Supporting Information for the manuscript:

An engineered aminoacyl-tRNA synthetase for cell-selective analysis of mammalian protein synthesis

Alborz Mahdavi[‡*], Graham D. Hamblin[‡], Granton A. Jindal[‡^], John D. Bagert[‡*^], Cathy Dong[*], Michael J. Sweredoski[¶], Sonja Hess[¶], Erin M. Schuman[ᵀ] and David A. Tirrell[‡]

[‡]Division of Chemistry and Chemical Engineering, [*]Division of Biology and Biological Engineering, [¶] Proteome Exploration Laboratory, Beckman Institute, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA, 91125.[ᵀ]Max Planck Institute for Brain Research, Frankfurt am Main, 60438, Germany

Materials and Methods:

Development of Mammalian Vectors for Expression of *E. coli* NLL-MetRS/tRNA^{Met}. For PCR and cloning purposes, unless otherwise stated, we used chemically competent *E. coli* MegaX DH10B (Zymo Research). Plasmid DNA was purified using a Miniprep kit (Qiagen); colony selection was performed on LB-agar plates with 100 µg/mL ampicillin. All plasmids were verified by sequencing (Laragen). The *E. coli* NLL-MetRS was obtained from pAM1 (Addgene plasmid 51401) through PCR amplification using a Nhe1 forward primer and an Xho1 reverse primer and inserted into the multiple cloning site of the mammalian expression vector pCDNA3.1+ (Invitrogen). This vector contains a CMV promoter, a bovine growth hormone (BGH) transcriptional stop sequence, and a neomycin resistance gene for G418 selection. The resulting plasmid was designated pMetRSNLL_G. For simultaneous expression of *E. coli* tRNA^{Met} and NLL-MetRS, the *E. coli* elongator tRNA^{Met} sequence was synthesized (Integrated DNA Technologies) with the 5' and 3' flanking sequences of human tRNA^{fMet} as well as flanking BglII restriction sites. This construct was inserted into the BglII site in pMetRSNLL_G to produce pMetRSNLLtRNA_G (sequence included in Figure S8). pMetRSNLLtRNA_G is a pCDNA3.1+-based vector expressing the NLL-MetRS under CMV promoter control as well as the *E. coli* tRNA^{Met}. A second tRNA sequence lacking the C-terminal CCA tail was synthesized (Integrated DNA Technologies) and inserted into the BglII restriction site of MetRSNLL_G to make pMetRSNLLtRNAdcca_G (sequence included in Figure S9).

Introduction of Mutations into MaRS and Development of Associated Mammalian Expression Vectors. MaRS was obtained from a cDNA clone from American Type Culture Collection clone ID 6414029 (ATCC). Site-directed mutagenesis (Agilent) was used to introduce the NLL, CLL, PLL and SLL mutations at residues L274, Y527 and H562 respectively. The L274G mutation was also introduced. These sequences were PCR amplified with a Nhe1 forward primer and an Xho1 reverse primer and inserted into pCDNA3.1+ (Invitrogen), resulting in plasmids pMaRSWT_G, pMaRSL274G_G/pMaRS_G, pMaRSSLL_G, pMaRSPLL_G, pMaRSCLL_G, pMaRSNLL_G (sequences in Figures S1-S6, respectively).

Development of Expression Vectors for Cell-Selective Proteomic Labeling and Cre-Lox Mediated Expression of L274G*Mm*MetRS. To prepare pMaRSC, the L274G MaRS coding sequence was connected to a mCherry sequence through a T2A linker by sewing PCR. Briefly, the MaRS sequence (obtained from pMaRSL274G_G/pMaRS) was PCR amplified using a Nhe1 forward primer containing a Flag-Tag sequence and a reverse primer containing a T2A linker. The mCherry coding sequence was amplified using a matching T2A sequence in the forward primer and a reverse primer encoding a C-terminal Myc-

tag and stop codon. Sewing PCR was used to amplify the final product with the following sequence components: Nhe1-FlagTag-MaRS-T2A-mCherry-MycTag-Xho1. This sequence was inserted between the Nhe1 and Xho1 sites of a pCDNA3.1+ vector containing a hygromycin resistance cassette (Invitrogen) to yield pMaRSC (sequence in Figure S12). For Cre-Lox mediated recombination, a LoxP-flanked transcriptional stop sequence was inserted after the CMV promoter in the pMaRSC plasmid. The LoxP-flanked transcriptional stop sequence consisting of forward LoxP (ATAACTTCGTATAGCATACATTATACGAAGTTAT) sequences flanking the transcriptional stop sequence was synthesized with Nhe1 restriction sites on both ends (Integrated DNA Technologies). The resulting fragment was ligated into the Nhe1 site of pMaRSC to yield the Cre-Lox plasmid pMaRSC_lox_H (sequence in Figure S13). The correct orientation of the insert into the Nhe1 cut site was verified by sequencing.

Cell Culture. Cells were passaged every three days on tissue-culture plates and incubated at 37°C and 5% CO_2. CHO-K1 cells were maintained in RMPI (Invitrogen) medium with 10% fetal bovine serum, and supplemented with Pen/Strep, L-glutamine and non-essential amino acids (Invitrogen). HeLa and COS7 cells were cultured in DMEM (Invitrogen) medium with 10% fetal bovine serum, and supplemented with Pen/Strep, L-glutamine and non-essential amino acids (Invitrogen).

Cell Transfection, Selection and Conditional Transgene Activation. Mammalian expression plasmids were amplified in *E. coli* strain MegaX DH10B and purified by using endotoxin-free plasmid Maxi-kits (Qiagen). Lipofectamine 2000 (Invitrogen) was used for all transfections according to the manufacturer's recommended procedures. For identification of MetRS variants that charge Anl and to study Anl incorporation, all cells were transiently transfected 30 hours prior to Anl labeling. For the Cre-Lox transgene activation study, pMaRSC_lox_H was linearized with BglII and transfected into CHO cells. After selection on hygromycin at 100 μg/ml for 10 days, surviving colonies were picked and expanded to yield a stable cell line. Cre-mediated recombination in these cells was achieved through transient transfection with a plasmid expressing eGFP-Cre under control of an EF1α promoter (Addgene plasmid 11923).

Synthesis of Azidonorleucine. Azidonorleucine synthesis was based on a previous protocol for azidohomoalanine synthesis, using Boc-lysine as the starting material.[1]

Copper-Catalyzed Reaction of Alkyne-TAMRA with Anl-labeled Proteins in Cell Lysates and Detection by In-gel Fluorescence. Cells were lysed with 4% SDS in phosphate-buffered saline (PBS).

Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Roche) was added to the lysates to reduce protease activity. PBS was added to dilute the SDS concentration to 1%, and cell lysates were centrifuged at 14,000 rcf for 10 minutes to remove cellular debris. Protein concentrations were measured by using a bicinchoninic protein quantification kit (BCA assay; Pierce). The same amount of protein was used for each condition; concentrations ranged from 0.1 to 0.4 mg/mL. Copper-catalyzed reactions were performed using the Click-IT TAMRA protein analysis kit (Invitrogen). Proteins were precipitated with chloroform/methanol, washed with methanol to remove unreacted dye and resuspended in protein loading buffer containing 2% SDS and 10 mM 2-mercaptoethanol. Proteins were separated by electrophoresis on 12% Bis-Tris polyacrylamide gels (Invitrogen). TAMRA ($\lambda_{excitation}$ = 555 nm and $\lambda_{emission}$ = 580 nm) was excited at 532 nm and detected with a 580 band-pass 30 nm filter. In-gel fluorescence images were acquired on a Typhoon 9400 molecular imager (GE Healthcare).

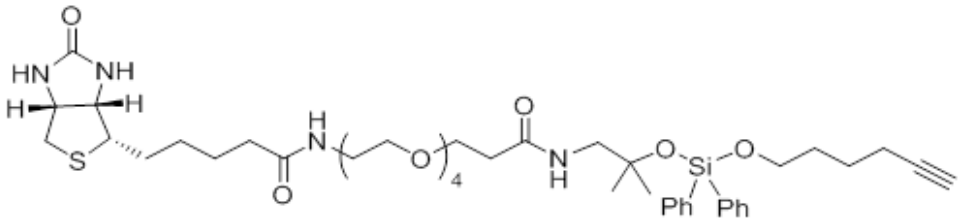
Detection of Proteins in Gels and Western Blots. Bicinchoninic acid protein quantification (Pierce) was used to equalize the amounts of proteins analyzed under different conditions. After dye labeling via the copper-catalyzed click reaction described above, proteins were washed with methanol to remove unreacted dye and then separated on Novex 12% Bis-Tris polyacrylamide gels (Invitrogen). Colloidal blue dye (Invitrogen) was used for nonspecific protein detection. For Western blots the proteins were transferred to nitrocellulose membranes (GE Healthcare), and probed with a Myc-tag-Alexa Fluor 488 conjugate monoclonal antibody (Cell Signal Technologies) at 1:1000 dilution in PBS with 0.2% v/w Tween20 (Sigma). Imaging of Western blots and gels was performed with a Typhoon 9400 molecular imager (GE Healthcare).

