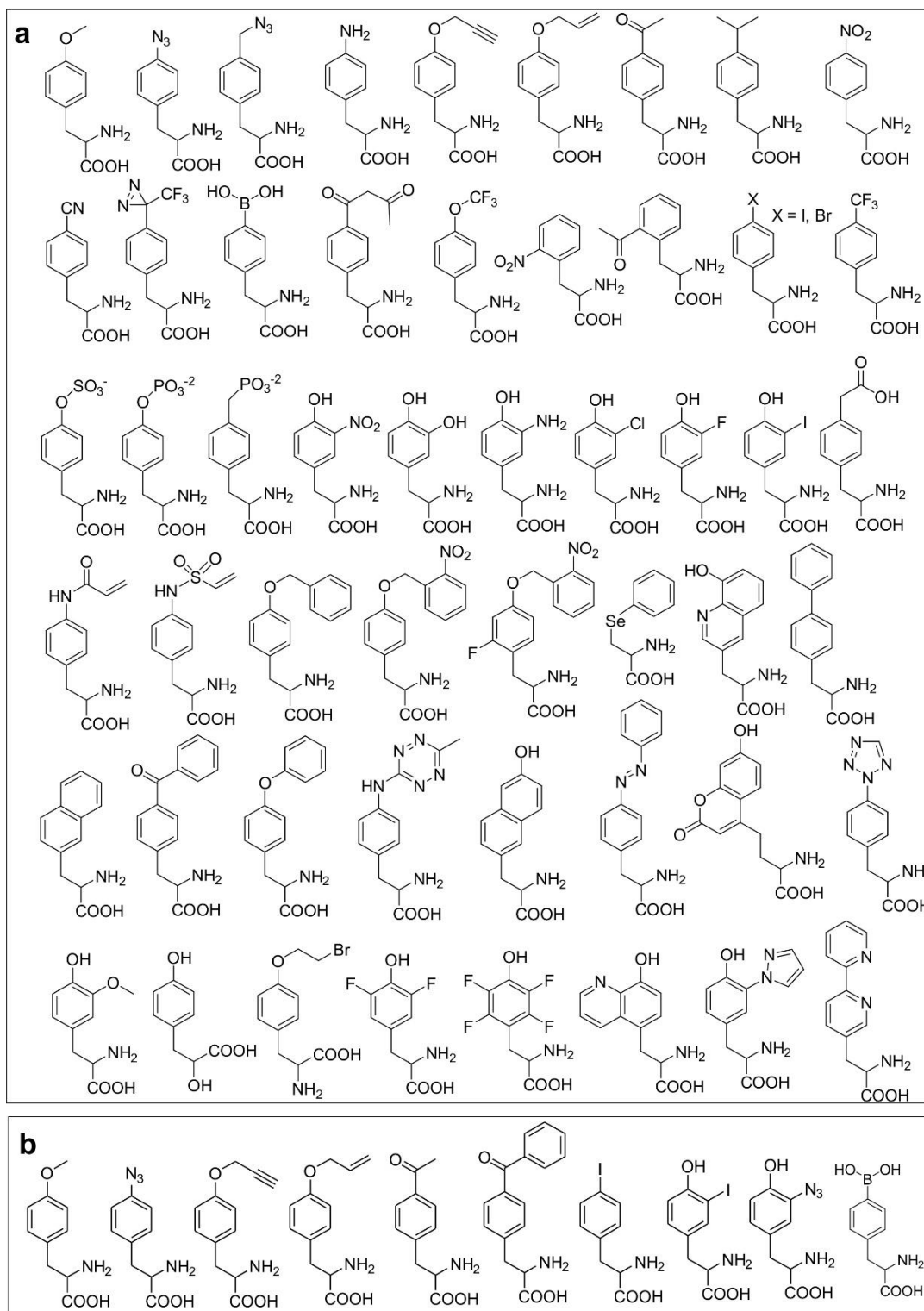
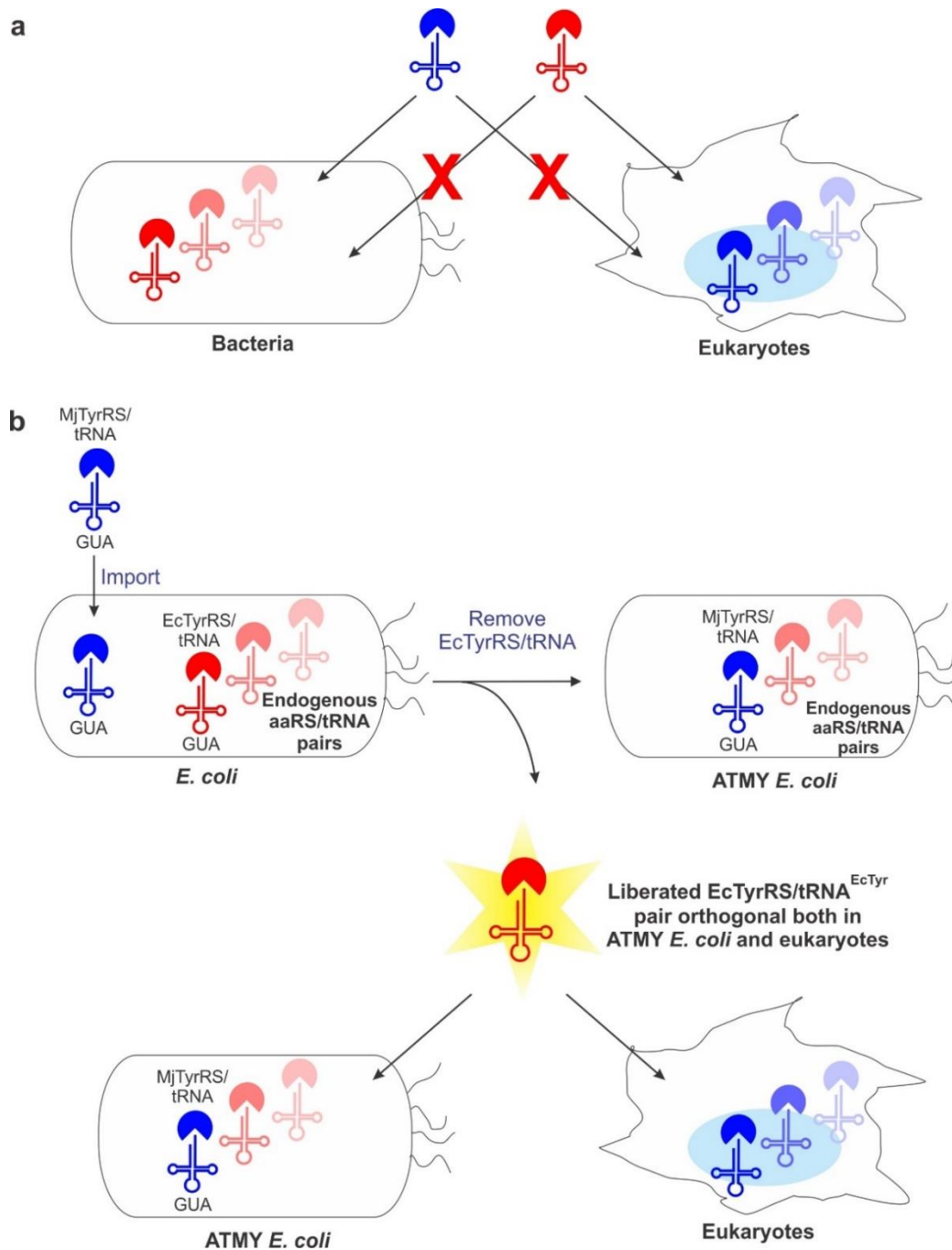


