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Letters

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Cell-specific Profiling of Nascent Proteomes Using Orthogonal Enzyme-mediated Puromycin Incorporation

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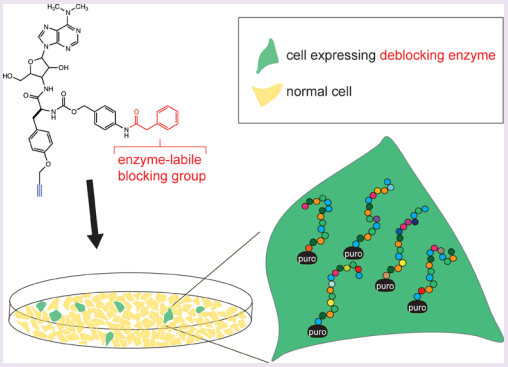
Supporting Information

ABSTRACT: Translation regulation is a fundamental component of gene expression, allowing cells to respond rapidly to a variety of stimuli in the absence of new transcription. The lack of methods for profiling nascent proteomes in distinct cell populations in heterogeneous tissues has precluded an understanding of translational regulation in physiologically relevant contexts. Here, we describe a chemical genetic method that involves orthogonal enzyme-mediated incorporation of a clickable puromycin analogue into nascent polypeptides. Using this method, we show that we can label newly synthesized proteins in a cell-specific manner in cells grown in culture and in heterogeneous tissues. We also show that we can identify the nascent proteome in genetically targeted cell populations using affinity enrichment and tandem mass spectrometry. Our method has the potential to provide unprecedented insights into cell-specific translational regulation in heterogeneous tissues.



The control of gene expression at the level of mRNA (mRNA) translation is essential for cell identity and function.[1] This mode of regulation is essential for many important physiological events, such as memory consolidation[2] and metabolic homeostasis,[3] and is dysregulated in several human diseases, such as autism[4] and cancer.[5] mRNA translation is a dynamic process that allows cells to respond rapidly to various stimuli, for example, electrical activity[2] and metabolic changes.[3] While most cells have the same genome, the assemblage of proteins that are synthesized can vary widely among different cell types in tissues and whole organisms. The heterogeneous nature of most tissues makes it challenging to study cell-specific translational regulation *in vivo*. Heintz and co-workers addressed this challenge by developing a technique called Translating Ribosome Affinity Purification (TRAP), which is based on the cell-specific immunopurification of epitope tagged ribosomes associated with actively translated mRNAs.[6] Although TRAP enriches protein-coding mRNAs in the process of being translated, it does not monitor protein synthesis directly, and the relationship between ribosome binding to mRNA and translation is not precise. Moreover, a recent study demonstrated that noncoding RNAs are enriched using TRAP, showing that not all RNAs bound to the ribosome are undergoing translation.[7] Other methods, including PUNCH-P, efficiently label and identify newly synthesized peptides but lack cell-type specificity.[8] Methods for directly labeling and identifying newly synthesized (nascent) polypeptides in specific cell populations are critically important for understanding how gene expression is regulated at the level of translation.

One strategy for labeling and identifying newly synthesized polypeptides exploits the mechanism of the antibiotic puromycin (puro), an aminonucleoside tRNA mimic. During active translation, puro binds to the translating ribosome where its α-amino group covalently attacks the carbonyl of the aminoacyl-tRNA ester, causing premature termination of translation. When puro is used at low micromolar concentrations, for a short period of time (minutes), it does not inhibit translation or induce a stress response.[9] Thus, the use of low concentrations of puro or puro analogues for a limited period of time provides a means to label and identify newly synthesized proteins. Recently, a clickable analogue of puro in which the *O*-methyl was replaced with an *O*-propargyl group (OP-puro) was synthesized and used to visualize nascent protein synthesis in cells and *in vivo* after conjugation of the OP-puro-labeled polypeptides to a fluorescent azide tag using click chemistry.[10] In principle, the OP-puro method could be used to identify newly synthesized proteins in specific cell populations *in vivo* by purifying the desired cells to homogeneity. This would be a daunting challenge, however, especially in heterogeneous tissues and for low abundance cell populations. Moreover, the process of cell purification likely alters the nascent proteome, complicating interpretations. To overcome these challenges, we developed a chemical genetic method that directs precise control of the cell-specific action of OP-puro. In this strategy, we designed an analogue of OP-puro