Copper-Catalyzed Reaction of Alkyne-TAMRA in Adherent Cells and Fluorescence Confocal Microscopy. Copper-catalyzed azide-alkyne cycloaddition reactions and synthesis of requisite THPTA ligand were performed as described previously[1,2]. Adherent CHO cells seeded onto glass bottom tissue culture plates (MatTek) were incubated in fresh CHO medium, as described above, supplemented with Anl at 1.5 mM, for 6 hours. Cells were washed twice with PBS, fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature, permeabilized with 4°C methanol for 10 minutes, and washed three times with PBS at room temperature. Labeling with alkyne-TAMRA (Invitrogen) was performed at room temperature in pH 7.4 PBS for 2 hours, using a final concentration of 0.1 mM copper sulfate, 0.5 mM THPTA, 5 mM sodium ascorbate, 5 mM aminoguanidine and 10 μM alkyne-TAMRA. To remove unreacted dye and other reaction components, cells were washed five times at 30-min intervals with PBS. Cell nuclei were stained with 300 nM DAPI in PBS for 30 min at room temperature and washed

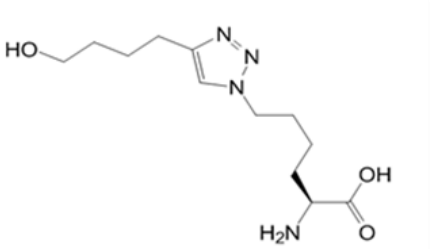
three times with PBS before imaging. Fluorescence confocal images were obtained on a Zeiss LSM 510 microscope.

Identification of Anl Incorporation Sites in Cellular Proteins.

HeLa cells expressing L274G*Mm*MetRS were labeled for a total of 10 hours with 2mM Anl before proteomic analysis. Cells were lysed with 1% SDS in PBS supplemented with EDTA-free protease inhibitor (Roche). Lysates was sonicated using a tip sonicator to reduce viscosity and centrifuged at 14,000 rcf for 15 min to remove cellular debris and reacted with an acid-cleavable biotin-alkyne enrichment tag for 2 hours, using a final concentration of 0.1 mM copper sulfate, 0.5 mM THPTA ligand, 5 mM sodium ascorbate, 5 mM aminoguanidine, and 100 µM alkyne probe. To identify sites of Anl incorporation in proteins, we used a cleavable enrichment tag that would allow detection of Anl and tagged-Anl residues at Met positions. This acid-cleavable biotin-alkyne enrichment tag was previously reported by us[3] and has the following structure:



After click reaction with this tag, proteins were precipitated with acetone, dissolved in 250 µl of 4% SDS in PBS, and diluted to 0.1% SDS by addition of PBS supplemented with EDTA-free protease inhibitor (Roche). Proteins were incubated with 400 µl Streptavidin Plus Ultralink resin (Pierce) for 1.5 hours at room temperature. Affinity purification was performed according to a previously published protocol[4] and the tag was cleaved in mild acidic solution, the resulting cleaved tag structure after reaction with Anl residue is as follows:



This tag structure introduces a mass shift of 121.12 amu at each methionine position, this variable mass modification includes the mass shift from methionine to Anl and the triazole conjugate containing the

cleaved tag moiety. We used this mass shift in our mass spectrometric analysis as a variable mass modification to search for Anl incorporation at Met positions in proteins.

Tryptic Digest for Identification of Anl Incorporation Sites. After enrichment as described above, elution fractions were combined with Amicon Ultra 0.5 centrifuge filters (3 kDa MWCO) (Millipore), and resuspended in 200 µL Tris-HCl pH 8.5. Lysyl endopeptidase (Wako; 10 µL of 0.1 µg/µL solution in 100 mM Tris-HCl pH 8.5) was added, and the sample was incubated for 4 hours at room temperature in the dark. Thereafter 20 µL of 0.5 µg/µL trypsin (Wako) in water was added and the sample was incubated in the dark overnight at room temperature. The eluent was centrifuged at 14000 rcf for 20 min using a 10 kDa molecular weight cutoff spin filter (Pierce) to remove undigested proteins as well as trypsin which remains in the filter. The flow-through, which contained tryptic peptides, was retained and acidified to 0.2% CF_3COOH. The peptide solution was desalted as described by Mann and coworkers[5] using a 3 mL MILI-SPE C18-SD extraction disk cartridge (3M) as follows. The cartridge was washed with 1 mL CH_3OH and centrifuged at 1500 rcf for 1 min, washed with 0.5 mL 0.1% CF_3COOH, 70% CH_3CN in water, and centrifuged at 1500 rcf for 1 min. The cartridge was washed with 0.1% CF_3COOH in water and centrifuged at 1500 rcf for 1 min. The peptide sample was loaded into the cartridge and passed through three times; each time the cartridge was centrifuged at 150 rcf for 3 min. The cartridge was washed twice with 0.5 mL 0.1% CF_3COOH in water and centrifuged at 150 rcf for 3 min. To elute the desalted peptides, the cartridge was washed with 0.5 mL of CH_3CN in water and centrifuged at 150 rcf for 3 min. The desalted peptides were lyophilized and stored at 4°C before analysis by mass spectrometry.

Affinity Enrichment of Anl-labeled Proteins for Shotgun Proteomics. Cells were labeled for a total of 24 hours with 2 mM Anl, then washed with PBS and lysed with 1% SDS in PBS supplemented with EDTA-free protease inhibitor (Roche) and 100 mM chloroacetamide. Lysates were boiled for 10 minutes at 95°C, and centrifuged at 14,000 rcf for 30 minutes to remove cellular debris. The supernatant protein content was quantified using the bicinchoninic acid assay. For each enrichment, 3 mg of lysate was combined with 50 µL of azadibenzocyclooctyne resin (50% slurry by volume; Click Chemistry Tools) that had been washed three times with 0.8% SDS in PBS. This copper-free on-resin cycloaddition reaction was incubated at room temperature on a rotating table for 3 hours, and then unreacted DBCO groups were quenched by addition of 20 µL 100 mM Anl for 30 minutes. The supernatant was then removed, and the beads washed with 1 mL H_2O, reduced with 0.5 mL DTT (1 mM, 15 minutes at 70°C), and alkylated with 0.5 mL iodoacetamide (40 mM, 30 minutes at room temperature, protected from light). The resin was

then washed extensively to remove non-specifically bound proteins, using 40 mL each of 0.8% SDS in PBS, 8 M urea in 100 mM tris (pH 8.0), and 20% acetonitrile (in 5 mL aliquots). After washing, the resin was transferred to an eppendorf with 100 µL trypsin digestion buffer (10% acetonitrile in 50 mM ammonium bicarbonate). This was supplemented with 0.1 ug of sequencing grade trypsin, and incubated with shaking at 37°C overnight to digest the resin-bound proteins. The supernatant was then collected, combined with two washes of the resin (150 µL 10% acetonitrile each), and lyophilized. To investigate the non-specific background of this enrichment, this same protocol was also carried out on cells that had been pulsed with 2 mM methionine instead of Anl.

Sample Preparation for Shotgun Proteomics. After affinity enrichment, digested peptides were resuspended in 100 uL of 50 mM ammonium bicarbonate, treated with a HiPPR detergent removal spin column (Pierce) to remove trace SDS, and desalted with a C18 ZipTip (EMD Millipore). The eluate was lyophilized, and resuspended in 0.2% formic acid for LC-MS/MS analysis. An aliquot of each sample was diluted in water and quantified using the LavaPep Fluorescent Protein and Peptide Quantification Kit (Gel Company), to estimate the appropriate volume to analyze by mass spectrometric analysis.