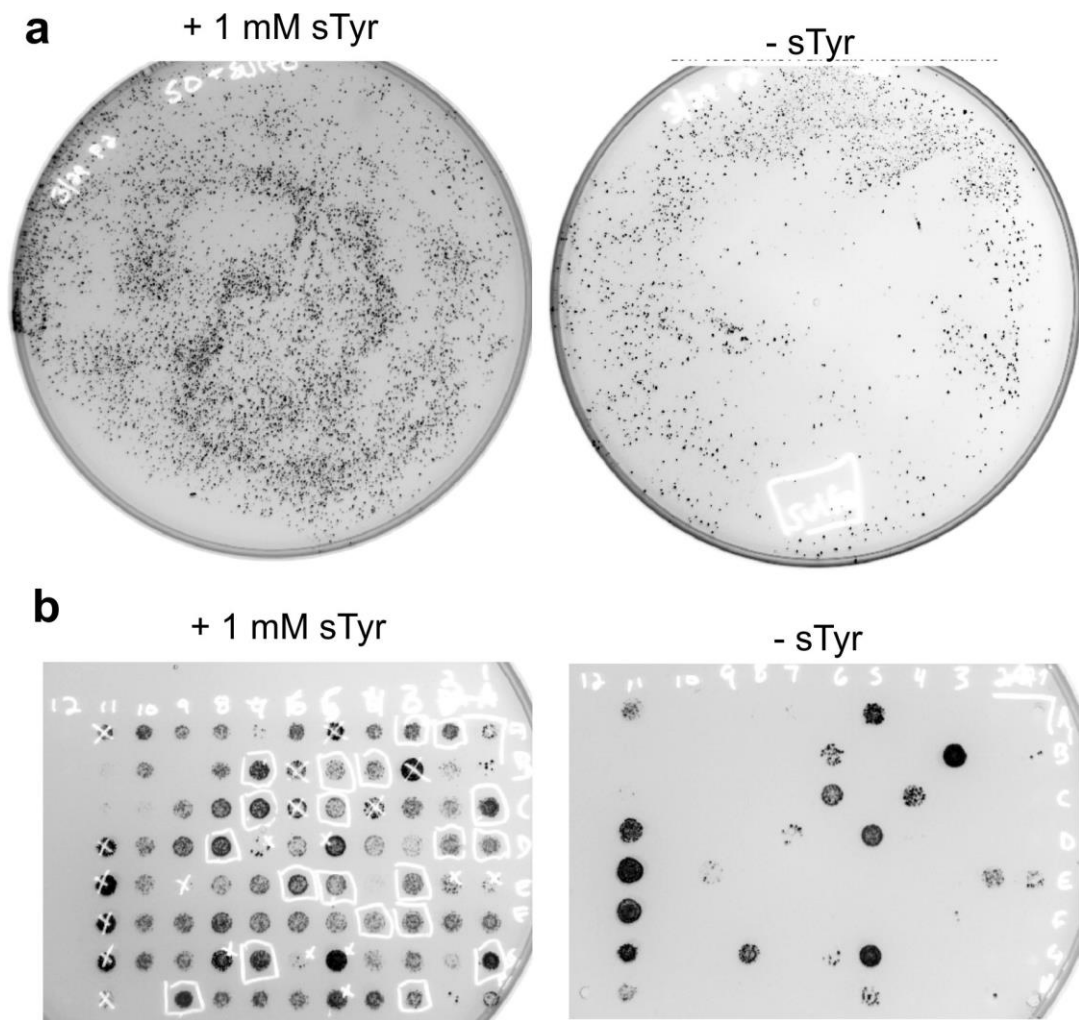
Supplementary Figure 1. Proteins processed through the trans-Golgi network in multicellular eukaryotes are subjected to tyrosine sulfation by two Tyrosylprotein Sulfotransferase (TPST) enzymes that use PAPS as the cofactor.



