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In-Depth Quantitative Proteomic Analysis of de Novo Protein Synthesis Induced by Brain-Derived Neurotrophic Factor

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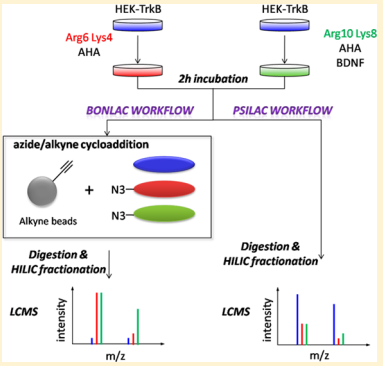
Supporting Information

ABSTRACT: Measuring the synthesis of new proteins in the context of a much greater number of pre-existing proteins can be difficult. To overcome this obstacle, bioorthogonal noncanonical amino acid tagging (BONCAT) can be combined with stable isotope labeling by amino acid in cell culture (SILAC) for comparative proteomic analysis of de novo protein synthesis (BONLAC). In the present study, we show that alkyne resin-based isolation of L-azidohomoalanine (AHA)-labeled proteins using azide/alkyne cycloaddition minimizes contamination from pre-existing proteins. Using this approach, we isolated and identified 7414 BONCAT-labeled proteins. The nascent proteome isolated by BONCAT was very similar to the steady-state proteome, although transcription factors were highly enriched by BONCAT. About 30% of the methionine residues were replaced by AHA in our BONCAT samples, which allowed for identification of methionine-containing peptides. There was no bias against low-methionine proteins by BONCAT at the proteome level. When we applied the BONLAC approach to screen for brain-derived neurotrophic factor (BDNF)-induced protein synthesis, 53 proteins were found to be significantly changed 2 h after BDNF stimulation. Our study demonstrated that the newly synthesized proteome, even after a short period of stimulation, can be efficiently isolated by BONCAT and analyzed to a depth that is similar to that of the steady-state proteome.



KEYWORDS: *BONCAT, pulsed SILAC, mass spectrometry, proteomics, BDNF, translation*

INTRODUCTION

Mass spectrometry (MS)-based quantitative proteomics is a powerful tool to study dynamic protein synthesis. Stable isotope labeling by amino acid in cell culture (SILAC)[1] is widely used in protein turnover (synthesis/degradation) studies.[2] This involves switching cells from regular culture medium to heavy SILAC medium that contains heavy isotope coded amino acids. All newly synthesized proteins from this point on are labeled by the heavy SILAC medium and thus can be easily distinguished in MS from pre-existing proteins. A variant of SILAC, pulsed SILAC[3] (pSILAC), uses two different heavy labels and thus allows comparison of newly synthesized protein levels between two experimental conditions. However, measuring the synthesis of new proteins over a relatively short period of time such as a few hours can be difficult due to a vast excess of pre-existing proteins.

Another method to distinguish newly synthesized proteins from old proteins is bioorthogonal noncanonical amino acid tagging (BONCAT).[4] In BONCAT, proteins are metabolically labeled with L-azidohomoalaine (AHA), a methionine surrogate. The azide moiety from AHA allows labeled proteins to be covalently conjugated to alkyne-containing tags through azide/alkyne cycloaddition click chemistry. These tags can be engineered to include fluorescent reporters to allow selective visualization of new proteins.[4,5] Alternatively, for proteomic analysis, they can include affinity tags, such as biotin, which can be used to isolate newly synthesized proteins from total proteins prior to MS analysis.[6,7]

Combining BONCAT and pSILAC techniques allows for comparative analysis of newly synthesized proteins in a high background of pre-existing proteins. This strategy was first applied to study secreted proteins[8] and translation in primary cells[9] and now is becoming increasingly popular.[10,11] Although one version of the combination[8] of BONCAT and pSILAC has been referred to as QuaNCAT,[9] we prefer the term BONLAC because SILAC is used for quantitation.

In this study, we performed a comprehensive characterization of the BONLAC approach in terms of its ability to identify and quantify the nascent proteome that is synthesized within a relatively short time period, and we used this approach to screen for brain-derived neurotrophic factor (BDNF)-induced

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proteins in HEK cells with stable expression of tropomyosin-related kinase B (TrkB). BDNF regulates many important aspects of neural function, including cell survival, cell fate decisions, axon growth, dendrite pruning, and long-term potentiation, a form of synaptic plasticity considered to be a cellular substrate for memory formation.[12,13] BDNF initiates intracellular signaling primarily through the receptor tyrosine kinase TrkB.[14] Activated TrkB eventually leads to translation of selected target mRNAs. However, the list of target mRNAs has yet to be completely characterized.

Our in-depth BONLAC and pSILAC analyses of newly synthesized proteins upon BDNF treatment characterized the performance of the BONCAT methodology in a large-scale proteomic study. A group of 53 proteins was found to have increased abundance after BDNF treatment.

