Supporting Information Appendix for:

Mutant Methionyl-tRNA Synthetase from Bacteria Enables Site-Selective N-terminal Labeling of Proteins Expressed in Mammalian Cells

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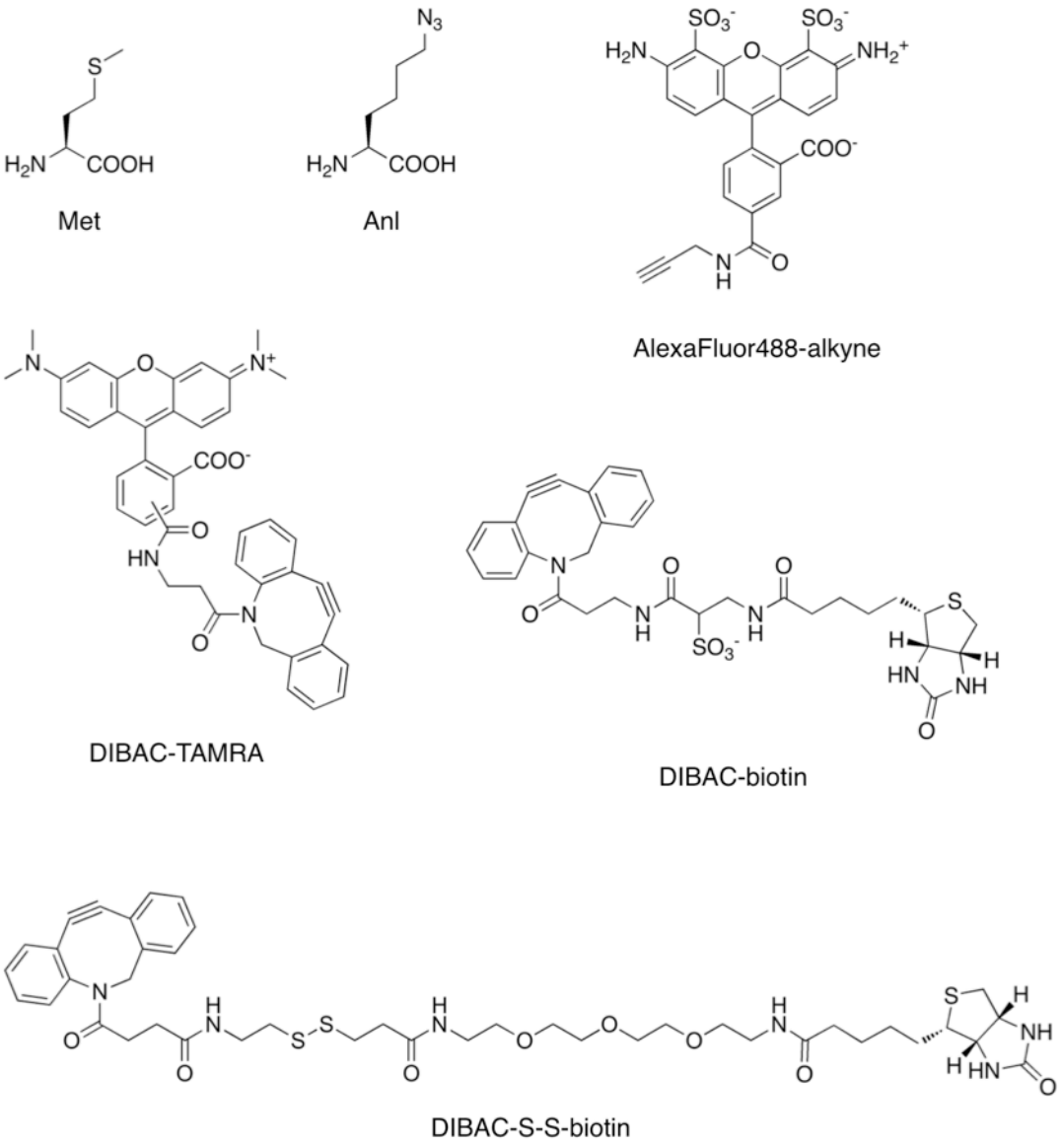

Fig. S1. Structures of the amino acids and probes used in this study.

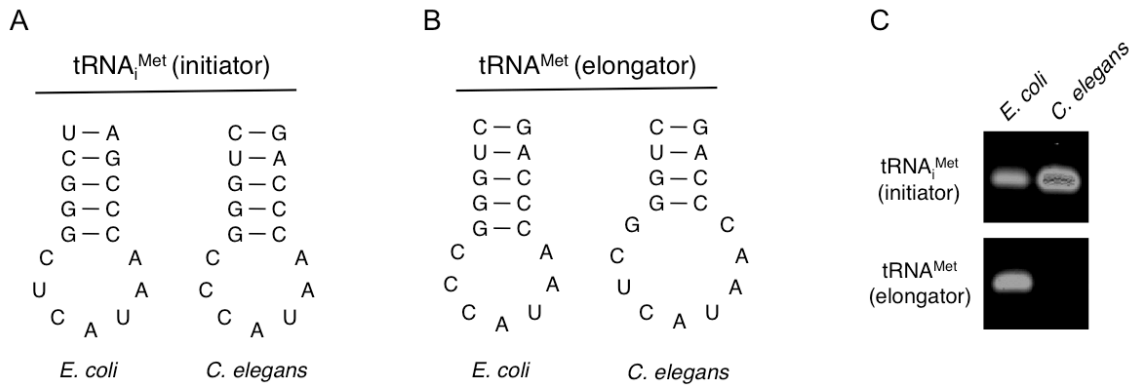

Fig. S2. Selective aminoacylation of initiator tRNA by EcMetRS. Synthetically prepared tRNAs tested with EcNLL-MetRS and Anl. The formation of Anl-tRNAs was examined by conjugation of DIFO-AlexaFluor488 to Anl residues followed by fluorescence scanning of agarose gels. The NLL-EcMetRS, like the wild-type *E. coli* MetRS, does not charge eukaryotic cytoplasmic elongator-tRNA^{Met}.

