QuaNCAT: quantitating proteome dynamics in primary cells

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Here we demonstrate quantitation of stimuli-induced proteome dynamics in primary cells by combining the power of bio-orthogonal noncanonical amino acid tagging (BONCAT) and stable-isotope labeling of amino acids in cell culture (SILAC). In conjunction with nanoscale liquid chromatography–tandem mass spectrometry (nanoLC-MS/MS), quantitative noncanonical amino acid tagging (QuaNCAT) allowed us to monitor the early expression changes of >600 proteins in primary resting T cells subjected to activation stimuli.

Our understanding of cell behavior at a systems level is largely incomplete because of limited knowledge of proteome dynamics. Changes in the expression of proteins and their cognate mRNAs exhibit positive yet only modest correlation[1,2], with the highest correlation observed for components of conserved molecular machines[3]. The abundance of cellular proteins may be predominantly controlled at the translation level[4]. Thus, knowledge of transcriptome dynamics alone is evidently insufficient to understand cell behavior. SILAC-based mass spectrometry analysis permits quantitation of steady-state proteome changes[5]. But monitoring the proteome response to stimuli remains challenging, especially for *ex vivo* primary cells, to which SILAC cannot be straightforwardly applied[6]. Likewise, quantifying immediate or early changes of protein networks that occur during complex biological processes (such as cell proliferation and differentiation), is problematic because short SILAC pulses of a few hours label only a small fraction of a protein pool[7], severely limiting mass spectrometry–based quantitation of changes in protein expression. Selective isolation of the fraction of SILAC-labeled proteins would help to overcome the above limitations. BONCAT exploits Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) for site-selective labeling of newly synthesized proteins[8], without a need to modify the translation machinery by genetic engineering. Cells are cultured in the presence of the azide-bearing methionine surrogate L-azidohomoalanine (AHA), which is efficiently incorporated into newly synthesized proteins without adverse effects on cellular functions[8,9]. After cell lysis, the AHA azide group reacts with an alkyne-bearing biotinylated tag in the presence of CuAAC catalyst, allowing for the selective enrichment of the tagged protein pool. BONCAT has been used for mass spectrometry–based nonquantitative monitoring of newly synthesized proteins in cultured mammalian cells[9]. However, the efficiency and rate of CuAAC is site-dependent and protein-dependent, and may not be homogeneous for all proteins[10,11]. Moreover, monitoring protein changes by BONCAT alone using label-free mass spectrometry–based quantitation would have low accuracy.

To overcome the technical limitations of BONCAT and pulsed SILAC, we combined their strengths in QuaNCAT (**Fig. 1**). To test the feasibility and sensitivity of QuaNCAT, we applied it to freshly isolated human CD4-positive ($CD4^+$) T cells stimulated by phorbol 12-myristate 13-acetate and ionomycin. These drugs closely mimic cellular activation by antigen and CD28 'co-stimuli', thereby eliciting proliferation and differentiation. We starved human $CD4^+$ T cells for 60 min in medium depleted of methionine, arginine and lysine and then cultured them for 2 h and 4 h with 1 mM AHA and either 'heavy' [$^{13}C_6$,$^{15}N_4$]L-Arg and [$^{13}C_6$,$^{15}N_2$]L-Lys for stimulated cells or with 'medium' [$^{13}C_6$]L-Arg and [$^2H_4$]L-Lys for nonstimulated cells (**Fig. 1**). This labeling strategy ensured straightforward distinction by mass spectrometry of stimulation-induced (heavy isotope–labeled) from steady-state (medium isotope–labeled) synthesized proteins. A pulse with 1 mM AHA for up to a maximum of 4 h did not cause discernible changes in morphology and viability of T cells (**Supplementary Fig. 1**). In Jurkat T cells, these pulse conditions had no significant effect on the rate of protein synthesis as compared to methionine-pulsed cells, and proteins containing AHA or methionine had very similar degradation rates (**Supplementary Fig. 2**).

