SUPPLEMENTARY MATERIAL FOR:

Cell-Selective Metabolic Labeling of Proteins

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SUPPLEMENTARY METHODS:

Reagents:

Azidonorleucine (**2**) and azidohomoalanine (**3**) were prepared as previously described[1], both were confirmed by 1H NMR and mass spectrometry, and stored at 100 mM in water at 4 °C. The triazole ligand (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) was prepared as described previously[2], confirmed by 1H NMR and mass spectrometry, and stored at 20 mM in DMSO at -20 °C. The biotin-FLAG-alkyne reagent (**4**) was synthesized by GenScript Corporation, was determined pure by mass spectrometry, and stored at 5 mM in water at -20 ^{o}C. The dimethylaminocoumarin-alkyne (**5**) was synthesized as previously described[3], confirmed by 1H NMR and mass spectrometry, and stored at 40 mM in DMSO at -20 °C. The Click-iT Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit dye was purchased from Invitrogen and the TAMRA-alkyne (**6**) dye was used as obtained. CuBr (99.999% purity) was purchased from Sigma and suspended in water at 20 mM immediately before use.

Strains and Plasmids:

Plasmid	IPTG Inducible Protein	MetRS Gene Cassette
pQE-80L	None	None
pJTN1	None	NLL-MetRS
pJTN2	GFP	NLL-MetRS
pJTN3	DHFR	None
pJTN4	GFP	None
pJTN5	DHFR	NLL-MetRS
pJTN6	GFP	WT-MetRS
pJTN7	DHFR	L13G-MetRS

pQE-80L: was used as obtained from Qiagen.

pJTN1: The gene encoding wild-type *E. coli* MetRS was isolated by digestion of pAJL61[1] with *Nhe*I. The purified fragment containing the gene cassette for constitutive expression of *E. coli* MetRS under control of its natural promoter was then ligated into pQE-80L. The mutations L13N, Y360L, H301L were generated by three sequential site-directed mutageneses according to the manufacturer's protocol from the commercially available QuikChange kit (Stratagene).

pJTN2: pJTN1 was digested with *Nhe*I and the gene cassette for constitutive expression of NLL-MetRS was isolated by gel purification. This fragment was inserted into the *Nhe*I site of pJTN4 to generate pJTN2.

pJTN3: Renamed from pAJL60 previously reported[4].

pJTN4: Renamed from pQE-80L/GFPrm_AM previously reported[5].

pJTN5: pJTN1 was digested with *Nhe*I and the gene cassette for constitutive expression of NLL-MetRS was isolated by gel purification. This fragment was ligated into the *Nhe*I site of pJTN3 to generate pJTN5.

pJTN6: pAJL61 was digested with *Nhe*I and the gene cassette for constitutive expression of WT-MetRS was isolated by gel purification. This fragment was inserted into the *Nhe*I site of pJTN4 to generate pJTN6.

pJTN7: pAJL61 was subjected to site-directed mutagenesis to generate the L13G mutation on the region that encodes the MetRS.

Sequencing of plasmids was performed by Laragen. All plasmids were transformed into the XL-1 Blue strain of *E. coli* (Stratagene) for cloning. Transformants were grown on plates containing 200 ug ml^{-1} ampicillin. For pulse-labeling experiments, plasmids were transformed into the DH10B strain of *E. coli* (Invitrogen). Transformants were grown on ampicillin agar plates or maintained in media with 200 ug ml^{-1} ampicillin.

