**Brief Communication**

**QuaNCAT: quantitating proteome dynamics in primary cells**

Andrew J.M. Howden1,5,6, Vincent Geoghegan1,5, Kristin Katsch1,5, Georgios Efstathiou1,5, Bhaskar Bhushan2, Omar Boutureira2,7, Benjamin Thomas1, David C. Trudgian1,8, Benedikt M. Kessler3, Daniela C. Dieterich4, Benjamin G. Davis2 and Oreste Acuto1

1 Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK. 2 Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford, OX1 3TA, UK. 3 Henry Wellcome Building for Molecular Physiology, Nuffield Department of Medicine, University of Oxford, Roosevelt Drive, Oxford ,OX3 7BN, UK. 4 Emmy Noether Research Group Neuralomics, Leibniz Institute for Neurobiology, Brenneckestr. 6, 39118 Magdeburg,and Otto-von-Guericke University, Institute for Pharmacology and Toxicology, Leipziger Strasse 44, 39120 Magdeburg, Germany.

Corresponding Authors:

Oreste Acuto, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK. (oreste.acuto@path.ox.ac.uk)

Ben Davis, Department of Chemistry, University of Oxford Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, UK. (Ben.Davis@chem.ox.ac.uk)

5 These authors contributed equally to this work.

Present Addresses:

6 Division of Plant Sciences, College of Life Sciences, University of Dundee, Errol Road, Invergowrie, Dundee DD2 5DA, UK.

7 Departament de Química Analítica i Química Orgànica, Universitat Rovira i Virgili, C/ Marcel·lí Domingo s/n. 43007 Tarragona, Spain

8 Biochemistry Department and Proteomics Core. UT Southwestern Medical Center, 5323 Harry Hines Boulevard Dallas, Texas 75390, USA

KEYWORDS: quantitative proteomics, SILAC, BONCAT, T cell activation

**Please cite the Eichelbaum et al. paper in the main text and briefly put it in context of your work (Done, please, see below).**

**and please cite the Supplementary Note in the text or Online Methods (Done, please, see below)**

**Abstract**

Here we demonstrate that quantitation of stimuli-induced proteome dynamics in primary cells is feasible by combining the power of Bio-Orthogonal Non Canonical Amino acid Tagging (BONCAT) and Stable Isotope Labelling of Amino acids in Cell culture (SILAC). In conjunction with nanoLC-MS/MS, QuaNCAT allowed us to monitor the early expression changes of > 600 proteins in primary resting T cells subjected to activation stimuli.

Understanding cell behaviour at a systems level is largely concealed due to limited knowledge of proteome dynamics. Changes in the expression of proteins and their cognate mRNAs display positive, yet only modest correlation[1](#_ENREF_1), [2](#_ENREF_2), the highest correlation is being observed for components of conserved molecular machines[3](#_ENREF_3). Indeed, abundance of cellular proteins may be predominantly controlled at the translation level[4](#_ENREF_4). Thus, knowledge of transcriptomes’ dynamics alone is evidently insufficient to understand cell behaviour. SILAC-based MS analysis permits quantitation of steady-state proteome changes[5](#_ENREF_5). However, monitoring the proteome response to stimuli remains challenging, especially for *ex vivo* primary cells, to which SILAC cannot be straightforwardly applied[6](#_ENREF_6). Likewise, quantifying immediate/early changes of protein networks occurring 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](#_ENREF_7), severely limiting MS-based quantitation of protein expression changes. Selective isolation of the fraction of SILAC-labelled proteins would help to overcome the above limitations. BONCAT exploits Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC) for site-selective labelling of newly synthesised proteins[8](#_ENREF_8), without modifying the translation machinery by genetic engineering. Cells are cultured in the presence of the azide-bearing methionine surrogate azidohomoalanine (AHA), which is efficiently incorporated into newly synthesized proteins without adverse effects on cellular functions[8](#_ENREF_8), [9](#_ENREF_9). After cell lysis, the AHA azide group is reacted 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 MS-based non-quantitative monitoring of newly synthesized proteins in cultured mammalian cells[9](#_ENREF_9). However, the efficiency and rate of CuAAC is site-and protein-dependent and may not be homogeneous for all proteins[10](#_ENREF_10), [11](#_ENREF_11). Moreover, monitoring protein changes by BONCAT alone using label-free MS-based quantitation would have low accuracy.