We mixed nonstimulated and stimulated cells in a 1:1 ratio and extracted proteins by SDS, followed by reduction, alkylation and CuAAC with an alkyne-containing biotinylated cleavable linker[12] (**Fig. 1**). An optimized CuAAC protocol with Cu(I)-ligand complex proved superior to using Cu(II)/ascorbate (Online Methods), with enhanced rate (<2 h) and reduced damage to proteins. After removing excess alkyne tag, we bound biotin-tagged proteins to

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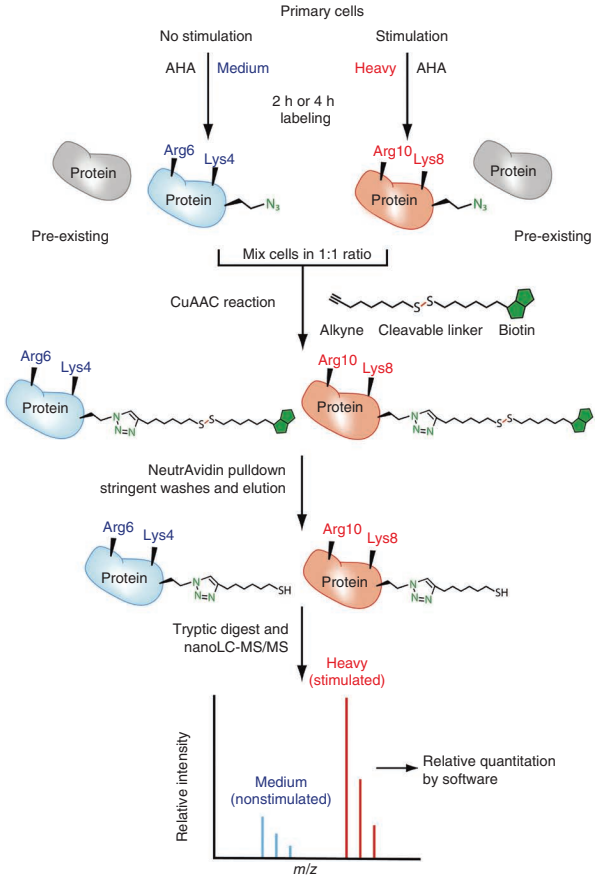

NeutrAvidin-immobilized beads and removed nonspecifically bound proteins by thorough washing. Immunoblotting with antibody to biotin confirmed effective biotin labeling of AHA-containing proteins and their enrichment after NeutrAvidin binding (data not shown). We eluted biotin-tagged proteins from NeutrAvidin with DTT (**Fig. 1**), substantially reducing the mass spectrometry signal from contaminating unlabelled ('light') proteins. We analyzed eluted proteins digested by trypsin by nanoLC-MS/MS by injecting each sample three times. Simultaneous AHA and stable isotope labeling with arginine and lysine, followed by biotin tagging, allowed for strong enrichment and quantitation of newly synthesized proteins to determine stimuli-induced changes in their abundance. We quantified peak intensities of 'medium' and 'heavy' peptide pairs (**Fig. 1**) using both Proteome Discoverer 1.3 Thermo Scientific and MaxQuant 1.3.0.5 software[13] (Online Methods and **Supplementary Fig. 3**). Fold induction for each quantitated protein after 2 h and 4 h stimulation and standard deviation, obtained from three independent

Figure 1 | Schematic QuaNCAT workflow. Cells were metabolically labeled with a combination of AHA and stable isotope–containing $[^{13}C_6]$L-Arg and $[^{2}H_4]$L-Lys ('medium') or $[^{13}C_6,^{15}N_4]$L-Arg and $[^{13}C_6,^{15}N_2]$L-Lys ('heavy'), were left unstimulated or stimulated for 2 h or 4 h, respectively, and were processed as indicated.

experimental replicates (and three mass spectrometry analyses for each one) is provided in **Supplementary Table 1**. Comparisons of nine individual data sets to each other (Pearson correlation coefficient; **Supplementary Fig. 4**), indicated good overall experimental reproducibility. Consistently, experimental replicates for 2-h and 4-h stimulations exhibited 95% and 89% coincidence for 410 and 550 quantified proteins, respectively (**Supplementary Fig. 5**). We created value-ordered plots for 410 and 550 proteins quantified at 2 h and 4 h of stimulation, respectively (**Fig. 2a**). We analyzed these data with the linear models for microarray data (LIMMA) statistical package[14], which provided fold change with associated *P* value for differential expression of each protein (**Fig. 2b**). To validate the quantitation of differentially expressed proteins ($P < 0.05$), we manually inspected the mass spectra. We validated 107 differentially expressed proteins, 93 of which exhibited an increase after stimulation of T cells (**Supplementary Table 2** and **Fig. 3**). Fifty-six proteins were differentially expressed already at 2 h after stimulation, 33 of which (~60%) were still differentially expressed (increased or decreased expression) at 4 h after stimulation (**Supplementary Table 2**), indicating the robustness of the QuaNCAT protocol. Of the 107 proteins, 50 were differentially expressed only after 4 h of stimulation. Fold increases showed a dynamic range extending from 1.6-fold to 589-fold, with 72 of 141 measured changes being ≥ 5-fold. Some of the largest fold changes likely represent instances of *de novo* protein expression. Gene ontology analysis of molecular function of differentially expressed proteins (**Supplementary Fig. 6**) revealed that many of these proteins are involved in regulation of transcription, including pre- and post-transcriptional processes. This is consistent with transcriptome studies of activated human T cells[15], which have revealed that T cells, which are weakly metabolically active in G0 phase of the cell cycle, proliferate and differentiate into immune response effectors. Consistently, QuaNCAT revealed substantial increases (5-fold to 500-fold) in expression at 2 h and/or 4 h after stimulation of several immediate early and early genes, many of which encode transcription factors and transcriptional regulators (such as Fos, Jun, NR4A, IRF and EGR family members, c-Rel,

