Efficient farnesylation of an extended C-terminal C(x)_3X sequence motif expands the scope of the prenylated proteome

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Protein prenylation is a post-translational modification that has been most commonly associated with enabling protein trafficking to and interaction with cellular membranes. In this process, an isoprenoid group is attached to a cysteine near the C-terminus of a substrate protein by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase type I or II (GGTase-I and GGTase-II). FTase and GGTase-I have long been proposed to specifically recognize a four amino acid CaaX C-terminal sequence within their substrates. Surprisingly, genetic screening reveals that yeast FTase can modify sequences longer than the canonical CaaX sequence, specifically C(x)3X sequences with four amino acids downstream of the cysteine. Biochemical and cell-based studies using both peptide and protein substrates reveal that mammalian FTase orthologs can also prenylate C(x)3X sequences. As the search to identify physiologically relevant C(x)3X proteins begins, this new prenylation motif nearly doubles the number of proteins within the yeast and human proteomes that can be explored as potential FTase substrates. This work expands our understanding of prenylation’s impact within the proteome, establishes the biological relevant reactivity possible with this new motif, and opens new frontiers in determining the impact of non-canonical prenylated proteins on cell function.

Post-translational modification of proteins is essential for biological functions such as cell signaling and regulation of enzyme activity. (1) Among the extensive and growing catalog of known post-translational modifications, protein lipidation (e.g. prenylation, palmitoylation, myristoylation) can influence protein trafficking to and interaction with cellular membranes. (1-9) Prenylation involves the covalent attachment of a hydrophobic isoprenoid group, either a 15-carbon farnesyl or 20-carbon geranylgeranyl group, to the side chain thiol of a cysteine near the C-terminus of certain proteins. (10-12) The sequence context of this cysteine residue determines the enzyme responsible for its modification, with a cysteine within a C-terminal CaaX sequence recognized by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase type I (GGTase-I). (11,13-17) Alternatively, a cysteine present in a C-terminal CC or CxX motif can be modified by protein geranylgeranyltransferase type II (GGTase-II), also known as Rab GGTase. (18-20) Cysteine prenylation alters the biophysical properties of proteins in several well-studied cases (e.g. Ras and Ras-related GTPases), resulting in protein association with cell membranes where these lipidated proteins are involved in cell signaling pathways. (21-25)

The prenylation pathway involving FTase or GGTase-I consists of number of modification steps that can precede substrate protein localization to cellular membranes (Figure 1). In the obligate first step of this pathway, FTase or GGTase-I catalyzes cysteine alkylation with a farnesyl or geranylgeranyl group using farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP), respectively. Following modification by FTase or GGTase-I, prenylated proteins bearing CaaX motifs are often subject to additional modifications involving the CaaX sequence amino acids. (26-29) 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 moiety is then methylated by the SAM-dependent enzyme isoprenylcysteine carboxyl methyltransferases (ICMT). All three modification steps are necessary for many prenylated proteins to function optimally, although there can be variation in the extent of, and dependence on, these subsequent modifications. (30,31) Recently, a shunt pathway for prenylated proteins in yeast has been reported in which prenylation is the endpoint of CaaX protein modification. In this case, post-prenylation processing is not required and is actually deleterious to protein function. (32)

The CaaX motif that has served as the de facto consensus motif for prenylation by FTase and GGTase-I was first described over three decades ago, when yeast mating factors, Ras GTPases, and nuclear lamins were found to be lipid modified. (33-38) 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 for recognition by FTase and/or GGTase-I. Biochemical and cell-based studies, including single amino acid mutations at each position of the
CaaX sequence and reengineering of FTase and GGTase-I substrate selectivity, have contributed to the development of rules for prenyltransferase selectivity at each amino acid position within the CaaX sequence. Crystallographic studies provide a valuable structural context for consideration of FTase and GGTase-I substrate selectivity. In addition to implicating certain FTase and GGTase-I residues as interacting with the CaaX substrate sequence side chains, the structural models of FTase and GGTase-I revealed contacts to the cysteine and terminal carboxylate group of the CaaX sequence that explain the observed preference for the four amino acid CaaX substrate sequence (Figure 1). Proteomic 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.

In the expansion of our understanding of FTase and GGTase-I substrate recognition, the creation of a novel thermotolerance screen for protein prenylation in yeast has augmented our ability to leverage yeast genetic methodology to identify prenyltransferase substrate sequences. This screen is based on the yeast protein Ydj1p, a cytosolic type I Hsp40 chaperone that is required for high-temperature growth. Ydj1p is a shunted CaaX protein, being farnesylated but not proteolyzed and methylated. This crucial feature has allowed direct investigation of protein prenylation without the requirement for concurrent sequence compatibility with Rce1p or Ste24p for subsequent proteolysis.

In this work, we describe the discovery of a five amino acid C(X)X sequence motif recognized by both yeast and mammalian FTase orthologs. Multiple C(X)X 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)X sequences derived from human proteins are efficiently prenylated by rat FTase, and that a reporter protein terminating in a C(X)X sequence exhibits sufficient reactivity to be farnesylated under biologically relevant conditions within a human cell.

