PROTOCOL

Proteome-wide identification of ubiquitin interactions using UbIA-MS

Xiaofei Zhang[1,2,5], Arne H Smits[3,5], Gabrielle BA van Tilburg[4,5], Huib Ovaa[4], Wolfgang Huber[3] 🔗 & Michiel Vermeulen[2] 🔗

[1]CAS Key Laboratory of Regenerative Biology, Guangdong Provincial Key Laboratory of Stem Cells and Regenerative Medicine, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [2]Department of Molecular Biology, Faculty of Science, Radboud Institute for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, the Netherlands. [3]Genome Biology Unit, European Molecular Biology Laboratory, Heidelberg, Germany. [4]Department of Chemical Immunology, Leiden University Medical Center, Leiden, the Netherlands. [5]These authors contributed equally to this work. Correspondence should be addressed to X.Z. (zhang_xiaofei@gibh.ac.cn), A.H.S. (arne.smits@embl.de) or M.V. (michiel.vermeulen@science.ru.nl).

Ubiquitin-binding proteins play an important role in eukaryotes by translating differently linked polyubiquitin chains into proper cellular responses. Current knowledge about ubiquitin-binding proteins and ubiquitin linkage-selective interactions is mostly based on case-by-case studies. We have recently reported a method called ubiquitin interactor affinity enrichment–mass spectrometry (UbIA-MS), which enables comprehensive identification of ubiquitin interactors for all ubiquitin linkages from crude cell lysates. One major strength of UbIA-MS is the fact that ubiquitin interactors are enriched from crude cell lysates, in which proteins are present at endogenous levels, contain biologically relevant post-translational modifications (PTMs) and are assembled in native protein complexes. In addition, UbIA-MS uses chemically synthesized nonhydrolyzable diubiquitin, which mimics native diubiquitin and is inert to cleavage by endogenous deubiquitinases (DUBs). Here, we present a detailed protocol for UbIA-MS that proceeds in five stages: (i) chemical synthesis of ubiquitin precursors and click chemistry for the generation of biotinylated nonhydrolyzable diubiquitin baits, (ii) in vitro affinity purification of ubiquitin interactors, (iii) on-bead interactor digestion, (iv) liquid chromatography (LC)–MS/MS analysis and (v) data analysis to identify differentially enriched proteins. The computational analysis tools are freely available as an open-source R software package, including a graphical interface. Typically, UbIA-MS allows the identification of dozens to hundreds of ubiquitin interactors from any type of cell lysate, and can be used to study cell type or stimulus-dependent ubiquitin interactions. The nonhydrolyzable diubiquitin synthesis can be completed in 3 weeks, followed by ubiquitin interactor enrichment and identification, which can be completed within another 2 weeks.

INTRODUCTION

The covalent attachment of ubiquitin molecules to protein substrates is essential for almost all eukaryotic cellular processes[1]. According to the number and topology of ubiquitin molecules attached to protein substrates, ubiquitination is classified as monoubiquitination, multi-monoubiquitination or polyubiquitination[2]. There are eight homotypic polyubiquitin chains, which are linked via the C terminus of distal (donor) ubiquitin molecules to any of the lysine residues or the first methionine residue of proximal (acceptor) ubiquitin molecules[3]. The ability of cells to interpret different ubiquitin linkages and to induce the protective responses relies on ubiquitin-binding domains (UBDs) within ubiquitin-binding proteins (UBPs)[4]. Previous studies have shown that some UBDs recognize specific ubiquitin linkages[2]. Most of these studies were performed using recombinant (truncated) proteins and in vitro binding assays. Investigation of ubiquitin linkage-selective interactions in vivo has been challenging, considering the assembly of homotypic, branched and mixed polyubiquitin chains in cells. Recently, we introduced UbIA-MS, which enables the enrichment and identification of interactors for in vitro-synthesized and immobilized ubiquitin linkages from crude cell lysates at near-physiological conditions[5]. This enables the proteome-wide identification of linkage-selective ubiquitin interactions in different biological contexts.

Overview of the procedures
This protocol contains five parts: (i) synthesis of nonhydrolyzable diubiquitin by click chemistry (**Boxes 1** and **2**), (ii) capture of

diubiquitin interactors from cell lysates (Steps 1–18), (iii) digestion of interactors to peptides (Steps 19–39), (iv) identification of interactors by mass spectrometry (Steps 40 and 41) and (v) mass spectrometry data analysis and data visualization (Steps 42–47). **Figure 1** illustrates the workflow of UbIA-MS. **Figure 2** describes the steps of data analysis.

Applications of the method
We have used UbIA-MS to study the ubiquitin interactions in multiple human and mouse cell types and in different biological contexts[5]. Instead of whole-cell extracts, lysates can also be generated from purified cellular organelles such as mitochondria or nuclei. Furthermore, lysates can be generated from cells that have been exposed to a certain stimulus or perturbation. In our initial description of the UbIA-MS method, we mainly made use of diubiquitin linkages[5]. In the future, it will be interesting to use the workflow to study interactions with different ubiquitin topologies, including mixed and branched ubiquitin chains, and phosphorylated or acetylated ubiquitin molecules. In addition, the effects of heterologous ubiquitin/ubiquitin-like chains, such as ubiquitin-SUMO or ubiquitin-Nedd8, will be interesting to investigate in vivo. Finally, the R package that we provide is not restricted to UbIA-MS experiments, but can be used to analyze different types of mass spectrometry experiments. The DEP package was developed to test for differentially enriched or expressed proteins in quantitative mass spectrometry-based proteomics data, and can be used to analyze, among others, affinity purification

Box 1 | Production of alkyne- (distal) and azide- (proximal) functionalized ubiquitin ⬤ TIMING < 16.5 d

The protocol and reagents for making ubiquitin with total linear Fmoc-based SPPS have been described in detail elsewhere[28]. For copper(I)-catalyzed azide alkyne cycloaddition (CuAAC), it is essential to have an azide and alkyne functionality incorporated into the moieties that are to be 'clicked' together in the procedure described here.

The ubiquitin amino acid sequences and modifications needed for the generation of nonhydrolyzable diubiquitin baits are shown in the image below. The upper panel shows dipeptide incorporation for total linear Fmoc-based ubiquitin synthesis. The lysine conjugation residues are depicted in bold, and the residues that are replaced by a pseudoproline dipeptide are colored in blue (L8T9, I13T14, L56S57 and S65T66). In addition, the residues that are replaced by a dimethoxybenzyl dipeptide are shown in red (A46G47 and D52G53). The middle panel shows the distal ubiquitin sequence, 1–75, which is to be equipped with propargylamine (PA, orange) on the C terminus and contains a biotin handle followed by a PEG spacer on the N terminus (green). The lower panel shows the proximal ubiquitin sequence, 1–76, which contains azide-equipped amino acids. α-Azidonorleucine is to be incorporated at the Met1 position (pink) and azidoornithine at any of the desired lysine conjugation sites (purple).

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Standard Ub sequence
 1  MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ  40
41  QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGG       76

Distal Ub sequence
 1  BIO-PEG-MQ IFVKTLTGKT ITLEVEPSDT IENVKAKIQD KEGIPPDQ  40
41  QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRG(PA)            75

Proximal Ub sequence
 1  MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ  40
41  QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGG       76
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Reagents

- Trityl resin (TentaGel R TRT-Gly Fmoc; Rapp Polymere, cat. no. RA1213)
- PP reactor, 5 ml with plunger (MultiSyntech, cat. no. V051TF062)
- Ethylene glycol spacer (8-(Fmoc amino)-3,6-dioxaoctanoic acid; AK Scientific, cat. no. 166108-71-0)
  ! CAUTION 8-(Fmoc amino)-3,6-dioxaoctanoic acid is an irritant. Always wear protective gloves when handling it.
- Biotin (Sigma-Aldrich, cat. no. B4501, CAS: 58-85-5)
- Fmoc-L-azidoornithine (Chiralix, cat. no. CX25404, CAS: 1097-192-04-5)
- Fmoc-L-norleucine (Chem-Impex International, cat. no. 02440, CAS: 77284-32-3)
- N,N-diisopropylethylamine (DIPEA; Biosolve, cat. no. 0004153332BS, CAS: 7087-68-5) **! CAUTION** DIPEA is flammable, corrosive and toxic. Handle it inside a chemical fume hood; always wear protective gloves when handling it and keep it away from ignition sources.
- N-methyl-2-pyrrolidone (NMP; Peptide-S; Biosolve, cat. no. 0013563302BD, CAS: 872-50-4) **! CAUTION** NMP is toxic and an irritant. Handle it inside a chemical fume hood and avoid breathing its vapors. Always wear goggles and protective gloves when handling it.
- 1-Hydroxybenzotriazole hydrate (HOBT, peptide synthesis grade) (Biosolve, cat. no. 08123391, CAS: 123333-53-9) **! CAUTION** HOBT is explosive. There is a risk of explosion upon shock, friction, fire or other sources of ignition. Handle it inside a chemical fume hood; always wear protective gloves when handling it and keep it away from ignition sources.
- O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU, peptide synthesis grade) (Biosolve, cat. no. 08013391, CAS: 94790-37-1) **! CAUTION** HBTU is flammable and an irritant. Handle it inside a chemical fume hood; always wear protective gloves when handling it and keep it away from ignition sources.
- N,N-dimethylformamid, analytical reagent (AR) (DMF; Biosolve, cat. no. 0004190501BS, CAS: 68-12-2) **! CAUTION** DMF is flammable, toxic and an irritant. It is a health hazard; it may damage fertility or the unborn child. Handle it inside a chemical fume hood and avoid breathing its vapors. Always wear goggles and protective gloves when handling it.
- 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP; Chem-Impex International, cat. no. 00080, CAS: 920-66-1) **! CAUTION** HFIP is an irritant, toxic and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it.
- Dichloromethane (stabilized with amylene), AR (DCM; Biosolve, cat. no. 0013790502BS, CAS: 75-09-2) **! CAUTION** It is a health hazard; it is suspected of causing cancer. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it.
- Benzotriazol-1-yloxy(tripyrrolidin-1-yl) phosphonium hexafluorophosphate (PyBOP; NovaBiochem, cat. no. 851009, CAS: 128625-52-5) **! CAUTION** PyBOP is an irritant. Always wear protective gloves when handling it.
- Triethylamine (TEA; Sigma-Aldrich, cat. no. T0886, CAS: 121-44-8) **! CAUTION** TEA is flammable, toxic and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves and goggles when handling it and keep it away from ignition sources.