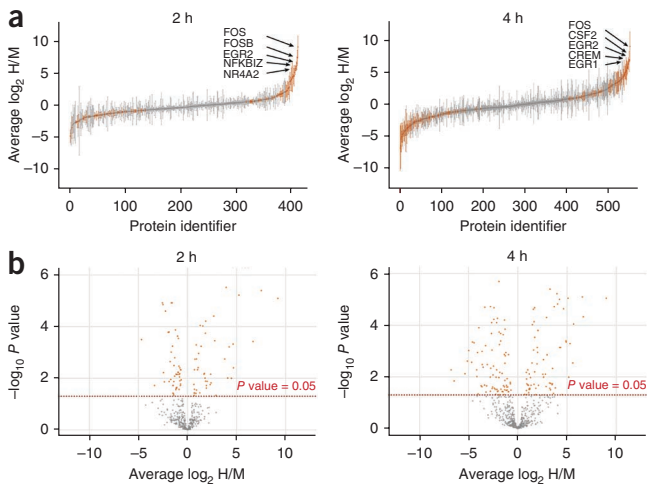

Figure 2 | Global changes of protein expression in primary human CD4+ T cells after 2-h and 4-h activation. (**a**) Value-ordered plots of $\log_2$ stimulated (heavy isotope–labeled proteins)/nonstimulated (medium isotope–labeled proteins) (referred to as $\log_2$ H/M) ± 1 s.d. for each quantified protein at 2 h and 4 h. H/M data were obtained from three independent experiments, each one including three separate nanoLC-MS/MS analyses. Data for differentially expressed proteins ($P < 0.05$) are colored orange. Data for the top five upregulated proteins are indicated in black, and the proteins are identified. (**b**) Volcano plots of $\log_2$ H/M versus $-\log_{10}$ *P* value for each quantified protein (threshold for $P < 0.05$ is indicated). Data for differentially expressed proteins ($P < 0.05$) are colored orange.