Mass Spectrometry. Analyses were performed on a hybrid LTQ-Orbitrap Elite (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source connected to an EASY-nLC II instrument (Thermo Fisher Scientific) as described previously[6]. Separation of peptides was performed using a 15-cm reversed phase analytical column (75 µm ID) with 3 µm C18 beads (ReproSil-Pur C18-AQ) with a gradient of 2% solvent B for 5 minutes followed by an increase from 2% to 30% in 115 min and lastly a sharp rise to 100% B in 1 min. Solvent A was 0.2% formic acid, 2% acetonitrile, 97.8% LC-MS water and solvent B was 0.2% formic acid, 80% acetonitrile, 19.8% LC-MS water. The mass spectrometer was operated in data-dependent mode. Survey full scan mass spectra were acquired with a resolution of 120,000 at 400 m/z. The top 20 most intense ions from the survey scan were isolated and, after the accumulation of 5000 ions, fragmented in the linear ion trap by collision induced dissociation. Precursor ion charge state screening was enabled and singly charged and unassigned charge states were rejected. The dynamic exclusion list was enabled with a relative mass window of 10 ppm. An additional exclusion list included common trypsin peptide masses. Data analysis was performed using MaxQuant software (v. 1.5.3.8).[7] Spectra were extracted from the raw files using MaxQuant with match between runs. Spectra were searched against UniProt Chinese Hamster database (23888 entries) and a contaminant database (246 entries). Digestion enzyme was specified as trypsin with up to two missed cleavages. Variable modifications

included methionine oxidation (+15.9949), N-terminal protein acetylation (+42.0106), Met to Anl (+23.0450), and Met to Anl to Lys modification (-2.9455) and a fixed modification of cysteine carbamidomethylation (+57.0215). The Met to Anl to Lys modification is for residues in which Anl is incorporated at Met codons, wherein the azide side chain of this Anl does not form a triazole linkage during cycloaddition reaction, and it is reduced to a primary amine during proteomics workup and sample preparation. Precursor mass tolerance was less than 4.5 ppm after mass recalibration by MaxQuant. Fragment ion tolerance was 0.5 Da. Protein and peptide false discovery rates were fixed at 1% and estimated using a decoy database search performed by MaxQuant. Annotation of proteins into different cellular components was performed using STRAP software.[8]

Figure S1. The pMaRSWT_G vector for expression of wild-type *Mm*MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence.

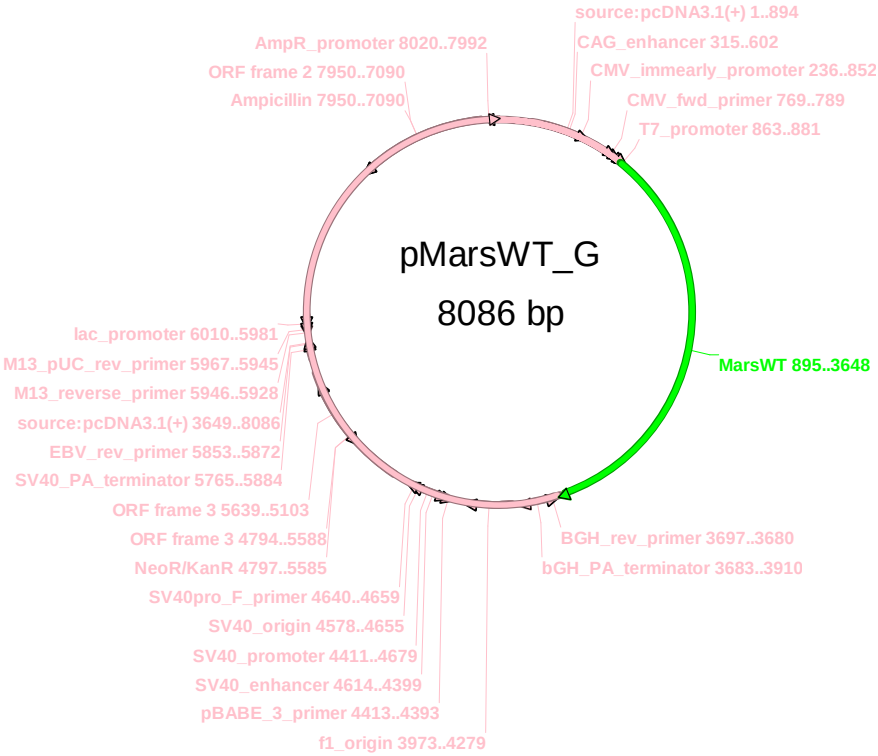


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Figure S2. The pMaRSL274G_G vector for expression of L274G*Mm*MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence. This construct is available through Addgene.

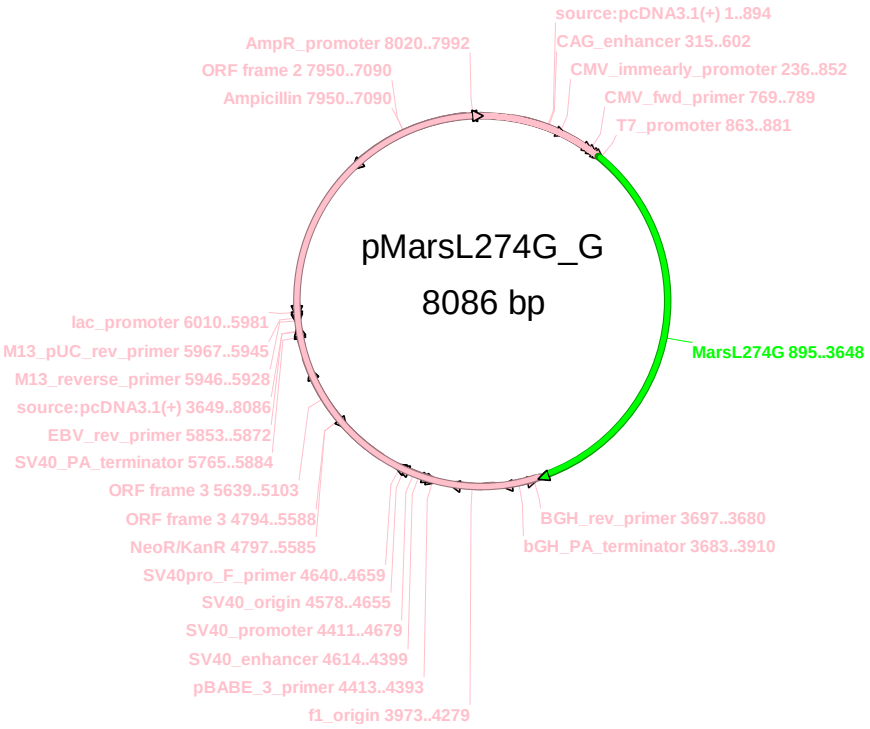

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Figure S3. The pMaRSSLL_G vector for expression of SLL-*Mm*MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence.

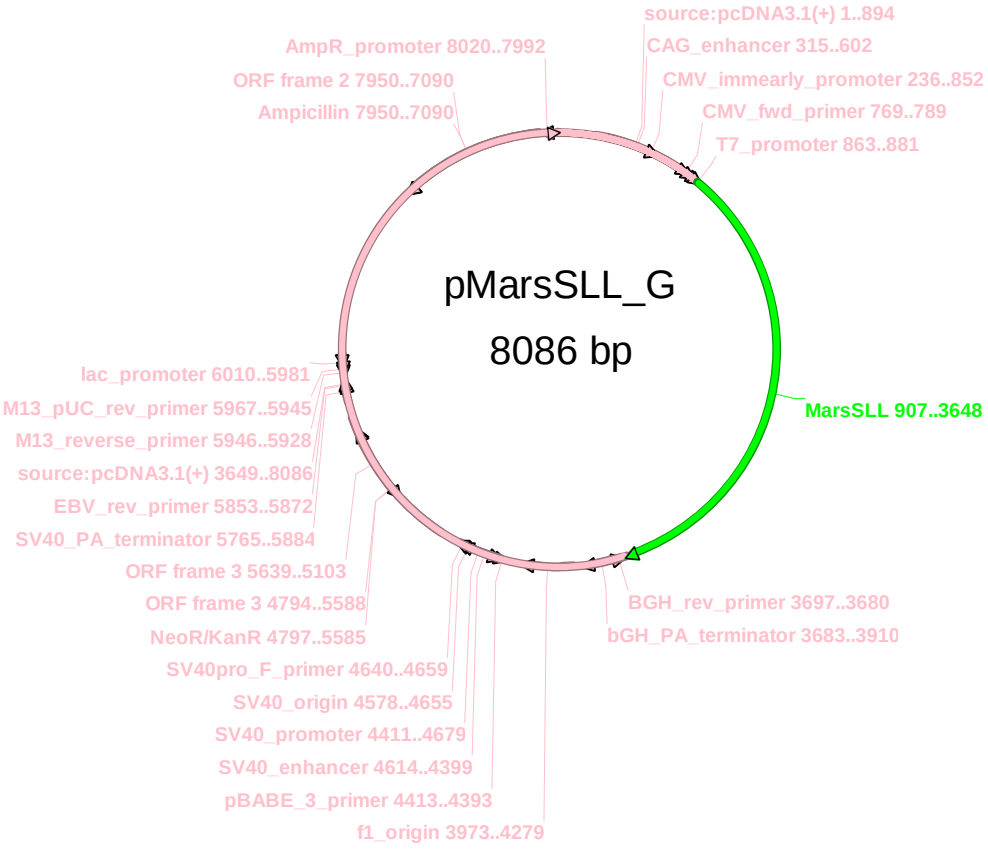

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Figure S4. The pMaRSPLL_G vector for expression of PLL-*Mm*MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence.