(continued)

Box 1 | Production of alkyne- (distal) and azide- (proximal) functionalized ubiquitin ● TIMING < 16.5 d (continued)

- Propargylamine (PA; Sigma-Aldrich, cat. no. P50900, CAS: 2450-71-7) ❗ **CAUTION** PA is flammable, toxic and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves and goggles when handling it and keep it away from ignition sources.
- Toluene (Biosolve, cat. no. 0020150501BS, CAS: 108-88-3) ❗ **CAUTION** Toluene is flammable, toxic and an irritant. It is a health hazard; it may damage fertility or the unborn child. Handle it inside a chemical fume hood and avoid breathing its vapors. Always wear goggles and protective gloves when handling it and keep it away from ignition sources.
- 1,2-Dichloroethane, AR (DCE; Biosolve, cat. no. 040504, CAS: 107-06-2) ❗ **CAUTION** DCE is flammable, harmful and potentially carcinogenic. Handle it inside a chemical fume hood and avoid breathing its vapors. Always wear goggles and protective gloves when handling it and keep it away from ignition sources.
- Potassium hydrogen sulfate (KHSO$_4$; Fisher Chemical, cat. no. 60-011-94, CAS: 7646-93-7) ❗ **CAUTION** KHSO$_4$ is an irritant and corrosive. Always wear protective gloves when handling it.
- Sodium sulfate (Na$_2$SO$_4$; Sigma-Aldrich, cat. no. 239313-M, CAS: 7757-82-6)
- Trifluoroacetic acid, peptide synthesis grade (TFA; Biosolve, cat. no. 0020233320BS, CAS: 76-05-1) ❗ **CAUTION** TFA is corrosive and toxic. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it.
- Triisopropylsilane (TIS; Sigma-Aldrich, cat. no. 233781, CAS: 6485-79-6) ❗ **CAUTION** TIS is flammable. Handle it inside a chemical fume hood and keep it away from ignition sources.
- Diethyl ether (stabilized with BHT), AR (DEE; Biosolve, cat no. 0005280501BS, CAS: 60-29-7) ❗ **CAUTION** DEE is toxic and flammable. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- *N*-Pentane (AR; Biosolve, cat. no. 0016050502BS, CAS: 109-66-0) ❗ **CAUTION** *N*-Pentane is flammable, toxic and dangerous to the environment. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources. Dispose of chemical waste properly.
- Formic acid (FA) (UPLC/MS grade; Biosolve, cat. no. 0006914143BS, CAS: 64-18-6) ❗ **CAUTION** Formic acid is flammable and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- Acetonitrile (ACN), AR (Biosolve, cat. no. 0001200502BS, CAS: 75-05-8) ❗ **CAUTION** Acetonitrile is flammable, toxic and an irritant. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- Sodium acetate (Sigma-Aldrich, cat. no. S8750, CAS: 127-09-3)
- Ultrapure Milli-Q water

Equipment
- Peptide synthesizer (MultiSyntech Automated Peptide Synthesizer; Biotage, model no. Syro II)
- Rotary evaporator (Büchi Rotavapor, model no. R-200; with B-300 heating bath and V-700 vacuum pump)
- LC–MS mass spectrometer (Waters Alliance, Micromass LCT Premier HPLC–ESI–MS model)
- Büchi purifier (Sepacore, model no. X50, equipped with a C-620 control unit, a C-660 fraction collector, a C-640 UV photometer and a C-605 pump unit)
- Workbeads 40S media/Workbeads 40S prepacked column (Bio-works, product no. 40 200 010)
- HPLC system (Waters, model no.1525EF Binary HPLC pump with 2487 dual λ absorbance detector)
- XBridge Prep C18 column, 5 µM optimum bed density, 30 × 150 mm (Chromex Scientific, part no. 186003284)
- Lyophilizer (Snijders Scientific, model no. LY7FM-UL)

Reagent Setup
DCM/toluene (1:1 (vol/vol)). This solution must be freshly prepared.
KHSO$_4$(1 M). This solution can be stored at room temperature for several months.
NaOAc (pH 4.65, 3 M). This solution can be stored at room temperature for several months.
TFA cleavage mix. This solution is 90.5% (vol/vol) TFA, 5% (vol/vol) dH$_2$0, 2.5% (vol/vol) triisopropylsilane (TIS) and 2% (vol/vol) phenol. ▲ CRITICAL This solution must be freshly prepared.
Lyophilization mix. This solution is 65% (vol/vol) Milli-Q water, 25% (vol/vol) acetonitrile and 10% (vol/vol) acetic acid. This solution can be stored at room temperature for several months.
Cation exchange buffer A (50 mM NaOAc, pH 4.65). This solution can be stored at room temperature for several months.
Cation exchange buffer B. This solution is 50 mM NaOAc, pH 4.65, and 1 M NaCl. This solution can be stored at room temperature for several months.

Procedure
N-terminal biotinylation of distal ubiquitin ● TIMING ~5 h
▲ CRITICAL If desired, biotin-Ahx-Ub-PA is commercially available (UbiQ, cat no. UbiQ-076), which circumvents the need for the first two steps of this section.
1. Synthesize Ub$_{1-75}$ (see the middle panel of the image above for the amino acid sequence) on a Trityl TentaGel resin on a 25 µmol scale. During the final step of SPPS, couple the ethylene glycol spacer to the N terminus of the ubiquitin chain using standard coupling reagents. A detailed protocol describing this procedure has been published[28]. Wash the resin 3× with 1 ml of NMP.

(continued)

Box 1 | Production of alkyne- (distal) and azide- (proximal) functionalized ubiquitin ● TIMING < 16.5 d (continued)

2. Prepare a biotin-coupling mixture by dissolving 47.4 mg of HBTU (5 equiv., 0.3 M) and 16.9 mg (5 equiv., 0.3 M) of HOBT in 417 µl of DMF. Dissolve 30.5 mg (5 equiv., 0.3 M) of biotin in 417 µl of NMP and add the HOBT/HBTU to the biotin solution. Add 43.6 µl of DIPEA, make up the total volume to 1 ml with DMF and pre-activate the reaction mixture for 5 min.

▲ CRITICAL STEP Dissolve the biotin in NMP before addition to the HBTU/HOBT solution and then add the biotin solution to the HBTU/HOBT solution. Sonicate the HBTU/HBTU/biotin/DIPEA reaction mixture if precipitation is seen.

? TROUBLESHOOTING

3. Add the biotin-coupling mixture (step 2) to the resin and react for 3 h on a shaker at room temperature. Wash the resin 3× with 1 ml of NMP.

4. Cleave the protein from the resin with 5 ml of HFIP/DCM (1:4 (vol/vol)) cleavage mix for 30 min at room temperature. Collect the flow-through in a 50-ml round-bottom flask, wash the resin with an additional 3–5 ml of DCM and collect the flow-through in the same round-bottom flask.

5. Evaporate the solvent under reduced pressure using a rotary evaporator.

6. Dissolve the peptide in 10 ml of DCE and evaporate under reduced pressure. Repeat this step two times to remove residual HFIP traces.

■ PAUSE POINT The ubiquitin peptide may be stored at 4 °C for several weeks before proceeding to the next step.

C-terminal glycine replacement by propargylamine of distal ubiquitin ● TIMING ~ 3.5 d

7. Dissolve the protected ubiquitin peptide in DCM (1 ml/5 µmol) and add 5 equiv. of PyBOP, 5 equiv. of triethylamine and 10 equiv. of propargylamine. React at room temperature while stirring for a total time of 16 h.

8. Concentrate the reaction mixture under reduced pressure using a rotary evaporator.

9. Remove excess propargylamine by co-evaporation with 10 ml of DCM/toluene (1:1 (vol/vol)). Repeat this step two times.

10. Dissolve biotin-PEG-Ub$_{1-75}$-PA in 3–5 ml of DCM and extract with 1 M KHSO$_4$. Repeat this step three times and collect all DCM layers.

11. Dry the DCM layer with sodium sulfate, filter off the sodium sulfate and concentrate the organic layer under reduced pressure. Dry the resulting peptide overnight under high vacuum using a lyophilizer.

■ PAUSE POINT The lyophilized ubiquitin polypeptide may be stored at 4 °C for several weeks before final deprotection.

12. To fully deprotect the polypeptide, dissolve biotin-PEG-Ub$_{1-75}$-PA in 5 ml of TFA cleavage mix and react for 2.5 h.

▲ CRITICAL STEP The cleavage mix solution must be freshly prepared.