To overcome the technical limitations of BONCAT and pulsed SILAC, we have combined their strengths in Quantitative Non Canonical Amino acid Tagging (QuaNCAT) (**Fig. 1**). To test its feasibility and sensitivity, we applied QuaNCAT to freshly isolated human CD4 T cells (hCD4+) stimulated by Phorbol 12-myristate 13-acetate (PMA) and ionomycin. These drugs closely mimic cellular activation by antigen and co-stimuli, thereby eliciting proliferation and differentiation. hCD4+ cells were starved for 60 min in Met-, Arg- and Lys-depleted medium and then cultured for 2 and 4 h with 1 mM AHA and either ‘heavy’ [13C6,15N4]-L-Arg and [13C6,15N2]-L-Lys, for stimulated cells, or with ‘medium’ [13C6]-L-Arg and [2H4]-L-Lys, for non-stimulated cells (**Fig. 1**). This labelling strategy ensured straightforward distinction by MS of stimulation-induced (‘heavy-labelled’) from steady-state (‘medium-labelled’) synthesised proteins. 1 mM AHA pulse for up to a maximum of 4 h did not cause significant changes in T cell morphology and viability (**Supplementary Fig. 1**). As examined in Jurkat T cells, these pulse conditions had no significant effect on the rate of protein synthesis as compared to Met-pulsed cells and proteins containing AHA or Met had very similar degradation rates (**Supplementary. Fig. 2**).

