype-FTase reaches a clear saturation point indicative of peptide binding saturation while the FL and YL FTase variants do not. This lack of saturation suggests that the FL and YL active site mutations interfere with formation of the initial FTase\*FPTII complex or the formation of the FTase\*FPTII\*peptide complex. To determine if FPTII inhibitor binding is affected in the YL and FL FTase variants, the inhibition constant ( $K_i$ ) for the FPTII inhibitor was measured with each variant with either dansyl-GCVLS or dansyl-GCVYS peptide substrates. Results are shown in Table 3. The increased

$K_i$  values of FPTII with the variants and the dansyl-CVLS substrate as compared to wildtype-FTase suggest a decreased affinity of FPTII inhibitor for FTase, which is consistent with our results. The  $K_i$  of FPTII of the FL variant with CVYS is considerably lower than the  $K_i$  of FPTII of the FL variant with CVLS. This suggests that inhibitor resistance maybe substrate dependent.

**Table 3.**  $K_i$  values of FPTII inhibitor with wildtype FTase, FL FTase and YL FTase with the peptides dansyl-CVLS and dansyl-CVYS

Enzyme	$K_i$ (uM) LS	$K_i$ (uM) YS
WW FTase	$0.3 \pm 0.2$	NA
YL Variant	$5.1 \pm 5.3$	NA
FL Variant	$13.5 \pm 15.6$	$0.23 \pm 0.042$

The increased resistance to the competitive inhibitor FPTII in the presence of a saturating amount of FPP suggests either a decrease in the binding affinity of FPTII and/or an increase in the binding affinity of FPP. The values of  $K_M$  of FPP with the variants, calculated from FPP titrations, are reported in Table 4. The decreased  $K_M$  of FPP with the variants as compared to wildtype-FTase is consistent with the variants exhibiting tighter FPP binding, which could lead to less effective competitive inhibitor by FPTII.

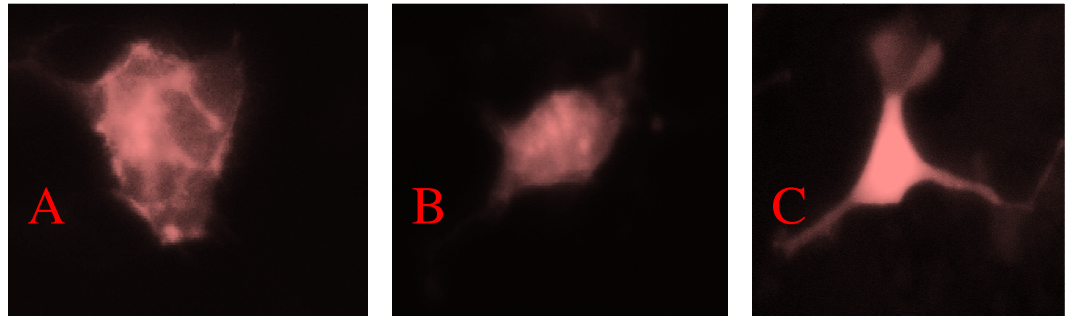
**Table 4.** The  $K_M$  values of FPP with wildtype FTase, W102F W106L FTase and W102Y W106L FTase with dansyl-CVLS

Enzyme	$K_M$ (M) LS
WW FTase	$7.56 \pm 3.0$
YL Variant	$0.92 \pm 0.37$
FL Variant	$1.0 \pm 0.17$

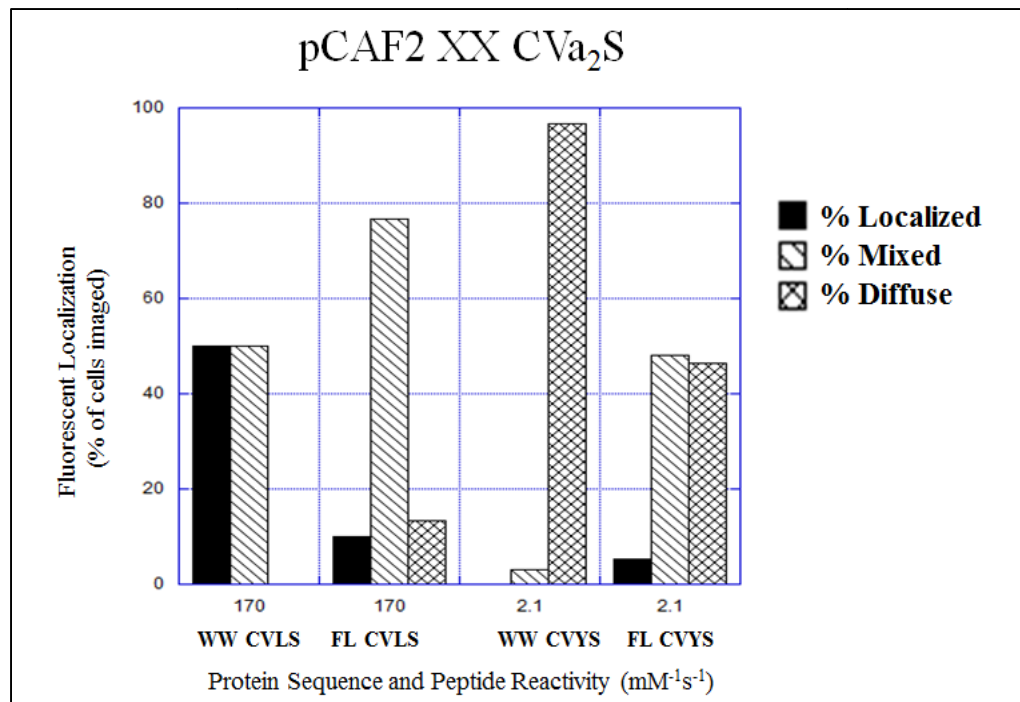
*Reactivity of Selected Variants in vivo.* To examine farnesylation activity of the W102F W106L and W102Y W106L variants *in vivo*, pCAF2 vectors containing the mutants were transfected into HEK293T cells. The pCAF2 vectors allowed for co-expression of a variant FTase along with a fusion protein tagged with a red fluorescent protein (TagRFP) at its N-terminal. The fusion protein comprises of the upstream sequence H-Ras (-KLNPPDESGPGCMSC-) and either -GCVLS or GCVYS at its C-terminal. Farnesylation activity was monitored by the localization of the fusion protein as determined from the distribution of fluorescence.