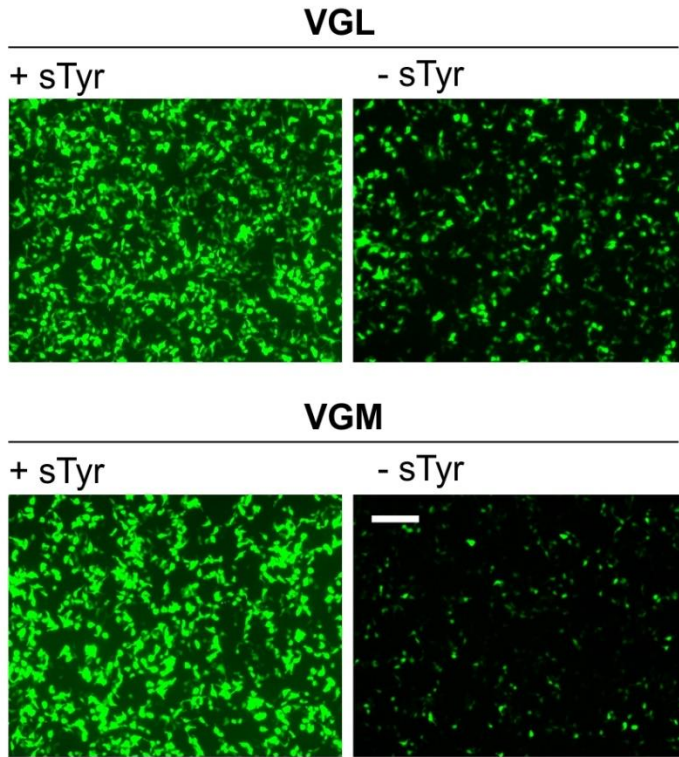
Supplementary Figure 2. Many more nCAAs have been genetically encoded in *E. coli* using the MjTyrRS/tRNA pair (**a**) than in eukaryotes using the EcTyrRS/tRNA pair (**b**).