Proteomic Pulse Labeling and Conjugation:

DH10B/pQE-80L and DH10B/pJTN1 were used for this experiment. Cells were diluted 1:50 from an overnight culture in LB into M9 minimal medium and grown at 37°C with agitation at 250 rpm. When OD_{600} = 0.5 was reached, cells were pulse-labeled by addition of 1 mM azidonorleucine to the medium for 10 m. A culture of DH10B/pQE-80L in which no azidonorleucine was added was used a control. As an additional control, a culture of DH10B/pJTN1 was treated with 100 ug $ml-^{1}$ of chloramphenicol for 10 m prior to addition of azidonorleucine, to inhibit protein synthesis. Cells were collected by centrifugation at 5,000 *g* at 4°C for 5 m, resuspended in PBS (pH 7.8) with 10% SDS, and heated at 95°C for 5 m for lysis. PBS (pH 7.8) was added to each sample to bring the concentration of SDS to 1%. Azidonorleucine-labeled proteins were then conjugated to probe by addition of 200 uM triazole ligand, 25 uM biotin-FLAG-alkyne, and 400 uM CuBr. The reaction was allowed to proceed for 16 h at room temperature with gentle agitation. Proteins were precipitated by addition of trichloroacetic acid (TCA) to 10%, and the resulting pellets were washed twice with cold acetone. Proteins were resuspended in SDS-PAGE loading buffer and separated on two parallel

12% Tris-Tricine gels. One gel was stained with Coomassie-Brilliant Blue R-250 for non-specific detection of proteins. Proteins from the second gel were transferred to nitrocellulose and blocked in PBS-Tween containing 5% milk for Western blot analysis with anti-FLAG-HRP conjugate (Sigma) at a dilution of 1:20,000. Detection was achieved by chemiluminesence with Super Signal West Pico Chemiluminescent Substrate (Pierce) and x-ray BioMax film (Kodak).

Comparison of Anl Incorporation in Strains Over-Expressing the WT-MetRS, L13G Met-RS, or NLL-MetRS

DH10B/pJTN6, DH10B/pJTN7 and DH10B/pJTN2 were used for this experiment. Procedures for growth and pulse labeling of cells, as well as conjugation chemistry, processing and Western analysis of proteins were performed as described above.

Model Protein Expression, Labeling, Conjugation, and Purification:

DH10B/pJTN2 and DH10B/pJTN3 were used in this experiment. Cells were diluted 1:50 from an overnight LB culture into M9 minimal medium and grown at 37°C with agitation at 250 rpm. When OD_{600} = 1.0 was reached a third culture was generated by mixing DH10B/pJTN2 and DH10B/pJTN3 in a 1:2 volumetric ratio. To initiate protein labeling and synthesis, 1 mM azidonorleucine and 1 mM IPTG were added to the individual cultures of DH10B/pJTN2 and DH10B/pJTN3, as well as to the mixed culture. After 3 h, cells were collected by centrifugation at 5,000 *g* for 5 m, resuspended in PBS (pH 7.8) with 10% SDS, and heated at 95°C for 5 m to lyse cells. PBS (pH 7.8) was added to each sample to bring the

concentration of SDS to 1%. Azidonorleucine-labeled proteins were conjugated to probe by addition of 200 uM triazole ligand, 25 uM biotin-FLAG-alkyne, and 400 uM CuBr suspension in water. The reaction was allowed to proceed for 16 h at room temperature with gentle agitation. Proteins were precipitated by addition of TCA to 10%, and the resulting pellets were washed twice with cold acetone. Proteins were resuspended in Buffer B[6] with 10 uM β-mercaptoethanol and His-tagged proteins were isolated on Ni-NTA Spin Kit columns (Qiagen) as directed by the manufacturer's protocol. Proteins were separated on 12% Tris-Tricine gels, transferred to nitrocellulose, blocked with PBS-Tween containing 5% milk, and analyzed by Western blotting with Penta-His Antibody (Qiagen) at a dilution of 1:10,000 and with Streptavidin-HRP (Pierce) at a dilution of 1:5,000. Antibodies were detected as described above.