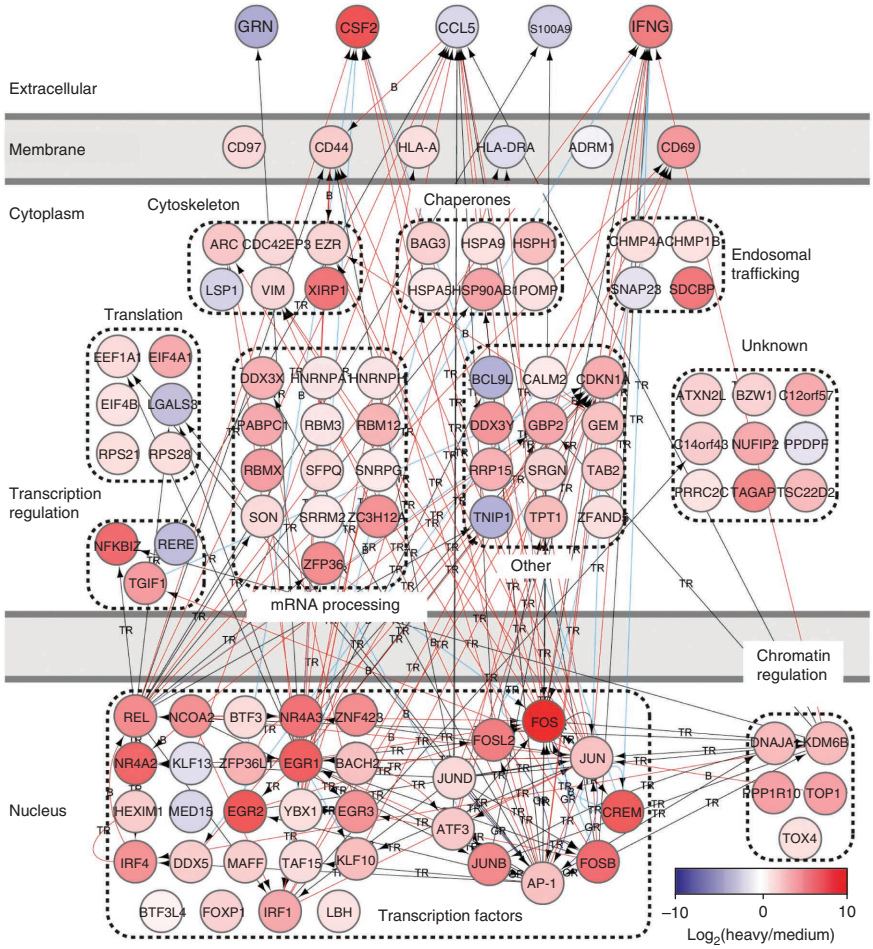

Figure 3 | Interaction network of differentially expressed proteins after activation of primary T cells for 2 h and 4 h. The network was constructed in MetaCore and visualized using Cytoscape. Data are colored red and blue, indicating up- and downregulation, respectively (see color bar). Red lines indicate positive regulation; blue lines indicate negative regulation, and black lines indicate unspecified interaction. TR, transcriptional regulation; B, binding; and GR, group relation.

FOXp1 and a lysine-specific demethylase; **Supplementary Fig. 6** and **Fig. 3**). Other identified proteins included cytokines (INFγ and CSF2) and activation surface markers (CD69, CD97 and HLA), protein chaperones, and proteins involved in cytoskeleton dynamics and vesicle transport. Of note was the detection of increases (2- to 20-fold) of several proteins that regulate mRNA metabolism, translation and the cell cycle. Some proteins were not annotated and/or not previously detected during T cell activation and it will be particularly interesting to unravel their role in T cell proliferation and/or differentiation (**Fig. 3**). QuaNCAT data also revealed decreased expression of several proteins, perhaps caused by attenuated transcription and/or translation or accelerated protein degradation. The latter two mechanisms, as well as their reverse trends (increase in mRNA translation and protein stability) would escape transcriptome analysis but can be detected by QuaNCAT. An interaction network of proteins differentially expressed at 2 h and 4 h illustrates that more than half of the proteins are interlinked by functional and biochemical networks (**Fig. 3**).

The use of 'medium' and 'heavy' SILAC overcame potential confounding effects in quantitation owing to persistent signals from 'light' peptides, likely derived from contaminating untagged pre-existing proteins. After affinity purification, 'heavy'- and 'medium'-labelled proteins typically comprised 10–20% of the identified proteins (data not shown), which is a substantial enrichment as only <<1% of total proteins were labeled after a 2 h pulse.

The ability to quantitate relative differences in abundance of many cellular proteins reveals the power of QuaNCAT, and this approach should be applicable to shorter and longer kinetics. However, long-term methionine starvation affects cellular methylation of proteins and nucleic acids, and prolonged AHA pulses could be sensed by the cellular machinery as some proteins may be affected by AHA incorporation. We did not notice stress in T cells when we pulsed them for up to 4 h with AHA, but longer pulses certainly require careful evaluation of potential toxicity effects. Application of QuaNCAT for relatively short times after cellular perturbations should enable quantitative monitoring of the most dynamic pool of the proteome, which is likely to be the one of most biological interest. Moreover, 2–4 h of AHA and SILAC pulse-labeling applied several hours after cell stimulation would also allow one to monitor proteomic changes at late stimulation time points and avoid adverse effects resulting from lengthy methionine starvation.