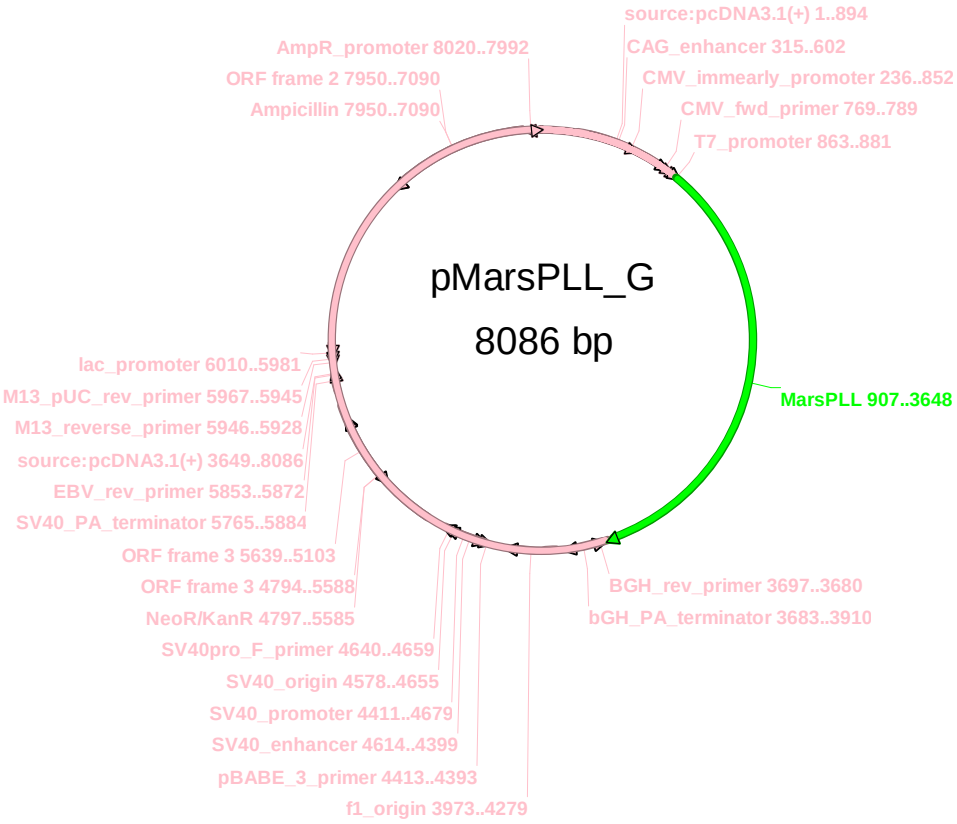

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Figure S5. The pMaRSCLL_G vector for expression of CLL-*Mm*MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence.

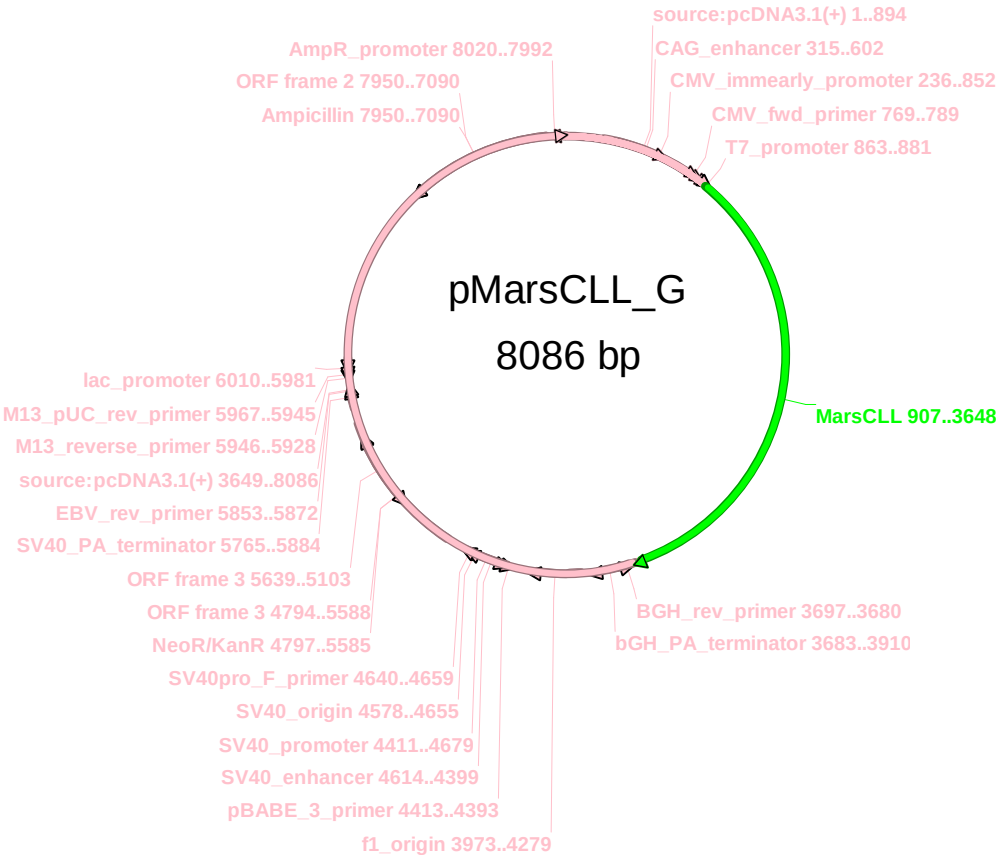

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Figure S6. The pMaRSNLL_G vector for expression of NLL-*Mm*MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence.

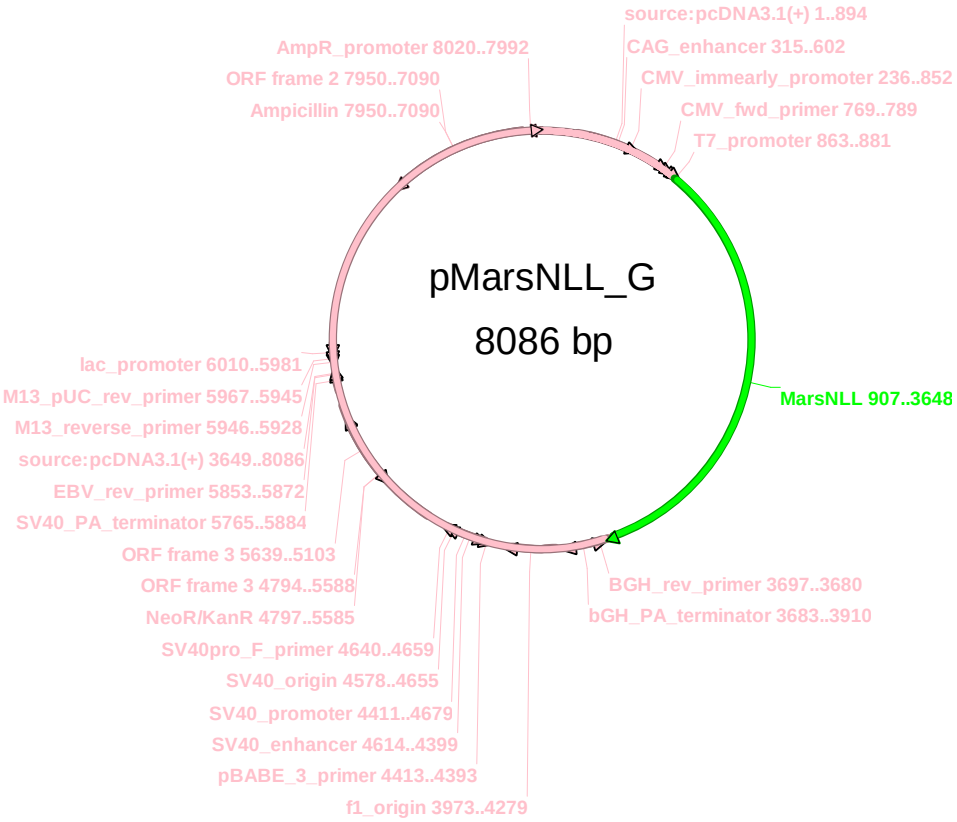

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Figure S7. The pMetRSNLL_G vector for expression of the *E. coli* NLL-MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow, start codon in red, and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence.

