Compartment-Specific Labeling of Bacterial Periplasmic Proteins by Peroxidase-Mediated Biotinylation

Uday S. Ganapathy, Lu Bai, Linpeng Wei, Kathryn A. Eckartt, Clarissa M. Lett, Mary L. Previti, Isaac S. Carrico and Jessica C. Seeliger

Table S1. Vectors and cloning strategies used in this study. Related to Figures 1-4.

#	Vector	Methodology	AbxR	Vector	Restriction Sites	Template
1	APEX	In-Fusion cloning	Kn	pRiboBsaIHindIII*	BsaI/HindIII	pcDNA3-mito-APEX
2	APEX2	Site-directed mutagenesis	Kn	N/A	N/A	APEX
3	Sec-APEX2	1) PCR1: *mpt63* signal, 3'BamHI		N/A		*Mtb* DNA
		2) PCR2: APEX2		N/A		APEX2
		3) PCR3: mpt63-APEX2		N/A		PCR1 And PCR2
		4) In-Fusion cloning: Sec-APEX2	Kn	pRiboBsaIHindIII*	NdeI/NheI	PCR3
4	Tat-APEX2	Ligation	Kn	Sec-APEX2	NdeI/BamHI	*Mtb* genomic DNA
5	pMV306-ribo-APEX2[§]	Subclone via digestion/ligation	Hyg	pMV306	XbaI/ClaI	APEX2
6	pMV306-ribo-Sec-APEX2[§]	Subclone via digestion/ligation	Hyg	pMV306	XbaI/ClaI	Sec-APEX2
7	pMV306-MsmegLprG-3XFLAG[†]	Subclone via PCR/In-Fusion	Hyg	pMV306	XbaI/ClaI	pSMT-MsmegLprG-3XFLAG***
8	pMV306-MsmegNA-LprG-3XFLAG[†]	Subclone via PCR/In-Fusion	Hyg	pMV306	XbaI/ClaI	pSMT-MsmegΔNLprG-3XFLAG***
9	pRibo-(Sec)-APEX2-Phsp60-3XFLAG	1) PCR1: Phsp60-EcoRI-ClaI		N/A		pMV306-MsmegLprG-3XFLAG
		2) PCR2: EcoRI-ClaI-3XFLAG		N/A		pMV306-MsmegLprG-3XFLAG
		3) PCR3: Phsp60-EcoRI-ClaI-3XFLAG		N/A		PCR1 and PCR2
		4) In-Fusion cloning	Kn	Sec-APEX2	ClaI/HpaI	pRIbo-APEX2 pRibo-Sec-APEX2
10	pRibo-(Sec)-APEX2-Phsp60-eGFP-3XFLAG[‡]	In-Fusion cloning	Kn	pMWS114*	ClaI/HpaI	pRibo-(Sec)-APEX2-Phsp60-3XFLAG
11	pMV261-eGFP-3XFLAG[§]	Digestion/T4pol polishing/ligation	Kn	pRibo-APEX2-Phsp60-eGFP-3XFLAG	ClaI/HpaI	
12	pMV261-MsmegLprG-3XFLAG[§]	Subclone via digestion/ligation	Kn	pMV261	XbaI/ClaI	pMV306-MsmegLprG-3XFLAG
13	pET24b-APEX2[#]	In-Fusion cloning	Amp	pET24b	NdeI/XhoI	APEX2

[§] = Used in Figure 3A; [†] = Used in Figure 4, [‡] = Used in Figure S13; [#] = Used in Figure S2

* Seeliger, J. C.; Topp, S.; Sogi, K. M.; Previti, M. L.; Gallivan, J. P.; Bertozzi, C. R., A riboswitch-based inducible gene expression system for mycobacteria. *PLoS ONE* **2012**, 7 (1), e29266.

** Cloning intermediate from this work; further information available upon request

*** Touchette, M. H.; Van Vlack, E. R.; Bai, L.; Kim, J.; Cognetta, A. B., 3rd; Previti, M. L.; Backus, K. M.; Martin, D. W.; Cravatt, B. F.; Seeliger, J. C., A Screen for Protein-Protein Interactions in Live Mycobacteria Reveals a Functional Link between the Virulence-Associated Lipid Transporter LprG and the Mycolyltransferase Antigen 85A. *ACS Infect Dis* **2017**, 3 (5), 336-348.

Table S2. Primers related to vectors in Table S1.* Related to Figures 1-4.

#	Forward Primer Sequence	Reverse Primer Sequence
1	AGGAGGCAACAAGATGCATATGGGCAAGCCCATCCCCAAC	CGACATCGATAAGCTTGGTCTCCATCTTCATTAGGCATCAGCAAACCCAAG
2	CTAACAACGGTCTTGACATCGCTG	GTCAAGACCGTTGTTAGCGCTG
3	GCAACAAGATGCATATGAAGCTCACCACAATGATCAAGACGGCAGTAGCGGTCG	GTTGGGGATGGGCTTGCCGGATCCAGCCAACGCGACCGGTGCCGCAAAGGTCGCGATGG
	CCATCGCGACCTTTGCGGCACCGGTCGCGTTGGCTGGATCCGGCAAGCCCATCCCCAAC	CGCTTTGTTGGCTAGCTGATCACCGCGGCCATG
	GCAACAAGATGCATATGAAGCTCACCACAATGATCAAGACGGCAGTAGCGGTCG	CGCTTTGTTGGCTAGCTGATCACCGCGGCCATG
	GCAACAAGATGCATATGAAGCTCACCACAATGATCAAGACGGCAGTAGCGGTCG	CGCTTTGTTGGCTAGCTGATCACCGCGGCCATG
4	GCAACAAGATGCATATGCGCAACAGAGGATTCGGTCGTCGCGAACTGCTGGTAG	GTTGGGGATGGGCTTGCCGGATCCCCCGCTCGCATGCCGTG
7	GATCTTTAAATCTAGAGGTGACCACAACGCGC	ACTACGTCGACATCGATACGCACGAAGCTCGCC
8	GATCTTTAAATCTAGAGGTGACCACAACGCGC	ACTACGTCGACATCGATACGCACGAAGCTCGCC
9	AGACCAAGCTTATCGGCCCGCTTTGATCGGGGAC	CGCTAGCGAATTCCTGATGTATCGATTGCGAAGTGATTCCTCCG
	CTTCGCAATCGATACATCAGGAATTCGCTAGCGACTACAAGGACG	GATCGTACGCTAGTTAACTACCTACTTGTCGTCGTCGTC
	GATCTTTAAATCTAGAGGTGACCACAACGCGC	GATCGTACGCTAGTTAACTACCTACTTGTCGTCGTCGTC
10	ATCACTTCGCAATCGATGGCCAAGACAATTGCGGATC	AGTCGCTAGCGAATTCCTTGTACAGCTCGTCCATGCC
13	GAAGGAGATATACATATGGGCAAGCCCATCCCCAAC	GGTGGTGGTGCTCGAGGGCATCAGCAAACCCAAGCTCG

* Restriction sites are underlined

Figure S1. Optimization of biotin-phenol labeling protocol in *Msm*. Related to Figure 1.
Msm expressing APEX2 was grown with or without theophylline and treated with varying concentrations of biotin-phenol for different incubation times. Streptavidin blot analysis of the lysates was used to assess protein biotinylation.