**Results**

**Yeast genetic screens suggest C(X)X sequences can be prenylated by yeast FTase**

While the four amino acid CaaX sequence has been widely accepted as the model for FTase and GGTase-I substrate selectivity, our investigation into the prenylation of C(X)X sequences was prompted in part by the observation that the CGGDD sequence of human annexin A2 and annexin A3 was identified as potentially prenylated in studies using in vivo farnesylation probes, although this reactivity was not supported by in vitro fluorescence-based peptide reactivity studies. 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 highly sensitive genetic reporter to identify whether C(X)X sequences could serve as prenylation motifs. Farnesylated a-factor is a secreted, diffusible signaling molecule produced by MATa haploid yeast that temporarily triggers cell cycle G1 arrest in nearby MATa haploid yeast so that mating can occur; this arrest is enhanced in certain mutant backgrounds (e.g. MATa sst2-1). We took advantage of the a-factor induced growth arrest phenotype to screen a plasmid-based library of a-factor mutants with C-terminal C(X)X sequences. Through standard colony replica techniques, a plate containing a population of MATa colonies expressing mutants was printed onto a thin lawn of MATa sst2-1 yeast, the printed plate incubated for a period of time, and individual colonies surrounded by a zone of MATa growth inhibition (i.e. halo) were scored as positive hits; associated plasmids were recovered and sequenced. We estimate 22.5% of the possible C(X)X combinations were evaluated (i.e. ~40,000 colonies; see Experimental Procedures for description of coverage estimate). With this limited coverage of the potential sequence space, the a-factor screen yielded two C(X)X hits (Table 1). The two C(X)X sequences were retested with other a-factor mutant sequences using the halo assay (Figure 2a). The halos associated with the C(X)X sequences were qualitatively smaller than 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. Of note, the halo associated with a-factor “CASQ”, which has a Rce1p / Ste24p CaaX protease cleavage resistant motif that severely compromises a-factor production (~1-2% relative to CVIA), still produced a halo similar to wild type. (32) This is due to the halo assay acting as an extremely sensitive and qualitative method for measuring a-factor production. To better assess the amounts of a-factor produced by mutants, we used a quantitative mating assay. We determined the C(x)X sequences promoted far fewer mating events relative to that observed for wild type and cleavage resistant a-factor (Figure 2a, numerical values below each panel). The reduced production of mature a-factor with the C(x)X hit sequences and lack of production with CGGDD could reflect deficiencies in either prenylation and/or proteolysis as both modification steps are required to yield fully active a-factor. To complement and expand upon the a-factor screen, we used the Ydj1p Hsp40 chaperone as an independent genetic reporter to select for C(x)X motifs compatible with farnesylation by yeast FTase. For proper function and support of yeast growth at elevated temperatures, Ydj1p requires cysteine farnesylation but does not require subsequent proteolytic and carboxylmethylation modifications typically associated with prenylated CaaX proteins. The Ydj1p-based approach provided several advantages over the a-factor screen, including higher throughput based on a temperature sensitive selection and elimination of the requirement for downstream proteolysis and carboxylmethylation following prenylation. (32,50) Through this approach, effects on thermotolerance can be directly related to changes in sequence modification by FTase and not impact on downstream processing steps. A library of Ydj1p mutants with C(x)X sequences was expressed in yeast, and individual colonies surviving growth at high temperature were identified. Based on codon redundancy and the number of colonies examined (~125,000), we estimate 48% of possible C(x)X sequences were evaluated. (58) The screen yielded 15 C(x)X sequence hits (Table 1). The Ydj1p C(x)X hits support growth at high temperature similar to wild type Ydj1p (CASQ C-terminal sequence). They are more effective than Ydj1p appended with the CVIA sequence that is cleaved by Rce1p and Ste24p, the CMIIM and CVLMM sequences that were derived from a-factor-based screening, and the annexin A2 CGGDD sequence identified by proteomics studies (Figure 2B). The thermostolerant Ydj1p C(x)X mutants were subsequently analyzed for evidence of prenylation by a mobility shift assay. In this assay, farnesylated Ydj1p has increased SDS-PAGE mobility relative to the unprenylated protein, which can be produced by expression in a FTase-deficient yeast strain (i.e. ram1). Using this assay, all C(x)X hits identified by Ydj1p-based screening exhibited prenylation, albeit only partially in most cases (Figure 2c). Of note, the CMIIM sequence appeared to be completely modified in the context of Ydj1p. By contrast, the CGGDD sequence did not appear to be modified.

Mammalian protein prenyltransferase activity with C(x)X peptide substrates

While these yeast experiments support the notion that prenylation of proteins terminating in C(x)X sequences occurs within yeast cells, these studies do not conclusively establish that the C(x)X motif can serve as a substrate for FTase or GGTase-I within yeast. For example, the identified C(x)X sequences could be processed by a carboxypeptidase to yield a shorter canonical CaaX sequence. This type of proteolytic “trimming” prior to modification by a prenyltransferase would present a new step within the established prenylation pathway, but would not reflect an expanded sequence length tolerance for the protein prenyltransferases. To address this ambiguity, we investigated the prenylation of C(x)X sequences in the context of synthetic peptides using purified mammalian FTase and GGTase-I in cell-free prenylation assays. These assays eliminate the possibility of peptide substrate processing and examine whether the reactivity with C(x)X sequences is a general feature of these enzymes or is exclusive to the yeast orthologs.

For these assays, fifteen C(x)X sequences identified by yeast screening were synthesized as 6-mer peptides with each C(x)X sequence preceded by a glycine residue with a pendant N-terminal dansyl fluorophore [dns-GC(x)X] (Table 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
peptide prenylation. Using this assay, incubation with rat FTase and FPP resulted in significant fluorescence enhancement (>5-fold, relative to negative “-FPP” control) with 5 of the 15 sequences (Figure 3a and Table 1). Among these sequences, two peptides (dns-GCMIIM and dns-GCAVGP) exhibited a plateau in fluorescence consistent with complete farnesylation within 4 hours. (Figure 3b). In parallel reactions with rat GGTase-I and GGPP, none of the dns-GC(x)3X peptides exhibited an increase in fluorescence suggesting no geranylgeranlylation activity. Notably, the annexin CGGDD sequences was also evaluated and observed to be unreactive under all conditions tested.