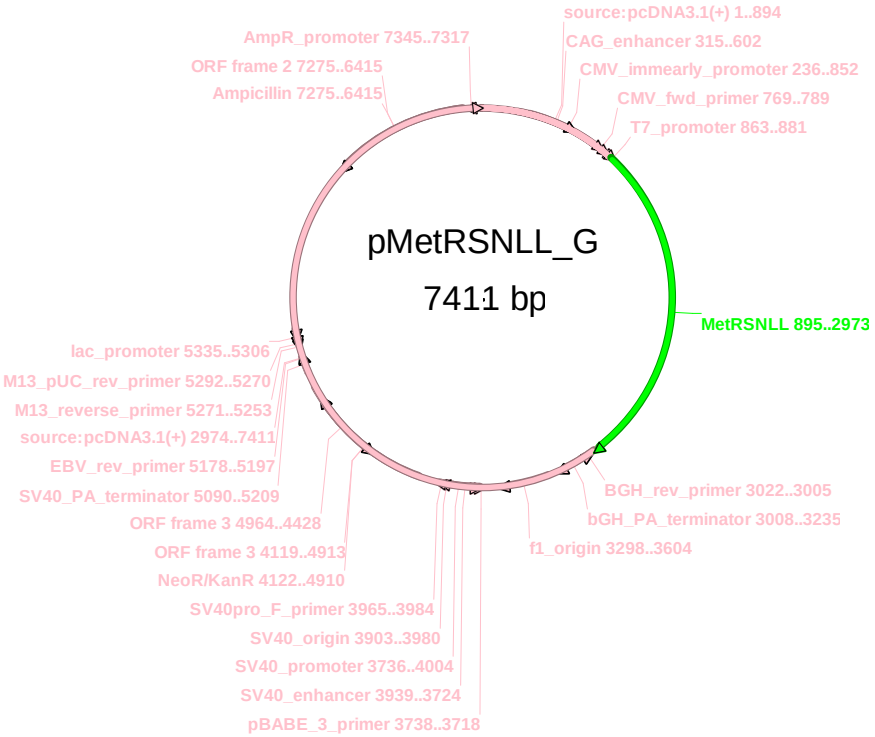

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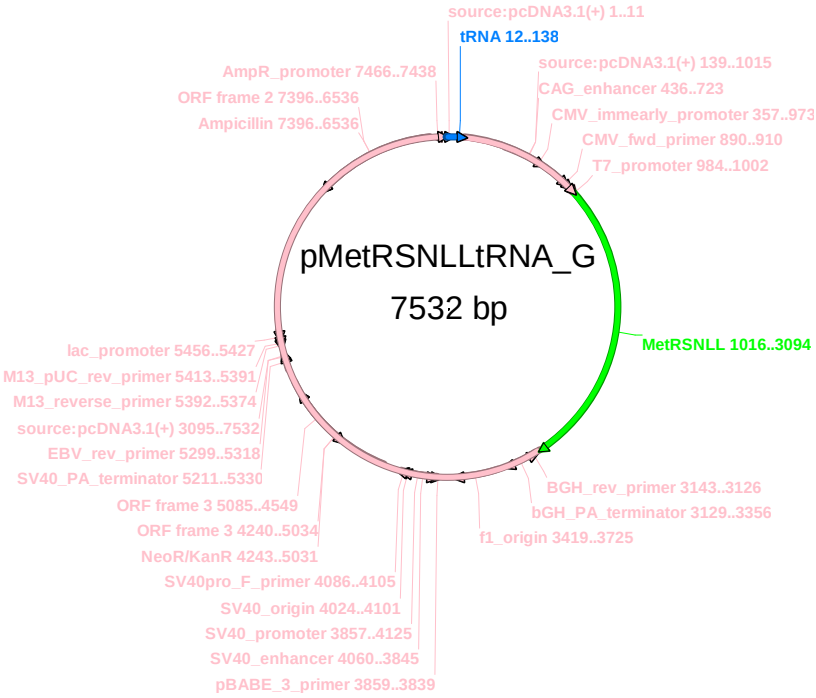
Figure S8. The pMetRSNLLtRNA_G vector for expression of the *E. coli* NLL-MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow, start codon in red, and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence. This plasmid also contains the tRNA expression cassette as outlined in Figure 1b of the main text. The tRNA cassette was inserted into the BglII restriction site in the plasmid and is lighted in pink.



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NRIDMRQVEALVEASKEEVKAAAAPVTGPLADDPIQETITFDDFAKVDLRVALIENAEFVEGSDKLLRLTLDLGGEK
RNVFSGIRSAYPDPQALIGRHTIMVANLAPRKMRFGISEGMVMAAGPGGKDIFLLSPDAGAKPGHQVK-

Figure S9. The pMetRSNLLtRNAdcca_G vector for expression of the *E. coli* NLL-MetRS under CMV promoter control. Restriction enzymes are highlighted in yellow, start codon in red, and the enzyme coding sequence is highlighted in green. Kozak sequence is highlighted in blue. The expressed protein sequence is included after the plasmid sequence. This plasmid also contains the tRNA expression cassette as outlined in Figure 1b of the main text, wherein the CCA tail of the tRNA was removed in this sequence. The tRNA cassette was inserted into the BglII restriction site in the plasmid and is lighted in pink.

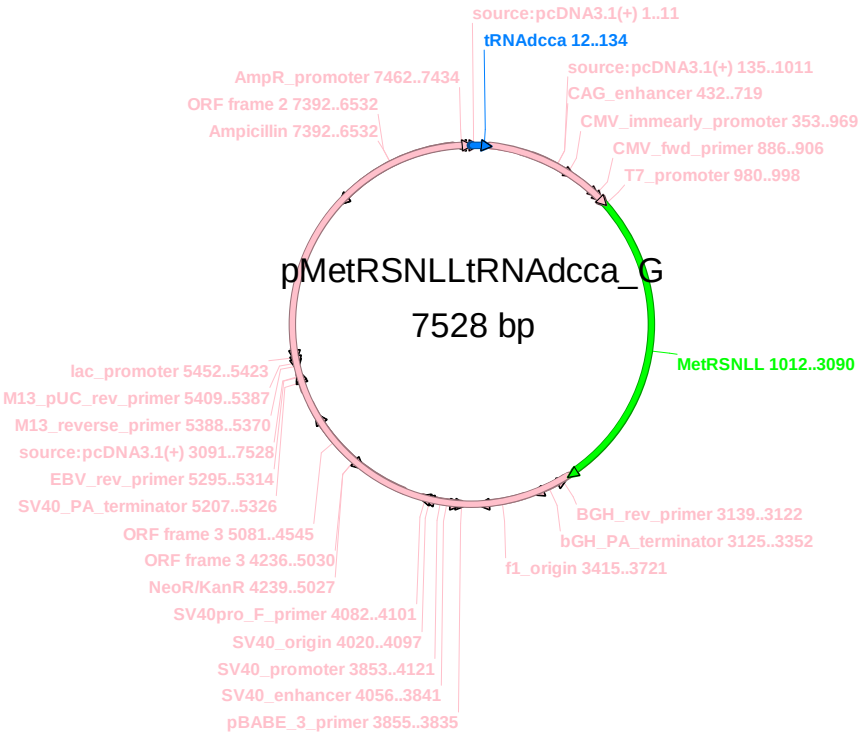

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Figure S10. Structure of the alkyne-TAMRA dye.

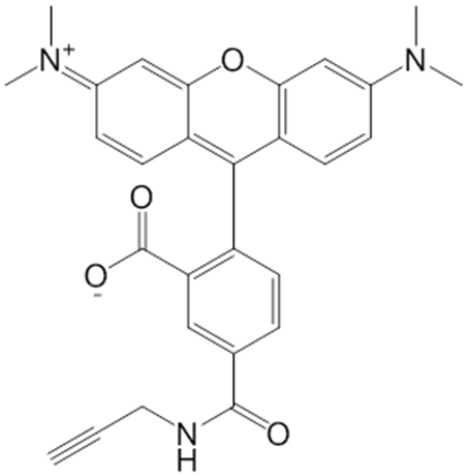


Figure S11.

Protein loading controls corresponding to Figure 1(d) of the main text. Cell lysate proteins which were labeled with TAMRA-alkyne dye and electrophoresed on a polyacrylamide SDS gel were subsequently stained with colloidal blue dye to determine the relative abundances of proteins in lysates derived from different labeling conditions (indicated above the gel) for each cell line.