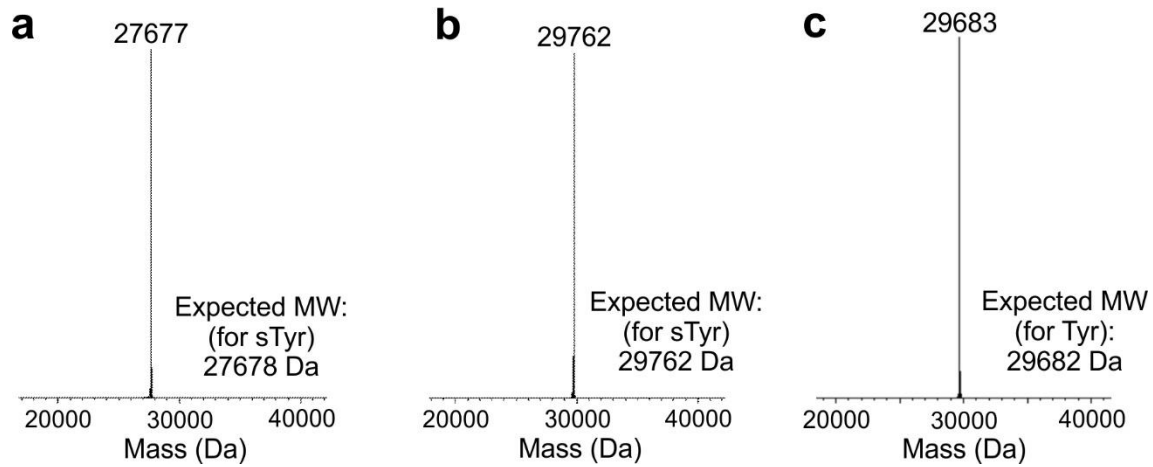
Supplementary Figure 3. a, Typically, the bacteria-derived aaRS/tRNA pairs (color-coded red) are orthogonal in eukaryotes and can be used for eukaryotic genetic code expansion, while eukaryote or archaea derived pairs (color-coded blue) are orthogonal in bacteria and are useful for bacterial genetic code expansion. **b**, Functionally substituting the EcTyrRS/tRNA pair in *E. coli* with the archaea derived MjTyrRS/tRNA pair creates an engineered ATMY strain. The ‘liberated’ EcTyrRS/tRNA pair can be established as an orthogonal nonsense suppressor in ATMY *E. coli*, and engineered in this strain for altering its substrate specificity.



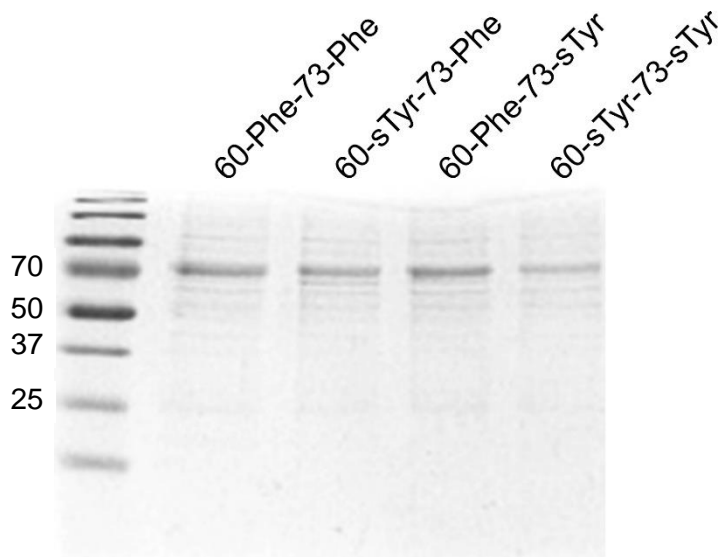
Supplementary Figure 4. **a**, The pool of EcTyrRS library of mutants selected through a single round each of positive and negative selection show substantial sTyr-dependent survival in a subsequent round of positive selection. **b**, Many individual clones isolated from these plates also show the same phenotype. These experiments were performed once.