13. Precipitate the biotin-PEG-Ub$_{1-75}$-PA in 20–25 ml of ice-cold diethyl ether/pentane (3:1 (vol/vol)) in a 50-ml Falcon tube. Centrifuge at 1,000g for 7 min at 4 °C and remove the supernatant.

? TROUBLESHOOTING

14. Wash the pellet with 20–25 ml of diethyl ether, vortex and centrifuge at 1,000g for 7 min at room temperature. Remove the supernatant. Repeat this step two times.

15. Air-dry the ubiquitin polypeptide for 30–60 min to remove the remaining diethyl ether traces and lyophilize under high vacuum.

▲ CRITICAL STEP Traces of diethyl ether may damage the peptide if still present when put on the lyophilizer.

■ PAUSE POINT The lyophilized ubiquitin peptide may be stored at 4 °C for several weeks before final purification.

Preparation of the proximal ubiquitin moiety ● TIMING ~9 d

16. Synthesize ubiquitin$_{1-76}$ (see lower panel of the image above for the amino acid sequence) as described previously[28], but incorporate Fmoc-L-azidoornithine at the desired lysine conjugation position and Fmoc-L-norleucine at the N-terminal methionine position. To make linear-linked nonhydrolyzable diubiquitin, incorporate á-azidonorleucine at the Met1 position[29].

17. Cleave ubiquitin from the resin by reacting with TFA cleavage mix for 2.5 h. Filter and precipitate the flow-through in a 50-ml Falcon tube containing ice-cold diethyl ether/pentane (3:1 (vol/vol)). Wash and dry the pellet as described in steps 13–15.

■ PAUSE POINT The lyophilized ubiquitin peptide may be stored at 4 °C for several weeks before final purification.

Purification of ubiquitin precursors ● TIMING ~3.5 d

18. Dissolve the ubiquitin polypeptides from steps 15 and 17 in 3–5 ml of warm DMSO (5% (vol/vol)).

? TROUBLESHOOTING

19. Add the ubiquitin solution dropwise to 60–100 ml of Milli-Q water while stirring.

? TROUBLESHOOTING

20. Add NaOAc, pH 4.65, dropwise to a final concentration of 50 mM.

21. Make a 40-ml cation exchange column using 40S Workbeads and equilibrate the column with cation exchange buffer A.

22. Perform a cation exchange run at a flow rate of 12 ml/min on a Büchi system, using the following gradient:

Time (min)	Gradient (%B in A)
0	0
7	0
47	100
54	100
57	0
64	0

(continued)

Box 1 | Production of alkyne- (distal) and azide- (proximal) functionalized ubiquitin ● TIMING < 16.5 d (continued)

23. Analyze the fractions on an LC–MS system (Waters Alliance, Micromass LCT Premier HPLC–ESI–MS system). Run the samples at a flow rate of 0.8 ml/min for 6 min at a temperature of 40 °C, using the following two mobile phases: A (1% (vol/vol) ACN, 0.1% (vol/vol) formic acid in Milli-Q water) and B (1% (vol/vol) Milli-Q water, 0.1% (vol/vol) formic acid in ACN), using the following gradient:

Time (min)	Gradient (%B in A)
0	5
0.5	5
4	95
5.5	95
6	5

24. Pool the fractions that contain the correct product.

25. Perform HPLC with the pooled fractions on a Waters chromatography system, using an XBridge Prep C18 column and the following mobile phases: A (Milli-Q water), B (ACN) and C (1% (vol/vol) TFA in Milli-Q water). Apply the following gradient in the run at a flow-rate of 37.5 ml/min, while keeping the percentage of solvent C at 5% in all steps:

Time (min)	Gradient (%B)
0	5
5	5
7	20
18	45
18.5	95
21.5	95
21.6	5
25	5

26. Analyze the peak fractions on an LC–MS system as described in step 24 of **Box 1**.

27. Combine the fractions that contain the correct product in a 50-ml Falcon tube and freeze the tubes using liquid nitrogen. Lyophilize polypeptides under high vacuum (~3 d).

■ PAUSE POINT The lyophilized ubiquitin precursors may be stored at 4 °C for several weeks.

followed by mass spectrometry experiments, protein expression profiling and nucleic acid–protein interaction screens.

Advantages and limitations

Comprehensive ubiquitin–UBP identification Most *in vitro* ubiquitin–UBP interaction studies are performed with recombinantly expressed and purified UBDs. The results obtained in these studies may not necessarily reflect the interactions between full-length UBPs and ubiquitin linkages *in vivo*, considering the fact that PTMs or interacting protein complexes may be important determinants of ubiquitin linkage-selective binding. Furthermore, multiple UBDs in a single protein may act together to achieve high levels of affinity binding to certain ubiquitin topologies, as we have shown for the interaction between tUIMs (tandem ubiquitin-interacting motifs) and K48 (i.e., lysine 48) and K63 linkages[5]. In UbIA-MS, ubiquitin interactions are investigated in the context of the complete cellular proteome. Cellular proteins are all endogenously expressed, contain relevant PTMs and are present in their native protein complexes. Therefore, we believe that UbIA-MS is a powerful method for identifying *in vivo*-relevant ubiquitin interactions.

Nonhydrolyzable diubiquitin UbIA-MS makes use of diubiquitin baits resistant to proteolytic cleavage by DUBs, which are abundantly present in cell lysates. The presence of such a nonhydrolyzable bond is thus a clear advantage of our method. Although these baits enabled the identification of most known ubiquitin-linkage-selective interactors and helped identify a plethora of new linkage-selective UBPs, we also observed length-dependent

interactors for linear polyubiquitin chains[5]. Therefore, the use of different polyubiquitin lengths should be considered. This could, for instance, be achieved semisynthetically through incorporation of the unnatural amino acids azidohomoalanine (Aha) and propargyl-derivatized pyrrolysine (Plk) into the ubiquitin sequence, which can then be used for CuAAC-mediated polyubiquitin synthesis. A detailed protocol for this procedure is available and could be readily used, provided that the necessary expertise, *Escherichia coli* strains and tRNA pairs are available[6]. The advantage of using a fully synthetic strategy, on the other hand, is that the building blocks needed for solid-phase peptide synthesis (SPPS) are all commercially available, and that the involved chemistry is straightforward.

Direct and indirect interactions In UbIA-MS, both direct and indirect ubiquitin interactions are detected. In the future, photoactivatable ubiquitin chains could be used to discriminate direct from indirect interactions[7]. The ability to detect a certain ubiquitin–UBP interaction in UbIA-MS experiments depends on certain parameters. First, the particular UBP must be expressed to sufficient amounts in the extract (median protein expression of detected interactors is ~10,000 copies per cell). Second, the UBP–ubiquitin interaction must have an adequate (micromolar range) affinity. Some UBDs are known to bind to ubiquitin with an affinity of <1 mM (ref. 8). It is unlikely that such low-affinity interactions would be detected in UbIA-MS experiments. Cross-linking MS or photoactivatable ubiquitin chains could be an option to study such low-affinity interactions. Whether such

Box 2 | Nonhydrolyzable diubiquitin synthesis ● TIMING <3.5 d

This box describes how to obtain purified nonhydrolyzable diubiquitin baits with click chemistry.

Reagents
- Biotin-PEG-Ub$_{1-75}$-PA (**Box 1**)
- Ub$_{1-76}$-N$_3$ with azidoornithine at desired Lys or with á-azidonorleucine at Met1 position (**Box 1**)
- NaH$_2$PO$_4$ (Sigma-Aldrich, cat. no. S8282, CAS: 7558-80-7)
- TBTA ester (1,1',1"-Tris (1H-1,2,3-triazol-4-yl-1-acetic acid ethyl ester) trimethylamine) (Carbosynth, cat. no. FT11240, CAS: 736958-00-2; or, alternatively, it can be prepared as described before[30])
- CuSO$_4$•5H$_2$O (Sigma-Aldrich, cat. no. 203165, CAS:7758-99-8)
- Sodium ascorbate (Sigma-Aldrich, cat. no. A7631, CAS: 134-03-2)
- Acetic acid (Merck Millipore, cat. no. 101830, CAS: 64-19-7) **! CAUTION** Acetic acid is flammable and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- Dimethylsulfoxide (DMSO; Biosolve, cat. no. 0004470501BS, CAS: 67-68-5) **! CAUTION** DMSO is an irritant. Always use protective gloves when handling it.
- EDTA (Sigma-Aldrich, cat. no. 431788, CAS: 60-00-4) **! CAUTION** EDTA is toxic. Always use protective gloves when handling it.
- Acetonitrile (ACN), AR (Biosolve, cat. no. 0001200502BS, CAS: 75-05-8) **! CAUTION** Acetonitrile is flammable, toxic and an irritant. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- Trifluoroacetic acid, peptide synthesis grade (TFA; Biosolve, cat. no. 0020233320BS, CAS: 76-05-1) **! CAUTION** TFA is corrosive and toxic. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it.
- Ultrapure Milli-Q water

Equipment
- LC–MS mass spectrometer (Waters Alliance, Micromass LCT Premier HPLC–ESI–MS)
- HPLC system (Shimadzu, with the following modules: CBM-20A, RF-20A, SPD-20A, FRC-10A, CTO-20AC and DGU-20A$_{3R}$)
- C18 RP-HPLC column (10 × 150 mm, 5-µm particle size; Atlantis, model Prep T3)
- Lyophilizer (Snijders Scientific, model no. LY7FM-UL)
- FPLC system (NGC Quest; BioRad)
- HiLoad S75 16/600 Superdex PG column (GE Healthcare, cat no. 28989333)
- Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane (Merck Millipore, cat. no. UFC900324)

Reagent Setup

Diubiquitin 'click' buffer. Diubiquitin 'click' buffer is 0.1 M NaH$_2$PO$_4$, pH 7.0, and 8 M urea. ▲ **CRITICAL** This buffer must freshly be prepared.