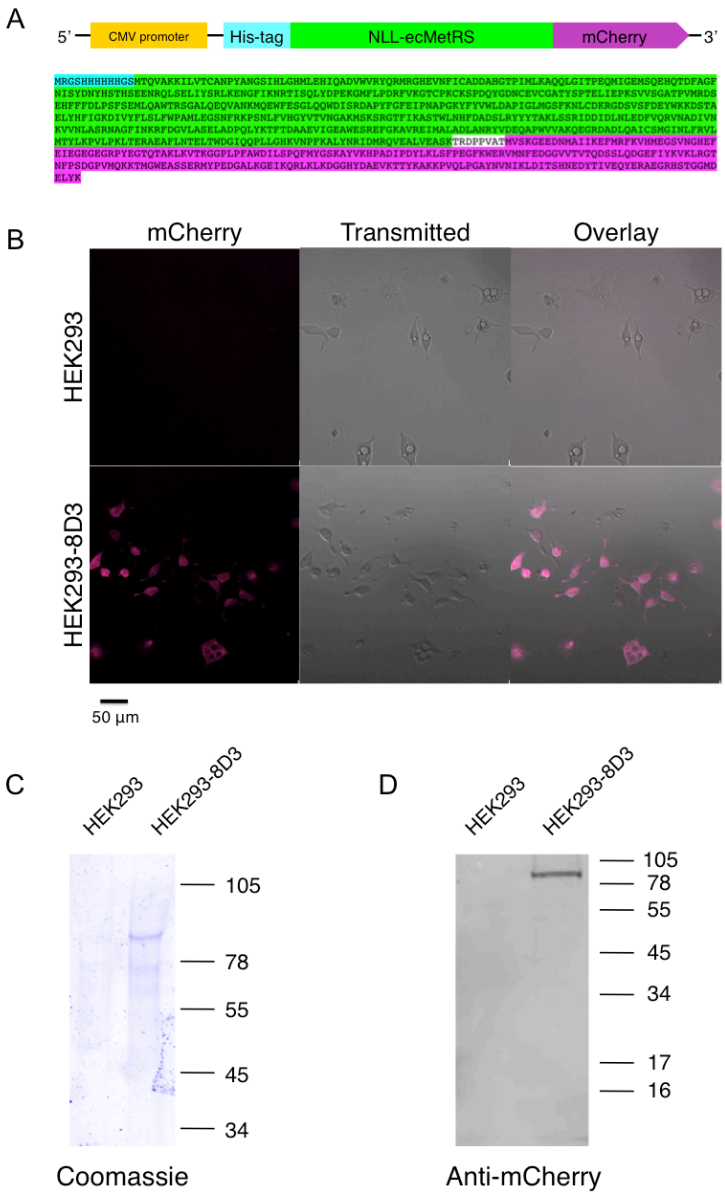

Fig. S3. NLL-EcMetRS is expressed as a full-length fusion with mCherry in the HEK293-8D3 cytoplasm. (*A*) The gene construct used to express a His-tagged NLL-EcMetRS-mCherry fusion (91.4 kDa). The DNA sequence for the bacterial synthetase was originally derived from the *E. coli* genome and expressed in truncated form (Δ548) with mutations corresponding to L13N/Y260L/H301L, which were generated by site-directed mutagenesis. (*B*) HEK293-8D3 cells exhibit broadly distributed fluorescence associated with expression of NLL-EcMetRS-mCherry. (*C*) Affinity purification of NLL-EcMetRS-mCherry from cytosolic proteins via its N-terminal His-tag verified that the protein was soluble and localized to the cytosol. (*D*) Western analysis of cytosolic proteins with anti-DsRed antibody for detection of C-terminal mCherry further confirmed that the protein fusion was fully intact following expression of the bacterial gene.

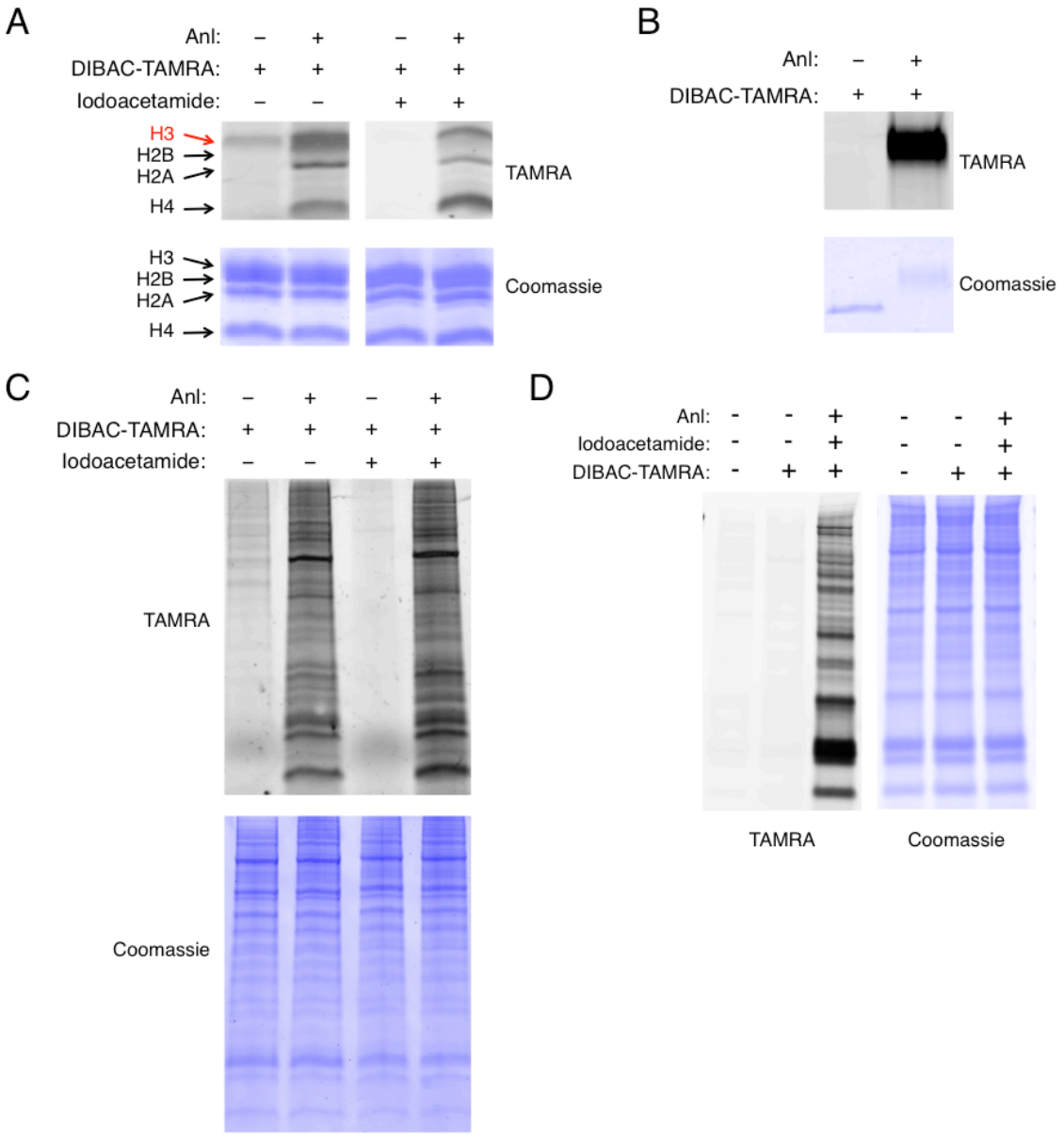

Fig. S4. Cysteine alkylation with iodoacetamide eliminates the background reaction of DIBAC-probes with protein thiols. (*A*) Histone H3 (the only histone that contains cysteine) from unlabeled cells was modified with DIBAC-TAMRA. The background reaction was eliminated following alkylation of cysteine with iodoacetamide. (*B*) Recombinant DHFR does not contain cysteine and unlabeled DHFR was not modified with DIBAC-TAMRA. A DHFR containing 7 Anl residues was modified with DIBAC-TAMRA; a complete band shift of the protein indicated that the reaction was efficient. (*C*) The background reaction between DIBAC-TAMRA and unlabeled proteins in cell lysates was eliminated following alkylation of cysteines with iodoacetamide. (*D*) DIBAC-TAMRA labeling is highly specific for Anl-labeled proteins following alkylation with iodoacetamide. A comparison with proteins not exposed to DIBAC-TAMRA is shown.