We mixed non-stimulated and stimulated cells in a 1:1 ratio and extracted proteins by SDS, followed by reduction, alkylation and performed the CuAAC reaction with an alkyne-containing biotinylated cleavable linker[12](#_ENREF_12) (**Fig.1**). An optimised CuAAC protocol with Cu(I)-ligand complex proved superior to using Cu(II)/ascorbate (Online Methods), with enhanced rate (<2 h) and reduced reactive oxygen species damage to proteins. After removal of excess alkyne tag, biotin-tagged proteins were bound to NeutrAvidin-immobilized beads and non-specifically bound proteins removed by thorough washing. Anti-biotin antibody immunoblots confirmed effective biotin labelling of AHA-containing proteins and their enrichment after NeutrAvidin binding (data not shown). Biotin-tagged proteins were eluted from NeutrAvidin with DTT (**Fig.1**), substantially reducing the MS signal from contaminating unlabelled (‘light’) proteins. Eluted proteins digested by trypsin were analysed by nanoLC-MS/MS by injecting each sample three times. Simultaneous AHA and stable isotope labelling with Arg and Lys, followed by biotin tagging, allowed for strong enrichment and quantitation of newly synthesised proteins to determine stimuli-induced changes in their abundance. The peak intensities of ‘medium’ (M) and ‘heavy’ (H) peptide pairs (**Fig. 1**) were quantified using both Proteome Discoverer 1.3™ and MaxQuant 1.3.0.5 software[13](#_ENREF_13) (see Online Methods and **Supplementary Fig 3**). Fold induction for each quantitated protein after 2 h and 4 h stimulation and standard deviation (SD), obtained from three independent experimental replicates (and three MS analysis for each one) is shown in **Supplementary Table 1**. Comparisons of individual data sets (9) to each other (Pearson correlation coefficient, **Supplementary** **Fig. 4 )**, indicated good overall experimental reproducibility. Consistently, experimental replicates for 2 h and 4 h stimulations showed 95% and 89% coincidence for 410 and 550 quantified proteins, respectively (**Supplementary Fig. 5a-c**). **Fig 2a** shows value-ordered plots for 410 and 550 proteins quantified at 2 h and 4 h of stimulation, respectively. These data were analysed with the LIMMA statistical package[14](#_ENREF_14), which provided fold change with associated *P*-value for differential expression of each protein, as shown in **Fig. 2b**. Quantitation of differentially expressed proteins (*P*-value < 0.05) was validated by manual inspection of the mass spectra. **Supplementary Table 2** and **Fig. 3** show the 107 manually validated differentially expressed proteins, 93 of which show an increase after T cell stimulation. 56 proteins were differentially expressed already at 2 h, 33 of which (~ 60%) were still differentially expressed (increased or decreased) at 4 h (Venn diagrams next to **Supplementary Table 2**), further indicating the robustness of the QuaNCAT protocol. 50 proteins of 107 were differentially expressed only after 4 h of stimulation. Fold increases showed a dynamic range extending from 1.6 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 a large number are involved in transcription regulation, including pre- and post-transcriptional processes. This is consistent with transcriptome studies of activated human T cells[15](#_ENREF_15), which have revealed the transition of weakly metabolically active T cells in G0 to proliferation and differentiation into immune response effectors. Consistently, QuaNCAT detected substantial increases (5 to 500 fold) in expression at 2 h and/or 4 h of several immediate early/early genes, many of which encode transcription factors and transcriptional regulators (such as Fos, Jun, NR4A, IRF and EGR family members, c-Rel, FOXp1 and a lysine-specific demethylase, **Supplementary Fig. 6 and Fig. 3**). Others included cytokines (INF and CSF2) and activation surface markers (CD69, CD97, HLA), protein chaperones, proteins involved in cytoskeleton dynamics and vesicle transport. Of note, was the detection of significant 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 transcription and/or translation attenuation or accelerated protein degradation. The latter two mechanisms, as well as their reverse trends (increase in mRNA translatability and protein stability) would escape transcriptome analysis but can be detected by QuaNCAT. An interaction network of proteins differentially expressed at 2 and 4 h illustrates that more than half are interlinked by functional and biochemical networks (**Fig. 3**).

The use of ‘medium’ and ‘heavy’ SILAC overcame potential confounding effects in quantitation due to persistent signals from ‘light’ peptides, likely derived from contaminating untagged pre-existing proteins. After affinity purification, ‘heavy’- and ‘medium’-labelled proteins typically represented 10-20 % in hCD4+ T cells (data not shown), a substantial enrichment, as only << 1% of total proteins were labelled after a 2 h pulse.

The ability to quantitate relative differences in abundance of many cellular proteins reveals the power of QuaNCAT and 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 pulsed for up to 4 h with AHA, but longer pulses certainly require one to carefully evaluate 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/SILAC pulse-labelling applied several hours after cell stimulation would also allow one to monitor proteomic changes at late stimulation time points and avoid adverse effects due to lengthy methionine starvation.

A recent work has reported the combination of SILAC and BONCAT to quantitavely analyse the cellular secretome.16 The method presented here allowed wider 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 MS-based quantitation could help to assess absolute changes in protein expression with further accuracy and sensitivity.

**Methods**

Methods and any associated references are available in the Supplementary information.

**Acknowledgments**

This work was supported by Wellcome Trust Grant WT094296MA and EU-FP7 “Sybilla” no. 201106 to O.A. D.C.D was supported by a DFG Emmy Noether Grant DI1512/1-1. B.G.D. and O.A. are recipients of Royal Society Wolfson Research Merit Awards. We thank Drs. Philip Charles, Steve Taylor and Eleni Giannoulatou for advice on the use of bioinformatics and statistics software. We thank Drs. Wolfgang Paster and Konstantina Nika for helpful suggestions and for critical feedback on the manuscript. We gratefully thank Matthias Selbach for helpful suggestions. V.G. was supported by a Ph.D. fellowship from BBSRC, O.B. by a Marie Curie Intra European Fellowship, B.B. by a Rhodes Scholarship. This paper is dedicated to Jamie.