Diubiquitin gel filtration buffer. Diubiquitin gel filtration buffer is 150 mM NaCl and 20 mM Tris-HCl, pH 7.6, in Milli-Q water; filter with a 0.22-µm filter. This buffer can be stored at 4 °C for a few months.

Procedure

▲ **CRITICAL** Purity of the starting material is key to CuAAC. Therefore, we recommend purifying the monoubiquitin precursors as described in steps 18–27 in **Box 1**.

1. Degas the diubiquitin 'click' buffer, acetonitrile and ultrapure Milli-Q water in a 50-ml Falcon tube by flushing through argon or nitrogen, using a glass Pasteur pipette.
▲ **CRITICAL STEP** This step serves to prevent untimely oxidation of the Cu$^+$–TBTA complex.

2. Dissolve 10 mg of CuSO$_4$ in degassed ultrapure Milli-Q water to a concentration of 100 mM.

3. Dissolve 12 mg of sodium ascorbate in degassed ultrapure Milli-Q water to a concentration of 600 mM.

4. Dissolve 13 mg of TBTA ester in degassed acetonitrile to a concentration of 100 mM.

5. Add 40 µl of CuSO$_4$ solution to 40 µl of sodium ascorbate solution and mix.

6. Add 40 µl of TBTA solution to the solution from step 5.

7. Weigh out 1 mg of biotin-PEG-Ub$_{1-75}$-PA (from **Box 1**) and 1.5 mg of Ub$_{1-76}$-N$_3$ (from **Box 1**) and dissolve in a minimal amount of DMSO (20 µl). Heat the sample in a heat block to 95 °C if the ubiquitin does not dissolve in the first instance.
? TROUBLESHOOTING

8. Add the ubiquitin–DMSO solution from step 7 slowly to 750 µl of diubiquitin 'click' buffer with regular vortexing in between.
? TROUBLESHOOTING

9. Add 30 µl of the 'click' mixture from step 6 to the Ub-solution from step 8.

10. React for 30–60 min at room temperature and follow the reaction by LC–MS as described in **Box 1** steps 23 and 24 (inject ±1 µg).
▲ **CRITICAL STEP** When preparing a sample for LC–MS follow-up, quench the reaction with 1 mM EDTA before applying it to a HPLC/LC–MS system.
? TROUBLESHOOTING

■ **PAUSE POINT** If the reaction is complete, the reaction mixture may be frozen by using liquid nitrogen and stored in a −80 °C freezer up to several weeks before proceeding with the HPLC purification. Quench the reaction with 1 mM EDTA before freezing.

(continued)

Box 2 | Nonhydrolyzable diubiquitin synthesis ● TIMING < 3.5 d (continued)

11. Perform HPLC on a Shimadzu chromatography system using an Atlantis Prep T3 C18 RP-HPLC column and the following mobile phases: (A) 95% (vol/vol) Milli-Q water, 5% (vol/vol) ACN and 0.05% (vol/vol) TFA; and (B) 5% (vol/vol) Milli-Q water, 95% (vol/vol) ACN and 0.05% (vol/vol) TFA. Elute the product over a linear gradient of 20–70% B in 12 min. Analyze the peak fractions on an LC–MS system as described in **Box 1**, steps 23 and 24.

12. Combine the fractions that contain the correct product in a 50-ml Falcon tube, rinse the tubes with lyophilization mix (65% (vol/vol) Milli-Q water, 25% (vol/vol) ACN and 10% (vol/vol) acetic acid) and add this to the 50-ml Falcon tube.

13. Use liquid nitrogen to freeze the tubes for 5–10 min. Lyophilize the diubiquitins under high vacuum (~3 d).

14. Dissolve (di)ubiquitin powder in 75 µl of DMSO. Heat the sample in a heat block to 95 °C if the (di)ubiquitin does not dissolve in the first instance.

▲ CRITICAL STEP The maximal final ubiquitin concentration should not exceed 15 mg/ml.

15. Add Ub-DMSO solution dropwise to 1.425 ml of gel filtration buffer with regular vortexing in between.

? TROUBLESHOOTING

16. Centrifuge the sample for 5 min at 18,800g at room temperature and apply to a HiLoad 16/60 S75 gel filtration column on an FPLC system.

17. Analyze the fractions on a 10% (vol/vol) SDS-PAGE gel, stain with Coomassie Brilliant Blue and pool the fractions that contain only diubiquitin (visible at 17 kDa).

18. Concentrate the pooled fractions using a 3-kDa cutoff Centricon filter to < 1 ml and collect the concentrate.

■ PAUSE POINT The purified nonhydrolyzable diubiquitins may be stored at −20 °C for several months or at −80 °C for several years.

low-affinity interactions are biologically meaningful, however, is questionable.

***In vitro* interaction screen** One clear limitation of UbIA-MS is the *in vitro* nature of the experimental approach. Interactions are studied in the context of crude cell lysates. Whether and to what extent *in vitro*-detected interactions are also relevant *in vivo* remains to be determined in follow-up experiments. One such follow-up approach is the proximity ligation assay, which we used to validate detected interactions with K6-linked ubiquitin chains upon induction of DNA damage in cells[5].

Experimental design

Starting material. The aim of this protocol is to comprehensively identify ubiquitin interactors for ubiquitin linkages at near-physiological conditions. Depending on the purpose of the experiment, cells can be maintained in a steady state or treated with a desired stimulus (e.g., growth factor stimulation, cytotoxic stress and different stages of the cell cycle). For stimulation experiments, lysates from both untreated and treated cells should be generated
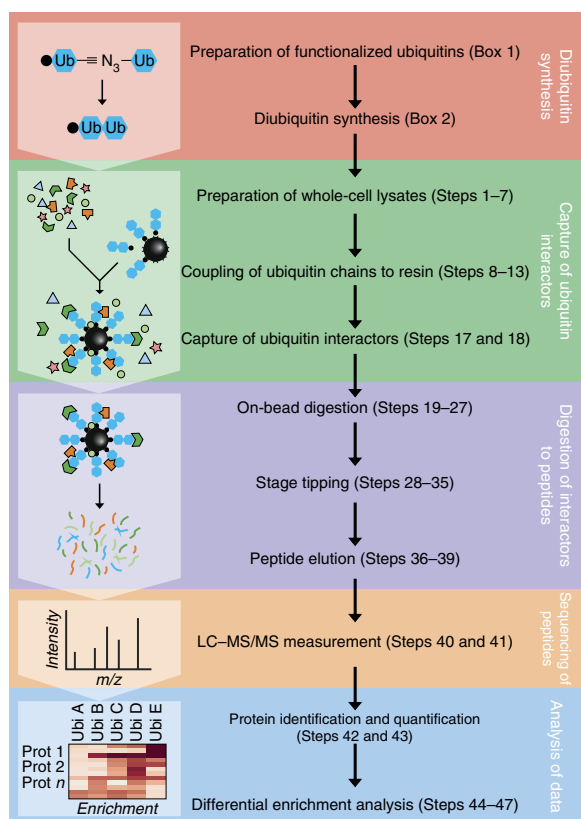
Figure 1 | Schematic overview of UbIA-MS workflow. UbIA-MS is divided into five stages: diubiquitin synthesis, capture of ubiquitin interactors, digestion of interactors to peptide, sequencing of peptides by LC–MS/MS, and data analysis/visualization. Prot, protein; Ubi, ubiquitin.
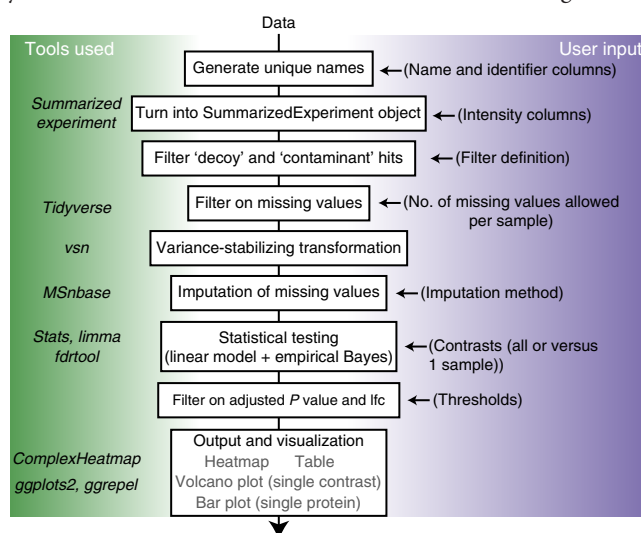
Figure 2 | Schematic representation of the DEP analysis workflow. The continuous vertical line represents the data flow. The R packages on which the analysis steps depend are listed on the left, and the required user input for the analysis steps is listed on the right. lfc, log$_2$ fold change.

to facilitate the identification of stimulation-induced interactions. The stimulus of interest may change the proteome. We therefore advise profiling the global proteomes of control and treated cells to discriminate expression-dependent from expression-independent stimulus-induced interactions[9]. In this protocol, we use 5 mg of whole-cell extracts from HeLa cells for each pull-down. Other materials, such as tissues and patient samples, are also applicable. Furthermore, cell fractionation assays can be used to study localization-dependent ubiquitin interactions (e.g., nuclear, cytoplasmic and mitochondrial fractions).