The cells were characterized as “Localized” where the reporter protein is located mainly to the plasma membrane, “Mixed” where the reporter protein located at cellular membranes and throughout the cytosol and “Diffuse” where the reporter protein is located throughout the cytosol (Figure 6). In the vector with wildtype FTase and TagRFP-CVLS, 50% of the cells exhibit localized fluorescence and 50% display mixed fluorescence. Changing the C-terminal sequence of the fluorescent protein to CVYS in the presence of wild-type FTase (WW CVYS) leads to a complete loss of “localized” fluorescence, with 97% of

cells exhibiting diffuse fluorescence and 3% mixed fluorescence. Introduction of the FL FTase variant in the presence of TagRFP-CVYS increased the number of cells displaying mixed fluorescence, but did not lead to localized fluorescence (Figure 7). These results indicate that the FL FTase variant is active within the cell.



**Fig 6.** Examples of TagRFP fluorescence localization in HEK293T cells: A. Diffused B. Mixed C. Localized



**Fig 7.** Fluorescent Localization of pCAF2 XX CV<sub>a</sub>2S in HEK293T cells.



## DISCUSSION

*The W102 $\beta$  and W106 $\beta$  residues of FTase play the key roles in recognizing the a<sub>2</sub> residue, one of the determinants of peptide selectivity by FTase.* The two developed FTase variants, W102F W102L and W102Y W106L, exhibit farnesylation activity with the CVLS and CVYS peptide. The chosen target peptide, CVYS, differs from the native substrate CVLS in that the amino acid at its a<sub>2</sub> position is larger while remaining hydrophobic. The two FTase variants selected in this work for their increased reactivity with the CVYS peptide both contain a tryptophan to leucine mutation at position 106 $\beta$ , which maintains hydrophobic character at this position while reducing amino acid size. Mutations observed at position 102 $\beta$  keep an aromatic amino acid at this position while also reducing amino acid size. These mutations suggest that increasing reactivity with the CVYS peptide requires relaxation of steric discrimination within the FTase active site pocket while preserving hydrophobic character in the a<sub>2</sub> binding pocket. The change in substrate selectivity observed in this work demonstrates the flexibility of the FTase active site and suggests that FTase selectivity is easily tunable with directed mutations at the W102 $\beta$  and W106 $\beta$  residues.

Notably, the FTase variants are capable of accelerating reactions with the –GCVYS peptide ~100 fold without losing reactivity with –GCVLS. This conservation of activity with GCVLS with W102 $\beta$  and W106 $\beta$  mutants has been observed in previous studies (7). The reactivity of the W102F W102L and W102Y W106L variants with CVLS reconfirm that the W102 $\beta$  and W106 $\beta$  residues are not vital in selecting for the natural substrate for prenylation. Instead,

the residues at the 102 $\beta$  and 106 $\beta$  positions select against substrates on the basis of size and polarity (7).

*Bioengineered prenylation pathway.* By mutating two residues within FTase, FTase variants with expanded substrate selectivity were developed. Studies of the enzymes involved in latter steps of the prenylation pathway have been restricted by the limited substrates of FTase. The novel variants identified in this study that can prenylate substrates with the CVYS as well as the CVLS motif lessens this constraint and can serve as tools for future prenylation studies.

In addition, studies of any single prenylation pathway within a cell have been limited because isolation of a single pathway was not possible; inhibitor treatment of FTase or any succeeding enzyme knockouts every farnesylation pathway within the cell. The unexpected finding that the novel variants are resistant to the inhibitor FPTII makes them all the more powerful as tools as they potentially can be implemented in a single specific prenylation pathway in *in-vivo* studies without the background of other prenylation pathways.