To confirm that the observed increase in fluorescence with dns-GC(x)3X peptides reflects peptide prenylation, samples generated using peptide incubation with FTase/FPP and GGTase-I/GGPP were analyzed by reverse-phase HPLC (RP-HPLC). In reactions without the C(x)X motif, there was no detectable fluorescence product observed, whereas reactions with the C(x)X motif exhibited significant fluorescence enhancement (>5-fold relative to negative “-FPP” control) with 5 of the 15 sequences (Figure 3a and Table 1). Among these sequences, two peptides (dns-GCMIIM and dns-GCAVGP) exhibited fluorescence consistent with complete farnesylation within 4 hours. (Figure 3b). In parallel reactions with rat GGTase-I and GGPP, none of the dns-GC(x)3X peptides exhibited an increase in fluorescence suggesting no geranylgeranlylation activity. Notably, the annexin CGGDD sequences was also evaluated and observed to be unreactive under all conditions tested.

Quantitative analysis of dns-GC(x)3X peptide reactivity with FTase and susceptibility to FTase inhibitor treatment

Steady-state kinetic analysis of the two peptides exhibiting the largest fluorescence enhancement upon prenylation, dns-GCMIIM and dns-GCAVGP, allows comparison of dns-GC(x)3X reactivity to peptides with canonical CaaX sequences such as dns-GCVLS derived from H-Ras. The dns-GCMIIM and dns-GCAVGP peptides exhibit similar values for kcat, with the Km for dns-GCMIIM approximately 10-fold lower than that for dns-GCAVGP (Table 2). Compared to dns-GCVLS (kcat = 0.3 s−1, Km = 1.5 µM), the kcat values for both dns-GC(x)3X peptides are reduced ~30-fold while Km increases ~4-fold for dns-GCAVGP and decreases ~3-fold for dns-GCMIIM. When compared to dns-GCMIIM and dns-GCAVGP, the potential sequences derived from proteolytic trimming, kcat/Km was increased 25-fold for dns-GCAVGP relative to dns-GCVLS and decreased 2-fold for dns-GCMIIM relative to dns-GCMIIM. These changes arise from changes in both kcat (dns-GCAVGP) and Km (dns-GCMIIM), indicating the last residue of the C(x)X can impact both binding to FTase and substrate turnover. Among these four peptides, only dns-GCMIIM serves as a GGTase-I substrate with a kcat/Km value comparable to dns-GCMIIM with FTase (1.4 +/- 0.1 x 104, Table 2).

We next determined the effect of tipifarnib, a farnesyltransferase inhibitor (FTI), on the farnesylation of these peptide substrates. Tipifarnib interacts with the peptide substrate binding site within FTase. Treatment with tipifarnib efficiently blocked FTase-catalyzed farnesylation of dns-GCMIIM (IC50 = 23 +/- 7 nM)
and dns-GCAVGP (IC$_{50}$ = 41 +/- 13 nM) (Figure S3). Tipifarnib inhibition of C(x)$_3$X peptide farnesylation suggests that these non-canonical sequences utilize many of the same interactions involved in CaaX peptide binding within the FTase active site.

An eGFP- C(x)$_3$X reporter protein serves as a FTase substrate

To address the relevance of our findings in the context of larger substrates, we next explored the ability of a C(x)$_3$X sequence to be modified by FTase in the more biologically relevant context at the C-terminus of a folded protein. We appended the reactive CAVGP sequence to the C-terminus of eGFP, purified the bacterially expressed protein, and evaluated the farnesylation potential of the reporter using an approach validated for detecting modification of eGFP fusion proteins bearing canonical CaaX sequences. (69,70) Farnesylation of this eGFP-CAVGP reporter protein was assessed via LC/MS (Figure 4). Following incubation in a reaction lacking the FPP co-substrate, only the unmodified eGFP-CAVGP protein is detected with a mass consistent with the expected unmodified protein (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 increased mass is the equivalent of eGFP-GCAVGP with the addition of a 205 Da (theoretical mass) farnesyl group, providing evidence of successful farnesylation of a full length protein terminating in a C(x)$_3$X 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 an FPP analogue (C15AlkOPP) bearing an alkyne group to allow for post-prenylation protein labeling. Following eGFP-CAVGP incubation with C15AlkOPP in the presence of FTase, the modified protein was derivatized with TAMRA-N$_3$ using Cu(II)-catalyzed alkyne-azide cycloaddition (CuAAC). (71,72) In-gel imaging of TAMRA fluorescence revealed a single band at the expected size of farnesylated eGFP-GCAVGP (~28 kDa, Figure 4c). The negative control reaction lacking the alkyne FPP analogue does not exhibit a TAMRA-fluorescent band, with Coomassie Blue staining confirming the presence of eGFP-CAVGP in both reactions. Together, these results provide additional evidence for the farnesylation of a C(x)$_3$X motif by purified FTase in the context of a full length protein.

Selection and reactivity analysis of C(x)$_3$X sequences derived from the human proteome

To address the relevance of our findings outside of yeast, we expanded our investigation to include C(x)$_3$X sequences derived from the human proteome to explore the potential biological relevance of this novel FTase substrate class. A scan of the Prosite database yielded 965 proteins in the human genome containing a C(x)$_3$X C-terminal sequences. Of these proteins, six candidate sequences were selected for further characterization based on comparison to highly active sequences from the yeast studies (see Experimental Procedures). Of the six human C(x)$_3$X sequences selected for analysis, three served as FTase substrates (Table 3). The two sequences resembling CAVGP, dns-GCQTGP and dns-GCSQGP, exhibited complete substrate farnesylation as determined by RP-HPLC, with the farnesylation peptide product of dns-GCFSKM modification by FTase also detected (Figure S2). None of the human-derived C(x)$_3$X peptides found to be substrates for FTase by RP-HPLC analysis exhibited sufficient fluorescence enhancement to allow steady-state characterization of peptide reactivity, underscoring that many C(x)$_3$X peptides appear refractory to detection of prenylation by fluorescence enhancement. As observed for the yeast C(x)$_3$X sequences, none of the human C(x)$_3$X sequences were substrates for rat GGTase I when examined by either fluorescence enhancement or RP-HPLC (data not shown).