MATERIAL AND METHODS

Cell Culture

HEK-293 cells overexpressing TrkB receptor (HEK-TrkB) were cultured in DMEM medium (Athena Environmental Sciences, Baltimore, MD) (methionine, 0.201 mM; lysine, 0.798 mM; arginine, 0.398 mM) supplemented with 10% FBS (Gibco) to confluence. The culture medium was removed, and cells were washed with PBS. Then, cells were incubated in a culture medium without methionine, lysine, and arginine (Athena Environmental Sciences, Baltimore, MD) for 30 min. One cell population was exposed to DMEM medium supplemented with L-azidohomoalanine (AHA, 1 mM, synthesized as described by Link et al.[15]), BDNF (25 ng/mL, Peptrotech), and $^{13}C_6$-$^{15}N_2$-lysine and $^{13}C_6$-$^{15}N_4$-arginine (Lys8/Arg10) for 2 h, and a control population was exposed to AHA and D_4-lysine/$^{13}C_6$-arginine (Lys4/Arg6). The SILAC amino acid concentrations were the same as those in the regular DMEM. In a replicate experiment, the SILAC labeling was reversed (BDNF was added to cells exposed to Lys4/Arg6).

Enrichment of AHA-Labeled Proteins

The enrichment procedures including cell lysis and tryptic digestion were performed using a kit (Click-iT Protein Enrichment Kit) from Invitrogen, following the protocol supplied with the kit with minor modifications.[8] Briefly, after labeling and BDNF treatment, cells were lysed in a buffer containing 8 M urea, 200 mM Tris, pH 8, 4% CHAPS, 1 M NaCl, and protease inhibitor cocktail (Sigma). Lysates of the BDNF treated/untreated cells were combined in equal volume. The mixed lysate was sonicated to reduce viscosity. AHA-labeled nascent proteins were cross-linked covalently to alkyne agarose beads using reagents provided by the kit. Beads were transferred to a SDS wash buffer (1% SDS, 100 mM Tris, pH 8, 250 mM NaCl, and 5 mM EDTA), and proteins on beads were then reduced with DTT at 70 °C and alkylated with iodoacetamide at room temperature. The beads then were washed sequentially with 100 column volumes of the following three buffers to remove nonspecific binding proteins: (1) SDS wash buffer, (2) 8 M urea, and (3) 20% acetonitrile. Cross-linked proteins were digested with trypsin on-resin at 37 °C overnight in 25 mM ammonium bicarbonate, and the resulting tryptic peptides were desalted using StageTips[16] and dried under vacuum in a SpeedVac device.

Digestion of Lysate Proteins

Cells were lysed with a solution containing 9 M urea, 20 mM Tris, pH 8, 0.2 mM EDTA, and protease inhibitors (Complete tablet; Roche, Mannheim, Germany). Proteins were then reduced with DTT and alkylated with iodoacetamide prior to overnight digestion with trypsin at 37 °C.[17] The tryptic peptides were desalted using StageTips and dried.

HILIC Fractionation of Tryptic Digests

The peptides were fractionated using hydrophilic interaction liquid chromatography (HILIC) according to a previously published protocol.[18] All HILIC experiments were performed on an Agilent 1100 HPLC system using a 1 × 250 mm TSKgel Amide-80 5 μm particle column (Tosoh Biosciences). Peptides were loaded in 90% solvent B (98% acetonitrile with 0.1% TFA). Solvent A consisted of 2% acetonitrile with 0.1% TFA. Peptides were eluted with an inverse gradient of 90% B to 85% B in 5 min followed by 85% B to 70% B in 40 min and finally a steep gradient to 0% B in 5 min at 0.05 mL/min. Twelve fractions were collected from HILIC separation and analyzed by LC–MS. Ten of these fractions contained peptides.

LC–MS

For LC–MS, a Thermo Scientific EASY-nLC 1000 coupled to a Q Exactive mass spectrometer[19] (Thermo Fisher Scientific) was used. A self-packed 75 μm × 25 cm reversed-phase column (Reprosil C18, 3 μm, Dr. Maisch GmbH, Germany) was used for peptide separation. Peptides were eluted by a gradient of 3–30% acetonitrile in 0.1% formic acid over 120 min at a flow rate of 250 nL/min at 45 °C. The Q Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 50 000 at m/z 400 (transient time = 256 ms). Up to the top 10 most abundant precursors from the survey scan were selected with an isolation window of 1.6 Thomsons and fragmented by higher-energy collisional dissociation with normalized collision energies of 27. The maximum ion injection times for the survey scan and the MS/MS scans were 60 ms, respectively, and the ion target value for both scan modes was set to 1 000 000.