**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., V.G., O.B, B.B., B.G.D. and O.A. designed and optimised the QuaNCAT procedure. B.B. and O.B. synthesised reagents for the CuAAC reaction and associated cell labelling; 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 labelling, CuAAC reactions in T cell extracts, protein affinity purification; optimised CuAAC protocol was initially performed by BB and OB on AJMH’s initial cell extracts; K.K., G.E., B.M.K. and O.A., performed radioactive labelling; K.K. performed FACS analysis. A.J.M.H., V.G. K.K., G.E. and B.T. carried out MS 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.

**Figure legends**

**Figure 1. Schematic QuaNCAT workflow**

Cells were metabolically labelled with a combination of Azidohomoalanine (AHA) and stable isotope-containing [13C6]-L-Arg and [2H4]-L-Lys (Medium,) or [13C6,15N4]-L-Arg and [13C6,15N2]-L-Lys (Heavy,) and left non-stimulated or stimulated for 2 or 4 h, respectively and processed as indicated [see also Online Methods]

**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 log2 stimulated (H)/non-stimulated (M) ratios ± 1 SD for each quantified protein at 2 and 4 h. H/M ratios were obtained from three independent experiments, each one including three separate nanoLC-MS/MS analyses. Differentially expressed proteins (P-value < 0.05) are coloured orange. The top five up-regulated proteins are indicated in black. (**b**) Volcano plots of log2 H/M ratios vs. –log10 P-value for each quantified protein (the threshold for P-value < 0.05 is indicated). Differentially expressed proteins (P-value < 0.05) are coloured orange. See online Methods for MS data quantitation and statistical analysis.

**Figure 3. Interaction network of differentially expressed proteins after T cell activation for 2 and 4 hours**

An interaction network of differentially expressed proteins in primary T cells after 2 and 4 h of stimulation was constructed in MetaCore and visualised using Cytoscape. Red and blue coloured proteins indicate up- and down-regulation, respectively. Red lines: positive regulation, blue lines: negative regulation, black lines: unspecified interaction. TR, transcriptional regulation. B, binding. GR, group relation

**Online Methods**

**Labelling cells with SILAC amino acids and Aha**

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 media (PAA) supplemented with 10% FBS (Gibco). Purity of isolated cells was assessed by analysing CD3 and CD4 surface expression using flow cytometry (data not shown). For metabolic labelling, cells were starved of methionine, arginine and lysine for 1 hour at 37 °C in RPMI SILAC media (Dundee Cell Products) supplemented with 10 % dialysed FBS at a cell density of 2×106/ml. After centrifugation, cells were equally divided into two sets. One set of cells was resuspended in fresh RPMI SILAC media containing 1.14 mM ‘medium’ arginine (13C6-L-arginine) and 0.219 mM ‘medium’ lysine (2H4-L-lysine) while the other set of cells was placed in 1.14 mM ‘heavy’ arginine (13C6,15N4-L-arginine) and 0.219 mM ‘heavy’ lysine (13C6,15N2-L-lysine) (CK Gas Products Ltd). In order to minimise arginine to proline conversion, RPMI SILAC media was supplemented with 1.7 mM L-proline. L-azidohomoalanine (AHA) was added to each set of cells to a final concentration of 1 mM. For details on the synthesis of AHA refer to Supplementary Information. Cells in the heavy medium were stimulated by adding PMA at 62.5 ng/ml (100 nM) and Ionomycin at 1 µg/ml (1.4 µM). Efficient stimulation was verified by CD69-surface staining and flow cytometry (data not shown). Cells were incubated for 2 and 4 hours at 37 °C before being harvested by centrifugation.