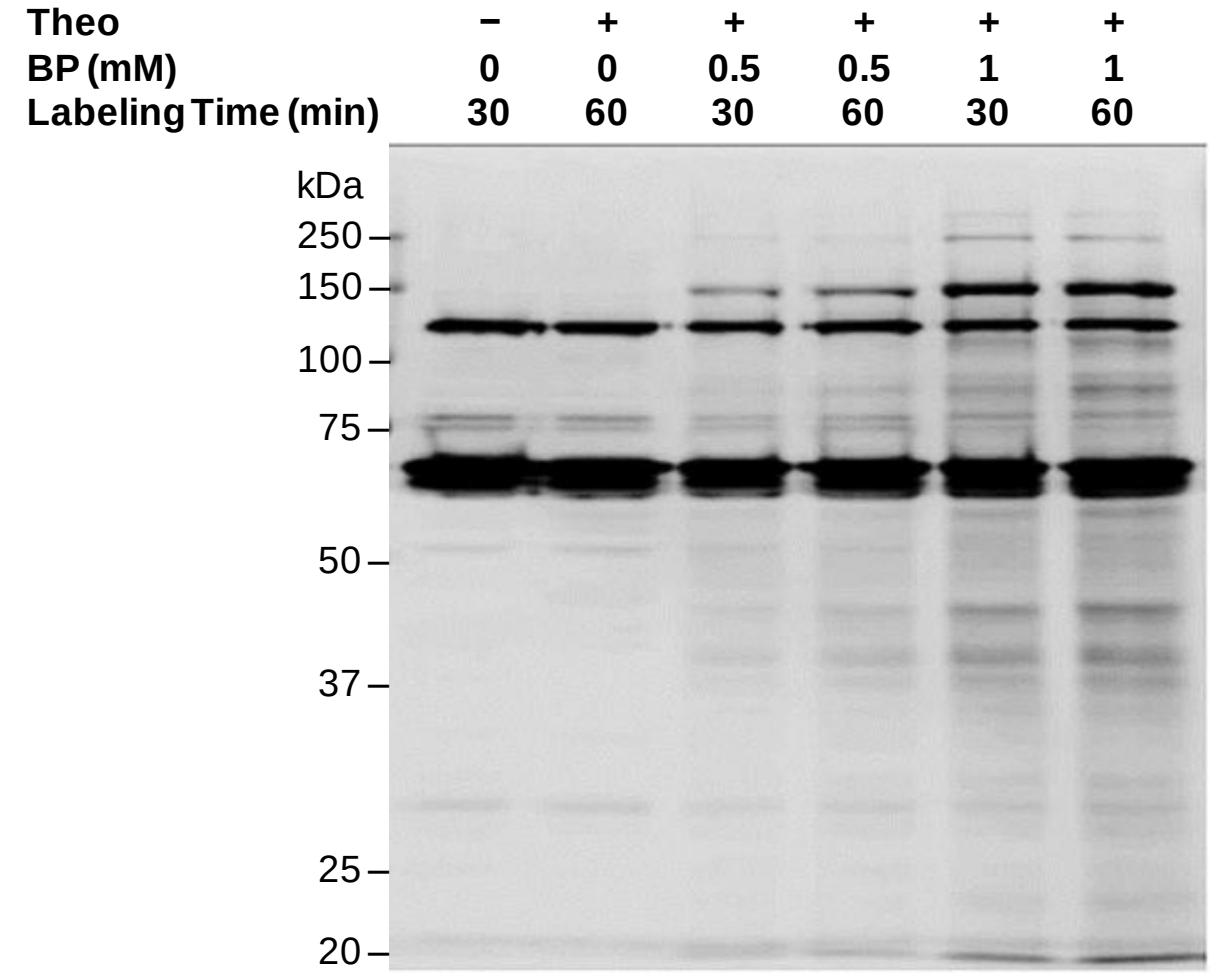

Figure S2. Protein biotinylation by APEX2 in *E.coli*. Related to Figure 1. *E. coli* expressing V5-tagged APEX2 (28.4 kDa) was grown without or with IPTG and subjected to the labeling protocol with biotin-phenol. Anti-V5 immunoblot analysis established APEX2 expression. Peroxidase activity was measured in whole cells using guaiacol. Streptavidin blot analysis of the lysates was used to assess protein biotinylation. Given the high level of APEX2 overexpression in this system, additional bands detected in the anti-V5 immunoblot may be due to aggregates not fully solubilized or reduced under the loading conditions used.

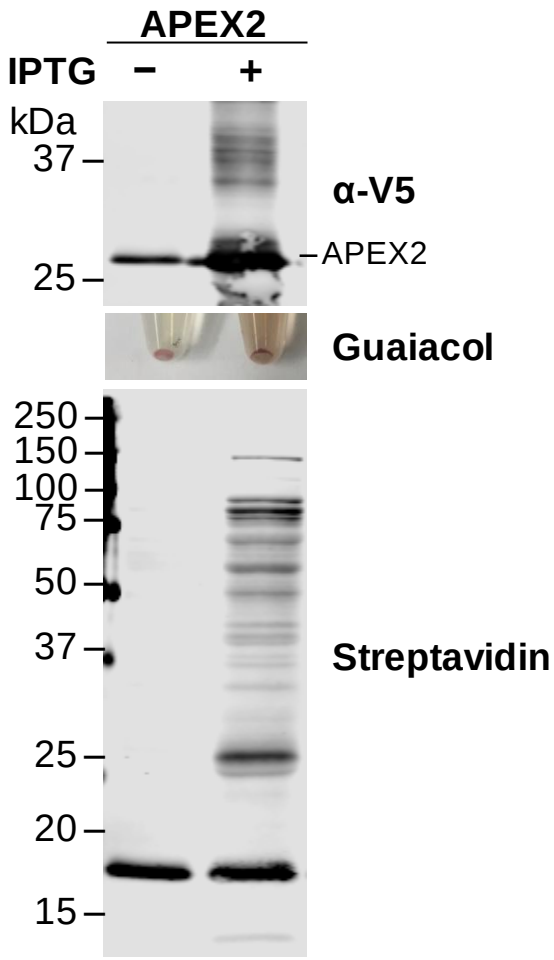

Figure S3. Validation of tyramide azide synthesis by NMR. Related to Figure 2. ^{1}H-NMR of the HPLC-purified tyramide azide. Asterisks indicate peaks from residual ethyl acetate (EtOAc)**.**