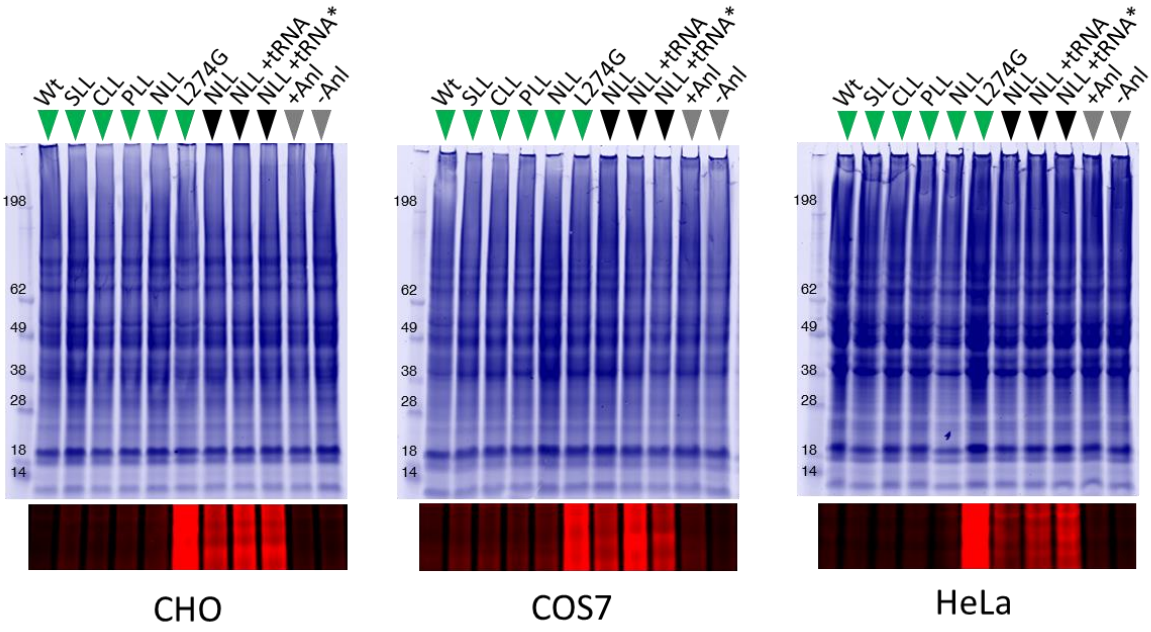


Figure S12. Selectivity of L274G*Mm*MetRS. In-gel fluorescence was used as a measure of Anl incorporation into cellular proteins. At a fixed concentration of Met, changes in Anl concentration determine the extent of protein labeling, which depends on the relative rates of activation of Anl and Met by L274G*Mm*MetRS.

Activation of Anl: $$E + A \leftrightarrow_{k_{-1}^A}^{k_1^A} ES_A \overset{k_2^A}{\rightarrow} E + P_A \quad (1)$$

Activation of Met: $$E + M \leftrightarrow_{k_{-1}^A}^{k_1^A} ES_M \overset{k_2^M}{\rightarrow} E + P_M \quad (2)$$

where ES_A is the complex of L274G*Mm*MetRS with Anl (A) and ES_M is the complex of L274G*Mm*MetRS with Met (M). P_A is tRNA^{Met} charged with Anl; P_M is tRNA^{Met} charged with Met.

$$\text{Let} \quad K^A = \left(\frac{k_{cat}}{k_M}\right)^{Anl} \text{ and } \quad K^M = \left(\frac{k_{cat}}{k_M}\right)^{Met}$$

Because A and M compete for L274G*Mm*MetRS (see Fersht (Eq. 3.44)[9]):

$$\frac{V_A}{V_M} = \frac{A}{M}\frac{K^A}{K^M} \quad (3)$$

where V_A and V_M are the rates of activation of Anl and Met, respectively.

For a fixed Anl concentration the total rate of substrate activation by L274G*Mm*MetRS is a constant (C):

$$C = V_A + V_M$$

Rearranging (3) gives: $K^A = \frac{V_A}{V_M}\frac{M}{A}K^M$

Eliminating V_M: $K^A = \frac{V_A}{C - V_A}\frac{M}{A}K^M$ or $V_A = \frac{K^A C}{\frac{M}{A}K^M + K^A}$ (4)

Dividing numerator and denominator by K^M yields:

$$V_A = \frac{A\left(\frac{K^A}{K^M}\right)C}{M + A\left(\frac{K^A}{K^M}\right)} \quad (5)$$

where $\left(\frac{K^A}{K^M}\right)$ is the selectivity, C is a constant and the Met and Anl concentrations are known. Measured values of in-gel fluorescence were fit to Equation 5 by least-squares methods at different concentrations of Anl and Met to determine the selectivity of L274G*Mm*MetRS.

The following Matlab functions were used to fit the data from Figure 2A in the main text. The resulting parameter fits yielded a selectivity of 0.2508 and C = 1.089.

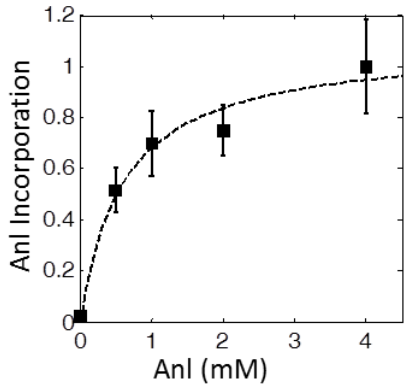


Figure S12A. Selectivity of L274G*Mm*MetRS. In-gel fluorescence was used as a measure of Anl incorporation into cellular proteins. At a fixed concentration of Met, changes in Anl concentration determine the extent of protein labeling, which depends on the relative rates of activation of Anl and Met by L274G*Mm*MetRS.

```
---------------------------------
function fitfluorescenceR
function m = FF(x , xdata)
for i=1:size(xdata)
m(i)=((xdata(i)*x(1)*x(2))/((0.15+xdata(i)*x(1))));
end
m=m';
end

A=[0; 0.5; 1;  2; 4];
fl=[0.022871044;    0.516731636;    0.699062794; 0.752107911;   1]
err=[0.002135075;   0.086032748;    0.127708537;    0.098922244;    0.183627695];
errorbar(A,fl,err,'ro');

% initial guess for parameters
x0=[0.5 1] % This is the selectivity parameters
% second 1 this is constant C
[x,resnorm, residual,~,exitflag,output]=lsqcurvefit(@FF,x0,A,fl);
hold on
counter=0; for i=0:0.1:4.5, counter=counter+1; II(counter)=i;
F(counter)=(i*0.2508*1.089)/(0.15+i*0.2508);end
plot(II,F)

SStotal = (length(fl)-1) * var(fl);
SSresid=sum(residual.^2);
rsq = 1 - SSresid/SStotal
end
-------------------------------------------------
```

We tested the selectivity parameters that we obtained from Figure 2A to compare the predicted and observed Anl incorporation levels at the different Met concentrations used in Figure 2B. The following Matlab functions were used for Figure 2B in the main text. The data in Figure 2B were first normalized so that at a Met concentration of 0.15 mM, the level of Anl incorporation corresponds to that of Figure 2A; the concentration of Met used in Figure 3A is a constant 0.15 mM.

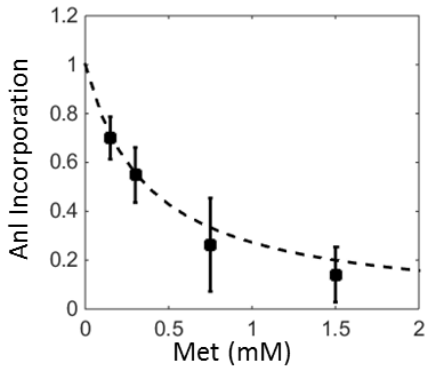


Figure S12B. Selectivity of L274G*Mm*MetRS. In-gel fluorescence was used as a measure of Anl incorporation into cellular proteins at a fixed Anl concentration with varying concentrations of Met. Selectivity parameters that were obtained from Figure 2A were used to obtain the predicted Anl incorporation levels shown in dotted line.