Diubiquitin baits. Purified, nonhydrolyzable diubiquitins are used as baits to isolate interactors from crude lysates. Recent advances in chemical biology have enabled the synthesis of ubiquitin chains and ubiquitinated proteins *in vitro*[6,10–13]. In previous work, we used click chemistry to synthesize all eight homotypic diubiquitin linkages in nonhydrolyzable form[5,14]. The procedures for obtaining the necessary monoubiquitin precursors and for the generation of the nonhydrolyzable diubiquitin baits are described in **Boxes 1** and **2**, respectively. **Box 1** describes how to make azide- and alkyne-containing ubiquitin moieties with SPPS, and **Box 2** details the workflow for the synthesis of nonhydrolyzable diubiquitins by click chemistry. An issue to be considered is the presence of highly abundant endogenous DUBs, which may cleave native ubiquitin chains during the incubation. To solve this problem, we recommend using a triazole linkage between ubiquitin molecules, which mimics the native ubiquitin linkage, but is resistant to cleavage by DUBs[5,11,14].

Bait resin preparation. To immobilize ubiquitin chains on a solid resin, an affinity tag is added to the N terminus of the distal ubiquitin. Common tags include Flag, HA, 6×His or biotin. We recommend using the biotin tag, because of the high affinity between biotin and streptavidin, as well as the low background binding of streptavidin Sepharose beads[15]. Two control samples are commonly included: the empty resin, to control for background binding proteins; and a monoubiquitin bait, to discriminate linkage-selective (poly)ubiquitin interactors from nonselective ubiquitin interactors.

Affinity purification and sample preparation. The resin-bound ubiquitin baits are subsequently incubated with crude cell lysates. We recommend using 5 mg of whole-cell extract for each pull-down experiment. To minimize nonspecific binding to the affinity resin, we recommend using 0.5 and 1% (vol/vol) IGEPAL CA-630 in the lysis and wash buffer, respectively. After incubation

and washes, proteins bound to the resin are digested with trypsin before LC–MS/MS analysis. We recommend on-bead protein digestion over in-gel protein digestion, because the on-bead workflow strongly reduces the number of fractions to be measured by mass spectrometry, as compared with in-gel protein digestion, while still identifying the most pronounced interactors[5]. Iodoacetamide is commonly used to alkylate cysteine residues after protein reduction. However, 2-chloroacetamide should be used if subsequent ubiquitin linkage analysis by MS is planned, because iodoacetamide causes the production of 2-acetamidoacetamide adducts on lysine residues that mimic ubiquitination[16].

LC–MS/MS analysis. Digested peptides are analyzed using a nano reverse-phase HPLC system directly connected to a mass spectrometer. The unbiased identification of ubiquitin interactors is facilitated by quantitative mass spectrometry-based proteomics[17]. UbIA-MS was established using label-free protein quantification, which allows any protein lysate as input, including lysates from tissues and primary material[18]. The protocol described here can also be implemented using other types of stable isotope labeling–based quantification.

Data processing and visualization. Different software packages are available to analyze raw mass spectrometry data. We make use of the MaxQuant software, which is freely available at http://www.maxquant.org. Detailed user protocols concerning MaxQuant are available elsewhere[19,20]. We developed the R package DEP, which uses the protein output files from MaxQuant as direct input and provides an integrated analysis workflow for differential protein expression or differential enrichment analysis. This package is integrated within the Bioconductor project[21] and includes functionalities to filter and normalize data, perform differential enrichment testing and visualize the results (**Fig. 2**). In addition, DEP comes with an easy-to-use graphical user interface, so it can be used without scripting or programming knowledge. In the analysis, the data are filtered for proteins with too many missing values, after which variance-stabilizing transformation using vsn[22] and data imputation using the methods from MSnbase[23] are applied. Subsequently, differential enrichment analysis is performed by applying empirical Bayes statistics on protein-wise linear models using limma[24,25]. Multiple quality-control plots are generated to visually inspect missing values, normalization and imputation. In addition, the results can be explored using different visualizations, including heatmap, volcano plot and bar plot representations. The output of the package consists of tab-delimited text files, PDF plots and reports.

MATERIALS

REAGENTS
- Cells of interest: We have used HeLa cells (ATCC, cat. no. CCL-2.2)
  ! CAUTION The cell lines used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- DMEM (Thermo Fisher Scientific, cat. no. 10569010)
- FBS (Thermo Fisher Scientific, cat. no. 10270106)
- Trypsin-EDTA (0.25% (wt/vol)) (Thermo Fisher Scientific, cat. no. 25200056)
- DPBS (Lonza, cat. no. BE17-512F)
- NaCl (Merck Millipore, cat. no. 106406, CAS: 7647-14-5)
- HCl (Merck Millipore, cat. no. 100317, CAS: 7647-01-0) **! CAUTION** HCl is toxic and corrosive. Handle it inside a chemical fume hood with protective gloves and avoid breathing its vapors.

- UltraPure Tris (Thermo Fisher Scientific, cat. no. 15504020)
- Glycine (Merck Millipore, cat. no. 357002, CAS: 56-40-6)
- SDS (Thermo Fisher Scientific, cat. no. 15525017, CAS: 151-21-3)
  ! CAUTION SDS is corrosive, toxic and flammable. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it, and keep it away from ignition sources.
- IGEPAL CA-630 (Sigma-Aldrich, cat. no. I8896, CAS: 9002-93-1)
  ▲ CRITICAL IGEPAL CA-630 is indistinguishable from NP-40, either of which can be used. **! CAUTION** IGEPAL CA-630 is toxic, corrosive and harmful to the environment. Always wear protective gloves when handling it.
- Glycerol (Merck Millipore, cat. no. 104092, CAS: 56-81-5)

- cOmplete, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, cat. no. 04693132001) **! CAUTION** cOmplete, EDTA-free Protease Inhibitor Cocktail is corrosive. Always wear gloves when handling it.
- Urea (Sigma-Aldrich, cat. no. U5378, CAS: 57-13-6)
- DTT (Sigma-Aldrich, cat. no. D9779, CAS: 3483-12-3) **! CAUTION** DTT is toxic. Handle it inside a chemical fume hood and avoid breathing its vapors.
- Ammonium bicarbonate (Sigma-Aldrich, cat. no. 09830, CAS: 1066-33-7) **! CAUTION** Ammonium bicarbonate is an irritant. Handle it inside a chemical fume hood with protective gloves and avoid breathing its vapors.
- Iodoacetamide (Sigma-Aldrich, cat. no. I1149, CAS: 144-48-9) **! CAUTION** Iodoacetamide is toxic and a mutagen. Always wear protective gloves when handling it.
- Acetic acid (Merck Millipore, cat. no. 101830, CAS: 64-19-7) **! CAUTION** Acetic acid is flammable and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors, always wear protective gloves when handling it and keep it away from ignition sources.
- Trypsin (MS grade; Promega, cat. no. V5280, CAS: 9002-07-7) **! CAUTION** Extra care should be taken when using acetic acid.
- Acetonitrile (ULC/MS grade; Biosolve, cat. no. 0001204101BS, CAS: 75-05-8) **! CAUTION** Acetonitrile is flammable, toxic and an irritant. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- Trifluoroacetic acid (TFA; ULC/MS grade; Biosolve, cat. no. 202341, CAS: 76-05-1)**! CAUTION** TFA is corrosive and toxic. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it.
- Methanol (ULC/MS grade; Biosolve, cat. no. 136841, CAS: 67-56-1) **! CAUTION** Methanol is flammable and an irritant. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- Formic acid (ULC/MS grade; Biosolve, cat. no. 069141, CAS: 64-18-6) **! CAUTION** Formic acid is flammable and corrosive. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it and keep it away from ignition sources.
- 3M Empore SPE C18 Disks (Sigma-Aldrich, cat. no. 66883-U)
- pH-Indicator strips (pH 0–14; Merck Millipore, cat. no. 109535)
- Streptavidin Sepharose (GE Healthcare Life Sciences, cat. no. 17-5113-01) **! CAUTION** Streptavidin Sepharose contains 20% (vol/vol) ethanol as a preservative; keep it away from ignition sources.
- BCA Assay Kit (Thermo Fisher Scientific, cat. no. 23225) **! CAUTION** The BCA Assay Kit contains reagents harmful to the environment.
- Coomassie Brilliant Blue R-250 (Bio-Rad, cat. no. 1610436)
- NuPAGE LDS sample buffer (4×; Thermo Fisher Scientific, cat. no. NP0007)
- HeLa Protein Digest Standard (Thermo Fisher Scientific, cat. no. 88329)
- Liquid nitrogen (Linde Gas)

EQUIPMENT
- Heracell CO_2 incubator (Thermo Fisher Scientific, cat. no. 51026280)
- 14.5-cm Cell culture dishes (Greiner Bio-One, cat. no. 639160)
- Safe-Lock tubes, 0.5 ml (Eppendorf, cat. no. 0030121.023)
- Safe-Lock tubes, 1.5 ml (Eppendorf, cat. no. 0030123.328)
- Safe-Lock tubes, 2 ml (Eppendorf , cat. no. 0030120.094)
- 15-ml Centrifuge tubes (Greiner Bio-One, cat. no. 188285)
- 50-ml Centrifuge tubes (Greiner Bio-One, cat. no. 227261)
- Serological pipettes, 5 ml (VWR, cat. no. 612-3702)
- Serological pipettes, 10 ml (VWR, cat. no. 612-3700)
- Serological pipettes, 25 ml (VWR, cat. no. 612-3698)
- Pipetboy (Integra Biosciences, cat. no. I155017)