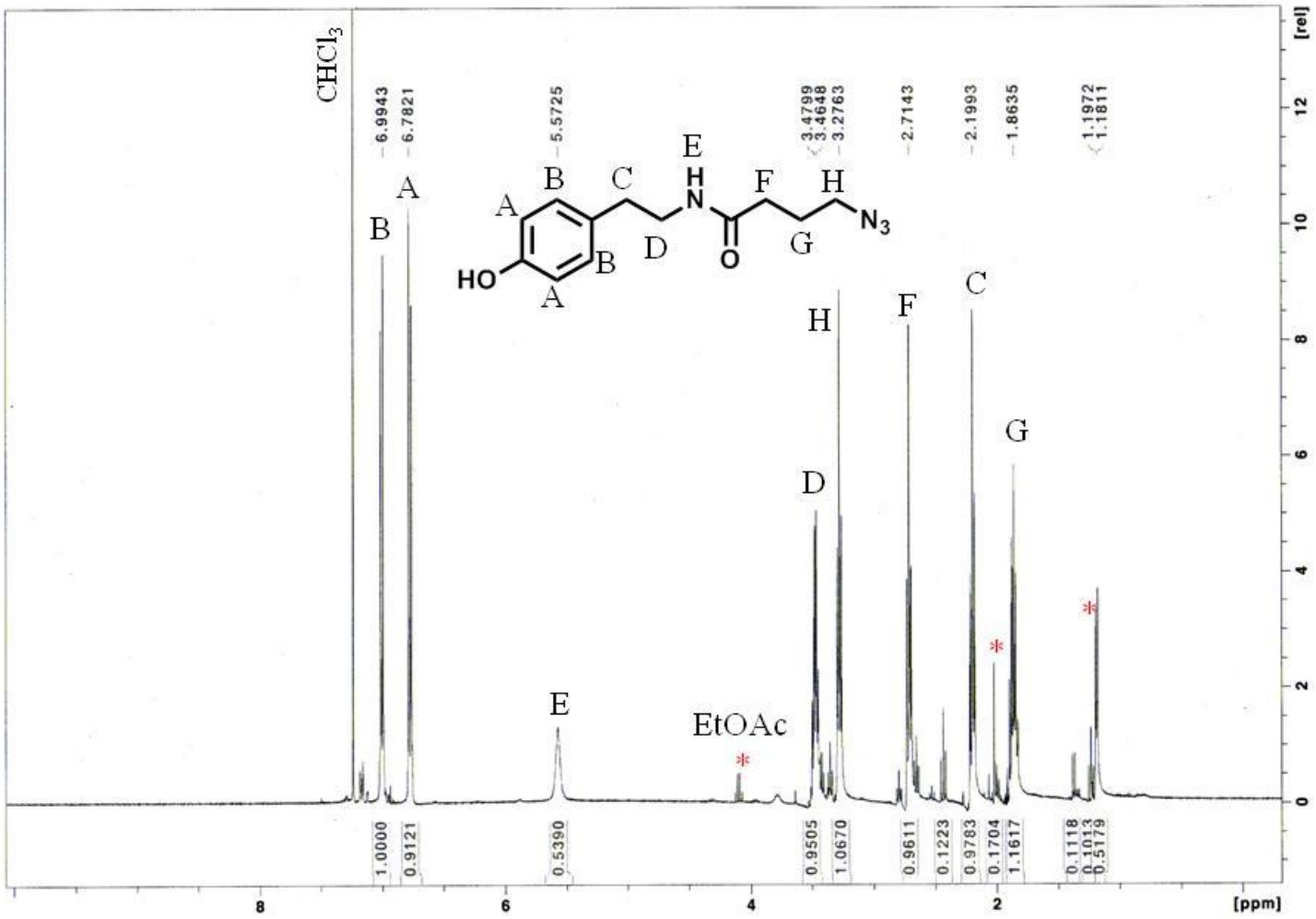

Figure S4. Validation of tyramide alkyne synthesis by NMR. Related to Figure 2. ^{1}H-NMR of HPLC-purified tyramide alkyne. "C3H8O" indicates peaks due to residual ethyl acetate.