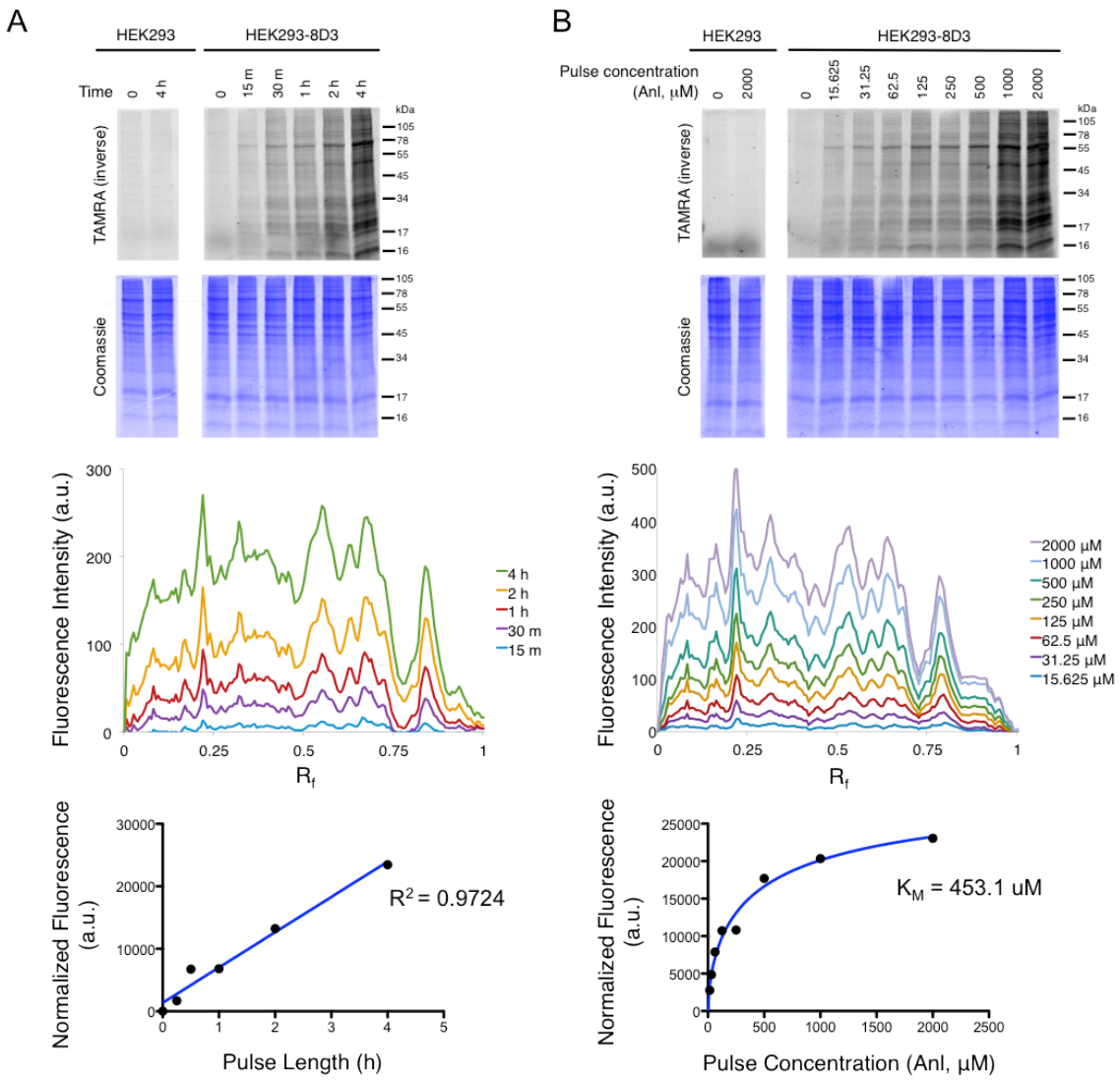

Fig. S5. Quantification of TAMRA intensity on proteins from cells pulsed for various times and with various concentration of Anl. (*A*)Labeled proteins could be detected from HEK293-8D3 cells following a 15 m pulse with 1 mM Anl; the amount of labeled protein increased as the pulse length was extended to 4 h. Proteins were not labeled in HEK293 cells treated with a mM Anl for 4 h. (*B*) Labeled proteins could be detected from HEK293-8D3 cells pulse-labeled with 15.625 μM Anl for 4 h; the extent of labeling increased as the Anl concentration was raised. Proteins were not labeled in HEK293 cells treated with 2 mM Anl for the same time.

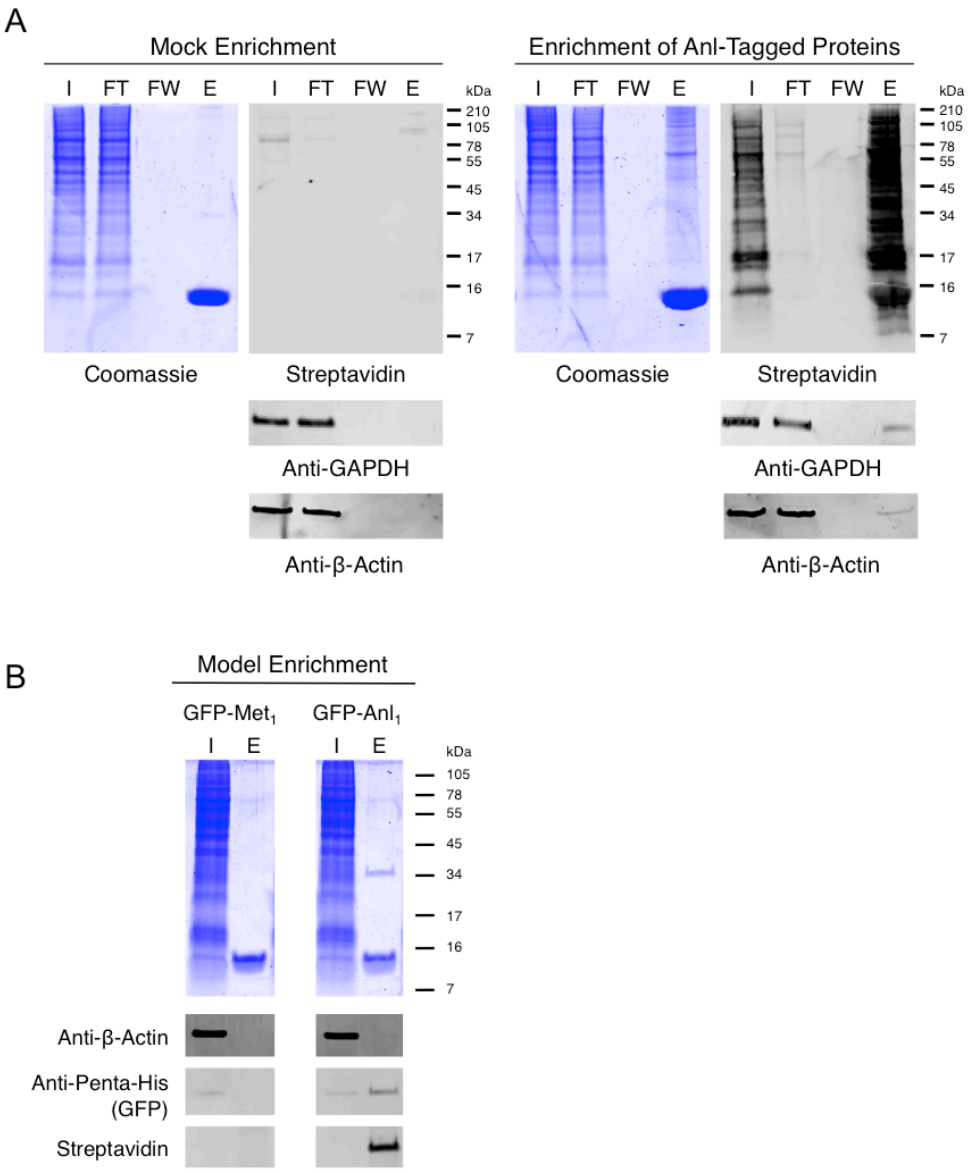

Fig. S6. Enrichment of labeled proteins. (*A*) Lysates from pulsed non-transfected cells ("mock enrichment") and pulsed HEK293-8D3 cells were treated with DIBAC-biotin (input, "I"), and biotinylated proteins were collected on immobilized streptavidin. The unbound fraction (flow-through, "FT"), and final wash "FW" were collected and compared to the elution "E." The enrichments were followed using Coomassie staining of SDS-PAGE gels and parallel detection of biotinylated proteins via Western blot with fluorescent streptavidin. (*B*) The extent of enrichment was estimated using purified GFPs (28.2 kDa) containing a single Anl or Met, which were mixed with unlabeled lysate at a ratio of 1:1,000 (GFP:lysate, w/w). The mixtures were subjected to the enrichment procedure using DIBAC-biotin and the extent of enrichment (more than 500-fold) was determined by comparing initial mixtures with eluted fractions.