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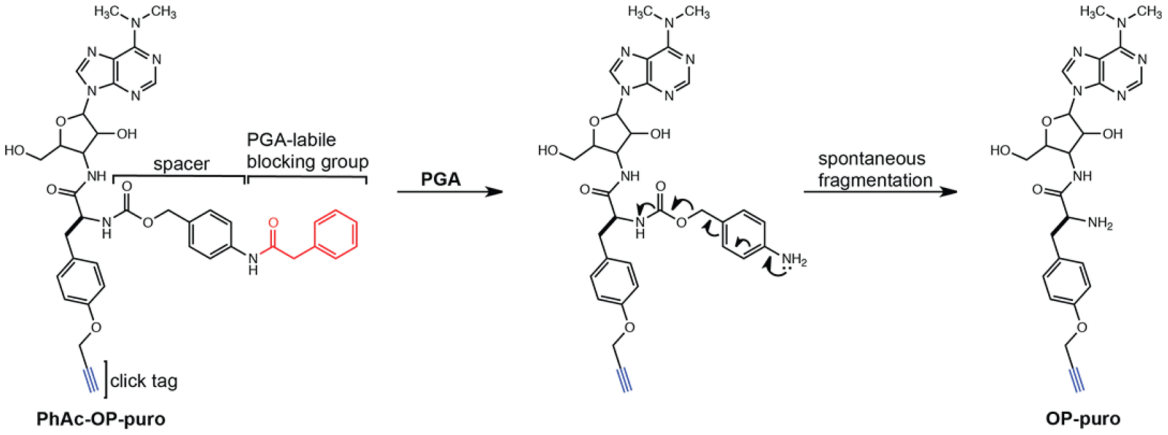
Scheme 1. Synthesis of PhAc-OP-puro[a]



[a]Reagents and conditions: (a) 1:1 TFA/CH_2Cl_2; (b) DMF, **2**, DIEA, CH_2Cl_2; (c) trimethyltin hydroxide, 1,2-dichloroethane; (d) pentafluorophenol, EDC, DMAP, THF; (e) puromycin aminonucleoside, TEA, CH_2Cl_2.

that contains an enzyme-labile blocking group on its α-amino group, rendering it inactive. By conditionally expressing an orthogonal enzyme that can selectively remove this blocking group from an inactive OP-puro analogue in genetically targeted cell populations, we can label and identify nascent polypeptides in a cell-specific manner in heterogeneous tissues.

We first sought an OP-puro analogue in which its α-amine could be modified with an enzyme-labile blocking group. One such blocking group for amines that has received particular attention is the phenylacetyl (PhAc) group, which can be removed by the enzyme penicillin G acylase (PGA).[11] PGA is normally expressed in *E. coli* where it removes the PhAc group from the β-lactam antibiotic, penicillin G, generating 6-aminopenicillanic acid (6-APA).[12] PGA is exquisitely selective for amides of phenylacetic acid and does not hydrolyze peptide bonds. In addition to its natural substrate penicillin G, PGA can remove the PhAc group from diverse compounds, including amino acids, nucleotides, and fluorescent reporters, in high yields.[11] The remarkably broad substrate tolerance of PGA makes it an ideal deblocking enzyme for our strategy to selectively activate an analogue of OP-puro containing the PhAc blocking group.

We therefore designed PhAc-OP-puro, an analogue of OP-puro that contains (i) a PhAc blocking group recognized by PGA and (ii) a stable *N*- (benzyloxy) carbamate spacer that will undergo a spontaneous fragmentation upon cleavage of the PhAc group by PGA to generate OP-puro (Scheme 1). We reasoned that the spacer would be important for minimizing potential steric hindrance. PhAc-OP-puro was synthesized according to Supporting Schemes 1 and 2. We hypothesized that PhAc-OP-puro should be inert in wild type mammalian cells because the α-amine group of OP-puro is blocked. By contrast, the blocking group of PhAc-OP-puro should be removed in mammalian cells expressing active PGA, allowing incorporation of OP-puro into nascent polypeptides.