Recently the combination of SILAC and BONCAT has been used to quantitatively analyze the cellular secretome[16]. The method we presented here allowed more in-depth analysis of protein changes in the entire cellular proteome and provides a much sought-after complement to the analysis of transcriptome dynamics. Its combination with targeted mass spectrometry–based quantitation could help to assess absolute changes in protein expression with additional accuracy and sensitivity.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.J.M.H., B.T., D.C.T., V.G. and O.A. initially conceived the QuaNCAT strategy. A.J.M.H., V.G., K.K., G.E., B.T., D.C.T., O.B., B.B., B.G.D. and O.A. designed and optimized the QuaNCAT procedure. B.B. and O.B. synthesized reagents for the CuAAC reaction and associated cell labeling; D.C.D. provided the cleavable tag and an improved protocol for affinity purification; A.J.M.H., V.G., K.K. and G.E. performed cell stimulation, metabolic labeling, CuAAC reactions in T cell extracts, protein affinity purification; optimized CuAAC protocol was initially performed by B.B. and O.B. on A.J.M.H.'s initial cell extracts; K.K., G.E., B.M.K. and O.A. performed radioactive labeling; K.K. performed flow cytometry analysis. A.J.M.H., V.G., K.K., G.E. and B.T. carried out mass spectrometry experiments and data analysis; A.J.M.H., V.G., K.K., G.E., B.T., B.B., B.G.D. and O.A. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

SILAC and azidohomoalanine labeling. Primary human $CD4^+$ T lymphocytes were isolated from blood by negative selection (Dynal isolation kit, Invitrogen). Primary T lymphocytes were rested overnight in RPMI 1640 medium (PAA) supplemented with 10% FBS (Gibco). Purity of isolated cells was assessed by analyzing CD3 and CD4 surface expression using flow cytometry (data not shown). For metabolic labeling, cells were starved of methionine, arginine and lysine for 1 h at 37 °C in RPMI SILAC medium (Dundee Cell Products) supplemented with 10% dialyzed FBS at a cell density of 2×10^6 ml^{-1}. After centrifugation, cells were equally divided into two sets. One set of cells was resuspended in fresh RPMI SILAC medium containing 1.14 mM 'medium' arginine ($[^{13}C_6]$L-Arg) and 0.219 mM 'medium' lysine ($[^2H_4]$L-Lys) and the other set of cells was placed in medium containing 1.14 mM 'heavy' arginine ($[^{13}C_6,^{15}N_4]$L-Arg) and 0.219 mM 'heavy' lysine ($[^{13}C_6,^{15}N_2]$L-Lys) (CK Gas Products). To minimize arginine-to-proline conversion, RPMI SILAC medium was supplemented with 1.7 mM L-proline. AHA was added to each set of cells to a final concentration of 1 mM. Details on the synthesis of AHA are provided in the **Supplementary Note**. Cells in the heavy medium were stimulated by adding phorbol 12-myristate 13-acetate at 62.5 ng/ml (100 nM final concentration) and ionomycin at 1 μg/ml (1.4 μM final concentration). Efficient stimulation was verified by CD69 surface staining and flow cytometry (data not shown). Cells were incubated for 2 h and 4 h at 37 °C before they were collected by centrifugation.

Protein extraction, reduction and alkylation. For each replicate, 90×10^6 'heavy', stimulated and 90×10^6 'medium', unstimulated labeled primary cells were collected by centrifugation, washed in PBS and combined. Cells were lysed in 500 μl of PBS with 1% SDS containing protease and phosphatase inhibitors (Complete EDTA-free, Roche, 2 mM sodium pervanadate) by rigorous vortexing. Lysate was boiled at 100 °C for 10 min, after which 1,000 units of benzonase (Sigma) was added, and the sample was incubated at room temperature for 15 min. The lysate was cleared by centrifugation at 20,000*g* for 10 min at 4 °C. To each sample, 10 mM tris(2-carboxyethyl) phosphine (TCEP) (Thermo) was added, and samples were incubated at 55 °C for 45 min to reduce proteins. After cooling down the sample, pH at room temperature was adjusted to 8 by adding sodium hydroxide before addition of iodoacetamide to a final concentration of 18 mM. Proteins were alkylated by incubation at room temperature for 30 min in the dark. Each lysate was desalted before biotinylation using spin desalting columns (Zeba 7-kDa MWCO, Thermo).