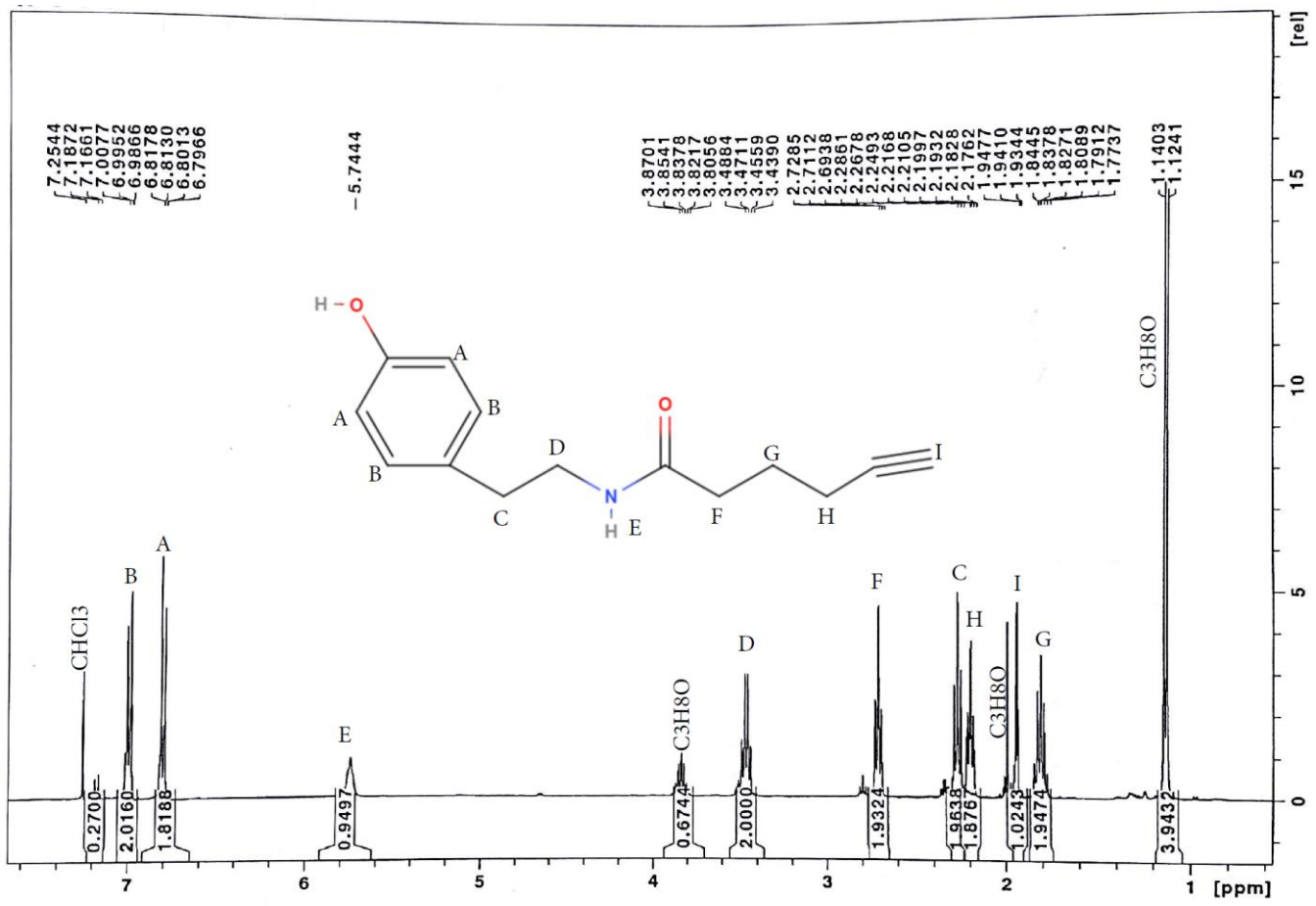

Figure S5. Coomassie stain confirms equal protein loading of tyramide azide- and tyramide alkyne-labeled samples. Related to Figure 2. *Msm* expressing APEX2, Sec-APEX2 or Tat-APEX2 were induced without or with theophylline, treated with either tyramide azide or tyramide alkyne labeling protocol and subsequent CuAAC click reaction with alkyne or azide-conjugated fluorescein. Gel is the same as that presented in Figure 2, but following fluorescence scanning for fluoroscein, the gel was stained with Coomassie and imaged.

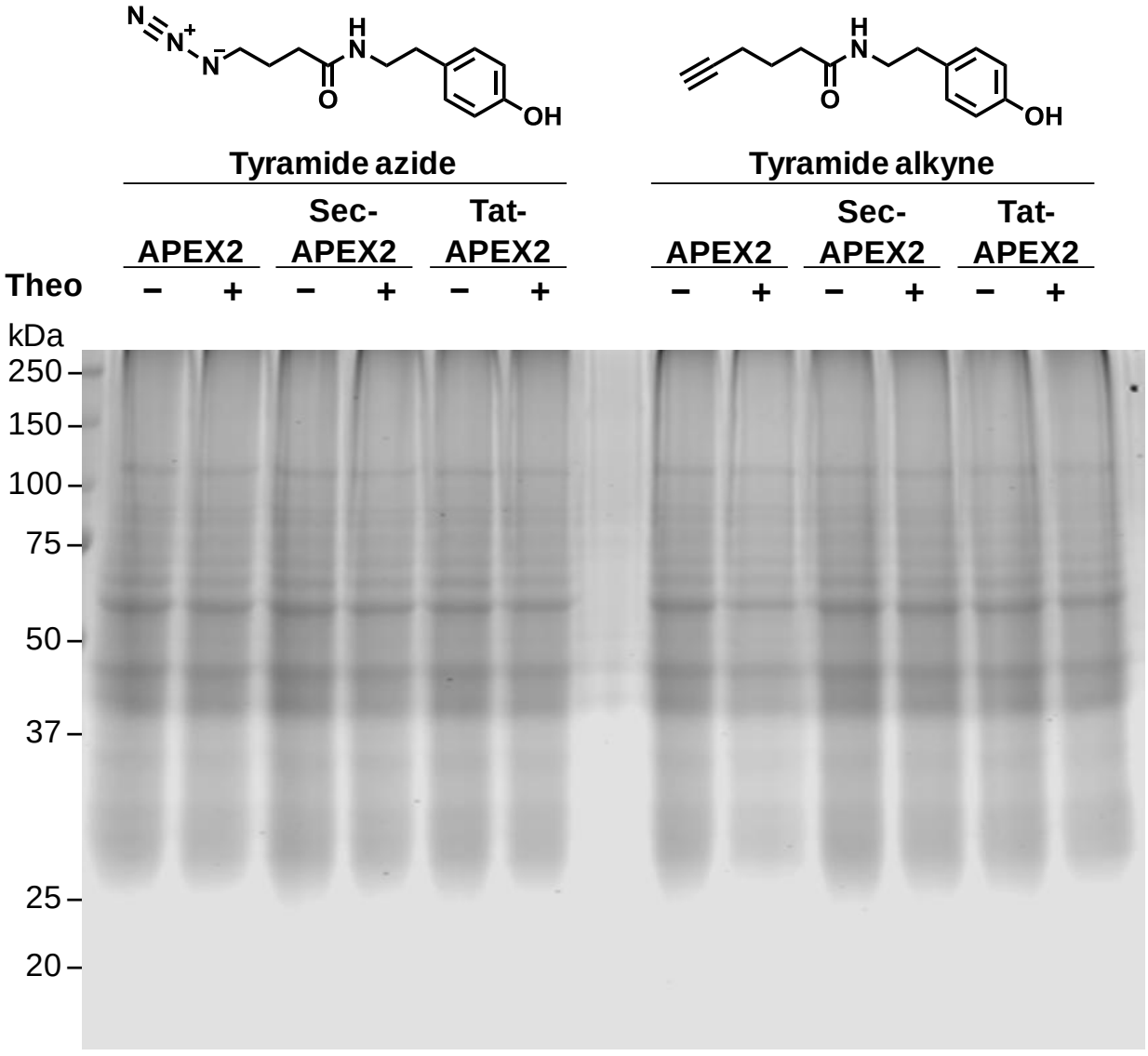

Figure S6. Protein labeling with tyramide azide is substrate-dependent and copper-dependent. Related to Figure 2. *Msm* expressing APEX2 was grown without or with theophylline and either left untreated or subjected to the tyramide azide labeling protocol, followed by a CuAAC click reaction with alkyne-conjugated fluorescein in the absence or presence of copper catalyst. In-gel fluorescence analysis of the clicked lysates was used to assess protein labeling.