Supplementary Figure 5. Fluorescence images of HEK293T cells expressing EGFP-39-TAG reporter using VGL- or VGM-EcTyrRS mutant in the presence or absence of sTyr (1 mM). These experiments were performed at least three times with similar results.

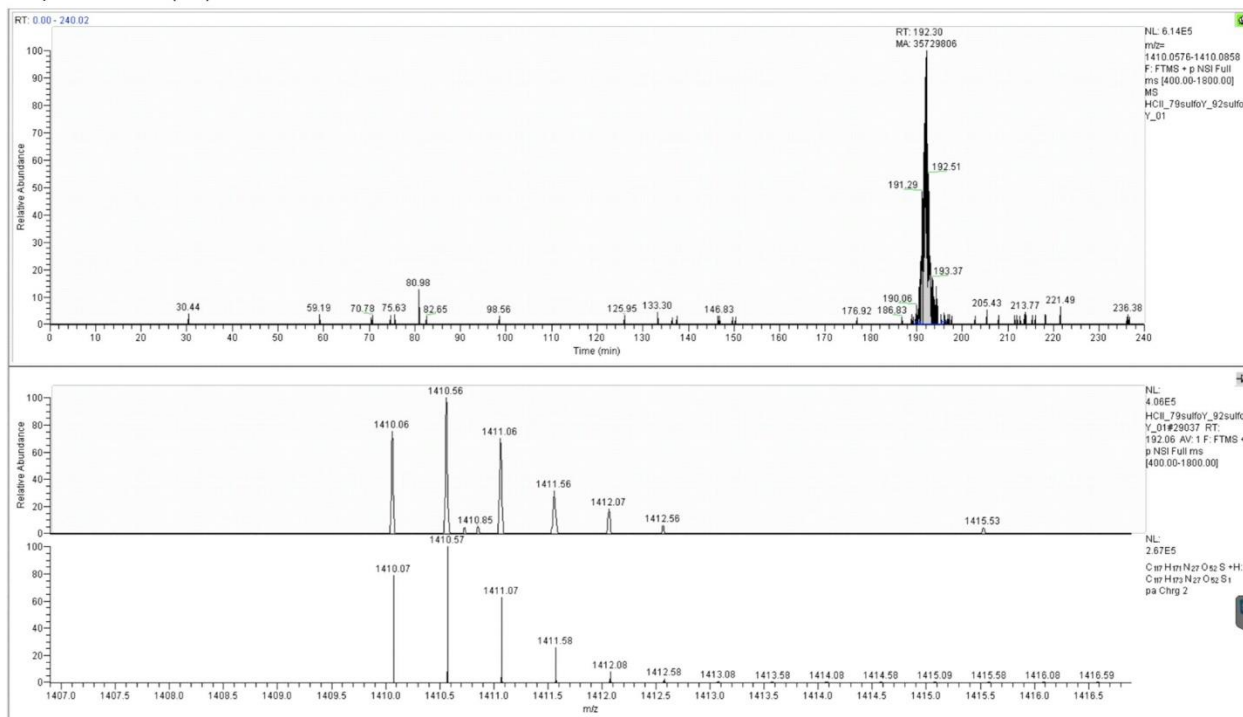


Supplementary Figure 6. ESI-MS analysis of the purified GFP reporter proteins. **a**, Deconvoluted mass of sfGFP-151-TAG reporter expressed in ATMY *E. coli* support incorporation of sTyr. **b**, EGFP-39-TAG reporter expressed in the presence of sTyr in mammalian cells show incorporation of sTyr; **c**, the same reporter expressed in the absence of sTyr show the incorporation of tyrosine. These MS analyses were performed at least twice with similar results.