We next designed a PGA construct for expression in mammalian cells. In *E. coli*, PGA is expressed as a precursor protein containing a signal sequence that targets it to the bacterial periplasm, where it is processed and activated.[13] Previous studies, however, have shown that by removing the signal sequence, PGA can be expressed in the cytoplasm of the methylotrophic yeast, *Pichia pastoris*.[14] Importantly, this cytoplasmic variant of PGA was as active as wild type PGA expressed in bacteria. These results suggested that it is possible to express active PGA in mammalian cells. We therefore designed a codon-optimized PGA variant that does not contain the signal sequence. Using a previously described fluorogenic coumarin PGA substrate,[15] we found that PGA produced in human embryonic kidney (HEK) 293T cells was active and not toxic to the cells (Supporting Figure 1).

To determine whether PGA can remove the PhAc blocking group on PhAc-OP-puro in mammalian cells, we monitored the incorporation of OP-puro into nascent polypeptides using click chemistry with biotin-azide. Treatment of HEK 293T cells expressing PGA with PhAc-OP-puro (10 μM) for 1 h followed by click conjugation with biotin-azide in cell lysates resulted in extensive biotin labeling across a spectrum of molecular weights (Figure 1a). Importantly, no labeling was observed in control transfected HEK 293T cells lacking PGA (Figure 1a). This labeling was blocked by cycloheximide, indicating that the biotin signal was due to active mRNA translation (Supporting Figure 2). Under these conditions, labeling was equivalent to a low dose of OP-puro that has been shown to cause little to no disruption of protein synthesis (Supporting Figure 2).[10] Further, treatment did not alter cellular morphology, and labeling was cumulative over time, indicating that labeled peptides are not quickly degraded (Supporting Figure 2). These results demonstrate that in mammalian cells, PGA can deblock PhAc-OP-puro to generate OP-puro, which can incorporate into nascent polypeptides. Importantly, PhAc-OP-puro is inert in cells that do not express PGA, demonstrating that the PhAc acts an effective blocking group.

To further demonstrate that the deblocking of PhAc-OP-puro occurs only in cells expressing PGA, we monitored the incorporation of OP-puro into nascent polypeptides in single cells using click chemistry with Alexa Fluor-568 azide. In HEK 293T cells transfected with a PGA-IRES-GFP plasmid (5% of cells were transfected with PGA) and treated with PhAc- OP-puro (5 μM) for 30 min, labeling of nascent polypeptides was detected exclusively in PGA-expressing cells (Figure 1b). Similar results were obtained in neurons, demonstrating that our approach is applicable to diverse cell types (Figure 1b). These results show that we can achieve cell-specific labeling of newly synthesized proteins in cells expressing PGA and treated with PhAc-OP-puro.

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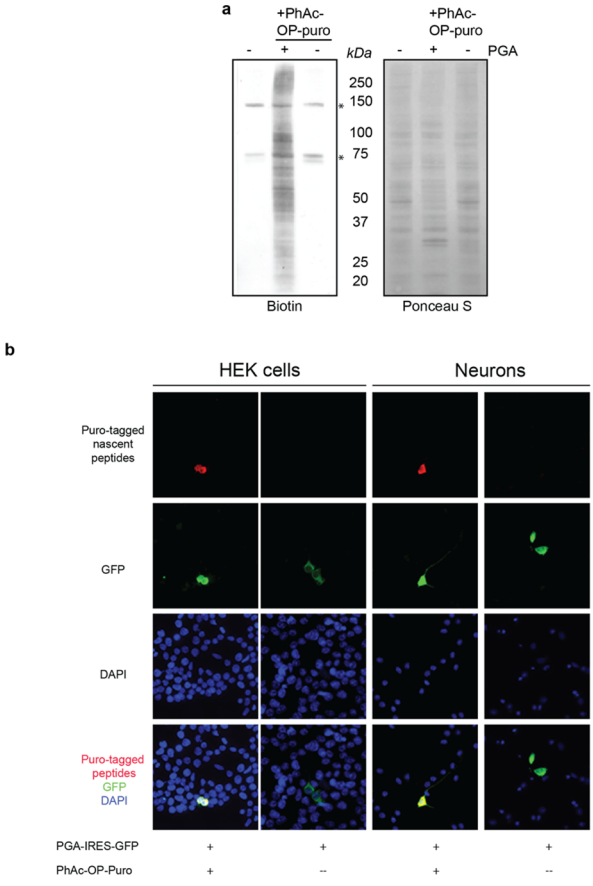