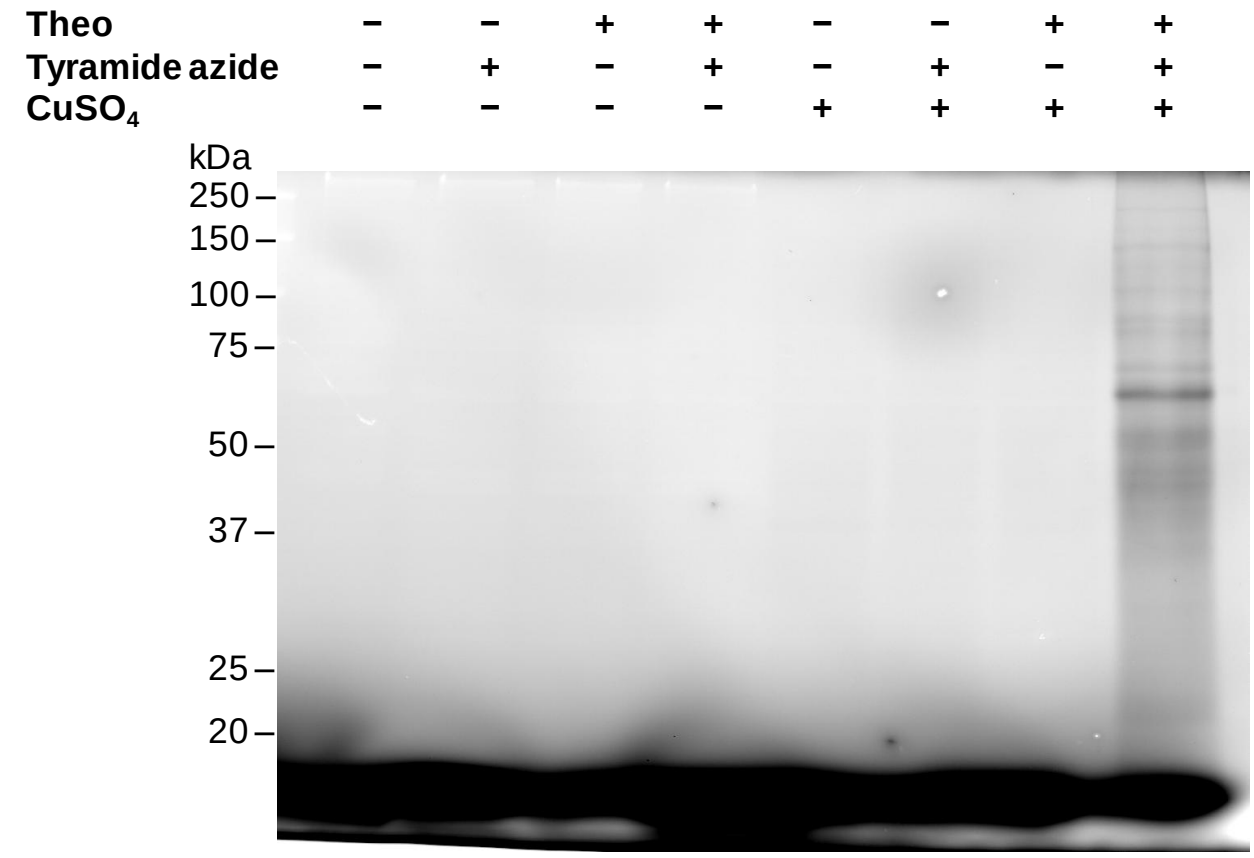

Figure S7. Optimization of tyramide azide labeling protocol in *Msm*. Related to Figure 2. *Msm* expressing APEX2 was grown without or with theophylline and treated with varying concentrations of tyramide azide for different incubation times and temperatures. Lysates were reacted via CuAAC with alkyne-conjugated fluorescein. In-gel fluorescence of the clicked lysates was used to assess protein labeling.

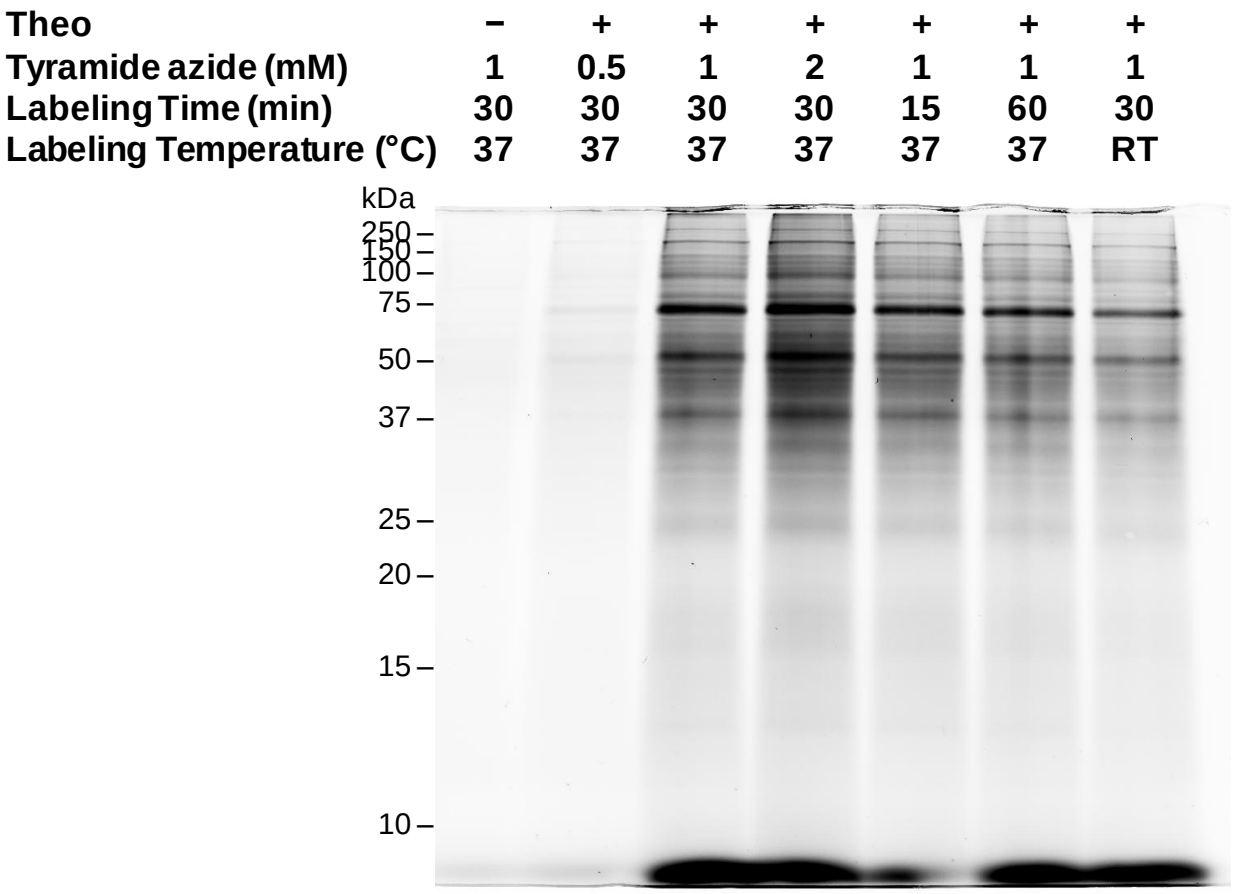

Figure S8. Optimization of tyramide alkyne labeling protocol in *Msm*. Related to Figure 2. *Msm* expressing APEX2 was grown without or with theophylline and subjected to the tyramide alkyne labeling protocol followed by a CuAAC click reaction with azide-conjugated fluorescein. In-gel fluorescence analysis of the clicked lysates was used to detect protein labeling.