Bacterial Dye Labeling and Imaging:

DH10B/pJTN4 and DH10B/pJTN5 were used in this experiment. Cells were diluted 1:50 from overnight cultures in LB into M9 minimal medium and grown at 37°C with agitation at 250 rpm. When OD_{600} of 1.0 was reached, a third culture was generated by mixing DH10B/pJTN5 and DH10B/pJTN4 in a 1:2 volumetric ratio. Each culture was then aliquoted into a clear-bottom, black 96-well plate and cells were allowed to settle for 2 h at 37°C. To initiate protein labeling and synthesis, 1 mM azidonorleucine and 1 mM IPTG were added to each well. After 4 h, cells were collected by centrifugation at 3,000 *g* for 5 m. Cells were washed twice and resuspended in PBS (pH 7.8). Dye labeling was performed by addition of 200 uM triazole ligand, 10 uM dimethylaminocoumarin-alkyne, and 400 uM

CuBr suspension with rocking for 16 h at 4°C. Prior to imaging, cells were washed twice with cold PBS (pH 7.8). Images were obtained using a Zeiss LSM510 confocal microscope (LSCM) with a 100x oil immersion lens. Images represent overlays of detected GFP and coumarin emission as previously described.[7,8]

Macrophage Infection Pulse with Azidonorleucine, Dye Labeling and Imaging:

The mouse alveolar macrophage cell line MH-S was obtained from ATCC and maintained in RPMI medium supplemented with 10% FBS, 0.05 mM β-mercaptoethanol, and 1% penicillin/streptomycin (Sigma) under standard culture conditions (37°C, 5% CO_2, humidified). DH10B/pJTN1 and DH10B/pJTN4 were inoculated 1:40 from an overnight culture into 2XYT medium. GFP synthesis was induced by the addition of 1 mM IPTG to the culture of DH10B/pJTN4 at an OD_{600} = 0.3. Bacterial cells were allowed to grow for 3.5 h after initial induction, diluted into 2XYT to obtain OD_{600} = 1.0, and placed on ice. At 20 h prior to infection, macrophages were passaged into wells on a LabTek II Chamber Slide System (Nalge-Nunc) at $4x10^5$ cells ml^{-1} in 0.5 ml maintenance media and kept under standard culture conditions. Medium was removed and replaced with antibiotic-free medium containing 100 nM Mitotracker Deep Red (Invitrogen). Mitochondrial staining took place for 30 m under standard culture conditions. Macrophages were rinsed twice with fresh antibiotic-free medium and chilled to 4°C for 15 m. Prior to infection, 2 mM of azidonorleucine was added to the cold macrophage cultures. To initiate infection, *E. coli* cells were immediately added

to the medium at a multiplicity of infection of 1:100. Cells were spun at 4°C at 100 g for 10 m to promote attachment of bacteria to macrophages and synchronize infection. To allow internalization of bacteria, cells were warmed to 37°C for 35 m. Following infection, cells were washed twice with PBS to remove unbound bacteria and fixed with 3.7% paraformaldehyde for 15 m at 37°C. Fixative was removed by washing twice with PBS. Cells were permeabilized with 0.1% Triton-X100 at room temperature for 3 m and washed three times with PBS (pH 7.8). The dye labeling solution was prepared 5 m before addition to each well and contained 200 uM ligand, 400 uM tris(2-carboxyethyl)phosphine hydrochloride, 200 uM $CuSO_4$, and TAMRA-alkyne diluted 1:750 as obtained from the manufacturer. Dye solution was added to each well and labeling was performed for 16 h at room temperature with gentle agitation. The following day, cells were rinsed three times with PBS containing 0.5 mM EDTA and 1% Tween-20. Well dividers were removed and samples were covered with mounting media (composed of 50% glycerol and 50% PBS) and coverslips. Samples were imaged on a Zeiss LSM 510 NLO three-channel confocal microscope with a 63x oil immersion lens. Mitotracker, TAMRA, and GFP were excited at 633, 543, and 488 nm, respectively. Images of Mitotracker and TAMRA or Mitotracker and GFP from the middle of a confocal stack were overlaid to create the figures. Bacteria appearing within the Mitotracker stain are internalized while those appearing on the edge are attached to the outside of macrophages.