Protein Identification and Quantitation

The raw files were processed using the MaxQuant[20] computational proteomics platform (version 1.2.7.0) for peptide identification and quantitation. The fragmentation spectra were searched against the UniProt human protein database (downloaded Feb 08, 2013) containing 87 647 protein sequences and allowing up to two missed tryptic cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine and protein N-terminal acetylation, D_4-lysine, $^{13}C_6$-arginine, $^{13}C_6$-$^{15}N_2$-lysine, and $^{13}C_6$-$^{15}N_4$-arginine were used as variable modifications for database searching. For the click-chemistry enriched BONCAT samples, AHA (−4.9863 Da) and L-2,4-diaminobutanoate (−30.9768 Da) were also used as variable modifications for methionine. The precursor and fragment mass tolerances were set to 7 and 20 ppm, respectively. Both peptide and protein identifications were filtered at 1% false discovery rate (FDR) and thus were not dependent on the peptide score. Perseus (version 1.4.1.3) was used to determine significantly changed proteins upon BDNF treatment based on significance B values for quantified proteins at a FDR of 1% using a Benjamini–Hochberg correction for multiple hypothesis testing. Significance B values are p values for detection of significant outlier protein ratios calculated on the protein subsets obtained by intensity binning.[20] Each BONLAC and pSILAC experiment was performed with two biological replicates, with labels reversed between replicates, and each biological replicate was analyzed twice by LC–MS. In order for a protein to be

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designated as having differential expression, it had to have passed the 1% FDR filter as described above for both the forward and reverse biological replicates. All raw LC−MS data files are freely available and can be downloaded from the following site: http://massive.ucsd.edu/ProteoSAFe/status.jsp?task=f80fc3ba51f54b0e815ac085eb50ce0c

Bioinformatic Analysis

To find statistically significantly overrepresented gene ontology (GO) terms and motifs, DAVID[21] (the database for annotation, visualization and integrated discovery, version 6.7) online function annotation tool was used. For retrieving protein interactions and constructing protein interaction networks, the STRING database[22] (version 9.1) was used. Cytoscape (version 3.1.0) was used to plot the protein interaction network exported by STRING.

RESULTS

BONCAT Enabled Near-Complete Removal of Non-AHA-Labeled Proteins

BONCAT has been used to isolate newly synthesized proteins from pre-existing ones in previous proteomic studies. In most of these published studies,[6,7,9−11,23,24] AHA-labeled proteins were coupled to a biotin-alkyne tag and then isolated through avidin-based affinity purification. It was reported that using such a strategy newly synthesized proteins comprised only 10−20% of the isolated proteins.[9] In the present study, we used a different enrichment method in which AHA-labeled proteins are coupled directly to alkyne agarose resin.[8] After stringent washes to remove nonspecific binding proteins, the resin-bound proteins were digested prior to LC−MS analysis.

To assess the level of the nonlabeled proteins in our BONCAT samples, we performed the alkyne-based BONCAT sample preparation using HEK-TrKB cells that were labeled with AHA for 2 h and compared these results to a control experiment in which the same sample preparation was performed with the same amount of lysate from unlabeled cells. Both the BONCAT and the control samples were analyzed by LC−MS. The sample preparation was repeated three times. We consistently identified dramatically more peptides from the BONCAT samples (2371, 2578, and 2681) than from the control samples (69, 19 and 83) at 1% FDR. Moreover, the peptides from the control samples generally had very low signals compared to those from the BONCAT samples (Figure 1; see Supporting Information Figure S1 for a replicate result). This result shows that the alkyne resin-based enrichment method has minimal contamination from non-AHA-labeled proteins and can be used to isolate high-purity nascent proteomes. This result represents a substantial improvement compared to the biotin tag-based approach because very stringent washing conditions can be used to efficiently remove nonspecific binding proteins without causing loss of target proteins.

Deep Coverage Analysis of the Nascent Proteome

To determine changes in protein synthesis induced by BDNF−TrkB signaling and to compare the performance of the two proteomic strategies, BONCAT/pSILAC(BONLAC) and pSILAC only (i.e., without enrichment of AHA-labeled proteins), we performed two experiments, as illustrated in Figure 2. For the BONCAT experiment, HEK cells over-expressing TrkB were incubated in two different types of SILAC media that contained two sets of heavy isotope-labeled

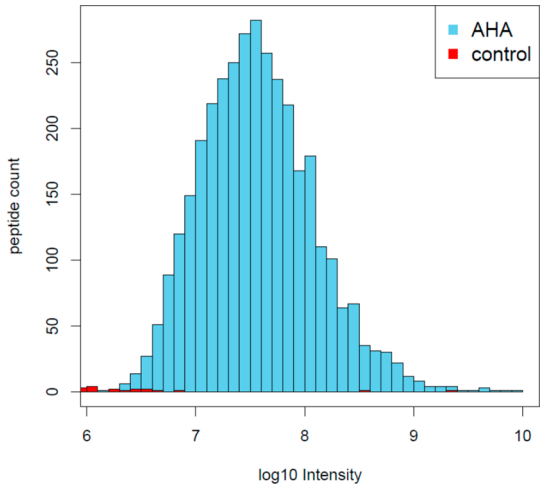

Figure 1. Assessing the level of nonspecific binding proteins for enrichment of AHA-labeled proteins using alkyne resin-based click chemistry. Equal amounts of AHA-labeled or unlabeled (control) HEK cell lysates were used for the enrichment. Histograms of peptide intensities for the AHA labeled and the control samples are shown.