Fluorescence localization and metabolic labeling studies indicate a C(x)$_3$X sequence can support protein farnesylation in mammalian cells

To be considered biologically relevant FTase substrates, proteins terminating in C(x)$_3$X sequences must exhibit sufficient intrinsic reactivity to be modified by endogenous FTase within an intact cell. The minimum reactivity for human substrates is estimated to be in the range of k$_{cat}$/K$_{m}$ = 0.5-2 x 10$^4$ M$^{-1}$s$^{-1}$ when peptide substrate reactivity is ascertained in an in vitro assay using purified FTase. (73) The reactivity of the dns-GCMII peptide with purified FTase (k$_{cat}$/K$_{m}$ = 1.9 +/- 0.6 x 10$^4$ M$^{-1}$s$^{-1}$, Table 2) suggests that 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.

To examine CMIIM reactivity within a mammalian cellular context, this sequence was introduced into a previously described fusion protein consisting of eGFP fused to the C-terminal domain of K-Ras4B. This reporter displays membrane localization upon prenylation of its canonical C-terminal CVIM sequence.(74) Following transient transfection into HEK293 cells, eGFP-KRas-CMIIM also displayed membrane localized fluorescence consistent with its farnesylation (Figure 5). 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-CMIIM) was also examined to determine if localization of C(x)3X proteins was due to endogenous proteolysis. eGFP-KRas-CMIIM also displays membrane localized fluorescence, but is unaffected by tipifarnib treatment. This behavior is consistent with the reactivity of the CVII sequence with GGTase-I (Table 2), which would lead to prenylation and membrane localization in the presence of tipifarnib. This result also underscores the FTase selectivity of the CMIIM sequence and further demonstrates that proteolytic trimming of the sequence does not occur within the cell.

To directly confirm prenylation of the eGFP-KRas-CMIIM reporter protein by endogenous FTase within a mammalian cell, we employed metabolic labeling of transiently transfected HEK293 cells using an alkyn-containing FPP analogue.(75) Following transient transfection with eGFP-KRas derived fusion proteins, cells were incubated with the C15AlkOPP FPP analog for alkyn functionalization of the expressed protein. Cell lysates were derivatized with TAMRA-N3 as described above. Transfection with eGFP-KRas-CVIM as a positive control followed by in-gel imaging of TAMRA fluorescence resulted in a single major band at expected size of this fusion protein (~50 kDa), with this band not observed in untransfected cells or transfected cells not treated with the C15AlkOPP analogue (Figure 5c). A similar predominant TAMRA-fluorescent band is observed at the expected size of eGFP-KRas-CMIIM in the presence of C15AlkOPP. This single predominant band was not observed when the CMIIM sequence in the reporter was mutated to SMIIM, with the SMIIM sample resembling the untransfected cell 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.

To provide further evidence that the eGFP-KRas-CMIIM protein is farnesylated in vivo, the alkyn-modified protein can be enriched owing to the presence of the appended alkyn isoprenoid analogue that is amenable to conjugation with an affinity handle. Thus, a quantitative chemical proteomic analysis was performed on lysates obtained from eGFP-KRas-CMIIM- vs eGFP-KRas-SMIIM-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-MS-MS. A volcano plot generated after performing a two-sample t-test (FDR = 0.05, s0 = 0.5) across three replicates strongly indicates that eGFP-KRas-CMIIM is enriched over eGFP-KRas-SMIIM, further evidencing the in vivo farnesylation of this protein (Figure 5d). 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) is insufficient to support direct detection of transfected protein prenylation within cells by metabolic labeling or imaging methods.(73) 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.

**Discussion**

Protein prenylation by FTase and GGTase-I is an important modification occurring on many targets within the proteome. Following more than two decades of biochemical, structural, and computational investigation of these enzymes and their protein substrates, a C-terminal CaaX
sequence has been defined as the signature recognition motif for both FTase and GGTase-I. In this work, we have identified multiple C-terminal C(x)_3X sequences representing a new class of substrates for FTase. These unanticipated substrates broaden the range of FTase substrate selectivity and expand the array of potential prenylation targets well beyond that predicted by previous biochemical, structural, and computational studies. Our findings provide the foundation for continuing studies towards identifying physiologically relevant C(x)_3X proteins within the proteome. This new substrate class carries with it the potential to expand the involvement of prenylated proteins within biology, with associated implications ranging across the biochemical, cell biology, and biomedical communities.

The ability of FTase to prenylate C(x)_3X sequences was not predicted based on the current understanding of FTase recognition of CaaX sequence substrates. 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.(45,76-78) Several of these interactions serve as constraints in the FlexPepBind approach for predicting peptide sequence reactivity with FTase. When applied to selected FTase-reactive C(x)_3X sequences FlexPepBind only predicts ~50% of the HPLC-verified FTase substrates (Table S4).(46) The ability of FTase to accept the longer C(x)_3X motif indicates that its active site is more flexible than previously proposed. In contrast, the inability of GGTase-I to accept these C(x)_3X substrates shows that it exhibits distinct length and/or sequence requirements, adding to the functional distinctions between these two closely related enzymes. We expect in future studies 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.(54,79) 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 965 proteins terminating in C(x)_3X sequences, with 773 of these sequences containing only a single cysteine residue. This representation nearly matches that of CaaX sequences in the human proteome (1166 sequences; 1021 with a single cysteine). Biochemical and computational studies indicate that a large fraction of these CaaX sequences can serve as FTase substrates, raising the possibility that a large fraction of C(x)_3X sequences could also be farnesylated.(16,46) Although fewer in number, 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) and 120 CaaX proteins (101 with a single cysteine). While this study verified farnesylation of human protein-derived C(x)_3X sequences in the context of small peptide substrates, inclusion of C(x)_3X proteins doubles the potential scope of prenylation within the human and yeast proteomes. Similar expansions of potentially farnesylated proteins could be expected in pathogenic organisms that employ either endogenous or host-mediated farnesylation, such as Plasmodium falciparum, Candida albicans, and Legionella pneumophila.(75,80-85) Our expansion of the potential substrates recognized by FTase, supported by the reactivity of human-derived C(x)_3X peptide sequences, 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 biological impact of prenylation of non-canonical FTase substrates. These cellular studies will be greatly aided by the demonstrated ability of
metabolic labeling to bioorthogonally functionalize a prenylated C(x):X protein. A key challenge in this work will be determining the minimum level of substrate protein reactivity required for efficient metabolic labeling. Defining this parameter will provide valuable insight into the applicability of this technique for identifying biologically relevant targets. 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.