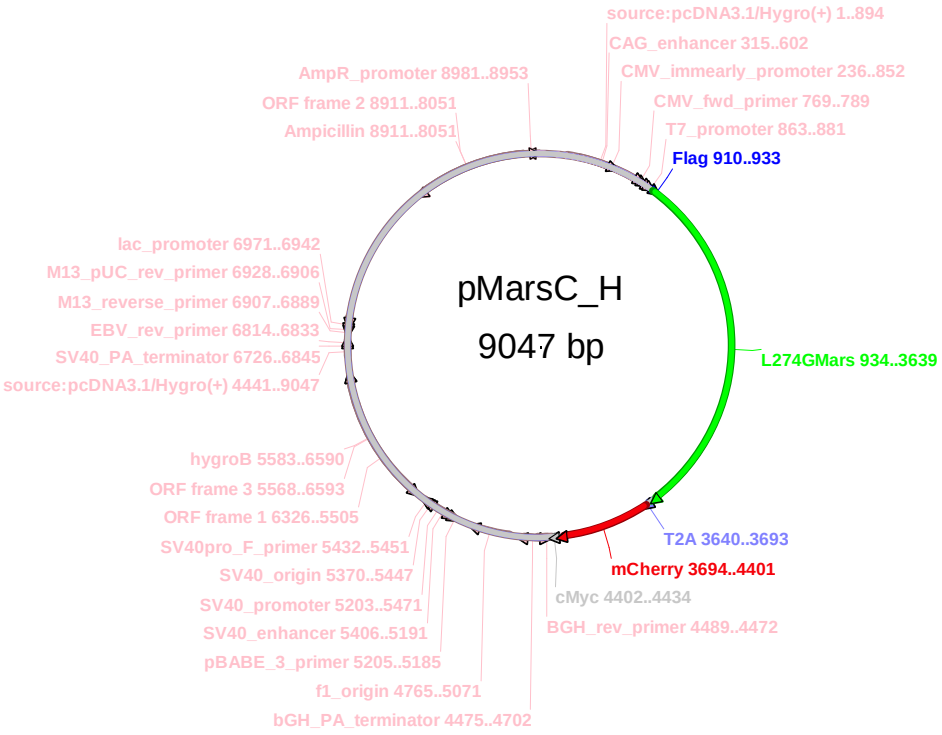
```
--------------------------------
function fitfluorescencetst
function m = FF(x , xdata)
for i=1:size(xdata)
m(i)=((1.5*x(1)*1)/((xdata(i)+1.5*x(1))))
end
m=m';
end

M=[0.15;    0.3;    0.75;   1.5];
fl=[0.7;    0.548851071;    0.263135683;    0.14119813];
err=[0.086722315;   0.112658654;    0.19100414; 0.11272512];
errorbar(M,fl,err,'ro');

counter=0; for i=0:0.1:2, counter=counter+1; II(counter)=i;
F(counter)=(1.5*0.25)/(i+1.5*0.25);end
plot(II,F)

SStotal = (length(fl)-1) * var(fl);
SSresid=sum(residual.^2);
rsq = 1 - SSresid/SStotal
end
-----------------------------------------------
```

Figure S13. The pMaRSC vector for expression of L274G*Mm*MetRS and mCherry proteins. The cassette inserted into the Nhe1/Xho1 restriction sites of the pcDNA3.1 plasmids is color coded corresponding to highlighted sequences. Kozak sequence is highlighted in light blue.The pMaRS plasmid is the same construct as pMaRSL274G, and below is the sequence for pMaRSC which contains a T2A-Mcherry sequence appended to the C-terminal of L274G*Mm*MetRS. This construct is available through Addgene.



Nhe1Flag-tagMaRS*T2AMcherryMyc-tagXho1

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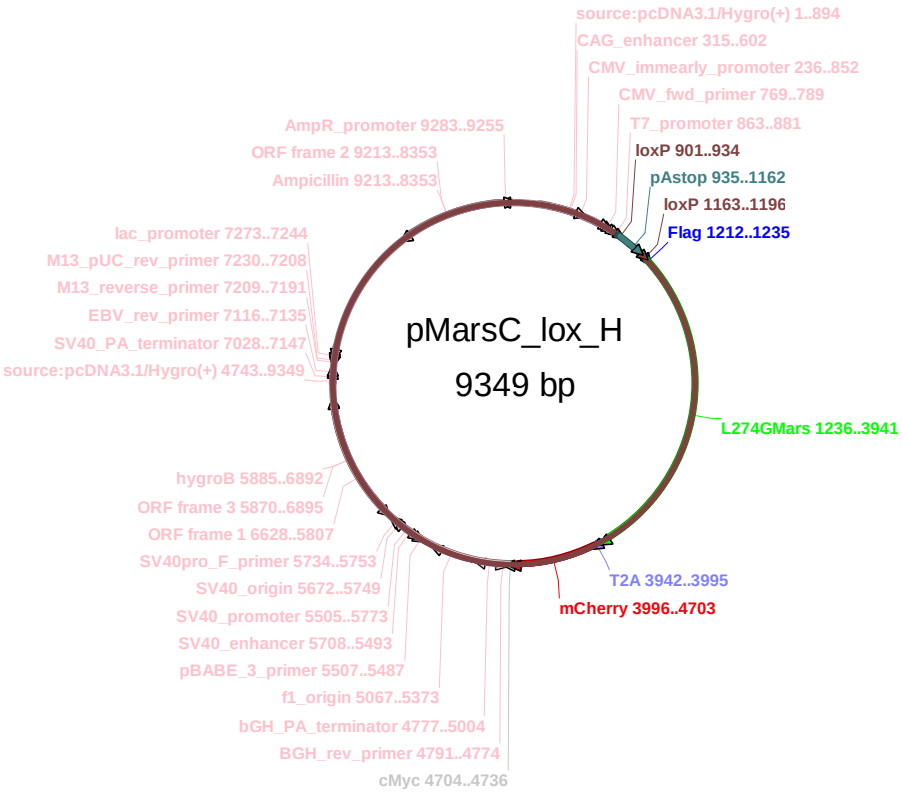
STGGMDELYKEQKLISEEDL-

Figure S14. The pMarlox vector for expression of L274G*Mm*MetRS and mCherry proteins. The cassette inserted into the Nhe1/Xho1 restriction sites of the pcDNA3.1 plasmids is color coded corresponding to highlighted sequences. Kozak sequence is highlighted in blue. The loxP sequences and transcriptional stop sequence are inserted into the Nhe1 site of this plasmid, thereby introducing a second Nhe1 site.



Nhe1loxpstoploxpFlagMaRS*T2AMcherryMycXho1

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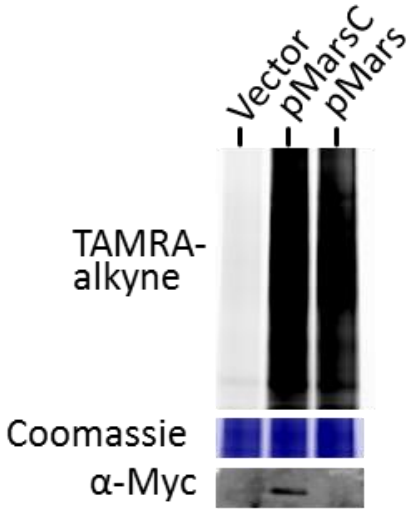
ASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRH
STGGMDELYKEQKLISEEDL-

Figure S15. Western blot for detection of L274G*Mm*MetRS and Anl labeled proteins. Metabolic incorporation of Anl by pMaRS- and pMaRSC-transfected CHO cells. The in-gel fluorescence image on top shows TAMRA labeling, which indicates Anl incorporation. Western blot at the bottom using anti-Myc antibody shows the detection of mCherry at approximately 25 kDa.



Western blot using a Myc-tag-Alexa Fluor 488 conjugate monoclonal antibody was used to probe for mCherry in lystes of CHO cells transfected with pMaRSC and pMaRS vectors. The pMaRSC lane shows a protein band at approximately 25 kDa corresponding to mCherry and no other bands at higher molecular weights indicating that mCherry is not fused to L274G*Mm*MetRS. The pMaRS vector lacks the mCherry sequence and as anticipated we do not observe a protein band corresponding to mCherry in the cell lysates.

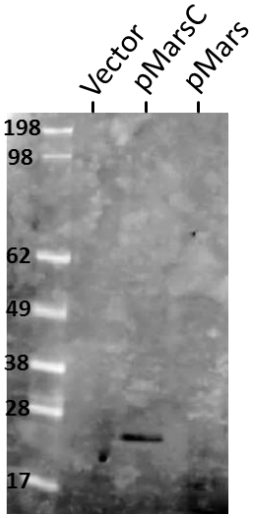
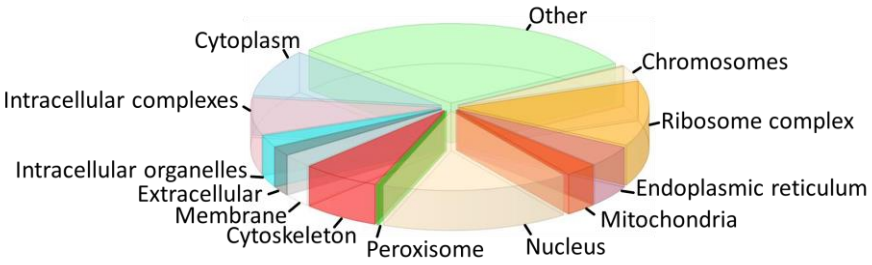
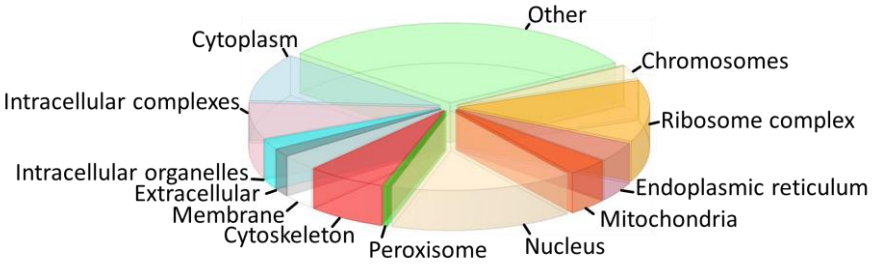


Figure S16. Identification of Anl-labeled proteins by tandem mass spectrometry and annotation in terms of cellular components using STRAP software. BONCAT was used to identify proteins made in CHO cells that constitutively express the L274G*Mm*MetRS under control of the CMV promoter. Cells were labeled in media containing either 2 mM Anl or with 2 mM Met. The Met samples were used to account for proteins that bind non-specifically to the resin during enrichment. Two biological replicates were performed for each amino acid with independent mass spectrometric runs for each. The charts below show the distributions of proteins identified in the Anl-labeled samples.