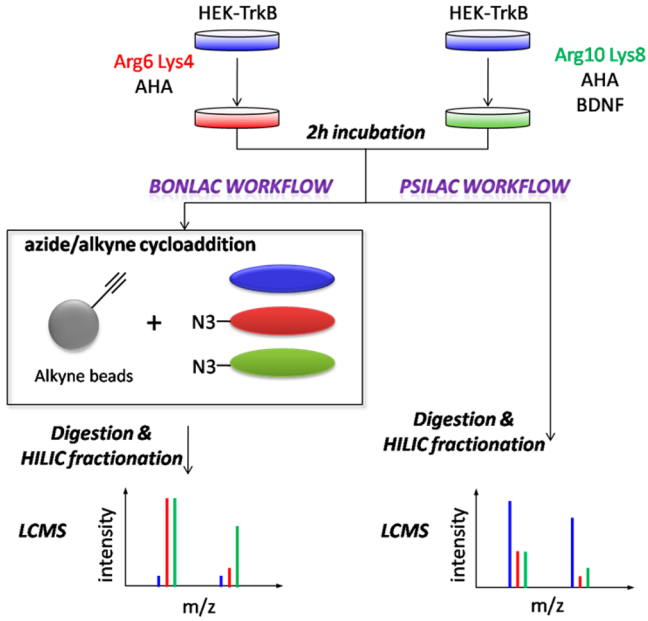

Figure 2. Schematic BONLAC and pSILAC workflows to investigate protein synthesis induced by BDNF signaling. HEK cells over-expressing TrkB were treated or left untreated with BDNF for 2 h. Concomitant with the treatment, the cells were labeled with SILAC and AHA. The samples were then mixed and analyzed using two workflows. In the BONLAC workflow, AHA-labeled proteins were separated from pre-existing proteins prior to LC−MS analysis, whereas in the pSILAC workflow, proteins were analyzed without the isolation step. Two biological replicates were performed with the SILAC labeling switched, and each biological replicate was analyzed twice by LC−MS.

arginines and lysines (Arg4/Lys6 and Arg10/Lys8) and AHA for 2 h. In one condition of the AHA-SILAC media, BDNF also was included to activate the TrkB pathway. The two cell populations were then combined in a 1:1 ratio and lysed. The newly synthesized proteins, which were labeled by both AHA and SILAC, were isolated using azide/alkyne cycloaddition click chemistry. After on-resin tryptic digestion of proteins, the

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resulting peptides were fractionated by hydrophilic interaction chromatography (HILIC), and the fractions were analyzed by LC−MS for protein identification and quantitation. This experiment is referred to as the BONLAC experiment hereafter.

For the purpose of comparison, a parallel experiment was performed using the same workflow described above except that the click chemistry enrichment step was omitted. This was essentially a pSILAC experiment. Approximately the same amount of BONLAC and pSILAC peptides (10 μg) was used for HILIC and LC−MS. This experiment is referred to as the pSILAC experiment.

From the pSILAC experiment result, SILAC label incorporation was estimated to be ~1.7% based on relative signal intensities of light and medium/heavy peptide signals. In the BONLAC proteins, SILAC incorporation was dramatically higher, with a median value of 76% (Figure 3; see Supporting Information Figure S2 for a replicate result). Essentially all proteins identified from the BONLAC experiment were SILAC-labeled, demonstrating its power to enrich for newly synthesized proteins. It is noted that the incorporation of labeled amino acids was lower than 100%, most likely caused by residual light lysine and arginine in the cells.

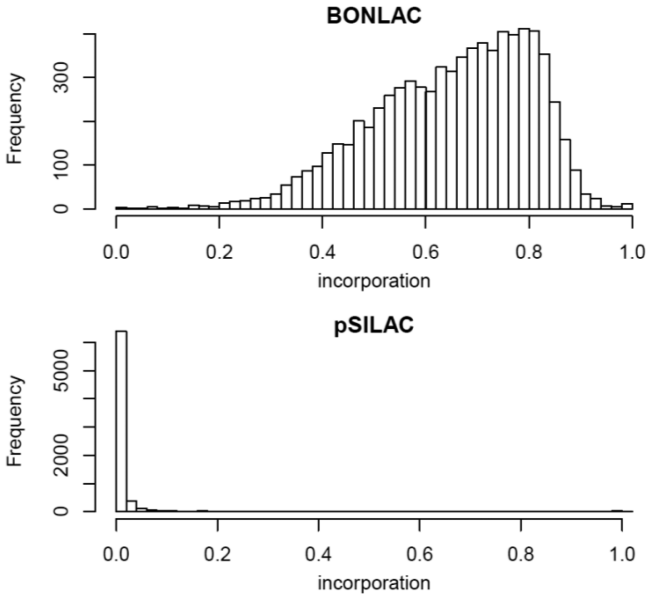

Figure 3. SILAC label incorporation of proteins identified from the BONLAC and pSILAC analyses.