In addition to expanding the scope of prenylation within the proteome, our findings further support the “shunt pathway” model for protein prenylation wherein a subset of farnesylated proteins can sidestep subsequent C-terminal processing steps.(32) Such proteins with canonical CaaX motifs include Ydj1p, Rab38, Phko/β, and Gyl5.(27,32,86,87) The majority of C(x):X 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):X sequences are present 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. Our findings inspire several important questions. First, how many naturally occurring C(x):X proteins are prenylated? Second, what fraction of naturally occurring prenylated C(x):X proteins are refractory to subsequent processing (i.e. shunted)? Third, how does subsequent processing, or lack thereof, affect the biological properties of prenylated C(x):X proteins? Identifying novel protein prenyltransferase C(x):X 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. membrane localization) may not be relevant to shunted proteins whether they are of the CaaX or C(x):X variety.

**Experimental Procedures**

**Miscellaneous Methods.** All in vitro FTase and GGTTase-I assays were performed at 25 °C. All curve fitting was performed with KaleidaGraph (Synergy Software, Reading, PA). Geranylgeranyl diphasate (GGPP) and farnesyl diphasate (FP) were purchased from Isoprenoid.com (Tampa, FL). Peptides were commercially synthesized (Sigma-Genosys, The Woodlands, TX) and exhibited >90% purity, as determined by RP-HPLC or after semi-prep purification via RP-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.(88) The isoprenoid analogue C15Alk-OPP was prepared as previously described.(64)

**Yeasts strains and plasmids:** The yeast strains used in this study were IH1793 (MATα lyst1), RC757 (MATα sst2-1 rme his6 met1 can1 cyh2), SM2331 (MATα trp1 leu2 ura3 his4 can1 mfa1 mfa2), yWS304 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydj1::KAN8), and yWS1632 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ram1::KAN8) and have been previously described.(32,57,89,90) These strains were routinely propagated at 30 °C (IH1793, RC757, SM2331) or room temperature (yW304, yWS1632) on either YPD or appropriate selective media when plasmid transformed. 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.(32,91-93) pWS705 (2μ URA3 MFA1-CCGDD), pWS1170 (CEN URA3 YDJ1-CVLM), pWS1488 (CEN URA3 YDJ1-CMIIM), pWS1111 (CEN LEU2 MFA1-CMIIM), and pWS1112 (CEN LEU2 MFA1-CVLM) were constructed by PCR-directed, recombination-mediated plasmid construction.(94) In brief, PCR products designed to amplify the 3’ ends of MFA1 and YDJ1 with encoding for desired C(x):X motifs were used to gap repair plasmids pSM1605 (2μ URA3 MFA1), pWS1024 (CEN URA3 MFA1-C*IA); * indicates a premature TAG stop codon at the a1 position of CaaX motif; this plasmid was created by recombination-based methods from pWS610, and pWS1132 (CEN URA3 YDJ1-SASQ) as previously described.(32,92) These plasmids and others.
identified through our screening methods were introduced into strains via a lithium acetate-based transformation procedure. (95)

**Oligo design for generation of randomized C(x)X sequences by PCR:** Synthetic oligonucleotides were designed to PCR amplify the CaaX encoding region and associated 3' untranslated region of either MFA1 or YDJ1 as encoded in pWS1024 and pWS1132, respectively. The forward PCR oligo was designed to encode the C(x):X sequences and was flanked on the 5' end by 39 nucleotides homologous to the appropriate gene immediately before the CaaX motif (to facilitate recombination), and on the 3' end by at stop codon and additional nucleotides homologous to the 3' untranslated region (to facilitate PCR priming). The oligo used for generation of CGGDD was designed to encode these specific amino acids. The oligo used to generate a library of C(x):X 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 a 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.

**a-factor mating pheromone screen, halo assay, and mating test:** SM2331 yeast were cotransformed with MluI and SphI digested pWS1024 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 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 MATα 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. (32) In brief, the MATα strains were independently cultured to saturation in selective media, and the IH1793 MATα λys strain was cultured in YPD. All strains were diluted to A600 ~ 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 MATα haploid cells and MATα 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 wild type a-factor.

**Thermotolerance screen:** yWS304 yeast was co-transformed with NheI digested pWS1132 and PCR product engineered to encode randomized 5-mer sequences. A percentage 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 colonies, indicative of the 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 reintroduced into yWS304 to confirm plasmid-linked thermotolerance prior to detailed thermotolerance analysis.

**Thermotolerance assay:** Saturated cultures grown in SC-uracil liquid media at room temperature were
seriously diluted into YPD and spotted onto YPD solid media (5 µl per spot) as previously described.(32) 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.

Estimate of C(x)₃X complexity in a-factor and thermotolerance screens: 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.(58) The number of colonies screened with the α-factor reporter was determined by direct colony counts on all plates evaluated. The number of colonies screened with the Ydj1p reporter was estimated from the colony numbers observed for the percentage of the transformation mix plated onto selective media at non-selective temperature. The number of false positive colonies were not counted toward the total number of colony-forming units (CFUs); the false positive numbers were determined from transformation mixtures containing the linearized plasmids and PCR products alone that typically yielded a smaller number of CFUs relative to co-transformed sample (total typically <2%).

Immunoblot analysis for protein prenylation in yeast: Whole cell lysates of mid-log yeast were prepared as previously described using alkaline hydrolysis and TCA precipitation.(32,96) 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).

Image analysis for yeast assays and immunoblots: 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.