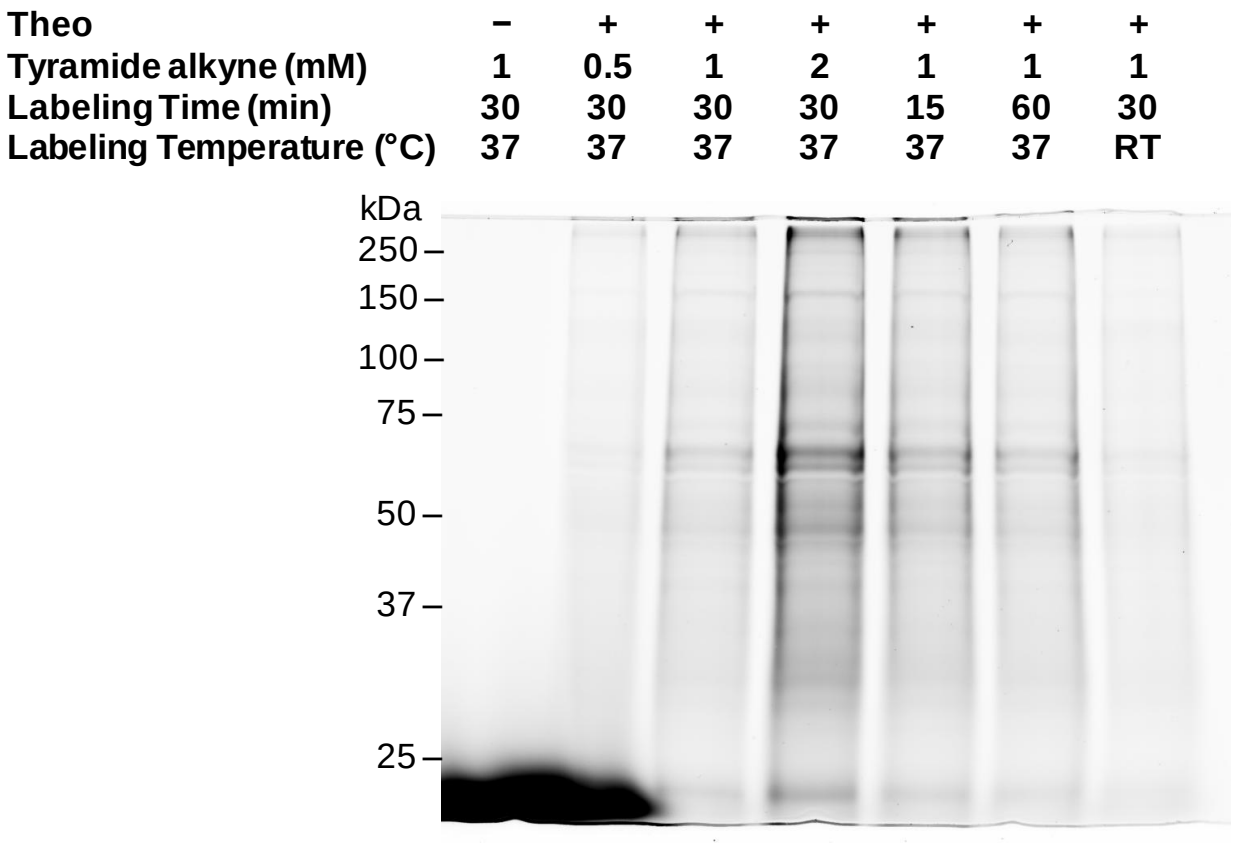

Figure S9. Differential labeling efficiency of alternative labeling substrates. Related to Figure 2. *Msm* expressing APEX2, Sec-APEX2 or Tat-APEX2 were induced without or with theophylline, treated with either tyramide azide or tyramide alkyne labeling protocol and subsequent CuAAC click reaction with alkyne or azide-conjugated fluorescein. In-gel fluorescence of the clicked lysates was used to detect protein labeling. Gel is the same as that presented in Figure 2, but with adjusted brightness and contrast settings in order to better observe labeling by tyramide alkyne. Arrowheads indicate examples of Sec- and Tat-APEX2 expression-dependent bands.