Figure 1. Orthogonal enzyme-mediated incorporation of OP-puro for cell-specific labeling of newly synthesized proteins. (a) Selective labeling of nascent polypeptides in PGA-expressing HEK 293T cells. Nascent protein synthesis was detected by click chemistry using biotin-azide. Samples were subjected to immunoblot detection with Streptavidin-HRP (left). Total protein is indicated by Ponceau S staining (right). *Endogenous biotinylated proteins. (b) Single cell labeling of nascent polypeptides in PGA-expressing HEK 293T cells and primary rat hippocampal neurons. Nascent protein synthesis was detected by click chemistry using Alexa Fluor-568-azide. Total cells were detected by DAPI.

We next wanted to determine whether we could achieve labeling of nascent polypeptides in a specific cell type in a heterogeneous tissue. We chose islets of Langerhans of the pancreas because they contain multiple cell types with well-characterized and highly distinct functions. One particular cell type that has received much attention is the beta cell because of its essential role in maintenance of blood sugar levels.[16] To achieve specific expression of PGA in beta cells, we used an adeno-associated virus serotype 8 (AAV8) that expresses PGA under the control of the mouse insulin promoter (AAV8-mIP2-PGA). When AAV8-mIP2 is delivered by intraperitoneal (IP) injection in mice, it transduces a fraction of beta cells of the pancreas.[17] We delivered AAV8-mIP2-PGA by IP injection in mice and after 2 weeks isolated islets and cultured them *in vitro*. Treatment of islets from AAV8-mIP2-PGA infected mice with 20 μM PhAc-OP-puro followed by click chemistry with Alexa Fluor-568 azide resulted in labeling of nascent polypeptides only in beta cells as demonstrated by colocalization with insulin (Figure 2). Importantly, no labeling of nascent polypeptides was detected in another cell type in islets, glucagon-expressing cells (Figure 2). Together, these results demonstrate our

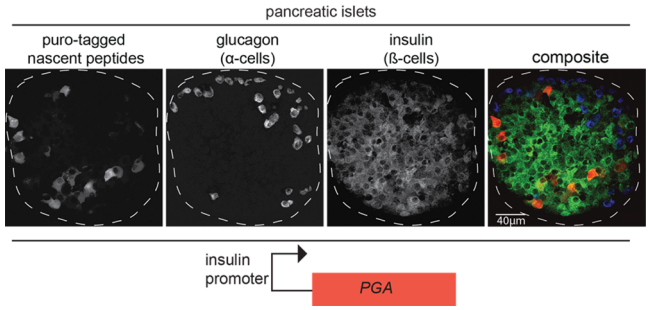

Figure 2. Selective labeling of nascent polypeptides in β cells expressing PGA. Pancreatic islets infected *in vivo* with β cell specific AAV-mIP2-PGA were treated *ex vivo* with PhAC-OP-puro (20 μM) for 2 h in high glucose following 1 h in low glucose. Tissue was fixed with 4% formaldehyde, and click chemistry with Alexa Fluor-568-azide was performed. Islets were immunostained for glucagon and insulin.

chemical genetic strategy can be used to label nascent polypeptides in genetically targeted cells in a complex cellular environment.

Having demonstrated that we can label nascent polypeptides with PhAc-OP-puro exclusively in PGA-expressing cells, we next sought to identify newly synthesized proteins in genetically targeted cells using our strategy. HEK 293T cells transfected with PGA were treated with PhAc-OP-puro (20 μM). Two control conditions were used: cells transfected with GFP and treated with PhAc-OP-puro (20 μM) and cells transfected with PGA and treated with vehicle control. After 1 h, click conjugation with biotin-azide was performed in cell lysates, and biotinylated proteins were enriched using streptavidin magnetic beads. Silver stain analysis showed an enrichment of proteins only in cells expressing PGA and treated with PhAc-OP-puro (Figure 3a), consistent with the extensive biotin labeling observed only under those conditions (Supporting Figure 3). Proteins were then proteolyzed, and eluted peptides were subjected to tandem mass spectrometry (LC-MS/MS). The total number of peptides and peptide signal intensity was