Activity screening of dns-GC(x)₃X peptides by fluorescence-based prenylation assay: Prenylation of dansylated dns-GC(x)₃X peptides was assessed by a time-dependent increase in fluorescence ($\lambda_{ex}$ 340 nm, $\lambda_{em}$ 520 nm) upon prenylation of the dansylated peptide.(16,44,60-62) 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 assess the reactivity of dns-GC(x)₃X peptides with FTase and GGTase-I, time-course reactions were performed in the presence and absence of FPP and GPP. Fluorescence was measured for each peptide at time zero ($F_0$) and four time points ($F_t$; $t = 30, 60, 90, 120$ min). Corrected fluorescence ($F_t - F_0$) values were calculated for each time point and condition (Table S1). 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]. None of the dns-GC(x)₃X peptides exhibited fluorescence enhancement in reactions with GGTase-I and GGPP.

Activity screening prenylation of dns-GC(x)₃X peptides via RP-HPLC: RP-HPLC analysis was performed on all dns-GC(x)₃X peptides to confirm farnesylation (FPP and FTase) or geranylglyceranylization (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 RP-HPLC (Zorbax Eclipse XDB-C18 column). Peptides and products were detected by fluorescence ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 496$ nm). In all cases, the peak for the dns-
GC(x)₃X peptide shifts to a longer retention time upon farnesylation, whereas parallel reactions performed without FPP showed no change in peptide retention time. Representative RP-HPLC traces are included as Figure S2 in the Supplemental Data. Analogous reactions with GGTase-I and GGPP provided no evidence for geranylgeranylation of dns-GC(x)₃X peptides.

**Steady-state characterization of dns-GC(x)₃X and dns-GCaaX peptides:** Steady-state kinetics were determined as previously described for prenylation of dns-GC(x)₃X and dns-GCaaX peptides by FTase or GGTase-I by monitoring the time-dependent increase in fluorescence (λₑₓ 340 nm, λₑₘ 520 nm) upon prenylation of the dansylated peptide in a Synergy HI multimode plate reader (Biotek). (43,59,63)

**Determination of tipifarnib inhibition of dns-GC(x)₃X peptide farnesylation:** Assays were performed as described above for fluorescence-based detection of peptide farnesylation, 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 IC₅₀ values.

\[
\text{Normalized Slope} = \frac{[\text{tipifarnib}]}{[\text{tipifarnib}]+IC_{50}}
\]

**ESI-MS analysis of farnesylated dns-GC(x)₃X peptides:** In vitro reactions with dns-GC(x)₃X peptides (5 μM) were prepared in prenylation buffer (50 mM NaHEPES, 5 mM TCEP, pH 7.8) 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 H₂O. A step-gradient elution was carried out with 6-mL volumes of 10% (CH₃CN/H₂O; 0.1% TFA), 35% (CH₃CN/H₂O; 0.1% TFA), and 100% CH₃CN with 0.1% TFA. The 100% CH₃CN fractions containing the modified peptides (confirmed by fluorescence under UV lamp) were evaporated and residues were redissolved in 30% CH₃CN/H₂O with 0.1% TFA. Samples were loaded to an LC column (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).

**Construction of pJExpress414 plasmid encoding the His₆-eGFP-GCAVGP reporter protein:** A gene encoding the His₆-eGFP-GCAVGP reporter protein was prepared by PCR using the pJExpress414-eGFP-CVIA vector as a template with the GCAVGP C-terminal sequence and HindIII restriction site encoded in the 3’ primer. (97) 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 NheI and HindIII, the His₆-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).

**Expression and purification of His₆-eGFP-GCAVGP:** Chemically competent BL21 (DE3) E. coli (Z-competent, Zymo Research, Irvine, CA) were transformed with pJExpress414_His₆-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. (98) Following overnight incubation at 28 °C with shaking, bacteria were harvested, lysed, and His₆-eGFP-GCAVGP purified as previously described. (70) Protein concentration was measured using absorbance of eGFP at 488 nm (λₑₘ = 55,000 M⁻¹cm⁻¹). (99)

**Farnesylation reactions with His₆-eGFP-GCAVGP:** Farnesylation of purified His₆-eGFP-
GCAVGP was performed by incubation of purified protein (5 µM) with 100 nM FTase, 10 µM FPP or C15AlkOPP, and 5 mM MgCl₂ 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-N₃ 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 (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% HCO₃ in H₂O) and buffer B (0.1% HCO₃H in CH₃CN) 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. Prior to in-gel fluorescence labeling, His₆-eGFP-GCAVGP was recovered using a protein precipitation kit (ProteoExtract, Calbiochem, San Diego, CA). Protein pellets were redissolved in PBS + 1% SDS and 14 µg of proteins was subjected to click reaction (25 µM TAMRA-N₃, 1 mM TCEP, 0.1 mM TBTA, and 1 mM CuSO₄) 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 analyzed using a 15% SDS-PAGE gel. In-gel fluorescence was detected using a fluorescence scanner (Typhoon FLA 9500, GE Healthcare; λₑₓ 542 nm, λₑₘ₅ 568 nm). Gels were stained with Coomassie Blue and destained to visualize protein loading. Images were processed on ImageJ.

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 CxX)(18,20) 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.

Construction of eGFP-KRas-C(x)₃X reporter protein plasmids: Gene inserts encoding eGFP-KRas-XMII (X=C or S) and eGFP-KRas-CMII reporter proteins were prepared by PCR using the pEGFP-KRas vector (Casey Lab, Duke University) as a template with the CMIIM, SMII, or CMII C-terminal sequences and KpnI restriction site encoded in the 3′ primers.(100) PCR products were purified using the BioBasic Inc. EZ-10 Spin Column PCR Purification Kit following the manufacturer’s instructions. Following digestion by NheI and KpnI, the eGFP-KRas-XMII or eGFP-KRas-XMI 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, South Plainfield, NJ).