Macrophage Infection Pulse with Azidohomoalanine, Dye Labeling and Imaging:

An additional control was performed for the *E.coli*/macrophage experiment to ensure that macrophages were indeed synthesizing protein and that the lack of macrophage fluorescence emission in the TAMRA channel was not due to an inability of the macrophages to synthesize protein in co-culture. The control experiment was performed as above except pulse labeling was performed with 2 mM azidohomoalanine instead of azidonorleucine. Azidohomoalanine can be incorporated by the endogenous protein synthesis machinery and can label proteins synthesized in macrophages. The experiment was performed in methionine-free medium (Dulbecco's Modified Eagle's Medium supplemented with 1% Glutamax (Sigma)). Conditions for infection, dye labeling, and analysis were identical to those described above.

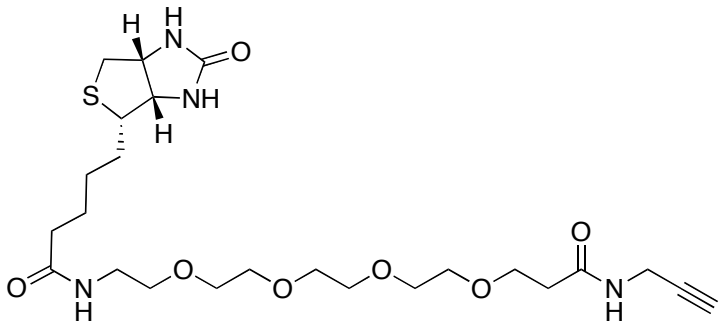
Isolation of Labeled, Bacterially Expressed Proteins from Infection:

The mouse alveolar macrophage cell line MH-S and the *E. coli* strain DH10B/pJTN2 were used for this experiment. DH10B/pJTN2 from an overnight culture in LB was inoculated 1:50 in fresh LB and grown for three hours. Marcophages (2.12×10^8 cells) were suspended in RPMI medium supplemented with 10% FBS, and 1% penicillin/streptomycin in a 125 mL shake flask and agitated gently with rotation at 60 rpm at 37°C. DH10B/pJTN2 cells were then pelleted by centrifugation at 6,000 *g* for 1 m, washed once in 0.9% NaCl, and resuspended in 0.9% NaCl. Azidonorleucine was added to the macrophage medium to 2 mM, DH10B/pJTN2 was then added to the macrophage culture (2.12×10^9 cells) and 1 mM IPTG was added immediately to induce bacterial expression of GFP. After a 35 m with gentle agitation at 60 rpm at 37°C, all cells

were collected by two sequential centrifugation steps at 100 *g* and 6000 *g* respectively. Cells were combined and lysed in PBS pH 7.4 with 2% SDS with heating to 85°C for 5 m. The SDS concentration was diluted to 0.5%, and insoluble lysate debris was removed by centrifugation at 15,000 *g* for 30 m at room temperature. The resulting solution was subjected to conjugation to the commercially available biotin-alkyne (**7**) (Invitrogen). Conjugation was performed with 200 uM triazole ligand, 100 uM biotin-alkyne, and 400 uM CuBr. The reaction was allowed to proceed over night with rotation. The following day, excess unreacted biotin was removed from the mixture using PD-10 (GE) desalting columns pre-equilibrated with PBS pH 7.4 with 0.5% SDS. The proteins were then subjected to affinity purification with Neutravidin Resin (Pierce). Prior to binding, 2 mL of resin was washed with PBS pH 7.4 with 0.5% SDS then added to the protein solution and allowed to bind at room temperature for 1 h with rotation. The unbound proteins were then removed and the resin was subjected to five 10 mL washes with PBS pH 7.4 with 0.2% SDS. The proteins were then eluted in 2 mL of PBS pH 7.4 with 2% SDS and 2 mM free biotin with boiling for 10 m. The unreacted sample, unbound proteins (flow through), wash 1, wash 3, wash 5, and the eluent were then subjected to immuno-blot using the Bio-Dot Slot-Format apparatus (Bio-Rad) to immobilize proteins on two nitrocellulose membranes according to the manufacturer's protocol. Both blots were blocked in PBS-Tween containing 5% milk. One blot was probed with Anti-Penta-His Alexa 647 Conjugate (Qiagen) according to supplier's protocols. The second blot was probed first with Mouse Monoclonal

Anti-β-Actin Antibody (Sigma) then with a secondary Rabbit Anti-Mouse Cy5 (Millipore) both according to suppliers' recommendations. Blots were then analyzed by fluorescence scanning using the Typhoon Trio (GE). Densitometric analysis was performed using the companion software ImageQuant (GE).