From the BONLAC experiment, 7474 proteins were identified (1% FDR). (See Supporting Information Table S1 for details.) To our knowledge, this represents the most comprehensive proteomic analysis of newly synthesized proteome isolated by BONCAT to date. From the pSILAC samples, 7144 proteins were identified. (See Supporting Information Table S2 for details.) Because the pSILAC samples had very low SILAC incorporation, proteins identified from these samples represent the steady-state proteome. There was a remarkable overlap between proteins identified from the BONLAC and pSILAC experiments: 6389 proteins were identified in both (85% of BONLAC proteins and 89% of pSILAC proteins). The MS intensities of proteins from the two samples were also strongly correlated (Figure 4; see Supporting Information Figure S3 for a replicate result). These results suggest that the proteome being actively synthesized closely

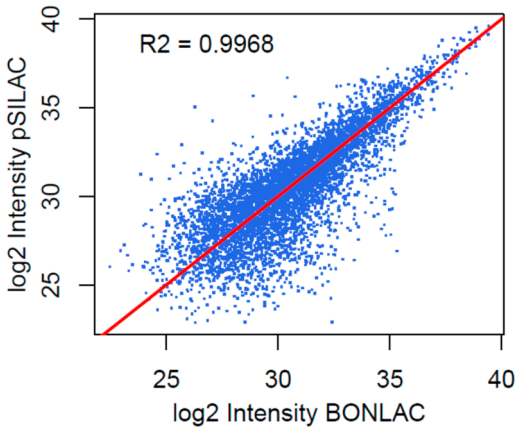

Figure 4. Correlation of protein intensities between the newly synthesized proteome (identified from the BONLAC experiment) and the steady-state proteome (identified from the pSILAC experiment).

mirrored the steady-state proteome. More importantly, our result shows that BONLAC can be used to study the whole nascent proteome with coverage similar to that of the steady-state proteome.

In total, 8229 proteins were identified from the two experiments, with 1085 identified only by BONLAC and 722 only by pSILAC. A gene ontology enrichment analysis was performed for the groups of proteins unique to BONLAC. The result shows that transcription factors are highly enriched in these proteins (p-value = 2.94×10^{-40}). Using a manually curated transcription factor database[25] that contains 1391 transcription factors, 483 transcription factors were found by BONLAC and 253 by pSILAC. Two-hundred eighteen transcription factors were found by both protocols. GO analysis on proteins unique to pSILAC revealed no significantly enriched GO terms. It is unlikely that the enrichment of transcription factors in the BONLAC samples was due to greater rates of synthesis for transcription factor proteins because the SILAC incorporation levels of transcription factors in general were not significantly higher than those of other proteins.

Taken together, these results suggest BONCAT is efficient in capturing transcription factors.

Assessing AHA Labeling Levels in BONCAT Samples

In theory, when using the alkyne resin-based approach, peptides with methionine in their sequence should not be identified from BONCAT samples because those peptides would remain attached to the resin via their AHA residues (substituted for methionine) after digestion, but, in fact, about 20% of all peptides identified from the BONLAC samples contain at least one methionine, which is only moderately lower than that of the pSILAC samples (26%). This suggests that methionines in the newly synthesized proteins were only partially replaced by AHA. To assess the level of AHA label, we compared peptide intensities measured from the BONLAC and pSILAC samples (Figure 5; see Supporting Information Figure S4 for a replicate result). There was a shift between the intensity ratio distributions of methionine-containing peptides and non-methionine-containing peptides, which was caused by partial replacement of methionine by AHA. On the basis of the shift, the estimated occurrence ratio of AHA to methionine in BONCAT samples is 3 to 7, i.e., only 30% of methionine in the newly synthesized proteins were replaced by AHA.

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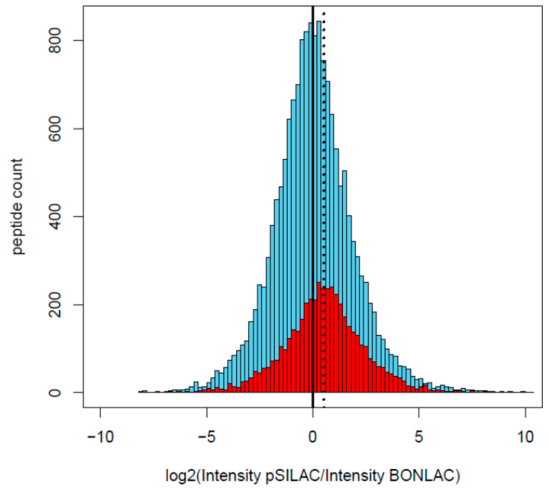

Figure 5. Assessing the level of AHA-methionine replacement. Intensity ratio histograms for non-methionine-containing peptides (blue) and methionine-containing peptides (red) are shown. Normalization was performed so that the median ratio for non-methionine-containing peptides is 1:1. The median $\log_2$ ratio for methionine-containing peptides is 0.524 (indicated by dashed line), which corresponds to an AHA/methionine ratio of 3:7 for BONLAC proteins.