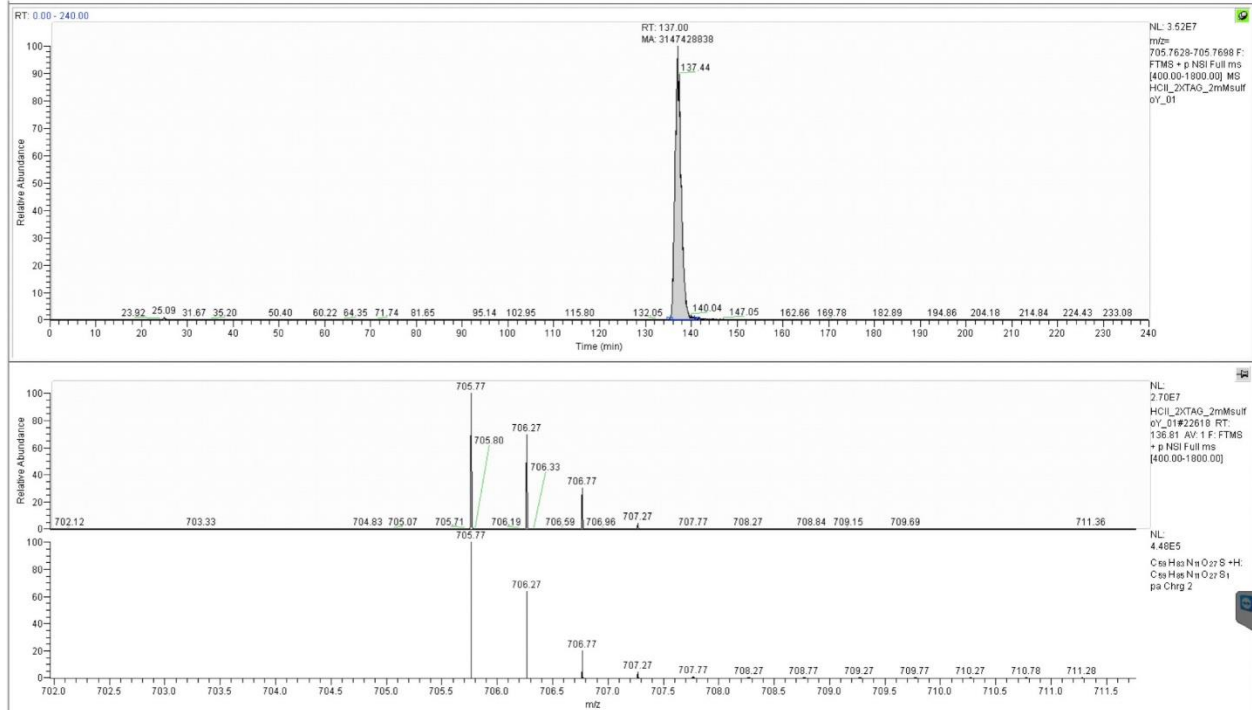
Supplementary Figure 7. SDS-PAGE analysis of secreted HCII mutants expressed in HEK293T cells and isolated from the culture media using a C-terminal polyhistidine tag. Due to well-established glycosylations, the observed molecular weight is significantly larger than what is predicted from the primary sequence (~57 kDa). SDS-PAGE analyses of various HCII preparations were performed at least three times with similar results.

Peptide harboring 60-sTyr
ENTVTNDWIPEGEEDDDY*LDLEK
Expected m/z (+2): 1410.07

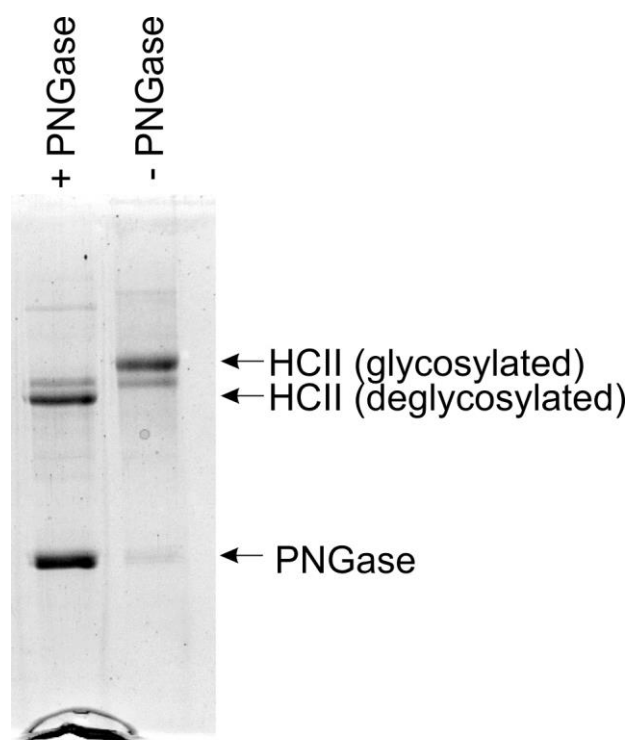


Supplementary Figure 8. Trypsin digestion followed by LC-MS analysis of HCII-60-sTyr-73-sTyr isolated from HEK293T cells identifies the presence of the peptide harboring 60-sTyr. These experiments were performed twice with similar results.