- Mini-PROTEAN Tetra cell for precast gels (Bio-Rad, cat. no. 1658005EDU)
- Milli-Q Advantage A10 Water Purification System (Merck Millipore, cat. no. Z00Q0V0WW) **▲ CRITICAL** Mass spectrometry is very sensitive to contaminants; ultrapure water (18.2 MΩ cm resistance, total organic carbon <12 parts per billion) is required for every buffer.
- Q-POD Ultrapure Water Remote Dispenser (Merck Millipore, cat. no. ZMQSP0D01) without polisher
- Vortex (IKA, cat. no. 0003340000)
- pH meter (HACH, cat. no. HQ411D)
- Heraeus Multifuge X3 centrifuge (Thermo Fisher Scientific, cat. no. 75004515)
- Sorvall Fresco 21 Centrifuge, refrigerated (Thermo Fisher Scientific, cat. no. 75002425)
- Heraeus Pico 21 Microcentrifuge (Thermo Fisher Scientific, cat. no. 75002415)
- Eppendorf ThermoMixer F1.5 (Eppendorf, cat. no. 5384)
- Intelli-Mixer (ELMI Laboratory Equipment, cat. no. RM-2L)
- 4–20% Mini precast protein gel (Bio-Rad, cat. no. 4561096)
- Speed vacuum (Concentrator Plus; Eppendorf, cat. no. 5305 000.509)
- 96-Well plate rotor (Eppendorf, cat. no. A-2-VC)
- 0.2-ml 8-Tube strips (Thermo Fisher Scientific, cat. no. AB0264)
- 10-μl Tips (Rainin, cat. no. 17005085) **▲ CRITICAL** Mass spectrometry is very sensitive to contaminants; in our experience, pipette tips from this brand cause the least contamination.
- 250-μl Tips (Rainin, cat. no. 17005087)
- 1,000-μl Tips (Rainin, cat. no. 17005083)
- 30-Gauge syringe (BD, cat. no. 3021607)
- 18-Gauge blunt-end syringe needle (Capital Medical Supplies, cat. no. TERDU1838)
- Laboratory bottle with blue cap and ring (Duran, cat. no. 218015455)
- Cylinder (Duran, cat. no. 213965405)
- EASY-nLC 1000 liquid chromatograph (Thermo Fisher Scientific, cat. no. LC120)
- Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific, cat. no. IQLAAEGAAPFADBMBCX)
- Nanospray Flex ion source (Thermo Fisher Scientific, cat. no. ES072)
- Eppendorf Combitips (Eppendorf, cat. no. 0030089456)
- Windows PC (Windows 7, 32 GB of RAM, 16 cores and a 1-TB hard drive)
- MaxQuant software package (v1.5.0.1 or newer, freely available at http://www.maxquant.org)
- R: Install the latest version of R, which can be downloaded from http://www.cran.r-project.org.
- Pandoc, which can be downloaded from http://www.pandoc.org/
- Rtools (for Windows users only), which can be downloaded from https://cran.r-project.org/bin/windows/Rtools/
- NetCDF (for Linux users only), which can be downloaded from https://www.unidata.ucar.edu/software/netcdf/
- DEP package (https://bioconductor.org/packages/release/bioc/html/DEP.html): Install the DEP package as described in Equipment Setup.
- Example data set: The diubiquitin interaction data set published by Zhang *et al.* is used as example data for the computational analysis[5]. The data are included in the DEP package. It consists of label-free protein quantification data from UbIA-MS experiments with ten different conditions, performed in triplicate.

REAGENT SETUP

Cell culture In the example described in this protocol, we perform pull-downs for ten conditions in triplicate (one negative control, one monoubiquitin and eight diubiquitins). This requires 20–30 90% confluent 14.5-cm dishes of HeLa cells in order to produce at least 150 mg of cell lysate. **▲ CRITICAL** For experiments with different cell models, use appropriate cell culture medium and detachment enzyme (e.g., use Accutase to detach embryonic stem cells).

5 M NaCl Dissolve NaCl in ultrapure Milli-Q water to a concentration of 5 M. After filtering with a 0.22-μM vacuum filter unit, it can be stored at room temperature (22 °C) for >1 year.

1 M Tris-HCl (pH 8.0/8.5) Dissolve ultrapure Tris in ultrapure Milli-Q water to a concentration of 1 M, and adjust the pH by addition of HCl. This buffer should be filtered with a 0.22-μM vacuum filter unit, and it can be stored at room temperature for a few months. ❗ **CAUTION** HCl is toxic and corrosive. Liquid and mist cause severe burns. The solution may be fatal if swallowed or inhaled. Always wear protective gloves when handling it.

IGEPAL CA-630 (10% (vol/vol)) Dilute 10 ml of IGEPAL CA-630 in 90 ml of ultrapure Milli-Q water to make 100 ml of 10% (vol/vol) IGEPAL CA-630. This can be stored at 4 °C for a few months. ❗ **CAUTION** IGEPAL CA-630 is toxic, corrosive and harmful to the environment. Always wear protective gloves when handling it.

cOmplete, EDTA-free Protease Inhibitor Cocktail (100×) Dissolve cOmplete, EDTA-free Protease Inhibitor Cocktail tablets in 500 μl of ultrapure Milli-Q water each. The solution can be stored at −20 °C for a few months. ❗ **CAUTION** cOmplete, EDTA-free Protease Inhibitor Cocktail is corrosive. Always wear gloves when handling it.

DTT (1 M) Dissolve DTT in ultrapure Milli-Q water to a concentration of 1 M. The solution can be stored in 100-μl aliquots at −20 °C for a few months. ❗ **CAUTION** DTT is toxic. Handle it inside a chemical fume hood and avoid breathing its vapors.

Ammonium bicarbonate (50 mM) Dissolve ammonium bicarbonate in ultrapure Milli-Q water to a concentration of 50 mM. ❗ **CAUTION** Ammonium bicarbonate is an irritant. Handle it inside a chemical fume hood with protective gloves and avoid breathing its vapors. ▲ **CRITICAL** Freshly prepare the buffer before use.

Iodoacetamide (0.55 M) Dissolve iodoacetamide in 50 mM ammonium bicarbonate to a concentration of 0.55 M. The solution can be stored at −20 °C for a few months. ❗ **CAUTION** Iodoacetamide is toxic and a mutagen. Always wear protective gloves when handling it. ▲ **CRITICAL** Store in the dark, as iodoacetamide is light-sensitive.

Trypsin Dissolve Trypsin in 50 mM acetic acid to a concentration of 100 ng/μl. It can be stored at −80 °C for a few months. ❗ **CAUTION** Extra care should be taken when using acetic acid.

TFA (10% (vol/vol)) Dilute 10 ml of TFA in 90 ml of ultrapure Milli-Q water to make 100 ml of 10% (vol/vol) TFA. ❗ **CAUTION** TFA is corrosive and toxic. Handle it inside a chemical fume hood and avoid breathing its vapors; always wear protective gloves when handling it.

Ubiquitin-binding buffer Ubiquitin-binding buffer is 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.1% (vol/vol) IGEPAL CA-630 in Milli-Q water. This buffer can be stored at 4 °C for a few months.

Cell lysis buffer Cell lysis buffer is 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% (vol/vol) IGEPAL CA-630 and 10% (vol/vol) glycerol in Milli-Q water. This buffer can be stored at 4 °C for a few months. ▲ **CRITICAL** Add 1× cOmplete, EDTA-free Protease Inhibitor Cocktail immediately before use.

Wash buffer Wash buffer is cell lysis buffer with 1% (vol/vol) IGEPAL CA-630. This buffer can be stored at 4 °C for a few months.

SDS running buffer SDS running buffer is 25 mM Tris, 192 mM glycine and 0.1% (vol/vol) SDS in Milli-Q water. This buffer can be stored at room temperature for a few months.

Elution buffer Elution buffer is 2 M urea, 10 mM DTT and 50 mM Tris-HCl, pH 8.5, in Milli-Q water. ▲ **CRITICAL** Elution buffer should not be heated above room temperature; prepare fresh buffer before use. ▲ **CRITICAL** Make sure that the pH of the buffer is ~8.5 to maximize the activity of trypsin.

Buffer A Buffer A is 0.1% (vol/vol) formic acid in Milli-Q water. This buffer can be stored at room temperature for a few months.

Buffer B Buffer B is 80% (vol/vol) acetonitrile and 0.1% (vol/vol) formic acid in Milli-Q water. This buffer can be stored at room temperature for a few weeks.

EQUIPMENT SETUP

LC setup Set the parameters for LC (Step 41) with a maximum pressure of 800 bar and a constant flow rate of 250 nl/min according to the following gradient:

Time (min)	Duration	Gradient (%B in A)
00:00	00:00	7%
01:00	01:00	7%
215:00	214:00	30%
220:00	05:00	60%
225:00	05:00	95%
230:00	05:00	95%
235:00	05:00	5%
240:00	05:00	5%

MS/MS setup Set the MS parameters for the LC–MS/MS analysis in Step 41 according to the following table.