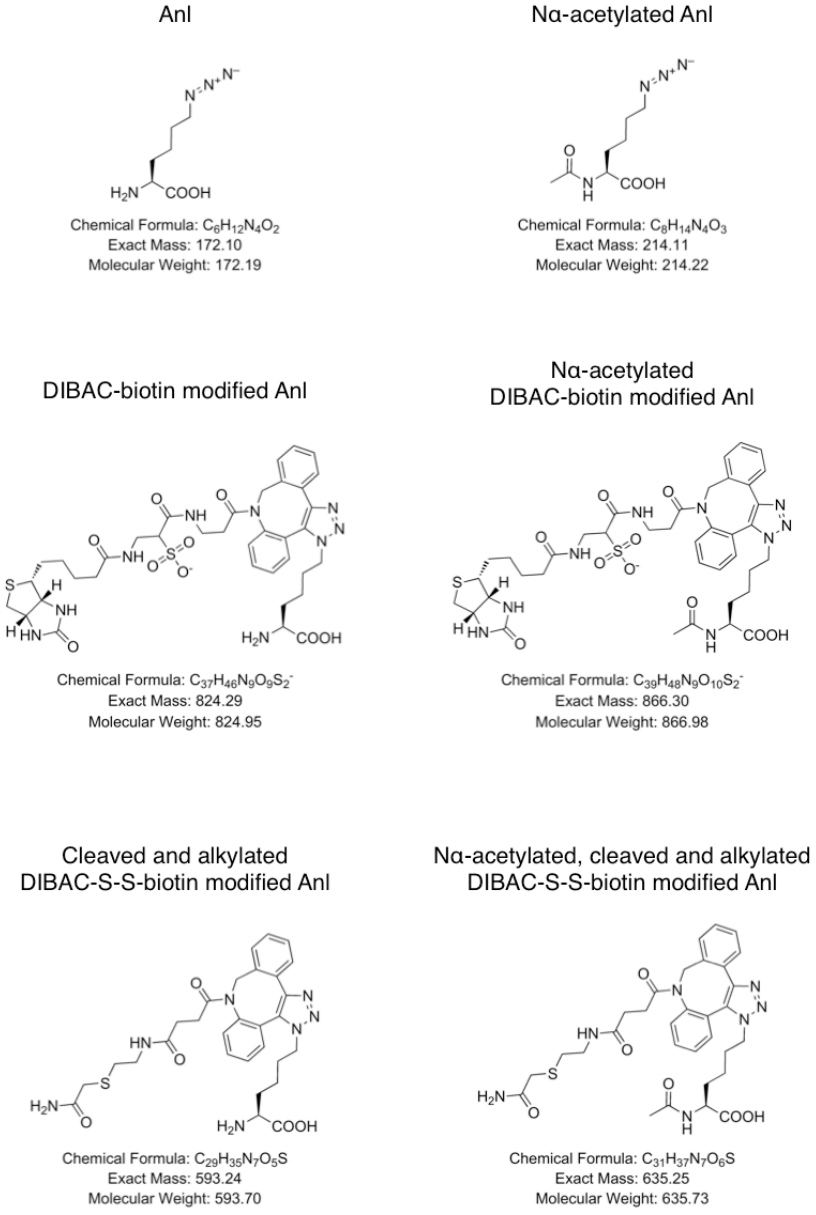

Fig. S7. Structures, masses, and formulas of the Anl-derived modifications used as variable substitutions in the peptide identification search.

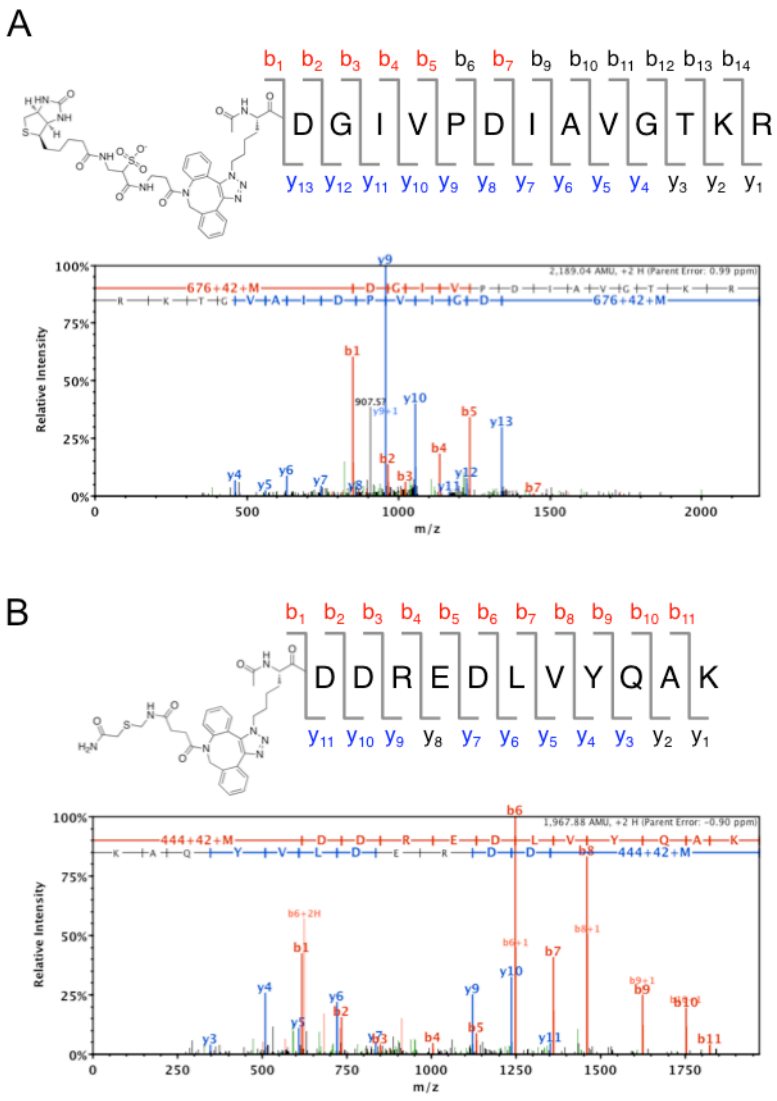

Fig. S8. Detection of N-terminal Anl residues by mass spectrometry. (*A*) Ion fragmentation spectrum of the N-terminal tryptic peptide of PTBP-1 containing modified Anl. Mass shifts corresponding to N^{α}-acetylation of Anl (+42) and modification with DIBAC-biotin (+676) are shown relative to Met (M). (*B*) Spectrum of the N-terminal peptide of 14-3-3 protein ε, which was identified by analysis of proteins enriched after treatment with DIBAC-S-S-biotin. The mass shifts caused by N^{α}-acetylation of Anl and by modification with the reduced and alkylated probe (+444) are shown relative to Met. Additional spectra of peptides containing N-terminal Anl modification are provided in Fig. S10.

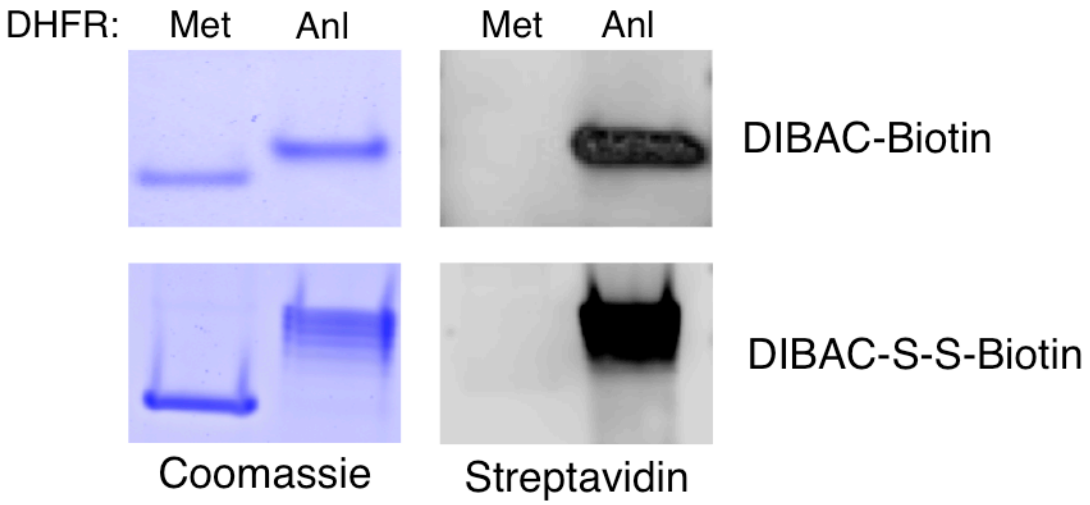

Fig. S9. Comparison of DIBAC-biotin and DIBAC-S-S-biotin. DIBAC-biotin and DIBAC-S-S-biotin specifically labeled Anl side chains in the absence of free thiols. The model DHFR used as the labeling substrate does not contain cysteine residues.