Replicate 1 (884 proteins)



Replicate 2 (959 proteins)



The proteins identified in either of the Met samples were removed from the list of proteins identified in either of the Anl samples. The result of this analysis is shown in the chart below and included in the main text in Figure 3d.

Proteins found only in Anl-labeled samples (786 proteins)

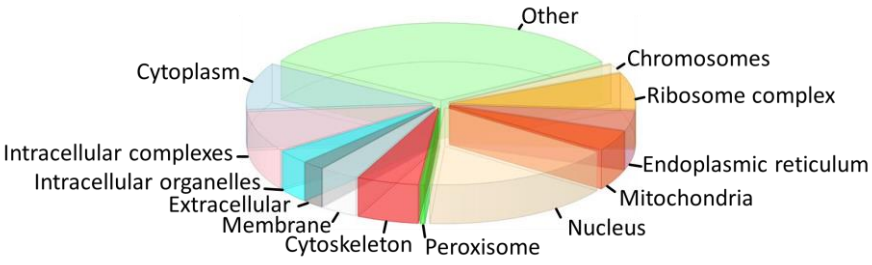


Figure S17. Assessment of enrichment and reproducibility in BONCAT analysis. The extent to which BONCAT allows enrichment of newly synthesized proteins was assessed by comparing the numbers of proteins found in Anl- and Met-treated samples, and by comparing spectral counts. We identified a total of 847 proteins that were found in both Anl replicates, of which 724 were not present in the Met controls. There were 129 proteins that were in both Met samples (a). Combining the total proteins identified in either of the Anl samples resulted in 996 proteins, 786 of which were not present in either of the Met samples (b). Comparison of the proteins identified in the two Anl replicates (c) and the two Met replicates (d) shows low sample-to-sample variability for the Anl replicates. Reproducibility of quantified protein levels across biological replicates was determined using MaxQuant's label-free quantification (LFQ) value. LFQ serves as a normalized measure of relative protein abundance. These results are shown as dot plots of LFQ levels between Anl replicates (e) and Met replicates (f). On average, we observed 19-fold more spectral counts for Anl samples than for Met samples (g). The numbers in panel g represent the spectral counts acquired in individual MS runs.

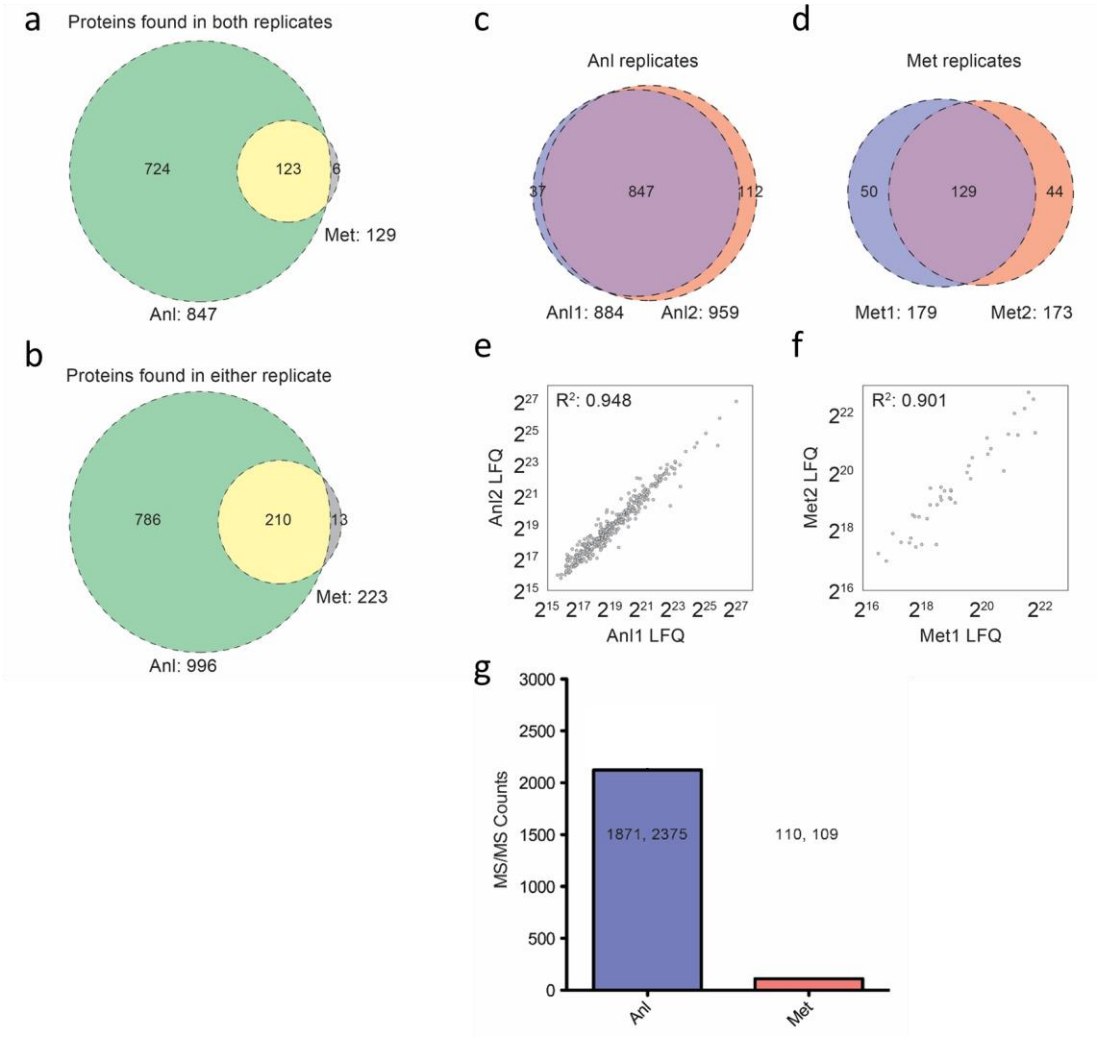
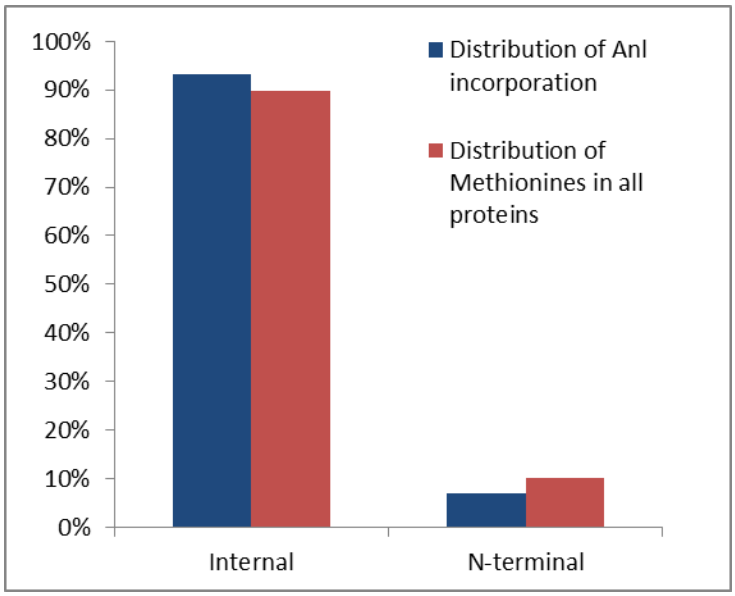


Figure S18. Incorporation of Anl at terminal and internal Met sites in HeLa cells. According to UniProt (http://www.uniprot.org/), there are 711,731 internal and 79,802 N-terminal Met residues in the human proteome; internal Met sites constitute 90% of the total. Mass spectrometric analysis of proteins made in HeLa cells expressing the L274G*Mm*MetRS (see table S2 for protein list) identified 161 sites of Anl incorporation; 150 (93%) at internal Met positions.



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