In conclusion, the W102 $\beta$  and W106 $\beta$  residues of FTase are easily tunable for controlled substrate selectivity. Variant FTases/fusion protein pairs are also powerful tools for studying prenylation modifications downstream of FTase farnesylation. This will ultimately lead to a better understanding of the prenylation pathway inhibitors effective against prenylate Ras-protein diseases such as cancer and cardiovascular disease.

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## Capstone Summary

Posttranslational modification, a critical step in protein biosynthesis, plays an essential role in regulating protein structure and function. Within a cell, posttranslational modifications are carried out by enzymes, catalytic biological molecules that accelerate the rate of chemical reactions. Protein prenylation is an important posttranslational modification required for the proper localization and function of many eukaryotic proteins. In the first step of the prenylation pathway, the enzyme farnesyltransferase (FTase) catalyzes the addition of a fifteen-carbon prenyl group to the cysteine of the “Ca<sub>1</sub>a<sub>2</sub>X” C-terminal sequence of a substrate protein. However, the interactions that lead to selectivity within the Ca<sub>1</sub>a<sub>2</sub>X sequence are still not well defined and FTase is proposed to modify a pool of substrates numbering more than one hundred.

Prenylated proteins are involved in vital cell signaling transduction pathways that control cell growth and differentiation, with these proteins linked to diseases including cancer and cardiovascular disease. Understanding how protein prenylation affects cellular health requires us to identify which proteins are prenylated and then characterize the impact of prenylation pathways modifications on protein structure and function. We seek to understand the interactions in the FTase active site that engender substrate selectivity.

In this study, we developed a library of ~400 FTase variants using a previously generated FTase plasmid library wherein the codons for the two active site amino acids, W102 $\beta$  and W106 $\beta$ , have been randomized. Codons, specific sequences of three nucleotides, determine the amino acid that is translated into the

peptide; thus, randomized codons at position W102 $\beta$  and W106 $\beta$  will result in random amino acids at those positions. 94 of the variants were screened for prenylation activity with three target peptides that bear Ca<sub>1</sub>a<sub>2</sub>X sequences that are less reactive than native FTase substrates such as Ras (-CVLS) in parallel to the natural substrate CVLS serving as a positive control. The peptide substrates were labeled with a dansyl group to allow monitoring of peptide prenylation activity by changes in dansyl fluorescence.

Two variant FTases that exhibited prenylation activity with the CVYS target peptide were isolated from this screen. The mutated vectors were sequenced and subsequently isolated and transformed into a strain of *E. coli* cells that is able to express the FTase protein. The protein was purified by ion exchange Fast Protein Liquid Chromatography (FPLC), a technique used to separate proteins by charge.

Following purification, we characterized their reactivity with the associated peptide targets to investigate how the mutations we observe lead to changes in substrate selectivity. This included measuring peptide substrate binding affinities, reaction velocities in the presence of both saturating and subsaturating peptide concentrations, and the maximum rate for substrate turnover by the selected FTase variants. We also tested the variants' resistance to the highly selective and potent inhibitor of wildtype FTase, FPTII.

In addition, to determine if the selected FTase variants exhibit biologically relevant levels of prenylation activity, we assessed the ability of these variants to prenylate a target fluorescent protein within a mammalian cell. Prenylation of the

target protein leads to protein localization to cellular membranes, which can be monitored by fluorescence microscopy. By co-expressing the given FTase variant with a fluorescent protein terminating the peptide sequence used to select the FTase variant, we determine if each variant is active enough to prenylate the target protein leading to a change from diffused to localized fluorescence.

These studies will ultimately lead to a better understanding of the selectivities and substrates for wildtype FTase. By mutating two residues within FTase, we have developed FTase variants with expanded substrate selectivity. The alteration in substrate selectivity observed in our variants suggests that FTase selectivity may depend on a small number of “tunable” active site contacts. Our work provides insight into how this multispecific enzyme recognizes its pool of substrates and will also aid in identifying additional FTase substrates. Studies of the enzymes involved in latter steps of the prenylation pathway have been restricted by the limited substrates of FTase. The novel variants identified in this study that can prenylated substrates with the CVYS as well as the CVLS motif lessens this constraint and can serve as tools for future prenylation studies. The unexpected finding that the novel variants are also resistant to the inhibitor FPTII makes them all the more powerful as tools as they can be implemented in a single specific prenylation pathway in *in vivo* studies without the background of other prenylation pathways.

In addition, the characterization of FTase substrate recognition derived from my research will provide insight for the development of better FTase inhibitors, an active area of drug research that is currently hindered because the

specific protein targets of FTase are unknown. FTase inhibitors have been designed and have been proven effective in the treatment of prenylated Ras-protein-related diseases such as various forms of cancer by suppressing angiogenesis and inducing apoptosis.