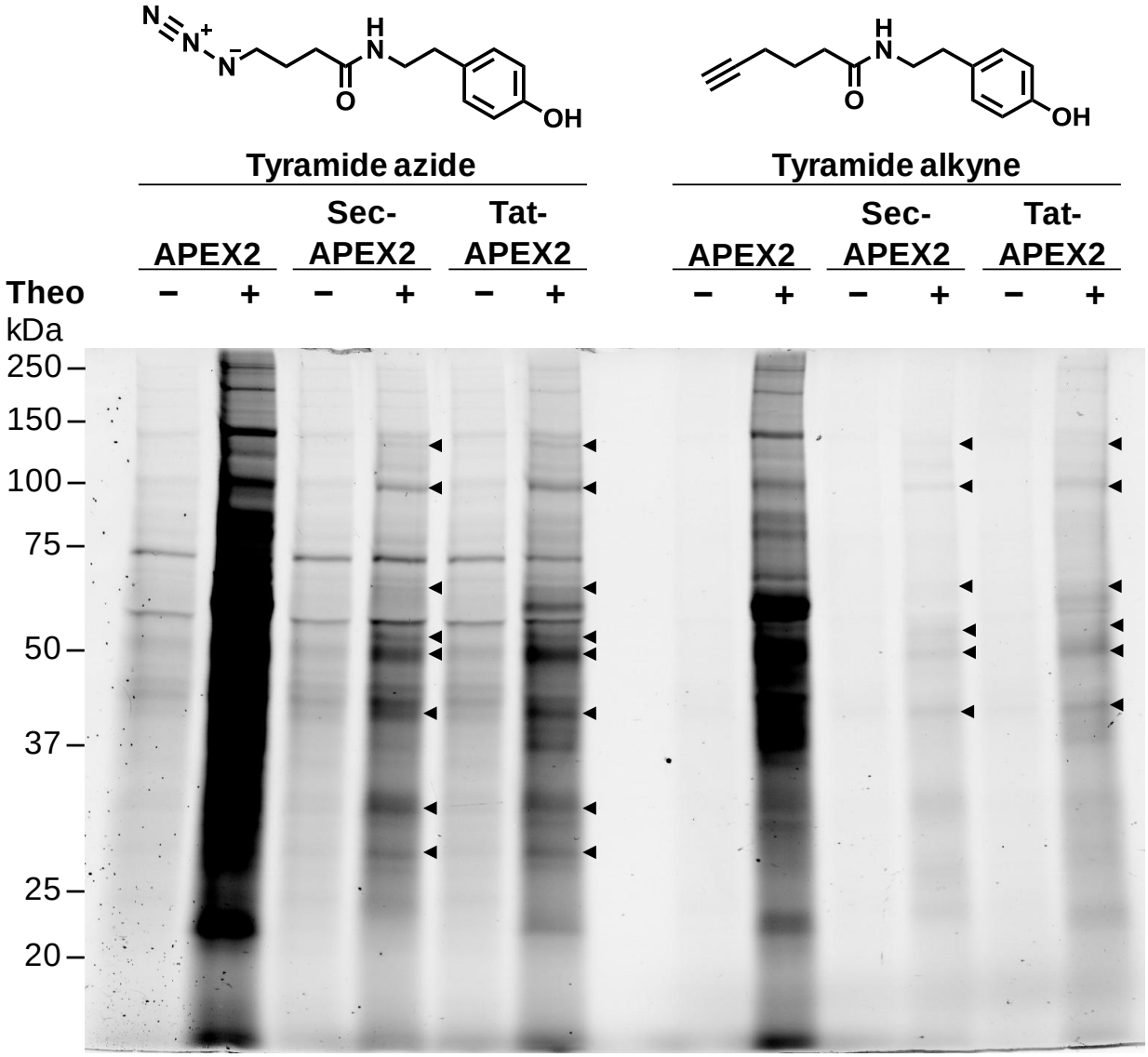

Figure S10. Optimization of CuAAC conditions for tyramide azide and tyramide alkyne labeling protocols in *Msm*. Related to Figure 2. *Msm* expressing APEX2, Sec-APEX2 or Tat-APEX2 was growth without or with theophylline and subjected to the labeling protocol with either tyramide azide or tyramide alkyne. Lysates underwent a CuAAC reaction with alkyne or azide-conjugated fluorescein using an optimized 1:1 ligand-to-copper ratio and 200 µM copper concentration. In-gel fluorescence detection of the treated lysates was used to assess protein labeling.

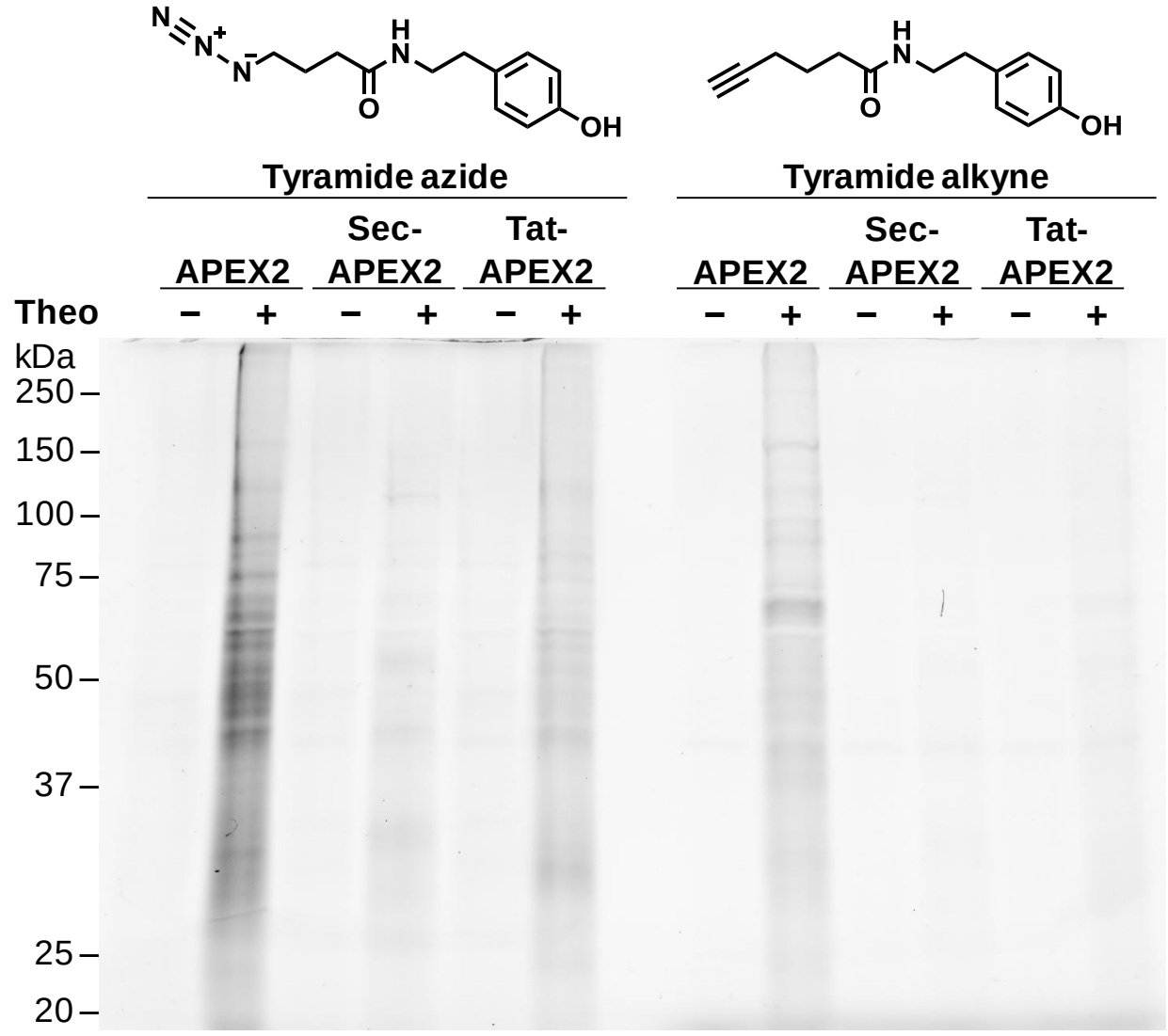

Figure S11. Optimization of CuAAC conditions for tyramide azide and tyramide alkyne labeling protocols in *Msm*. Related to Figure 2. *Msm* expressing APEX2, Sec-APEX2 or Tat-APEX2 was growth without or with theophylline and subjected to the labeling protocol with either tyramide azide or tyramide alkyne. Lysates underwent a CuAAC reaction with alkyne or azide-conjugated fluorescein using an optimized 1:1 ligand-to-copper ratio and 200 µM copper concentration. In-gel fluorescence detection of the treated lysates was used to assess protein labeling. Gel is the same as that presented in Figure S9, but with adjusted brightness and contrast settings in order to better observe labeling by tyramide alkyne.