Peptide harboring 73-sTyr
FSEDDDY*IDIV
Expected m/z (+2): 705.77

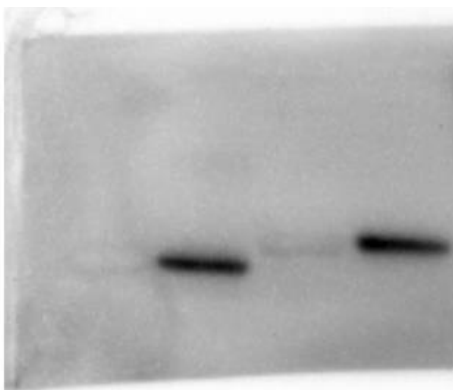


Supplementary Figure 9. Trypsin + elastase double digestion followed by LC-MS analysis of HCII-60-sTyr-73-sTyr isolated from HEK293T cells identifies the presence of the peptide harboring 73-sTyr. We were unable to find the HCII fragment harboring the 73 residue through trypsin digestion alone, likely due to its large predicted size. These experiments were performed twice with similar results.

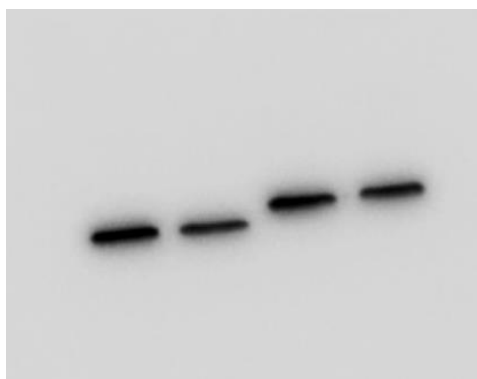


Supplementary Figure 10. PNGase F treatment of purified HCII-60-sTyr-73-sTyr substantially reduces its molecular weight by removing N-linked glycans. This experiment was performed only once.

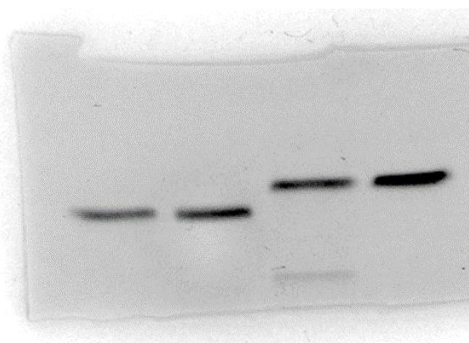
Anti-sTyr WB:



Anti-polyhistidine WB:



Coomassie:



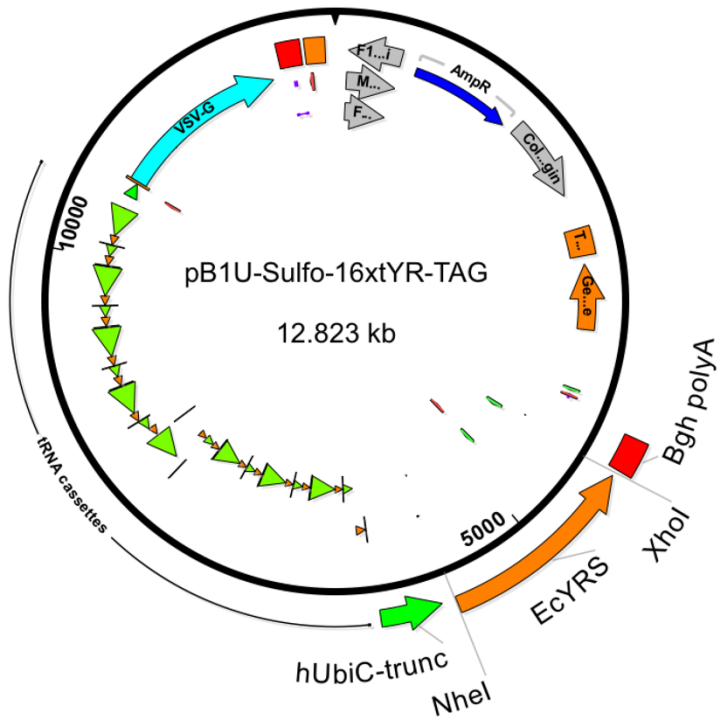
Supplementary Figure 11. Picture of full gels associated with Figure 1d.