**Protein extraction, reduction and alkylation**

For each replicate, 90×106 ‘heavy’-stimulated and 90×106 ‘medium’-unstimulated labelled primary cells were harvested by centrifugation, washed in PBS, and combined. Cells were lysed in 500 μL 1% SDS in PBS containing protease and phosphatase inhibitors (Complete EDTA-free, Roche, 2mM sodium pervanadate) by rigorous vortexing. Lysate was boiled at 100°C for 10 minutes, after which 1000 units of benzonase (Sigma) was added and the sample was incubated at room temperature for 15 minutes. The lysate was cleared by centrifugation at 20,000g for 10 minutes at 4 °C. 10 mM TCEP (Thermo) was added to each sample and incubated at 55 °C for 45 minutes to reduced proteins. After cooling down the sample, pH was adjusted to 8 by sodium hydroxide before addition of iodoacetamide to a final concentration of 18 mM. Proteins were alkylated by incubation at room temperature for 30 minutes in the dark. Each lysate was desalted before biotinylation using spin desalting columns (ZebaTM 7kDa MWCO, Thermo).

**Copper-catalysed Huisgen cycloaddition of protein samples with biotinylated-alkyne label:**

The protocol used has previously been optimized by van Kasteren *et al.*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). For details on the synthesis of the tris-triazolyl amine ligand refer to Supplementary Information. For cycloaddition 7 µL of the biotin-alkyne disulfide probe12 (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. 30 µL of the preformed Cu(I)-Ligand complex solution 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 minutes at room temperature. Tubes were centrifuged at 2,000g for 5 minutes and the supernatant was purified by desalting columns (ZebaTM 7kDa MWCO, Thermo).

**Pull-down of biotinylated proteins**

For each replicate, 40 µL of High Capacity Neutravidin® resin (Thermo) was washed three times in 1 mL 0.05 %SDS in PBS. After the final wash the supernatant was discarded and 577 µl of the desalted lysate was added to the reaction tubes containing the beads and 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, 20 % acetonitrile. For each washing step, beads were incubated for 5 min before spinning and collecting. 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 (NH4)HCO3) was added to the column and incubated with the beads for 10 minutes before spinning down and collecting the eluted proteins. This step was repeated two times. CaCl2 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/ 2%ACN and sonicated before analysis by mass spectrometry.

**Mass spectrometry and data analysis**

Samples were analysed 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) C18-reversed 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/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 300-1650 Th) were acquired at a resolution of 70,000 at 200 Th with the ten most abundant multiply charged (z ≥ 2) ions selected with a 3-Th isolation window for HCD fragmentation. MS/MS scans were acquired at a resolution of 17,500 at 200 Th.

Raw data were processed using MaxQuant13 1.3.0.5 and Proteome Discoverer 1.3 (Thermo Scientific). Searches against the UniProt17 human database were performed using Andromeda18 and Mascot19 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 R 2.15.120. Protein extracted ion chromatograms (XICs) were used from MaxQuant without further modification. For Proteome Discoverer, “Medium” and “Heavy” protein XICs were calculated from the sum of the individual unique peptides XICs in R21. 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 LIMMA 3.14.1/Bioconductor14. After protein area distributions were quantile normalized22, a linear model was fitted and a moderated t-test used to assess the statistical significance of Heavy-to-Medium Protein fold changes23. Proteins with a corresponding fold-change *P* value (adjusted for multiple hypothesis testing with the Benjamini-Hochberg method24) lower than 0.05 were accepted as differentially expressed.

**References**

1. Ghazalpour, A. *et al.* Comparative analysis of proteome and transcriptome variation in mouse. *PLoS Genet* **7**, e1001393 (2011).

2. Foss, E.J. *et al.* Genetic basis of proteome variation in yeast. *Nat Genet* **39**, 1369-1375 (2007).

3. Rogers, S. *et al.* Investigating the correspondence between transcriptomic and proteomic expression profiles using coupled cluster models. *Bioinformatics* **24**, 2894-2900 (2008).