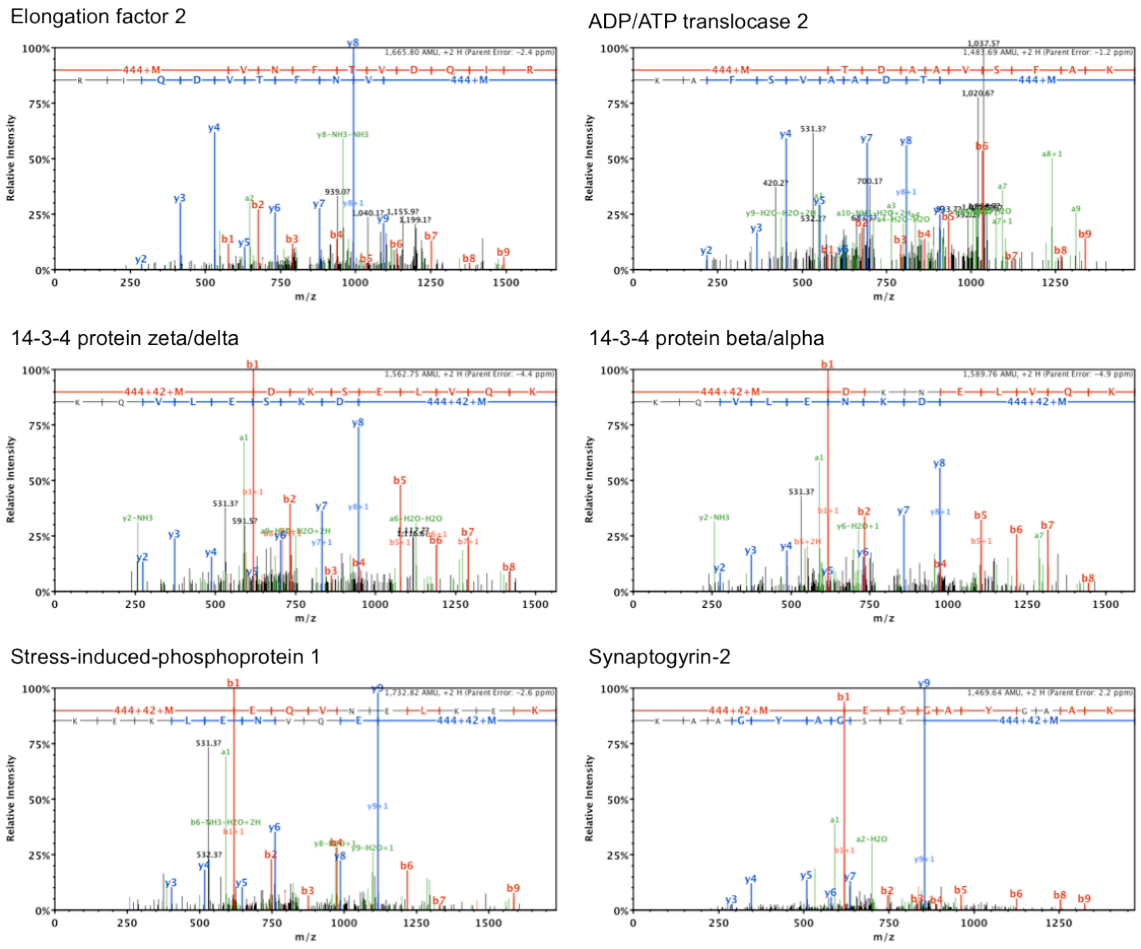

Fig. S10. Spectra of additional N-terminal peptides identified with Anl modifications.

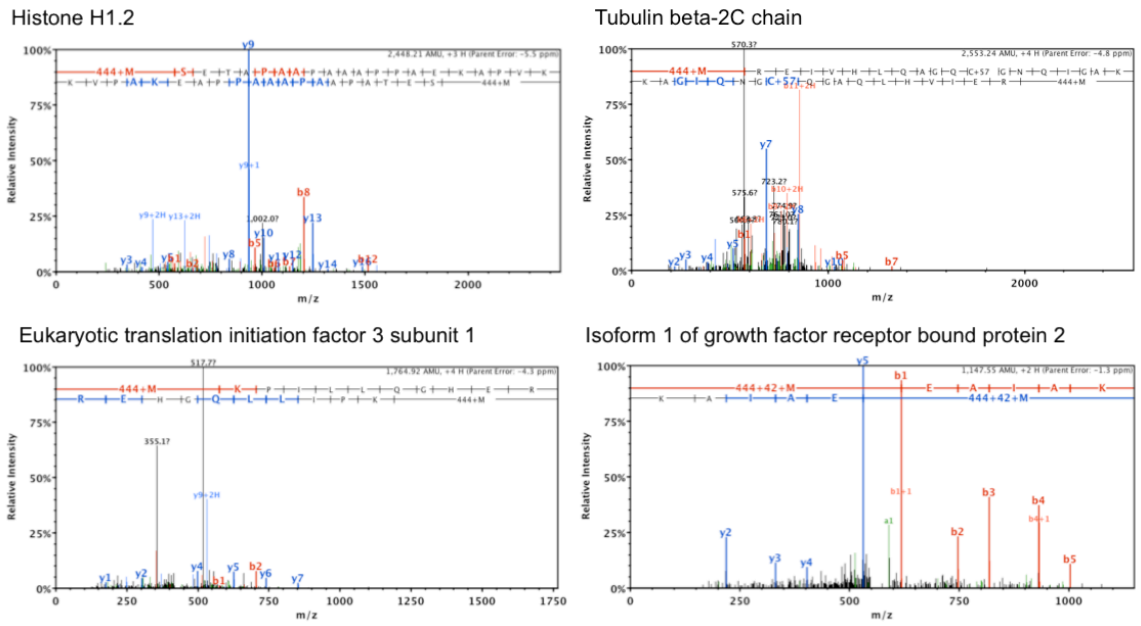

Fig. S10 (continued) Spectra of additional N-terminal peptides identified with AnI modifications.

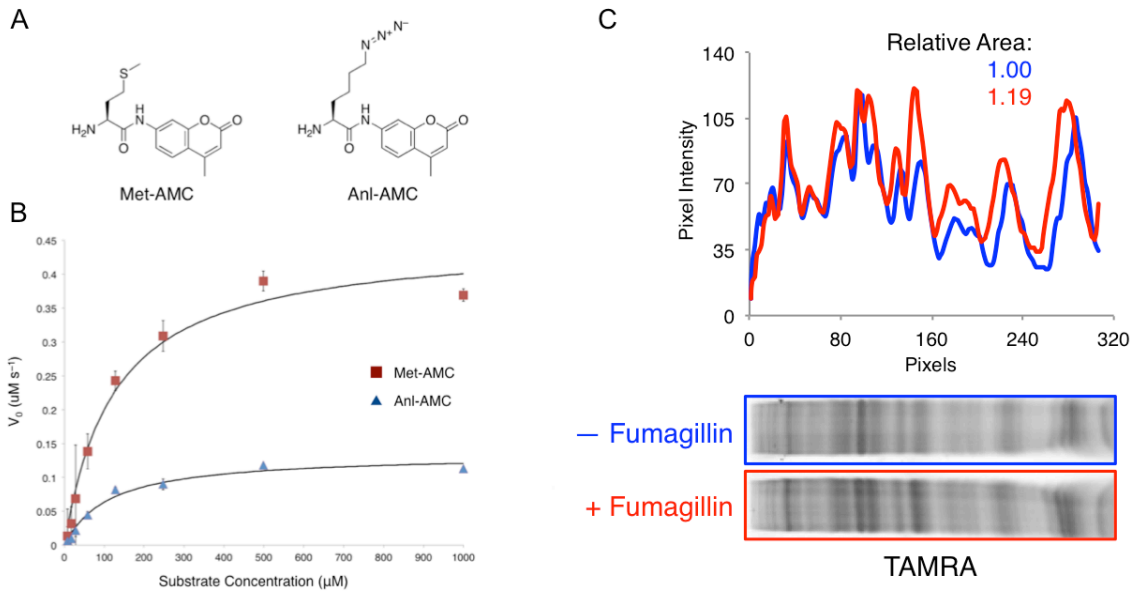

Fig. S11. MetAP exhibits attenuated excision activity toward N-terminal Anl. (*A*) Structures of fluorogenic model substrates tested with recombinant MetAP-2. (*B*) Saturation curves of Met-AMC and Anl-AMC in reaction with recombinant MetAP-2. Kinetic constants for the Met-AMC substrate were: K_m = 124 μM, k_{cat} = 31.19 s^{-1}, k_{cat}/K_m = 0.26 s^{-1}μM. For Anl-AMC: K_m = 111 μM, k_{cat} = 9.59 s^{-1}, k_{cat}/K_m = 0.33 s^{-1}μM. (*C*) Comparison of Anl-labeled proteins from cells treated with Anl in the presence or absence of 200 nM fumagillin, an irreversible MetAP-2 inhibitor.