In addition to methionine peptides, we also identified some AHA peptides, as a result of incomplete click chemistry reaction. However, these peptides account for only 0.4% of all identified peptides, suggesting very high efficiency of the azide/alkyne cycloaddition click chemistry reaction. In addition, in 19% of these AHA peptides, AHA was in the reduced form of L-2,4-diaminobutanoate, suggesting that during the sample preparation reduction of AHA is a common, but not dominant, side reaction.

BONCAT Has No Bias against Low-Methionine Proteins

A potential concern of the BONCAT approach is that proteins with low numbers of methionines are under-represented because the enrichment step requires the presence of AHA (substituted for methionine) in target proteins. This bias can be aggravated by low AHA labeling levels. A previous BONCAT study has reported that there is no such bias.[8] However, in that study, only secreted proteins were considered. In addition, most of the proteins were identified after 24 h of AHA labeling; thus, the level of AHA-methionine substitution is expected to be much higher than that in this study, which used only 2 h of AHA labeling. We compared the distributions of proteins containing different numbers of methionines for the BONLAC and pSILAC experiments (Figure 6; see Supporting Information Figure S5 for a replicate result). The result shows that the BONLAC and pSILAC proteins had almost identical density curves, indicating that, at the whole proteome level, there was no bias against low-methionine count proteins even with an AHA/methionine ratio of 3:7 in newly synthesized proteins.

SILAC Quantitation

Of the identified BONLAC proteins, 7176 out of the 7414 were quantified by SILAC, and 53 proteins showed consistent and significant SILAC ratio changes upon BNDF treatment at 1% FDR (Figure 7 and Table 1; for details, see Supporting Information Tables S1 and S2). For the pSILAC analysis, only 1840 out of 7144 identified proteins were quantified due to the low signals for the medium and heavy SILAC-labeled peptides.

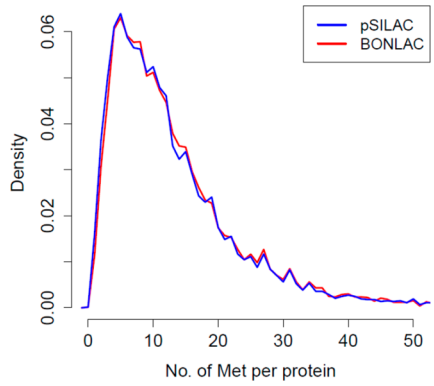

Figure 6. BONCAT did not over- or under-represent methionine-containing proteins. Distribution of identification frequency of proteins with different numbers of methionines is shown for the BONLAC and pSILAC experiments.

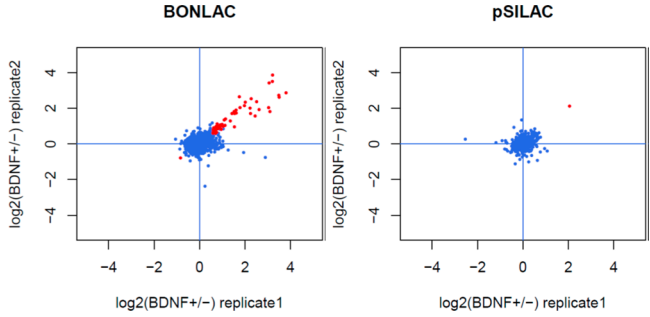

Figure 7. Identification of proteins whose synthesis rates were significantly changed upon BDNF treatment. Protein SILAC ratios from two biological replicates are shown, with significantly changed proteins marked in red (FDR = 0.01 in both replicates).

Only one protein (ADAMTS1) had significant SILAC ratio changes. This protein also was identified as a changing protein by BONLAC. The dramatic difference between the two experiments in the ability to quantify newly synthesized proteins highlights the importance of BONCAT enrichment prior to quantitation.

Among the 53 proteins were some well-known BDNF-induced proteins such as Jun,[26] Fos,[26] CDKN1A,[27] Arc,[28] and early growth response proteins (EGR1 and EGR2).[29] MKD-1 is a phosphatase known to be induced by BDNF in primary neurons.[30] This confirmed that the BONLAC approach was able to detect previously confirmed and biologically relevant BDNF target proteins. Most of the proteins in Table 1 have not heretofore been associated with BDNF signaling. A gene ontology analysis of the 53 changing proteins revealed that transcription regulator activity is highly enriched: 24 of the 53 proteins have transcription regulator activity (p-value: 5×10^{-11}) and 16 of the 53 proteins are transcription factors according to the transcription factor database.[25] To find the connections between the changing proteins, we used these proteins as an input to retrieve known protein interactions from the STRING protein interaction database. This resulted in a strongly connected network, which includes 36 of the 53 proteins (Figure 8), suggesting that the list of changing proteins identified in BONLAC is not a random list. Most proteins have not been previously reported to be involved in BDNF signaling and thus represent novel candidates for BDNF-induced proteins.