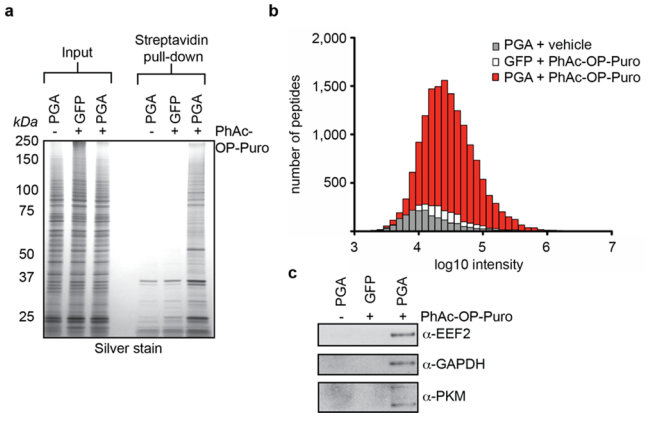

Figure 3. Identification of newly synthesized proteins in genetically targeted cells. (a) Enrichment of nascent polypeptides in PGA expressing cells treated with PhAc-OP-puro. Newly synthesized proteins were labeled with biotin using click chemistry with biotin-azide. Biotinylated proteins were enriched using Streptavidin magnetic beads and detected by silver stain. (b) Intensity distribution ($\log_{10}$) of the number of identified HEK 293T peptides for conditions described in a. (c) Western blot validation of LC-MS/MS results using specific antibodies. Conditions are as described in a.

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much greater in the sample derived from cells expressing PGA and treated with PhAc-OP-puro compared to both control samples (Figure 3b). From these peptides, we identified 1165 different human proteins that were greater than 5-fold enriched in the sample derived from cells expressing PGA and treated with PhAc-OP-puro (Supporting Table 1). To confirm our LC-MS/MS results, we performed enrichment using streptavidin magnetic beads, followed by immunoblot detection. We selected three proteins for our analysis: elongation factor 2 (EEF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and pyruvate kinase (PKM). Cells expressing PGA and treated with PhAc-OP-puro showed enrichment in all three proteins (Figure 3c). By contrast, no detectable enrichment was detected in samples derived from control conditions. Taken together, these results demonstrate the feasibility of identifying newly synthesized proteins from genetically targeted cells using our strategy.

In this study, we developed a chemical genetic strategy for labeling and identifying newly synthesized proteins in genetically targeted cell populations. The attachment of PhAc to the α-amine of OP-puro prevented it from incorporating into nascent polypeptides. By expressing PGA, an enzyme that can remove the PhAc blocking group from PhAc-OP-puro to generate OP-puro, we showed that we could label and identify the nascent proteome in a cell-specific manner.

Although recent strategies based on unnatural amino acid technologies have been reported for cell-specific proteome labeling,[18,19] they do not provide the temporal resolution required to examine translational regulation, which occurs on a rapid (minutes) time scale. The chemical genetic strategy described in this study should be useful for investigating the role of translational regulation in any cell population that can be specifically targeted using genetic techniques. Future studies will be focused on optimizing the chemical genetic strategy described herein for investigating cell-specific translational regulation in heterogeneous tissues *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b01076.

Supporting Figures 1−3 and supporting Schemes 1 and 2 showing the synthesis of PhAc-OP-puro, compound characterization, and experimental methods (PDF)

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Author Contributions

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Notes

The authors declare the following competing financial interest(s): We have a patent application on the technology described in this manuscript.

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■ REFERENCES

(1) Kong, J., and Lasko, P. (2012) Translational control in cellular and developmental processes. *Nat. Rev. Genet. 13*, 383−394.

(2) Costa-Mattioli, M., Sossin, W. S., Klann, E., and Sonenberg, N. (2009) Translational control of long-lasting synaptic plasticity and memory. *Neuron 61*, 10−26.

(3) Gomez, E., Powell, M. L., Greenman, I. C., and Herbert, T. P. (2004) Glucose-stimulated protein synthesis in pancreatic beta-cells parallels an increase in the availability of the translational ternary complex (eIF2-GTP.Met-tRNAi) and the dephosphorylation of eIF2 alpha. *J. Biol. Chem. 279*, 53937−53946.