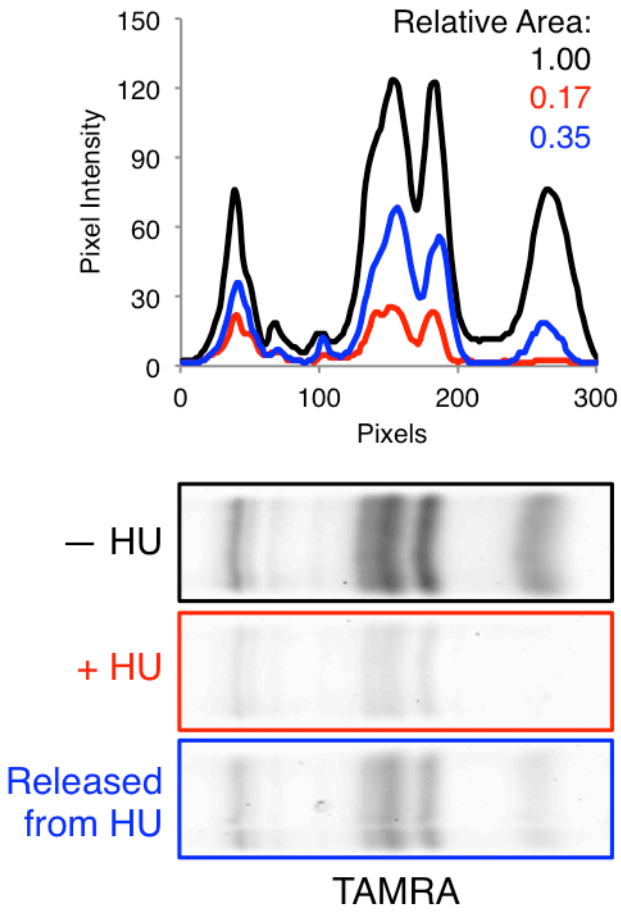

Fig. S12. Effect of HU on histone synthesis. HEK293-8D3 cells were pulsed with 1 mM AnI in the absence (black) or presence (red) of 4 mM HU, or treated with 4 mM HU and released from the agent 10 minutes before the pulse (blue). Following extraction of histones, proteins were modified with DIBAC-TAMRA and quantified by in-gel fluorescence scanning.

Protein	Protein Accession Number	Met AP Substrate
GRB2 Isoform 1 of Growth factor receptor-bound protein 2	IPI00021327	No
SYNGR2 Synaptogyrin-2	IPI00013946, IPI00654744, IPI00789763	No
HIST1H1C Histone H1.2	IPI00217465	Yes
YWHAB Isoform Short of 14-3-3 protein beta/alpha	IPI00759832	Partial
YWHAE 14-3-3 protein epsilon	IPI00000816	No
YWHAZ 14-3-3 protein zeta/delta	IPI00021263	No
SLC25A5 ADP/ATP translocase 2	IPI00007188	Yes
EIF3I Eukaryotic translation initiation factor 3 subunit I	IPI00012795	No
TUBB2C Tubulin beta-2C chain	IPI00007752	No
STIP1 Stress-induced-phosphoprotein 1	IPI00013894, IPI00871856	No
EEF2 Elongation factor 2	IPI00186290	Yes
PTBP1 polypyrimidine tract-binding protein 1 isoform a	IPI00183626, IPI00334175	No

Table S1. N-terminal peptides that contain Anl are derived both from proteins that are substrates of MetAPs, and from proteins that are not.

Protein	Protein Accession Number	N-α-acetyl-Met	N-α-acetyl-Anl
GRB2 Isoform 1 of Growth factor receptor-bound protein 2	IPI00021327	Yes	Yes
SYNGR2 Synaptogyrin-2	IPI00013946, IPI00654744, IPI00789763	Yes	Yes
HIST1H1C Histone H1.2	IPI00217465	n/a	No
YWHAB Isoform Short of 14-3-3 protein beta/alpha	IPI00759832	Yes	Yes
YWHAE 14-3-3 protein epsilon	IPI00000816	Yes	Yes
YWHAZ 14-3-3 protein zeta/delta	IPI00021263	Yes	Yes
SLC25A5 ADP/ATP translocase 2	IPI00007188	n/a	No
EIF3I Eukaryotic translation initiation factor 3 subunit I	IPI00012795	No	No
TUBB2C Tubulin beta-2C chain	IPI00007752	No	No
STIP1 Stress-induced-phosphoprotein 1	IPI00013894, IPI00871856	Yes	Yes
EEF2 Elongation factor 2	IPI00186290	n/a	No
PTBP1 polypyrimidine tract-binding protein 1 isoform a	IPI00183626, IPI00334175	Yes	Yes

Table S2. N-terminal peptides identified with Anl in place of Met are properly N^{α}-acetylated.

SUPPORTING INFORMATION MATERIALS AND METHODS:

Tagging and Detection Reagents

All materials from commercial suppliers were used without further purification unless otherwise indicated. Anl was synthesized as previously described (1) (using N^{α}-Boc-L-lysine in place of N^{α}-Boc-L-diaminobutyric acid as the starting material) and verified by ^{1}H NMR and mass spectrometry. Anl is commercially available and can be purchased from Jena Bioscience (as 6-azido-L-lysine HCl), and from MolPort (as (2S)-2-amino-6-azidohexanoic acid hydrochloride). DIBAC-TAMRA, DIBAC-Biotin, and DIBAC-S-S-biotin were purchased from Click Chemistry Tools (see Fig. S1 for structures). The Click-iT AlexaFluor488 Protein Synthesis HCS Assay was purchased from Life Technologies. [$^{13}C_6$, $^{15}N_2$]-lysine was purchased from Cambridge Isotopes.

Anl Labeling Procedure

HEK293 cells were grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS), penicillin and streptomycin, and 50 μg mL^{-1} geneticin at 37°C, with 5% CO_2 using standard procedures. The culture medium was exchanged with fresh medium either several hours or the night before each experiment. Cells at 70 – 80% confluence were labeled by addition of Anl to the culture medium at the indicated concentrations and incubated for the indicated times at 37°C under 5% CO_2. Labeling was terminated by aspiration of the culture medium, and cells were washed with phosphate buffered saline (PBS) and collected by trypsinization and centrifugation. Pelleted cells were washed with PBS to remove trypsin and stored at -20°C until further processing.

Preparation of Cell Lysates

Cell pellets were re-suspended in 100 mM Tris buffer (pH 8.0) containing protease inhibitor cocktail (eComplete ETDA-free, Roche, 1 tablet/50 mL) and 0.2% SDS (diluted from a 10% stock solution in water). Cells were lysed by pipetting and agitation, and chromosomal DNA was sheared by sonication on ice (three 10 s bursts at 9 W with 10 s pauses between bursts). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology) and normalized to 1 mg mL^{-1} with lysis buffer. Free thiols were alkylated by adding iodoacetamide to a final concentration of 25 mM from a freshly prepared 500 mM stock in water; samples were mixed by gentle rotation for 1 h while protected from light.

Protein Modification and Detection with DIBAC-functionalized Probes

DIBAC-probe stocks (5 – 20 mM) were prepared in DMSO and conjugation reactions were initiated by dilution of probe into solutions of alkylated proteins to a final concentration of 20 µM. Reactions with DIBAC-TAMRA were incubated at room temperature for 20 m with gentle mixing and protection from light. Reactions with DIBAC-biotin and DIBAC-S-S-biotin were mixed gently at room

temperature for 1 h. Reactions were quenched by addition of a five-fold excess (with respect to probe) of free Anl and immediately mixed by vortexing.

Dye Labeling and Imaging

HEK293-8D3 and non-transfected HEK293 cells were grown to ~75% confluence on 100 mm cell culture treated petri dishes, trypsinized and seeded in 8-well Lab-Tek II Chamber Slides (ThermoScientific) at a density of ~2.5 x 10^4 cells per well. Cells were allowed to adhere overnight at 37°C under 5% CO_2 in either 293FreeStyle (Life Technologies) or maintenance medium (see Cell Culture Maintenance). Cells in 293FreeStyle medium were less adherent than those grown in maintenance medium. The following day, the medium was removed and replaced with pre-warmed medium with or without 1 mM Anl. For control experiments in which protein synthesis was inhibited, anisomycin was added to a final concentration of 40 μM 30 m prior to the Anl pulse and was included in the pulse at the same concentration. After 4 h, the medium was removed and cells were rinsed gently with PBS. Cells were fixed with 3% formaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and labeled with the Click-iT Alexa Fluor 488 Protein Synthesis HCS Assay kit according to the manufacturer's protocol (Life Technologies). Cells were stained with Hoechst 33342 prior to imaging. For detection of mCherry, HEK293-8D3 and HEK293 cells from liquid suspension cultures in 293FreeStyle medium were plated in black-well clear-bottom 96-well poly-D-Lysine plates (Fisher) at ~5 x 10^3 wells per well. Cells were allowed to adhere overnight and imaged the next day. Images were obtained on a Zeiss LSM510 microscope with 20X, 40X, or 63X (oil immersion) lenses.