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Table 1. Proteins with Changed SILAC Ratios upon BDNF Treatment Identified from the BONLAC Analysis

gene names	protein names	ratio BDNF+/−	TF?[a]
Regulation of Transcription			
ATF3	Cyclic AMP-dependent transcription factor ATF-3	4.3	TF
BCL10	B-cell lymphoma/leukemia 10	1.9	
BHLHE40	Class E basic helix−loop−helix protein 40	3.2	
BTG2	Protein BTG2	5.0	
CBX4	E3 SUMO-protein ligase CBX4	1.6	
EGR1	Early growth response protein 1	10.3	TF
EGR2	E3 SUMO-protein ligase EGR2	6.0	TF
FOS	Proto-oncogene c-Fos	10.6	TF
FOSB	Protein fosB	8.7	TF
FOSL1	Fos-related antigen 1	4.2	TF
FOSL2	Fos-related antigen 2	2.0	TF
HES1	Transcription factor HES-1	1.6	TF
HEXIM1	Protein HEXIM1	1.9	
HEY1	Hairy/enhancer-of-split related with YRPW motif protein 1	1.9	TF
ID2	DNA-binding protein inhibitor ID-2	1.8	TF
ID4	DNA-binding protein inhibitor ID-4	2.5	
JUN	Transcription factor AP-1	4.0	TF
JUNB	Transcription factor jun-B	8.9	TF
KLF10	Krueppel-like factor 10	2.4	TF
NR4A1	Nuclear receptor subfamily 4 group A member 1	4.5	TF
PNRC1	Proline-rich nuclear receptor coactivator 1	1.6	
SERTAD1	SERTA domain-containing protein 1	5.4	
SOX9	Transcription factor SOX-9	1.5	TF
TRIB1	Tribbles homologue 1	11.9	
ZCCHC12	Zinc finger CCHC domain-containing protein 12	2.0	
ZNF217	Zinc finger protein 217	1.6	TF
Cell Signaling			
CTGF	Connective tissue growth factor	4.2	
DUSP1	Dual specificity protein phosphatase 1	4.8	
GADD45B	Growth arrest and DNA damage-inducible protein GADD45 beta	6.2	
Cell Signaling			
RHOB	Rho-related GTP-binding protein RhoB	1.7	
RND3	Rho-related GTP-binding protein RhoE	2.1	
ZFP36	Tristetraprolin	5.3	
CYR61	Protein CYR61	3.1	
FBXO5	F-box only protein 5	1.9	
PPP1R10	Serine/threonine-protein phosphatase 1 regulatory subunit 10	1.9	
SPRY4	Protein sprouty homologue 4	1.9	
Cell Proliferation/Apoptosis			
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	3.8	
CDC25B	M-phase inducer phosphatase 2	0.6	
CDKN1A	Cyclin-dependent kinase inhibitor 1	3.2	
TOB1	Protein Tob1	1.8	
TOB2	Protein Tob2	1.8	
MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1	3.0	
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	2.0	
TNFSF9	Tumor necrosis factor ligand superfamily member 9	3.4	
ARC	Activity-regulated cytoskeleton-associated protein	9.5	
SLFN11	Schlafen family member 11	1.5	
TUFT1	Tuftelin	1.6	
Metabolism			
DNAJB1	DnaJ homologue subfamily B member 1	2.1	
HSPA1A; HSPA1B	Heat shock 70 kDa protein 1A/1B	1.5	
IDI1	Isopentenyl-diphosphate Delta-isomerase 1	2.3	
MIDN	Midnolin	2.4	
PCF11	Pre-mRNA cleavage complex 2 protein Pcf11	1.7	
RBM12B	RNA-binding protein 12B	1.7	

[a]TF, transcription factor.

DISCUSSION

In proteomic analysis, the strength of BONCAT lies in its ability to separate newly synthesized proteins from pre-existing ones. Therefore, a key question is how clean the BONCAT protein preparation is. Our results indicate that it is possible to isolate newly synthesized proteins using BONCAT with minimal contamination from pre-existing proteins. This not only facilitates proteins identification/quantitation for the isolated nascent proteome but also opens the door to combining BONCAT with quantitation methods other than SILAC such as chemical labeling methods and label-free methods. This will be important for BONCAT applications for which it is difficult to use SILAC labeling.

We used BONCAT to purify a newly synthesized proteome and characterize it to a depth of 7414 proteins. To our knowledge, this is the most comprehensive MS analysis of a newly synthesized proteome. This result demonstrates that BONCAT enables in-depth proteomic analysis of newly synthesized proteins with coverage similar to that of a steady-state proteome. Our result also shows that the majority of the steady-state proteome can be readily captured by BONCAT. An interesting observation is that transcription factors were enriched efficiently by BONCAT. Thus, BONCAT can be used as a tool to investigate this important category of proteins. This observation might have been caused by the following: (1) transcription factors have fast degradation rates. This is supported by a previous study that shows transcription factor mRNAs have significantly increased average decay rates compared with those of other transcripts.[31] (2) Some transcription factors were induced by BDNF. However, this should not be a major contribution, as we found that a relatively small number of transcription factors were activated by BDNF.