Instrument	Parameter	Setting
Ion source	Spray voltage: positive ion	2,000 V
	Ion transfer tube temperature	275 °C
	Default charge status	2
MS¹	Orbitrap resolution	120.000
	Automatic gain control (AGC)	300.000 (ions)
	Scan range	400–1,500 m/z
	Maximum injection time	50 ms
MS²	Isolation window	1.6 m/z
	Ion trap resolution	30.000
	AGC	20.000 (ions)
	Maximum injection time	40 ms
	Acquisition mode	Top speed

DEP package Install the DEP package by running the following commands in R:

```
> source("https://bioconductor.org/biocLite.R")
> biocLite("DEP")
```

The package can now be loaded by running the following line:

```
> library("DEP")
```

All analysis steps are described in the protocol. Additional examples and explanations are available in a detailed vignette, which can be viewed using the following command:

```
> browseVignettes("DEP")
```

PROCEDURE
Preparation of whole-cell lysates ● TIMING ~4 h
▲ **CRITICAL** Throughout the protocol, we follow an experiment using biotin-tagged diubiquitin molecules to identify ubiquitin interactors from HeLa whole-cell lysate. The procedures for obtaining nonhydrolyzable diubiquitin baits are described in **Box 1** (ubiquitin precursor generation) and **Box 2** (click reaction/CuAAC). In this example, we perform pull-downs for ten conditions in triplicate (one negative control, one monoubiquitin and eight diubiquitins). This requires 20–30 90% confluent 14.5-cm dishes of HeLa cells in order to produce at least 150 mg of cell lysate.

▲ **CRITICAL** To minimize polymer contamination, do not autoclave pipette tips, buffers or glass bottles.

▲ **CRITICAL** To minimize keratin contamination, wear gloves throughout the PROCEDURE.

1| Discard the cell medium and wash the HeLa cells ('Reagent Setup') two times with 4–6 ml of PBS.

▲ **CRITICAL STEP** Calculate how much cell lysate is required for the experiment. We normally obtain ~5 mg of whole-cell lysate from one 90% confluent 14.5-cm plate of HeLa cells.

2| Add 3 ml of trypsin-EDTA to trypsinize cells at 37 °C for 3–5 min. Check the detachment by microscopy.

▲ **CRITICAL STEP** We recommend using microscopy to ensure that all cells are detached after trypsinization.

3| Add 5 ml of cell culture medium to quench the trypsinization, and collect the cells into 50-ml tubes. Centrifuge the cells at 400g for 5 min at 4 °C. Remove the supernatant.

4| Resuspend the cell pellets with ten volumes of DPBS, combine all cell suspensions in one 50-ml tube and centrifuge the cells (400g for 5 min at 4 °C) and remove the supernatant. Repeat this step once.

▲ **CRITICAL STEP** Use a Pipetboy to resuspend the cell pellets a few times to disrupt cell clumps, as such clumps interfere with efficient cell lysis.

5| Add five volumes of cell lysis buffer to the cell pellets, vortex the tube for 30 s and incubate the tube on a rotator (40–60 r.p.m.) for 2 h at 4 °C.

▲ **CRITICAL STEP** Vortex the sample for efficient and comprehensive cell lysis.

6| Centrifuge the sample at 4,816g with a swinging bucket rotor for 30 min at 4 °C in order to separate the soluble cell lysate from the insoluble material. Transfer the supernatant to a new 50-ml tube.

7| Use the BCA kit to determine the protein concentration of the lysate according to the manufacturer's instructions. We usually obtain 8 –12 mg/ml.

? TROUBLESHOOTING

■ **PAUSE POINT** Use liquid nitrogen to snap-freeze the crude cell lysate for long-term storage. The crude cell lysate can be stored at –80 °C for several years.

Coupling of biotin-tagged ubiquitin chains to streptavidin beads ● TIMING ~5 h
▲ **CRITICAL** This section of the PROCEDURE should be performed at room temperature.

8| Pipette 900 μl of streptavidin Sepharose beads (50% slurry; 15 μl of beads is needed for each pull-down) into a 15-ml tube and add five volumes of ubiquitin-binding buffer. Invert the tube a few times to resuspend the streptavidin beads, centrifuge the beads at 1,500g for 2 min at room temperature and remove the supernatant completely. Repeat the washing procedure three times.

▲ **CRITICAL STEP** Use a clean razor blade to cut the pipette tip to avoid clogging of the tip by the beads.

9| Add 3 ml of ubiquitin-binding buffer to the streptavidin beads and divide the beads equally among ten 1.5-ml Eppendorf tubes (for one negative control, one monoubiquitin and eight diubiquitins).

10| Add 75 μg of biotin-tagged mono- or diubiquitin (**Boxes 1** and **2**) to each of the corresponding tubes and add ubiquitin-binding buffer to a final volume of 600 μl.

▲ **CRITICAL STEP** Save 10 μl of supernatant as input for SDS-PAGE gel in Step 12 to check the depletion efficiency.

11| Incubate the tubes on an Intelli-Mixer tube rotator at a constant speed of 12 r.p.m. for 1 h at room temperature.
▲ CRITICAL STEP Determine the optimal rotation speed for other beads to keep them in suspension while avoiding forceful agitation.

12| Centrifuge the samples at 1,500*g* for 2 min at room temperature and load 10 µl of the supernatant on an SDS-PAGE gel. Stain the gel with Coomassie Brilliant Blue R-250 according to the manufacturer's instructions and check the coupling efficiency of the ubiquitin molecules to streptavidin beads.
▲ CRITICAL STEP Stop the experiment if the ubiquitin chains are not efficiently depleted from the supernatant after the incubation. We usually obtain ~100% depletion efficiency checked by Coommassie staining.
? TROUBLESHOOTING

13| Add 1 ml of ubiquitin-binding buffer to each tube and invert the tube a few times to resuspend the beads. Centrifuge the samples at 1,500*g* for 2 min at room temperature and remove the supernatant, repeat this step three times.
▲ CRITICAL STEP Make sure that the beads are resuspended thoroughly to ensure efficient washing.

Incubation of the coupled ubiquitin chains with whole-cell lysates ● TIMING ~20 h
▲ CRITICAL This part of the protocol must be performed at 4 °C. Use ice, a refrigerated centrifuge and 4 °C room.
14| Add 900 µl of cold cell lysis buffer to each tube and use a cut pipette tip to equally divide the beads among three new 1.5-ml tubes.
▲ CRITICAL STEP Decrease the volume of added lysis buffer when the protein concentration of the lysate is low, in order to make sure that the total volume after Step 15 does not exceed 1 ml.

15| Add 5 mg of cleared soluble cell lysate obtained in Step 6 of the PROCEDURE to the beads, and add lysis buffer to a final volume of 1 ml. Incubate the samples overnight (16–18 h) at 4 °C in an Intelli-Mixer tube rotator at a constant speed of 12 r.p.m.
▲ CRITICAL STEP Thaw on ice or at 4 °C to minimize protein degradation.
▲ CRITICAL STEP The amount of protein used per sample should be optimized experimentally for different types of lysate. For example, we use 2 mg of nuclear extracts for ubiquitin pull-downs.

16| Centrifuge the samples at 1,500*g* for 2 min at 4 °C and remove the supernatant completely.

17| Add 1 ml of wash buffer to each tube and invert the tube a few times to resuspend the beads. Incubate the beads for 3 min at 4 °C in an Intelli-Mixer tube rotator at a constant speed of 12 r.p.m., centrifuge the samples at 1,500*g* for 2 min at 4 °C and remove the supernatant completely. Repeat this step three times.

18| Add 1 ml of cold PBS to each tube and invert the tube a few times to resuspend the beads. Centrifuge the samples at 1,500*g* for 2 min at 4 °C and remove the supernatant completely. Repeat this step once.
▲ CRITICAL STEP PBS washes are necessary to remove the detergent IGEPAL CA-630 from the samples, as IGEPAL CA-630 polymer traces suppress peptide signals in LC–MS/MS analyses.

On-bead protein digestion ● TIMING ~1 d
19| Centrifuge the beads again at 1,500*g* for 2 min at 4 °C and use a 30-gauge syringe to remove all remaining PBS.

20| Add 100 µl of elution buffer to each tube and incubate for 20 min at 25 °C in a thermoshaker at 1,400 r.p.m.
▲ CRITICAL STEP Avoid heating the elution buffer to >25 °C, as heating urea can cause peptide carbamylation.

21| Add 10 µl of 0.55 M iodoacetamide (final concentration: 0.05 M) to each tube and incubate for 10 min at 25 °C in a thermoshaker at 1,400 r.p.m. in the dark.
▲ CRITICAL STEP Make sure to keep the sample in the dark, as iodoacetamide is light-sensitive.
▲ CRITICAL STEP Use 2-chloroacetamide instead of iodoacetamide if subsequent ubiquitin linkage analysis by MS is planned, as iodoacetamide causes the production of 2-acetamidoacetamide adducts on lysine residues that mimic ubiquitination[16].

22| Add 2.5 µl of trypsin (100 ng/µl) to each tube and incubate for 2 h at 25 °C in a thermoshaker at 1,400 r.p.m. in the dark.

23| Centrifuge the sample at 1,500g for 2 min at room temperature and pipette the supernatant into a new 1.5-ml tube.
▲ **CRITICAL STEP** Avoid pipetting the beads into the new tube, as this may clog the C18 column during stage-tipping (see below).

24| Add 100 µl of elution buffer to each tube and incubate for 5 min at 25 °C in a thermoshaker at 1,400 r.p.m. in the dark.

25| Centrifuge the samples at 1,500g for 2 min at room temperature and pipette the supernatant into the 1.5-ml collection tube from Step 23.
▲ **CRITICAL STEP** Avoid pipetting the beads into the new tube, as this will clog the C18 column in the Stage-tipping section of the PROCEDURE.

26| Add 2 µl of trypsin (100 ng/µl) to each tube and incubate overnight (16–18 h) at 25 °C in a thermoshaker without shaking in the dark.