4. Schwanhausser, B. *et al.* Global quantification of mammalian gene expression control. *Nature* **473**, 337-342 (2011).

5. Mann, M. Functional and quantitative proteomics using SILAC. *Nat Rev Mol Cell Biol* **7**, 952-958 (2006).

6. Geiger, T. *et al.* Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. *Nat Protoc* **6**, 147-157 (2011).

7. Schwanhausser, B., Gossen, M., Dittmar, G. & Selbach, M. Global analysis of cellular protein translation by pulsed SILAC. *Proteomics* **9**, 205-209 (2009).

8. Dieterich, D.C., Link, A.J., Graumann, J., Tirrell, D.A. & Schuman, E.M. Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). *Proc Natl Acad Sci U S A* **103**, 9482-9487 (2006).

9. Dieterich, D.C. *et al.* Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. *Nat Protoc* **2**, 532-540 (2007).

10. Van Kasteren, S.I., Kramer, H.B., Gamblin, D.P. & Davis, B.G. Site-selective glycosylation of proteins: creating synthetic glycoproteins. *Nat Protoc* **2**, 3185-3194 (2007).

11. van Kasteren, S.I. *et al.* Expanding the diversity of chemical protein modification allows post-translational mimicry. *Nature* **446**, 1105-1109 (2007).

12. Szychowski, J. *et al.* Cleavable biotin probes for labeling of biomolecules via azide-alkyne cycloaddition. *J Am Chem Soc* **132**, 18351-18360 (2010).

13. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology* **26**, 1367-1372 (2008).

14. Smyth, G.K. in *Bioinformatics and Computational Biology Solutions using R and Bioconductor* (eds. Gentleman, R., Dudoit, S., Irizarry, R., Huber, W.) 397-420 (Springer, New York; 2005).

15. Diehn, M. *et al.* Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc Natl Acad Sci U S A* **99**, 11796-11801 (2002).

# 16. Eichelbaum, K., Winter, M., Diaz, M. B., Herzig, S. & Krijgsveld, J.. Selective enrichment of newly synthesized proteins for quantitative secretome analysis. *Nat Biotechnol* 30, 984-990 (2012).

17. The UniProt Consortium, Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic Acids Res* **40**, D71-75 (2012).

18. Cox, J. *et al.* Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* **11**, 1794-1805 (2011).

19. Perkins, D. N., Pappin, D.J., Creasy, D.M., Cottrell, J.S.Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551-3567 (1999).

20. Ihaka, R. & Gentleman, R. R: A language for data analysis and graphics. *J Comput Graph Stat* **5**, 299-314 (1996).

21. Carrillo, B., Yanofsky, C., Laboissiere, S., Nadon, R. & Kearney, R.E.Methods for combining peptide intensities to estimate relative protein abundance. *Bioinformatics* **26**, 98-103 (2010).

22. Smyth, G. K. & Speed, T. P. Normalization of cDNA microarray data. *Methods* **31**, 265-273 (2003).

23. Smyth, G. K. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article 3 (2004).

24. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Statist Soc B*, **57**, 289-300 (1995).

25. Fernandez-Gonzalez, M. *et al.* Site-Selective Chemoenzymatic Synthesis of Glycoproteins using Endoglycosidases. *Chem Sci* **1**, 709-715 (2010).

26. Lundquist, J.T. & Pelletier, J.C. Improved Solid-Phase Peptide Synthesis Method Utilizing α-Azide-Protected Amino Acids. *Org Lett* **3**, 781-783 (2001).

27. Zhou, Z. & Fahrni, C.J. A Fluorogenic Probe for the Copper(I)-Catalyzed Azide−Alkyne Ligation Reaction:  Modulation of the Fluorescence Emission via 3(n,π\*)−1(π,π\*) Inversion. *J Am Chem Soc* **126**, 8862-8863 (2004).

Editorial Summary:

Combining a method to label newly synthesized proteins and a strategy to distinguish two populations in cell culture allows quantitative assessment of protein dynamics.