Structure of biotion-alkyne reagent (Invitrogen, SKU# B10185)



Chemical Formula: $C_{24}H_{40}N_4O_7S$
Molecular Weight: 528.66

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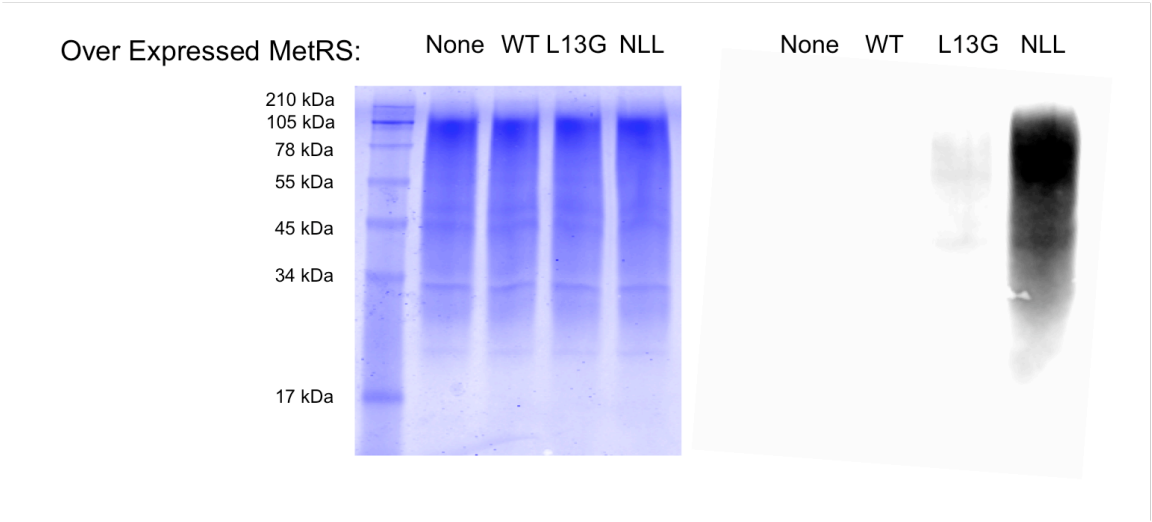
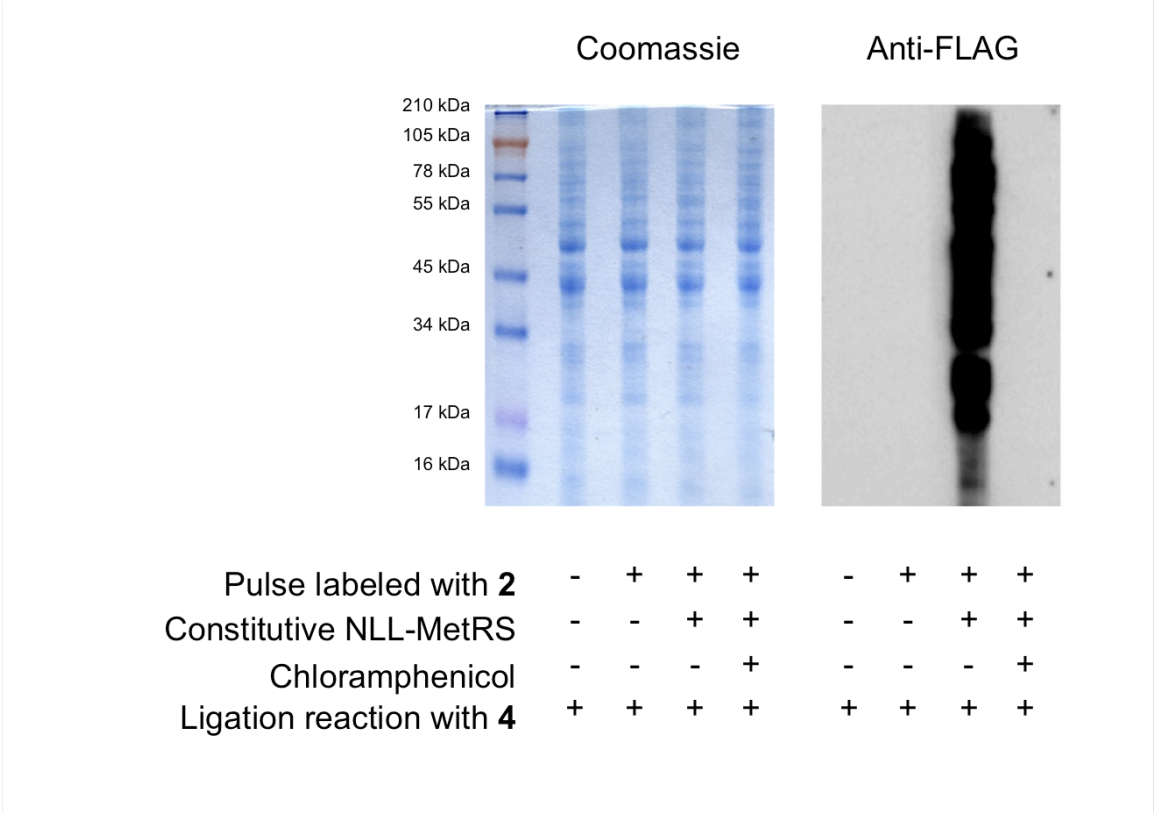
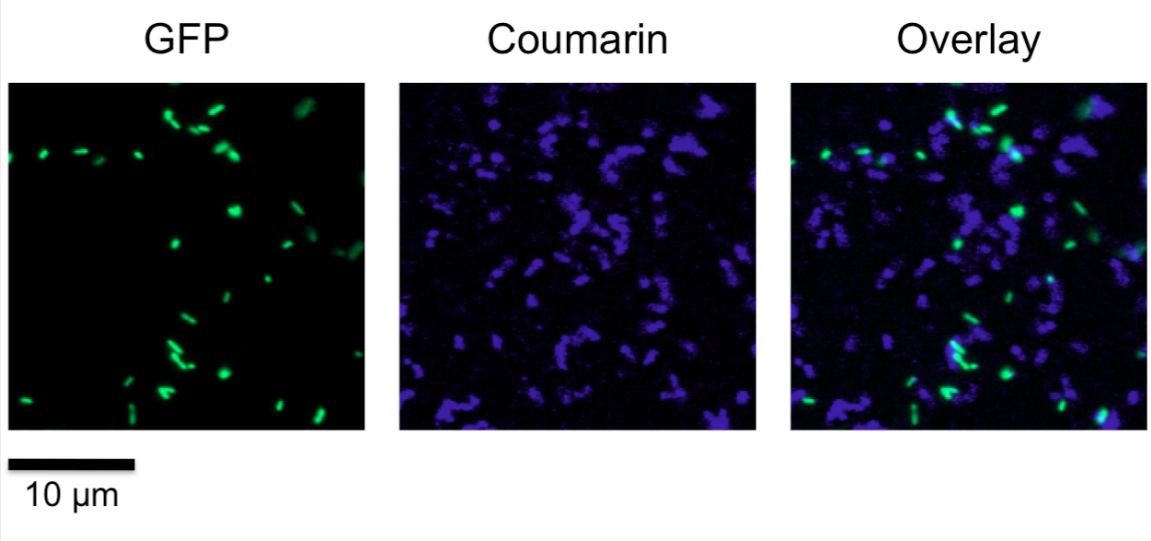