Copper-catalyzed Huisgen cycloaddition of protein samples with biotinylated-alkyne label. The protocol used has previously been optimized[10] and was applied universally to all samples as follows. A freshly prepared solution of copper(I) bromide (99.999% purity) in acetonitrile (163 μl, 10 mg/ml) was premixed with an acetonitrile solution of tris-triazolyl amine ligand (63 μl, 127 mg/ml). Details of the synthesis of the tris-triazolyl amine ligand are provided in the **Supplementary Note**. For triazole-formation, 7 μl of the biotin-alkyne disulfide probe[12] (25 mM stock solution in PBS at pH 7.8, 87.5 nmol) was added to 540 μl of reduced, alkylated and desalted protein lysate from each replicate and mixed. The preformed Cu(I)-ligand complex solution (30 μl) was added to the mixture and mixed thoroughly. The reaction was performed in 1.5-ml reaction tubes on an end-over-end rotator for 90 min at room temperature. Tubes were centrifuged at 2,000*g* for 5 min, and the supernatant was purified by desalting columns (Zeba 7 kDa MWCO).

Pull-down of biotinylated proteins. For each replicate, 40 μl of high-capacity Neutravidin resin (Thermo) was washed three times in 1 ml of PBS containing 0.05% SDS. After the final wash, the supernatant was discarded, 577 μl of the desalted lysate was added to the reaction tubes containing the beads, and the reaction was incubated at room temperature overnight on an end-over-end rotator. Samples were loaded into Pierce Spin Columns (Thermo), and beads were collected by centrifugation. Beads were washed three times with 600 μl of each of the following buffers: 4 M urea, 6 M urea, 10% acetonitrile and 20% acetonitrile. For each washing step, beads were incubated for 5 min before they were centrifuged and collected. Proteins were then eluted from the Neutravidin beads through reductive cleavage as follows: 50 μl of elution buffer (20 mM DTT, 1 M urea, 50 mM $(NH_4)HCO_3$) was added to the column and incubated with the beads for 10 min before spinning down and collecting the eluted proteins. This step was repeated two times. $CaCl_2$ was added to a final concentration of 1.25 mM in the eluate before overnight in-solution digestion of proteins with trypsin at 37 °C. Peptides were desalted using C18 desalting tips. Desalted, dried peptides were then resuspended in 0.1% TFA with 2% acetonitrile and sonicated before analysis by mass spectrometry.

Mass spectrometry and data analysis. Samples were analyzed on an Ultimate 3000 RSLCnano (Dionex) system run in direct injection coupled to a QExactive mass spectrometer (Thermo Scientific). Samples were resolved on a NanoEASY (Thermo Scientific) C_{18} reverse-phase column (50 cm long, 75 μm internal diameter, 2 μm beads) at a flow rate of 300 nl min^{-1} using a linear gradient of 5–44% buffer B (80% acetonitrile and 0.1% formic acid) over 190 min. The mass spectrometer was operated in a 'top 10' data-dependent acquisition mode with dynamic exclusion enabled (40 s). Survey scans (mass range 300–1,650 Th) were acquired at a resolution of 70,000 at 200 Th with the ten most abundant multiply charged ($z \geq 2$) ions selected with a 3-Th isolation window for fragmentation by higher-energy collisional dissociation. MS/MS scans were acquired at a resolution of 17,500 at 200 Th.

Raw data were processed using MaxQuant[13] 1.3.0.5 and Proteome Discoverer 1.3 (Thermo Scientific). Searches against the UniProt[17] human database were performed using Andromeda[18] and Mascot[19], respectively. Search parameters were two missed trypsin cleavage sites, cysteine carbamidomethylation fixed modification, methionine oxidation and N-terminal protein acetylation variable modifications. Peptide results were filtered to 1% false discovery rate by MaxQuant and Proteome Discoverer.

Protein and peptide quantitation information were extracted from MaxQuant and Proteome Discoverer and imported into the program R 2.15.1 (ref. 20). Protein extracted ion chromatograms (XICs) were used from MaxQuant without additional modification. For Proteome Discoverer, 'medium' and 'heavy' protein XICs were calculated from the sum of the individual unique peptides XICs in the program R[21].

Peptides with zero medium and heavy XICs were not considered for the calculation.

Proteins that were not reproducibly quantified across replicates were removed from downstream analysis. We required proteins to have a minimum of three quantifiable unique peptides in at least two of the three biological replicates.

Differential protein expression analysis was performed with the Bioconductor package LIMMA 3.14.1 (ref. 14). After protein area distributions were quantile-normalized[22], a linear model was fitted and a moderated *t*-test used to assess the statistical significance of heavy-to-medium protein fold changes[23]. Proteins with a corresponding fold-change *P* value (adjusted for multiple hypothesis testing with the Benjamini-Hochberg method[24]) lower than 0.05 were accepted as differentially expressed.

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