We also found that the extent of AHA replacement of methionine residues in our BONCAT samples was about 30%. This was most likely due to residual methionine inside the cell even after 30 min of methionine starvation before AHA labeling. Because methionine is preferred over AHA in vivo for protein synthesis, even low levels of methionine in the cell can significantly decrease the level of AHA labeling. However, we believe incomplete AHA labeling is beneficial for BONCAT

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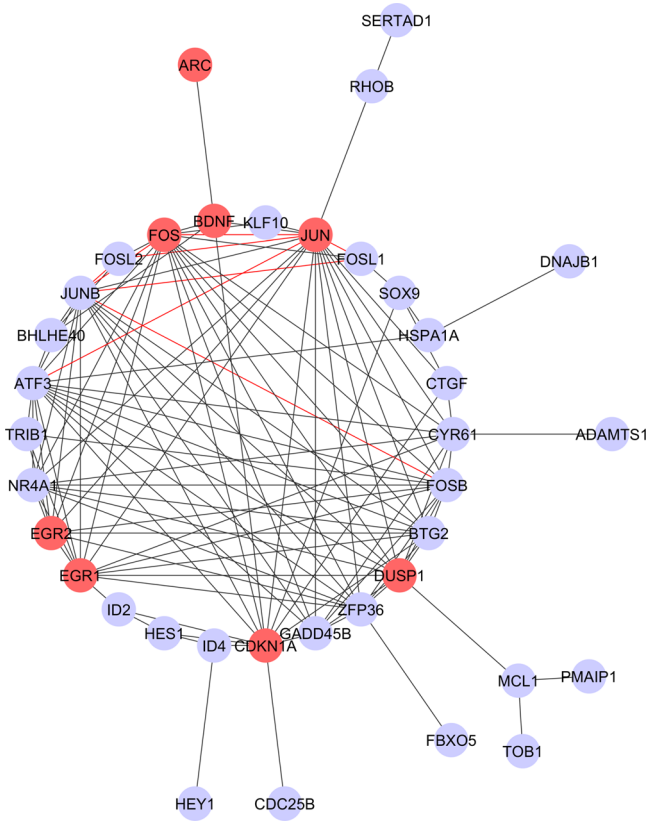

Figure 8. Protein interaction network for proteins with changed SILAC ratios from the BONLAC analysis. BDNF is included to form a more complete network. Nodes in red are previously reported BDNF-induced proteins. Lines connecting nodes represent protein interactions documented in the STRING database used. Red lines represent physical protein−protein binding. Black lines represent functional regulation/association.

analysis because methionine-containing peptides, which account for about one-quarter of all peptides, can be identified and thus contribute to protein identification and quantitation. These peptides would not be detected in the case of 100% AHA labeling because they would be attached to alkyne beads by click chemistry and cannot be recovered for LC−MS analysis. A high AHA labeling level could be considered beneficial because, in theory, it might improve BONCAT protein recovery. However, our results indicate that the azide/alkyne cycloaddition click chemistry yield is very high and thus a higher AHA level does not necessarily translate into higher recovery. On the contrary, for the alkyne resin-based approach, excessive AHA can cause a reduction in identification of methionine-containing peptides, which in turn compromises protein identification and quantitation. Our data show that at a 30% AHA level, protein identification was not affected by AHA labeling and little bias was observed against low-methionine count proteins. Another potential disadvantage of high AHA levels in labeling media is that AHA can change intracellular signaling and protein expression profiles. Therefore, AHA labeling parameters (including AHA concentration and methionine starvation time) are worthy of attention when designing an experiment to ensure sufficient protein recovery and at the same time minimize adverse effects.

Application of the BONLAC (BONCAT/pSILAC) approach to the study of the BDNF signaling pathway resulted in the identification of 53 proteins whose translation was significantly changed by BDNF treatment. This not only provides novel candidates of BDNF signaling for further biological investigation but also confirms the capability of BONLAC to reveal biologically relevant translational events with deep coverage and high accuracy.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: Identification and quantitation of BONCAT proteins to compare protein synthesis upon BDNF treatment. Table S2: Identification and quantitation of pSILAC proteins to compare protein synthesis upon BDNF treatment. Figure S1: Replicate analysis of the experiment to assess the level of nonspecific binding proteins for enrichment of AHA-labeled proteins using alkyne resin-based click chemistry. Figure S2: Replicate analysis of SILAC label incorporation for the BONLAC and pSILAC experiments. Figure S3: Replicate analysis of correlation of protein intensities between the newly synthesized proteome (identified from the BONLAC experiment) and the steady-state proteome (identified from the pSILAC experiment). Figure S4: Replicate analysis of AHA-methionine replacement. Figure S5: Replicate analysis to show BONCAT did not over- or under-represent methionine-containing proteins. This material is available free of charge via the Internet at http://pubs.acs.org. All raw data files can be downloaded from the following site: http://massive.ucsd.edu/ProteoSAFe/status.jsp?task=f80fc3ba51f54b0e815ac085eb50ce0c.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AHA, L-azidohomoalanine; BONCAT, bioorthogonal non-canonical amino acid tagging; SILAC, stable isotope labeling with amino acids in cell culture; pSILAC, pulsed SILAC; BONLAC, combined BONCAT and SILAC; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; BDNF, brain-derived neurotrophic factor

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