(4) Darnell, J. C. (2011) Defects in translational regulation contributing to human cognitive and behavioral disease. *Curr. Opin. Genet. Dev. 21*, 465−473.

(5) Siddiqui, N., and Sonenberg, N. (2015) Signalling to eIF4E in cancer. *Biochem. Soc. Trans. 43*, 763−772.

(6) Heiman, M., Schaefer, A., Gong, S., Peterson, J. D., Day, M., Ramsey, K. E., Suarez-Farinas, M., Schwarz, C., Stephan, D. A., Surmeier, D. J., Greengard, P., and Heintz, N. (2008) A translational profiling approach for the molecular characterization of CNS cell types. *Cell 135*, 738−748.

(7) Zhou, P., Zhang, Y., Ma, Q., Gu, F., Day, D. S., He, A., Zhou, B., Li, J., Stevens, S. M., Romo, D., and Pu, W. T. (2013) Interrogating translational efficiency and lineage-specific transcriptomes using ribosome affinity purification. *Proc. Natl. Acad. Sci. U. S. A. 110*, 15395−15400.

(8) Aviner, R., Geiger, T., and Elroy-Stein, O. (2013) Novel proteomic approach (PUNCH-P) reveals cell cycle-specific fluctuations in mRNA translation. *Genes Dev. 27*, 1834−1844.

(9) Schmidt, E. K., Clavarino, G., Ceppi, M., and Pierre, P. (2009) SUnSET, a nonradioactive method to monitor protein synthesis. *Nat. Methods 6*, 275−277.

(10) Liu, J., Xu, Y., Stoleru, D., and Salic, A. (2012) Imaging protein synthesis in cells and tissues with an alkyne analog of puromycin. *Proc. Natl. Acad. Sci. U. S. A. 109*, 413−418.

(11) Kadereit, D., and Waldmann, H. (2001) Enzymatic protecting group techniques. *Chem. Rev. 101*, 3367.

(12) Volpato, G., Rodrigues, R. C., and Fernandez-Lafuente, R. (2010) Use of enzymes in the production of semi-synthetic penicillins and cephalosporins: drawbacks and perspectives. *Curr. Med. Chem. 17*, 3855−3873.

(13) Kasche, V., Lummer, K., Nurk, A., and Piotraschke, E. (1999) Intramolecular autoproteolysis initiates the maturation of penicillin amidase from Escherichia coli. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1433*, 76−86.

(14) Maresová, H., Marková, Z., Valesová, R., Sklenár, J., and Kyslík, P. (2010) Heterologous expression of leader-less pga gene in Pichia pastoris: intracellular production of prokaryotic enzyme. *BMC Biotechnol. 10*, 7.

(15) Ninkovic, M., Riester, D., Wirsching, F., Dietrich, R., and Schwienhorst, A. (2001) Fluorogenic assay for penicillin G acylase activity. *Anal. Biochem. 292*, 228−233.

(16) Triplitt, C. L. (2012) Examining the mechanisms of glucose regulation. *Am. J. Manag Care 18*, S4−10.

(17) Tonne, J. M., Sakuma, T., Munoz-Gomez, M., El Khatib, M., Barry, M. A., Kudva, Y. C., and Ikeda, Y. (2015) Beta cell regeneration after single-round immunological destruction in a mouse model. *Diabetologia 58*, 313−323.

(18) Elliott, T. S., Townsley, F. M., Bianco, A., Ernst, R. J., Sachdeva, A., Elsasser, S. J., Davis, L., Lang, K., Pisa, R., Greiss, S., Lilley, K. S., and Chin, J. W. (2014) Proteome labeling and protein identification in specific tissues and at specific developmental stages in an animal. *Nat. Biotechnol. 32*, 465−472.

(19) Yuet, K. P., Doma, M. K., Ngo, J. T., Sweredoski, M. J., Graham, R. L., Moradian, A., Hess, S., Schuman, E. M., Sternberg, P. W., and

DOI: 10.1021/acschembio.5b01076
ACS Chem. Biol. 2016, 11, 1532−1536

Tirrell, D. A. (2015) Cell-specific proteomic analysis in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A. 112,* 2705−2710.

DOI: 10.1021/acschembio.5b01076
ACS Chem. Biol. 2016, 11, 1532−1536