Cell culture, transfection, and imaging. HEK293 cells (ATCC) were 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% CO₂ at 37 °C. Transfections and imaging were performed as previously described,(73) 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 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. Images were...
captured using a Zeiss Axiocam MRm camera and analyzed using Zen Pro software (Zeiss).

**Metabolic labeling of HEK293 cells expressing eGFP-KRas reporter proteins with C15AlkOPP analogue** HEK293 cells were maintained as described above. Upon 80% confluency, 2.5 x 10^5 cells were grown in 2-6 well plates (Corning) for each construct to be transfected, with incubation of each plate 24 hours to allow cells to adhere. Turbofect was then added to each well according to the manufacturer’s protocol. C15AlkOPP analogue (25 μM) was added 12 hours after the addition of Turbofect and cells were incubated an additional 12 hours prior to harvesting. Cells were harvested using gentle scraping in PBS, pelleting at 150 x g for 5 min, and cell pellets were stored at -80 °C.

**CuAAC reactions on protein lysates.** Cell pellets that were metabolically labeled with C15AlkOPP were lysed in lysis buffer (10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl, 2.4 μM PMSF, 65 units benzonase nuclease, protease inhibitor cocktail) and protein concentration was determined by BCA assay. For in-gel fluorescence analysis, proteins (100 μg) were subjected to click reaction with TAMRA-N₃ under conditions described above. Proteins were recovered by precipitation (ProteoExtract, Calbiochem), redissolved in 1X Laemlli buffer and resolved on an 10% SDS-PAGE gel (40 μg proteins). In-gel fluorescence and Coomassie staining were performed as described above. For biotinylation of C15AlkOPP-labeled protein lysates from HEK293 cells transfected with eGFP-KRas-CMIIM and eGFP-KRas-SMIIM, 2 mg of lysate protein in 1 mL lysis buffer were reacted with 100 μM biotin-N₃ in the presence of 1 mM TCEP, 0.1 mM TBTA, and 1 mM CuSO₄ for 1.5 hours at room temperature. Proteins were recovered by precipitation (1 x CHCl₃, 4 x CH₃OH, 3 x H₂O) and redissolved in 1 mL of 1X PBS with 1% SDS.

**Enrichment and sample preparation for proteomic analysis.** The biotinylated protein lysates were incubated with pre-washed Neutravidin® (100 μL beads) for 1.5 hours and washed with 1X PBS + 1% SDS (3x), 1X PBS, 8 M urea in 50 mM TEAB (3x), and 50 mM TEAB (3x). Proteins were reduced with DTT (10 mM) and alkylated with iodoacetamide (10 mM) followed by digestion with 0.5 μg sequencing grade trypsin overnight. Tryptic peptides were collected, lyophilized and redissolved in 100 mM TEAB. Peptides (10 μg) were labeled with TMT duplex (Thermo Scientific) following the manufacturer’s protocol (eGFP-KRas-SMIIM: TMT-126; eGFP-KRas-CMIIM: TMT-127). The TMT-labeled peptides were fractionated under high pH reversed phase conditions on STAGE tips into fractions containing 5, 10, 15, 20, 25, and 80% acetonitrile in 200 mM ammonium formate pH 10.

**LC-MS/MS and data analysis.** LC-MS/MS analyses were performed using a RSLCnano System (Dionex) and Orbitrap Elite Hybrid mass spectrometer. Each fraction was fractionated using a column (75 μm i.d., 35 cm) manufactured in-house and eluted at 300 nL/min using an 80-min gradient with 0.1% formic acid in water and 0.1% formic acid in acetonitrile. A data-dependent acquisition was performed set at 60,000 resolution over 350-1500 m/z range. HCD fragmentation was carried out with 1.4 m/z isolation window and NCE of 40%. The maximum injection times were 100 and 500 ms and ion targets were 10⁶ and 5 x 10⁴ for MS and MS/MS (at 15,000 m/z resolution), respectively.

The raw files were analyzed with MaxQuant version 1.6.0.16 and searched against nonredundant human proteome database (UP000005640) and common contaminants. Data output was further processed using Perseus version 1.6.0.7 with protein identification based on only identified by site and reverse sequences and contaminants (except GFP) were removed. A two-sample t-test was performed across three replicates (FDR = 0.05, s0 = 0.5) and the volcano plot generated was exported to Microsoft Excel for formatting.
C(x)_X sequences as non-canonical FTase substrates

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

MJB performed peptide- and protein-based prenylation assays, designed and expressed protein substrates, performed cell imaging experiments, and prepared metabolic labeling samples. KFS performed mass spectrometry and gel 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. MJB, KSF, MDD, WKS, and JLH designed experiments. MJB, KFS, MDD, WKS, and JLH wrote the manuscript.
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Table 1. Mammalian FTase reactivity with C(x)₃X sequences derived from yeast α-factor and thermodurance screening.

| Peptide sequence | Reporter / source | Fluorescence enhancement of dns-GC(x)X<sup>a</sup> | HPLC detection of farnesylated dns-GC(x)X |
|------------------|-------------------|-----------------------------------------------|------------------------------------------|
| CGGDD            | (55,56)           | -                                             | -                                        |
| CMIIM            | α-factor          | +                                             | +                                        |
| CVLMM            | α-factor          | <i>not determined</i>                         | <i>not determined</i>                    |
| CAVGP            | Ydj1              | +                                             | +                                        |
| CAYVL            | Ydj1              | +                                             | +                                        |
| CCAGH            | Ydj1              | <i>not determined</i>                         | <i>not determined</i>                    |
| CFFYI            | Ydj1              | +                                             | +                                        |
| CFNSL            | Ydj1              | -                                             | +                                        |
| CIPVQ            | Ydj1              | -                                             | +                                        |
| CLPIV            | Ydj1              | -                                             | +                                        |
| CQGFL            | Ydj1              | -                                             | +                                        |
| CSIQG            | Ydj1              | +                                             | +                                        |
| CSRLQ            | Ydj1              | -                                             | +                                        |
| CSSLQ            | Ydj1              | -                                             | +                                        |
| CVSFG            | Ydj1              | -                                             | +                                        |
| CWAGG            | Ydj1              | -                                             | +                                        |
| CWGEV            | Ydj1              | -                                             | +                                        |
| CWGGA            | Ydj1              | -                                             | +                                        |