Enrichment of Anl-labeled Proteins

T-75 flasks containing HEK293-8D3 or HEK293 cells at ~75% confluence were labeled with 2 mM Anl for 6 h. For labeling with heavy lysine, cells were rinsed once with lysine-free DMEM (Life Technologies) and then labeled in lysine-free DMEM supplemented with 800 μM [$^{13}C_6$, $^{15}N_2$]-lysine and 2 mM Anl. Lysates were prepared as described above on a 2 mL scale and protein content (~6 mg) was normalized. Proteins were alkylated and conjugated to DIBAC-biotin as described above. Excess DIBAC-biotin was removed by two successive passages of the reaction mixture through PD-10 desalting columns equilibrated with 100 mM Tris buffer, pH 8.0 with 0.2% (w/v) SDS. The first passage was performed according to the "spin protocol;" the second passage was performed according to the "gravity flow" protocol described by the manufacturer. Biotinylated proteins were collected on Streptavidin Plus UltraLink Resin (Pierce Biotechnology) by adding 250 μL of the 50% slurry to each protein solution. Proteins were bound to the resin by gentle agitation of the suspension overnight at room temperature. The following day, the suspension was loaded into a disposable chromatography column and the flow-through was collected. The resin was washed once with 100 mM Tris buffer, pH 8.0 containing 0.2% (w/v) SDS; once with 100 mM Tris, pH 8.0 containing 1.0% (w/v) SDS; once with a 6 M

urea solution in 100 mM Tris, pH 8.0; once with a 1 M NaCl solution in 100 mM Tris, pH 8.0; and once again with 100 mM Tris, pH 8.0 containing 0.2% (w/v) SDS. Each wash step was performed with 2.5 mL of solution and the entire wash procedure was performed twice before eluting resin-bound proteins by boiling in SDS-PAGE loading buffer containing 2% 2-mercaptoethanol (v/v)

Hydroxyurea Treatment

Hydroxyurea was added to cultures to a final concentration of 2.5 mM. Anl was added to a concentration of 1 mM 10 m later. Cells were labeled with Anl for 4 h before proteins were collected and analyzed as described in preceding sections.

Aminoacylation of synthetically prepared tRNA

NLL-EcMetRS was expressed and purified as previously described (2) Synthetic tRNAs were prepared by the run-off transcript method and aminoacylated with Met or Anl as previously described (3). We tested cytoplasmic $tRNA_i^{Met}$ and $tRNA^{Met}$ sequences from *C. elegans* (which are similar to the corresponding mammalian sequences); *E. coli* $tRNA_i^{Met}$ and $tRNA^{Met}$ sequences were used as controls. Aminoacylated tRNAs were stabilized by acetic anyhydride as previously described (4). The tRNAs were precipitated with ice-cold 70% ethanol and resuspended in 100 mM Tris pH 7.0. Normalized tRNAs were labeled with DIFO-AlexaFluor488 (a gift from Carolyn Bertozzi, UC Berkeley) by incubation with 10 µM dye for 10 m. Excess dye was removed using BioSpin gel filtration columns (BioRad). tRNAs were separated on 2% aragose and imaged on a Typhoon Trio+ (GE Healthcare).

Cloning

The plasmid pmCherry-N1-His6-EcNLL-MetRS was constructed by inserting a gene encoding an N-terminally His-tagged NLL-EcMetRS between the *XhoI* and *XmaI* sites of the pmCherry-N1 vector (Clontech). pJS2-NLL is a bacterial expression vector for IPTG-induced expression of a His-tagged GFP encoding a single Met at the N-terminal position followed by an arginine. pJS2-NLL was cloned by inserting the NLL-EcMetRS expression cassette from pJTN1 (5) into pJS2 (6) at the *NheI* site.

Generation of stable cell lines

A 30 mL suspension culture of HEK293-F cells (Life Technologies) was grown to a density of 1×10^6 cells/mL in 293FreeStyle medium (Life Technologies) in a humidified incubator at 37°C with 8% CO_2 and 130 rpm shaking. Cell were transfected with 150 µg of pmCherry-N1-His6-EcNLL-MetRS using polyethylenimine as the transfection reagent. The culture was allowed to grow for 48 h at which point an aliquot of cells was inspected for mCherry fluorescence by microscopy. Roughly half the surviving cells exhibited mCherry fluorescence. A 10 mL aliquot of the cell suspension was transferred to a T-75 culture flask and cells were allowed to adhere by incubation at 37°C with 8% CO_2 without shaking.

After 24 h, the culture medium was exchanged with fresh 293FreeStyle medium containing 50 μg mL^{-1} of geneticin sulfate to select for transfectants. Individual clones were isolated into 96-well plates and maintained in pre-conditioned 293FreeStyle medium before transfer into increasingly larger culture vessels. Cell stocks were prepared in 293FreeStyle medium containing 5% dimethylsulfoxide and frozen overnight at -80°C in a slow-freezing container before submersion in liquid N_2 for long-term storage. In total, ten lines were generated with varying degrees of mCherry fluorescence. The HEK293-8D3 clone used in Anl labeling experiments displayed a median value of mCherry fluorescence relative to the other lines generated, and was selected on the basis of growth rate (within 10% of the parent line) and high viability.

Cell Culture Maintenance

Cell stocks of HEK293-8D3 and non-transfected HEK293-F prepared as described above were thawed and plated in T-75 flasks containing 15 mL of low-glucose Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS), penicillin, and streptomycin. After 48 h, geneticin sulfate was added to the HEK293-8D3 culture to a final concentration of 50 μg mL^{-1}. The cultures were maintained in this medium (and with 50 μg mL^{-1} geneticin in the case of HEK293-8D3) at 37 °C, with 5% CO_2 using standard procedures.

Analysis of the NLL-EcMetRS from HEK 293F-8D3 Cells

To collect soluble cytoplasmic proteins, HEK293-8D3 cells were lysed in 1% (v/v) Triton-X100 solution in PBS with protease inhibitors. The N-terminally His-tagged NLL-MetRS-mCherry was purified from the lysate of HEK293-8D3 cells using magnetic Ni-NTA agarose beads (Qiagen) according to the manufacturer's protocol. As a control, the purification was also performed using a lysate of non-transfected HEK293 cells. Proteins were analyzed by separation on 9% polyacrylamide SDS-PAGE gels and stained using Coomassie Brilliant Blue G-250. We confirmed that the protein purified from HEK293-8D3 cells was indeed NLL-MetRS-mCherry by in-gel tryptic digestion of the gel fragment containing the band and LC/MS/MS analysis of extracted peptides as described in subsequent sections.

Western Blotting

Western blots were performed using standard procedures. Antibodies were used at the concentrations suggested by the manufacturers. Mouse monoclonal anti-GAPDH, mouse monoclonal anti-p53, and mouse monoclonal anti-β-actin were from Sigma-Aldrich. Rabbit polyclonal anti-DsRed (for mCherry detection) was from Clontech. Rabbit monoclonal anti-histone H4 (pan) was from Millipore. Anti-PentaHis-AlexaFluor647 conjugate was from Qiagen. Secondary antibodies included rabbit anti-mouse AlexaFluor647 conjugate from Invitrogen, and goat anti-rabbit DyLight680 conjugate from Cell Signaling Technology. Streptavidin-AlexaFluor488 and Streptavidin-AlexaFluor647 conjugates were from Invitrogen.

Antibody and streptavidin conjugates were detected by fluorescence scanning on a GE Healthcare Typhoon Trio+. Mass estimates were made by comparison to the SeeBlue Plus2 molecular weight marker from Invitrogen.

Model GFP Expression and Purification

E. coli strain DH10B (Invitrogen) transformed with pJS2-NLL (for GFP containing a single Anl) or pJTN5 (for DHFR) (5) as well as the repressor plasmid pREP4 (Qiagen) were grown overnight in 5 mL LB-broth containing 100 mg L^{-1} ampicillin and 35 mg L^{-1} kanamycin. The following day cells were diluted 1:50 into M9 minimal medium supplemented with 40 mg L^{-1} of each of the 20 amino acids, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 30 mg L^{-1} thiamine hydrochloride, and 0.4% (w/v) glucose. When the culture reached an OD_{600} of ~1.0, cells were collected by centrifugation at 8,000 x g for 5 m at 4° C and washed twice with chilled 0.9% NaCl (w/v) solution. Subsequently, cells were resuspended in either fresh M9 medium (as described above), or M9 medium containing 1 mM Anl in place of Met. To induce protein synthesis, IPTG was added to each culture to a final concentration of 1 mM. The cultures were incubated at 37 °C with 250 rpm shaking for 4 h. Cells were collected by centrifugation and stored at -20 °C. The next day, the Met- and Anl-containing model proteins were affinity purified under native conditions using Ni-NTA Agarose (Qiagen) according to the manufacturer's protocol. Purified proteins were buffer exchanged into PBS using PD-10 Desalting Columns (GE Healthcare) and normalized by protein concentration before use.