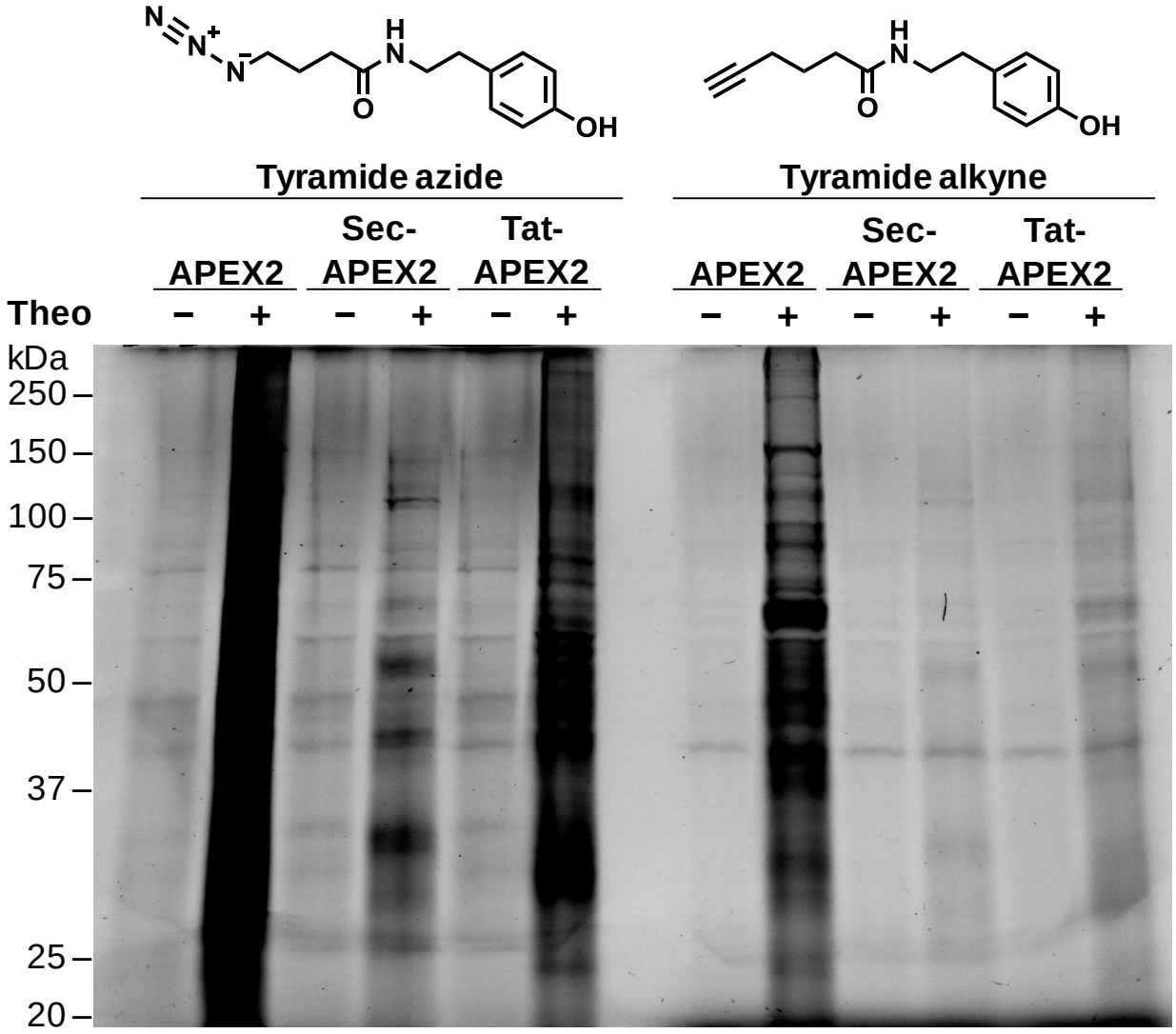

Figure S12. Truncating the predicted secretion signal relocalizes LprG to the cytoplasm. Related to Figure 4. *Msm* co-expressing APEX2 or Tat-APEX2 with LprG-3XFLAG (27 kDa) or NA-LprG-3XFLAG (24 kDa) from a multicopy episomal plasmid were grown without or with theophylline and subjected to the biotin phenol labeling protocol. Biotinylated proteins were enriched by avidin affinity. Anti-FLAG immunoblot analysis with chemiluminescence detection of the input and output fractions was used to determine expression and biotinylation of LprG-3XFLAG and NA-LprG-3XFLAG.

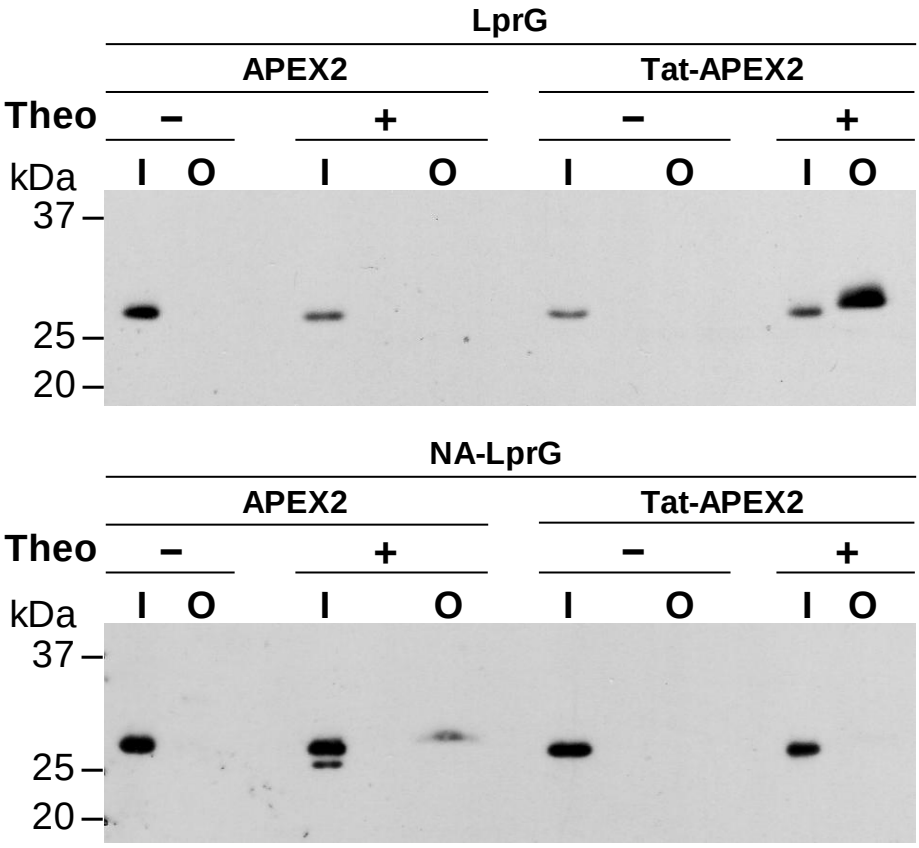

Figure S13. Labeling of eGFP by Sec-APEX2 is likely due to diffusion of biotin-phenoxyl radical into the cytoplasm. Related to Figure 3. *Msm* co-expressing APEX2 or Sec-APEX2 and eGFP-3XFLAG (31 kDa) were grown without or with theophylline and subjected to the labeling protocol with biotin-phenol. In contrast to Figure 3, both APEX2 and eGFP were expressed from multiple gene copies via a single dual-expression episomal plasmid (i.e., APEX2 was expressed at higher levels relative to Figure 3A). Biotinylated proteins were enriched by avidin affinity purification. Anti-FLAG immunoblot analysis with fluorescence detection was used to quantify expression and biotinylation. The fold increase in biotinylation upon induction of APEX2 or Sec-APEX2 ("Ratio") was calculated by taking the ratio of the + / − theophylline output signals after normalizing to the input. Lane labels I and O indicate input and output from the avidin enrichment. The image contrast parameters are different than those used in Figure 3A, but all intensities are from the raw images and can be compared directly between figures. Overall eGFP expression appears higher here than in Figure 3A (input lanes) likely due to higher transcript levels driven by the additional promoter on the upstream APEX2 gene (no terminator was placed between the two operons).