<sup>a</sup> 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.
### Table 2. Steady-state kinetic parameters for peptide reactivity with mammalian FTase and GGTase-I

| Peptide          | Reactivity with FTase | Reactivity with GGTase-I |
|------------------|-----------------------|--------------------------|
|                  | $k_{cat}$ (s$^{-1}$)  | $K_m$ (µM)               | $k_{cat}$ / $K_m$ (µM$^{-1}$s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_m$ (µM) | $k_{cat}$ / $K_m$ (µM$^{-1}$s$^{-1}$) |
| dns-GCVLS$^b$    | 0.3                   | 1.5                      | 2 x 10$^5$                          | Not reported        | Not reported | Not reported |
| dns-GCAVGP       | 0.009 ± 0.001         | 5.6 ± 1.0                | 1.6 ± 0.3 x 10$^3$                  | No activity         | No activity  | No activity   |
| dns-GCAVG        | 0.14 ± 0.01           | 3.5 ± 0.3                | 4.0 ± 0.2 x 10$^4$                  | No activity         | No activity  | No activity   |
| dns-GCMIIM       | 0.009 ± 0.001         | 0.5 ± 0.1                | 1.9 ± 0.6 x 10$^4$                  | No activity         | No activity  | No activity   |
| dns-GCMII        | 0.040 ± 0.002         | 4.6 ± 0.6                | 8.6 ± 0.6 x 10$^3$                  | 0.0070 ± 0.0003     | 0.50 ± 0.07  | 1.4 ± 0.1 x 10$^4$ |

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

$^b$ reference (64)
Table 3. Mammalian FTase reactivity with C(x)₃X sequences derived from the human genome.

| Peptide       | Human protein                                      | HPLC detection of farnesylated dns-GC(x)₃X |
|---------------|----------------------------------------------------|-------------------------------------------|
| dns-GCLLHP    | Ras association domain-containing protein 5        | -                                         |
| dns-GCSQGP    | Sushi, nidogen and EGF-like domain-containing protein 1 | +                                         |
| dns-GCQTGP    | Putative glycosylation-dependent cell adhesion molecule 1 | +                                         |
| dns-GCSVKM    | Olfactory receptor protein                         | -                                         |
| dns-GCFSKM    | Sorting nexin 4                                    | +                                         |
| dns-GCDREV    | Prostamide/prostaglandin F synthase                | -                                         |
Figure 1. Prenylation pathway recognition and modification of proteins terminating in CaaX sequences. a) Protein modification steps observed within the prenylation pathway, including a shunt pathway for proteins undergoing only prenylation without subsequent proteolysis.(32) b) Structural model of FTase recognition of a CVLS substrate sequence. Recognition of the length of the CVLS tetrapeptide (green) involves coordination of the cysteine side chain thiol to the catalytic zinc ion (orange sphere) and both direct and water-mediated (teal spheres) hydrogen bonding between the peptide C-terminal carboxylate group to FTase residues in both the alpha (Q167α) and beta (H149β) enzyme subunits. Image generated from PDB 1TN8 using PyMol.(77)
C(x)_3X sequences as non-canonical FTase substrates

Figure 2. Phenotypes and isoprenylation status of C(x)_3X motifs identified by yeast-based screening. a) α-factor C(x)_3X variants encoded in CEN LEU2 plasmids and transformed into SM2331 (MATα mfa1 mfa2) were evaluated for their ability to produce α-factor using a spot halo assay; the CGGDD variant was encoded in a high-copy 2µ URA3 plasmid but otherwise treated identically. Strains were spotted onto YPD, cultured for 48 hours at 30 °C, and replica transferred onto a thin lawn of RC757 (MATα sst2-1). Plates were imaged after 16 hours incubation at 30 °C. The same strains were subjected to quantitative mating analyses, which yielded the numerical values indicated below each image, where values are reported as percent relative to control (CVIA). b) Ydj1p C(x)_3X variants encoded in low-copy CEN plasmids were evaluated for their ability to rescue growth of yWS304 (ydj1Δ) 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 β subunit.
Figure 3. Dansyl-GC(x)₃X peptides can be efficiently farnesylated by mammalian FTase. a) Fluorescence-based screening for FTase-catalyzed farnesylation of dns-GC(x)₃X peptides. b) Farnesylation of dns-GCMIIIM (top) and dns-GCAVGP (bottom) by FTase as monitored by fluorescence enhancement. Red trace, farnesylation reaction; blue trace, control reaction lacking FPP. c) RP-HPLC analysis of FTase-catalyzed farnesylation of dns-GCMIIIM (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-GCMIIIM (left) and dns-GCAVGP (right); C(Fr) indicates farnesylated cysteine. Reactions were performed and analyzed as described in Experimental Procedures; tables of fluorescence screening data and ESI MS/MS ion assignments are included in the Supplemental Data.
**Figure 4.** A C(x)_3X sequence is efficiently farnesylated in the context of a protein substrate. a) LC/MS analysis of farnesylation of an eGFP reporter protein terminating in a C(x)_3X 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). c) 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.
Figure 5. eGFP-KRas-CMIIM is efficiently modified by FTase within a mammalian cell. a) Representative images of HEK293 cells transfected with eGFP-KRas-XMIIM or eGFP-KRas-CMIIM 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. Detailed scoring data is provided in Table S2. c) 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. d) Volcano plot for TMT-labeled quantitative proteomic analysis of eGFP-KRas-CMIIM- 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.
Efficient farnesylation of an extended C-terminal C(x)₃X sequence motif expands the scope of the prenylated proteome
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