Model Protein Enrichment

The purified GFP containing a single Anl or Met (3 µg) was added to 2 ml of HEK293 lysate (prepared as described above) containing 1.5 mg mL^{-1} protein. The mixture was alkylated, modified with DIBAC-biotin, and enriched using immobilized streptavidin as described in preceding sections. Western blots and Coomassie Brilliant Blue G-250 stained gels and were analyzed using ImageJ.

Mass Spectrometry and Peptide Identification

Enriched Anl-tagged proteins were separated by 1D SDS-PAGE and gel fragments were digested with protease according to previously described procedures (7). Extracted peptides were dried under rotary vacuum and resuspended in 0.1% formic acid in water (v/v) before analysis on a Thermo Scientific LTQFT mass spectrometer. MS/MS samples were analyzed using Mascot (Matrix Science). Mascot was set up to search the IPI_human_wDEC.v3.54j database (151385 entries) assuming the digestion enzyme trypsin or chymotrypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 ppm. The iodoacetamide alkylation of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine, replacement of Met by Anl, and various Anl derivatives (see SI) were specified in Mascot as variable modifications. Scaffold (version

Scaffold_3_00_07, Proteome Software) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm (8). Protein identifications were accepted if they could be established at greater than 90.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (9). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Sub-cellular Fractionation

Cells pulse-labeled with Anl for 4 h were collected as described earlier. Fractionation was performed using the Qproteome Cell Compartment Kit (Qiagen). Proteins were precipitated from the cytoplasmic and nuclear fractions and by chloroform-methanol and resuspended in 100 mM Tris, pH 8.0 containing 0.2% (w/v) SDS. Proteins were alkylated and treated with DIBAC-TAMRA as described above. Cytosolic and nuclear fractions were compared to total protein, which was prepared by lysing cells in RIPA buffer (supplemented with SDS to a final concentration of 0.2% (w/v) from a 10% (w/v) solution in water) followed by sonication. Proteins were alkylated and modified with DIBAC-TAMRA as described in preceding sections.

Histone Extraction

Frozen HEK298-8D3 cell pellets were resuspended in 3 mL PBS containing 0.5% (v/v) Triton X-100 and eComplete EDTA-free protease inhibitor cocktail (1 tablet per 50 mL). Cells were lysed by pipetting and agitation and incubated on ice for 10 m. Nuclei were collected by centrifugation at 2,000 x g for 10 m at 4° C. The supernatant was removed and pelleted nuclei were resuspended in an additional 1.5 mL of the lysis solution before centrifugation again. Histones were extracted from the nuclear pellet by resuspension in 500 μL of 0.2 N HCl followed by rotation overnight at 4 °C. The following day, nuclear debris was sedimented at 2,000 x g for 10 m at 4 °C and the histone-containing supernatant was collected. The solution pH was raised by addition of an equal volume of 1 M Tris pH 8.0 containing 1% (w/v) SDS. Proteins were alkylated and modified with DIBAC-TAMRA as described in preceding sections.

MetAP Enzymatic Assay

The fluorogenic substrates Met-AMC (Enzo Life Sciences) and Anl-7-amino-4-methylcoumarin (custom synthesis from Anaspec) were used in kinetic assays with purified recombinant human MetAP-2 2 (Enzo Life Sciences). Reactions were performed in black 96-well plates in 30 mM HEPES buffer (pH 7.4, 150 mM NaCl, 100 μM $CoCl_2$). Substrate was added to each reaction well at concentrations between 0 - 1000 μM. To ensure substrate solubility, each reaction contained DMSO at a final concentration of 4%. Reactions were initiated

by addition of 1 µL of MetAP-2 from a 0.5 mg/mL stock. The increase in AMC fluorescence was measured with excitation at 380 nm and emission detection at 460 nm using a Tecan Safire II fluorescence plate reader. Triplicate measurements were averaged and fit to the Michaelis-Menten equation using Origin software.

Fumagillin Treatment

Cells were pulse-labeled with 1 mM Anl for 4 h in the absence or presence of 200 nM of the irreversible MetAP-2 inhibitor fumagillin (Sigma-Aldrich). Following the pulse, cells were collected and Anl-labeled proteins were modified with DIBAC-TAMRA and analyzed by in-gel fluorescence scanning as described in preceding sections.

Sequence of the Mutant Synthetase Gene:

His_6-NLL-EcMetRS-mCherry

ATGAGAGGATCGCATCACCATCACCATCACGGATCCATGACTCAAGTCGCGAAGAAAATTCTGGTGACGTGCGCAAACC
CGTACGCTAACGGCTCAATCCACCTCGGCCATATGCTGGAGCACATCCAGGCTGATGTCTGGGTCCGTTACCAGCGAAT
GCGCGGCCACGAGGTCAACTTCATCTGCGCCGACGATGCCCACGGTACACCGATCATGCTGAAAGCTCAGCAGCTTGGT
ATCACCCCGGAGCAGATGATTGGCGAAATGAGTCAGGAGCATCAGACTGATTTCGCAGGCTTTAACATCAGCTATGACA
ACTATCACTCGACGCACAGCGAAGAGAACCGCCAGTTGTCAGAACTTATCTACTCTCGCCTGAAAGAAAACGGTTTTAT
TAAAAACCGCACCATCTCTCAGCTGTACGATCCGGAAAAAGGCATGTTCCTGCCGGACCGTTTTGTGAAAGGCACCTGC
CCGAAATGTAAATCCCCGGATCAATACGGCGATAACTGCGAAGTCTGCGGCGCGACCTACAGCCCGACTGAACTGATCG
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ATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGT
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CCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAG
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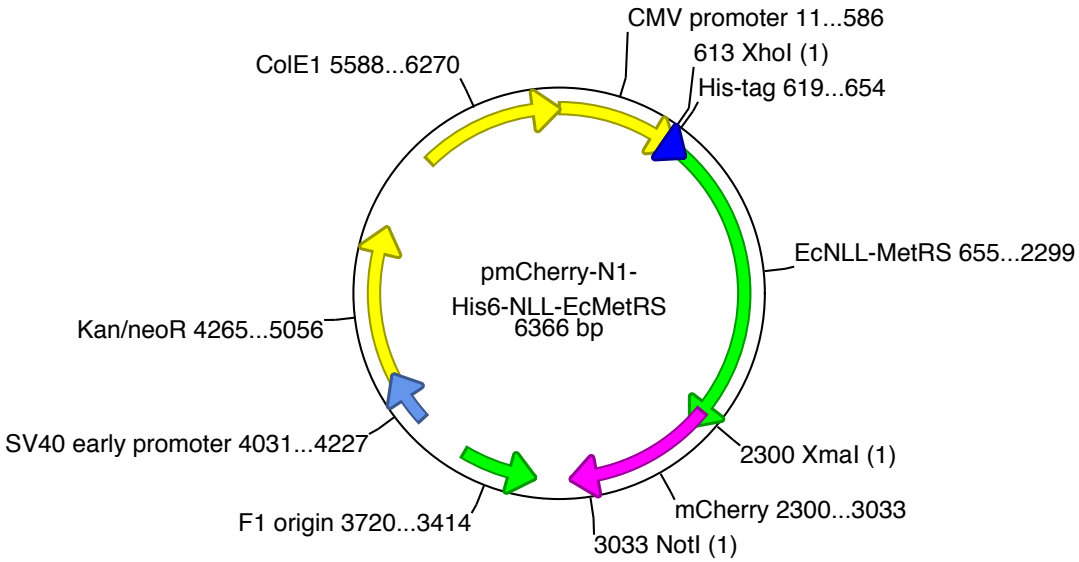
TGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGG
CGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCC
GAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAG

Plasmid map of the pmCherry-N1-His6-EcNLL-MetRS vector:



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