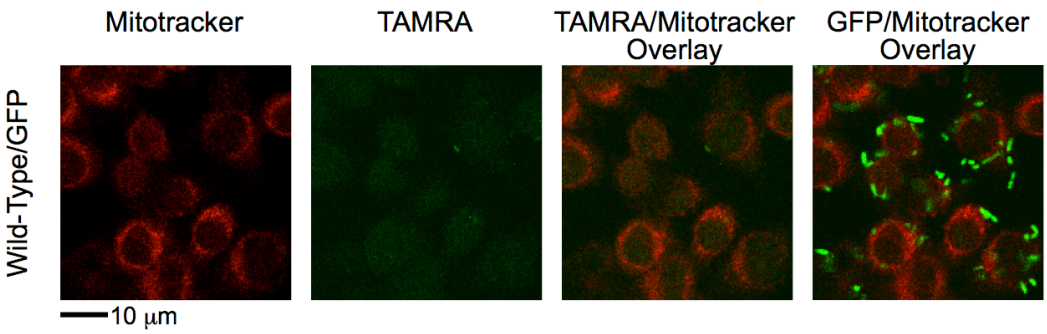
Supplementary Figure 1 Cells over-expressing plasmid-borne copies of either WT-MetRS, L13G-MetRS, or NLL-MetRS were compared with respect to incorporation of Anl into cellular proteins. The NLL mutant MetRS (NLL-MetRS) carries the mutations Leu13Asn, Tyr260Leu, and His301Leu and was isolated by methods described in Reference 11. Multiple rounds of high-throughput screening were performed in 1 mM azidonorleucine with increasing concentrations of methionine (from 0.01 to 0.1 mM) for isolation of highly active clones capable of charging azidonorleucine in the presence of methionine. In the work described in Reference 11 (Link *et al*, 2006), screening was done in methionine-depleted medium. The L13G mutant identified in that work affords only low levels of protein labeling with azidonorleucine in media supplemented with methionine. Over-expression of WT MetRS is insufficient for incorporation of Anl as shown by the absence of signal in western blots visualized with Anti-FLAG-HRP. Previously-isolated MetRS mutant L13G activates Anl, but yields low levels of Anl incorporation in media supplemented with Met.



Supplementary Figure 2 Coomassie detection of proteins from cells pulse-labeled with azidonorleucine (left) and Western blot detection of proteins conjugated to biotin-FLAG-alkyne (right). Azidonorleucine labels newly synthesized proteins in cells expressing the NLL-MetRS. Wild-type cells, cells not exposed to azidonorleucine, and mutant cells exposed to the protein synthesis inhibitor chloramphenicol are not labeled with azidonorleucine; proteins from these cells do not conjugate with biotin-FLAG-alkyne.



Supplementary Figure 3 Individual panels from imaging of mixed bacterial cultures from Figure 2b. Cu-catalyzed ligation to coumarin dye is restricted to cells expressing the NLL-MetRS. GFP-expressing wild-type cells not expressing the mutant enzyme do not exhibit fluorescence from the coumarin dye.



Supplementary Figure 4 Infection of macrophages is performed with control bacteria expressing GFP, but not the NLL-MetRS (Wild-Type/GFP). The absence of emission from the TAMRA indicates that both macrophages and bacteria not expressing the NLL-MetRS are unable to utilize **2** in protein synthesis. The presence of Wild-Type/GFP bacteria is confirmed through detection of GFP fluorescence.

Supplementary Movie 1 (available online) Infection of macophages (Red) is performed with bacteria expressing the NLL-MetRS and pulse labeled with 2. Upon conjugation with 6, fluorescence emission from 6 (Green) is observed in bacteria on the surface of macrophages as well as those that have been internalized via phagocytosis. Movie was made and exported with OsiriX. Confocal slices of both Mitrotracker and TAMRA emission from Fig 3a were combined into a 3D stack and rotated to different viewpoints.