Supplementary Table 1: List of oligonucleotides

Primer Name	Sequence
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EcYRS-L71-NBT-F	GCAGGCGGGCCACAAGCCGGTTGCnbtGTAGGCGGCGGACGGGTCTGATTG
EcYRS-N126-oR	GTTCCGCCGATAGCAGAGTTTTTC
EcYRS-N126x-F	GAAAACTCTGCTATCGCGGCGAACnnnTATGACTGGTTCGGCAATATGAATGTGCTGAC
pBK MCS JlsqR	GAGATCATGTAGGCCTGATAAGCGTAGC
EcYRS-NheI-F	GCTAGCGCCACCATGGCAAGCA
EcYRS-XhoI-R	aataatCTCGAGTTATTTCCAGCAAATCAGACAGTAATTCTTTTTACC
HCII-SfiI-F	TGGCAAAGAATTGGCCAAGGAGGCCACCATGAAACACTCATTAAACGCACTTC
10xHis-TGA-SfiI-R	TGGCGGCCGGCCAGGCCTCAATGATGGTGGTGATGATGATGGTGATGATG
HCII-79-Phe-R	GTCGTCGTCTTCACTGAATATCTTCTCCAGGTCCAGaaaGTCGTCGTCTCTCTCCCC
HCII-79-TAG-R	GTCGTCGTCTTCACTGAATATCTTCTCCAGGTCCAGctaGTCGTCGTCTCTCTCCCC
HCII-92-Phe-F	CTGGACCTGGAGAAGATATTCAGTGAAGACGACGACtttATCGACATCGTCGACAGTCTG
HCII-92-TAG-F	CTGGACCTGGAGAAGATATTCAGTGAAGACGACGACtagATCGACATCGTCGACAGTCTG
HCII-80-iF	CTGGACCTGGAGAAGATATTCAGTGAAGACGACGAC
HCII-80-iR	GTCGTCGTCTTCACTGAATATCTTCTCCAGGTCCAG

Supplementary note: Plasmid maps and sequences

pBIU-Sulfo-16xtYR-TAG:



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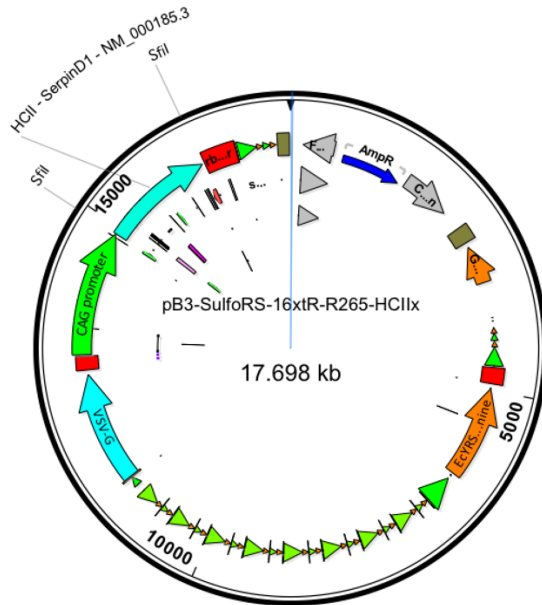
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pB3-SulfoRS-16xYtR-TAG-HCII: pAcBac3 OMeYRS was used as a starting vector to construct this plasmid.¹ pB3 (abbreviated pAcBac3) is identical to pB1u except it contains a CAG promoter upstream from an SfiI site as well as 4 additional tRNA cassette copies. OMeYRS was replaced with SulfoRS via NheI/XhoI as previously described in pB1U cloning description. The SfiI site was used to insert HCII. HCII-SfiI-F and 10xHis-TGA-SfiI-R were used to amplify HCII from pCMV-SerpinD1 (Origene, SC120039). Mutations were introduced via overlap extension (see primer list for 79, 92, and 80 overlap primers – 79 and 92 correspond to 60 and 73 sites, respectively).



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