27| Add 10 µl of 10% (vol/vol) TFA to the sample to stop the digestion by acidification and, using pH paper, check the pH of the solution (pH should be ≤2). Centrifuge the samples at 1,500g for 2 min at room temperature.
■ **PAUSE POINT** The digested peptides can be stored at 4 °C for a few weeks or at −20 °C for a few months.

Stage-tipping ● **TIMING ~1 h**
▲ **CRITICAL** This section of the PROCEDURE describes all the necessary steps to desalt the peptides using the so-called StageTips. The protocol should be adapted according to Rappsilber et al.[26].
▲ **CRITICAL** Make sure that all liquid passes through the StageTip during each step.
28| Fold a C18 disk and use a blunt-end syringe needle to puncture the folded disk twice to pick up four plugs of C18 material. Push these into a 250-µl pipette tip using a rod.
▲ **CRITICAL STEP** Be careful in regard to the amount of force used to fix the plugs into the tip. Applying too much force will result in a tight StageTip through which liquid passes slowly.
■ **PAUSE POINT** StageTips can be stored in a pipette box at room temperature for months.

29| Use scissors to remove the lid of a 2-ml Eppendorf tube, the lid and bottom of a 0.5-ml Eppendorf tube and the body part of a 250-µl pipette tip. Assemble a spin adaptor by putting the tip into the 0.5-ml tube and placing this into the 2-ml tube.

30| Transfer the StageTip to the spin adaptor, add 50 µl of methanol to each StageTip and centrifuge at 1,500g for 5 min at room temperature.

31| Add 50 µl of buffer B to each StageTip and centrifuge at 1,500g for 5 min at room temperature.

32| Add 50 µl of buffer A to each StageTip and centrifuge at 1,500g for 5 min at room temperature. Repeat this step once and discard the flow-through that accumulated in the 2-ml Eppendorf tube.

33| Pipette the samples from Step 27 into the StageTips and centrifuge at 400g for 10 min at room temperature.

34| Add 50 µl of buffer A to each StageTip and centrifuge at 1,500g for 5 min at room temperature.
? TROUBLESHOOTING

35| Transfer the StageTips to a tip box and store them at 4 °C.
■ **PAUSE POINT** Peptides on StageTips can be stored at 4 °C for a few weeks or at −20 °C for a few months.

Peptide elution ● **TIMING ~1 h**
36| (Optional) If StageTips have been stored at 4 °C or −20 °C, rehydrate the samples by adding 30 µl of buffer A to each StageTip, and centrifuge at 1,500g for 5 min at room temperature.

37| Add 30 µl of buffer B to each StageTip and assemble a 10-ml air-filled Eppendorf Combitip on top of the StageTip. Slowly force buffer B through the C18 tips and collect the peptides in 8-strip PCR tubes.

38| Place the 8-strip PCR tubes with open lids into a 96-well plate and evaporate the liquid using a speed vacuum concentrator for 15 min at room temperature to a final volume of 5 µl.
▲ **CRITICAL STEP** Balance the concentrator with an identical 96-well plate with the same number of PCR tubes filled with water.
▲ **CRITICAL STEP** Avoid heating the eluted peptides, as this increases the oxidation of amino acid residues[27].

39| Pipette 10 µl of buffer A into each PCR tube and mix well. Close the lid and centrifuge the samples at maximum speed (248*g*) using the speed vacuum for 30 s at room temperature.
▲ **CRITICAL STEP** We recommend checking each tube after centrifugation to make sure that there is no air bubble at the bottom of the PCR tube.
■ **PAUSE POINT** Eluted peptides can be stored at −20 °C for a few months.

LC–MS/MS measurement ● **TIMING** ~6.5 d
▲ **CRITICAL** This section of the PROCEDURE describes all steps of the LC–MS/MS analysis. The measurement time for each sample is ~5 h, including injection time, actual measurement time and time of the washes between sets of replicates.
▲ **CRITICAL** We recommend calibrating the LC–MS/MS system before the measurement, and running a HeLa Protein Digest Standard to check the performance of the instrument.
40| Place the PCR tubes into the LC instrument.

41| Analyze 5 µl of each sample by LC–MS/MS. Use the settings described in the Equipment Setup.
■ **PAUSE POINT** The remaining sample can be stored for a few months at −20 °C.

Protein identification and quantification using MaxQuant ● **TIMING** ~8 h
42| Check the raw mass spectrometry files for obvious problems, such as strong polymer contamination, a lack of peptide signals or partial trypsin digestion.
? TROUBLESHOOTING

43| Run the MaxQuant analysis. Detailed information and protocols for MaxQuant can be found in previous work from Cox *et al.*[19] and Tyanova *et al.*[20]. The analysis time depends on the configuration of the analysis computer on which the analysis is performed. We recommend using one core for each raw file to speed up the analysis. Use the default configuration of MaxQuant, with a few changes as specified in the table below:

Parameter	Settings
Version	1.5.1.0 (or newer)
Number of threads	30 (one for each raw file, if possible)
Variable modifications	Acetyl (protein N-terminal)
	Oxidation (M)
Enzyme	Trypsin/P (trypsin cleaves at the carboxyl side of the amino acid lysine or arginine, also if a proline follows)
Max. missed cleavages	2
Label-free quantification	On (default setting)
FASTA files	Human FASTA file
Match between runs	Match time window: 2 min
Label-free quantification	IBAQ (intensity-based absolute quantification) on

そ

Differential enrichment analysis ● TIMING ~0.5 h

44| Start R and load the DEP package (installation information in the Equipment Setup section) by running the following command:

```
> library("DEP")
```

? TROUBLESHOOTING

45| For the analysis, the MaxQuant output table 'proteingroups.txt' and an experimental design file (tab-delimited .txt file) are needed, which should contain the columns and content as described in the table below:

Column	Content	Examples
label	Identifiers for the different samples. These correspond to the 'Experiment' names in the MaxQuant analysis	diubiquitin_K11_1, diubiquitin_K11_2, diubiquitin_K11_3, diubiquitin_K27_1 and so on
condition	The condition to which the sample belongs	*K11, K27, K29* and so on
replicate	The replicate number to which the sample belongs	*1, 2, 3* and so on

46| Identify differentially enriched proteins, i.e., selective ubiquitin interactors, by using an interactive application (option A), by using the R command line with a single wrapper function (option B) or by using the R command line with detailed step-by-step functions (option C).

(A) Analysis of the data using the interactive application

(i) Start the application by running the following command:

```
> run_app("LFQ")
```

? TROUBLESHOOTING

(ii) Select the tab 'Files' (upper-left corner) and load the MaxQuant output table 'proteingroups.txt' in the designated box.

(iii) Load the experimental design of the experiment in the designated box (upper-left corner).

(iv) Select the 'Columns' tab and choose the columns that depict the 'Gene name' and 'Protein ID', select the proper columns for filtering (default 'Reverse' and 'Potential contaminant' columns) and select the sample name that corresponds to the control sample.

(v) Change the parameters for imputation methods in the 'imputation options' tab, if needed.

(vi) Run the analysis by clicking 'Analyze'. The resulting tables and figures are presented in the right panel.

? TROUBLESHOOTING

(vii) Adjust the rejection thresholds, i.e., cutoffs to call a protein differentially enriched, by changing the adjusted *P* value and $\log_2$ fold change thresholds (top) if needed.

▲ **CRITICAL STEP** The *P* value threshold is generally expected to be ≤0.1 and the $\log_2$ fold change threshold can be ≥0. These thresholds should always be reported. The analysis workflow is based on the assumption that the majority of proteins are not differentially enriched. Therefore, the fraction of differentially enriched proteins should not exceed 10–15% of the quantified proteins.

(viii) Specify the contrasts, i.e., the direct sample comparisons, for which to test differential enrichment. These can be specified to be all possible comparisons or only the comparisons versus the control samples ('all' or 'control', respectively, in the 'Contrasts' options in the upper-right corner).

(ix) Determine data scaling for visualization purposes in the 'Data presentation' box at the top. Use 'contrast' for visualization of direct ratios, and use 'centered' for the visualization of row-centered enrichment, i.e., sample enrichments that are subtracted by the mean protein enrichment over all baits.

(x) The data table contains all differentially enriched proteins. Select one of the rows to visualize the enrichment values for this protein in the 'Selected Protein' panel on the right.

(xi) Select the other tabs on the right for additional visualizations, including a heatmap depicting the enrichments of differentially enriched proteins for all baits (**Fig. 3**), and a volcano plot depicting the enrichment and *P* value for proteins in a single contrast (**Fig. 4**). Quality-control plots can be found in the lower-right corner, and these depict

the number of identified proteins per sample, the coverage of these proteins over different samples, the data normalization and pattern of missing values (**Supplementary Fig. 1**).

(xii) Select the appropriate table and click 'save' in the lower-left corner to save the results table. In addition, click the 'save' buttons directly below the figures to save all figures as PDF files.

(B) Analysis of the data using the R command line (wrapper function)

(i) Set your working directory to the folder containing your data.
? TROUBLESHOOTING

(ii) Load the MaxQuant output table 'proteingroups.txt' (if needed, change the file path) by running the following command:

```
> df <- read.csv(file =
        "proteingroups.txt",
header = TRUE, stringsAsFactors
        = FALSE, sep = "\t")
```

Alternatively, to load example data, run the following command:

```
> df <- DiUbi
```

(iii) Load the experimental design (if needed, change the file path) by running the following command:

```
> exp_design <- read.csv(file =
        "experimental_design.txt",
header = TRUE, stringsAsFactors
        = FALSE, sep = "\t")
```

Alternatively, to load example data, run the following command:

```
> exp_design <- DiUbi_ExpDesign
```

(iv) Using the LFQ function, run the complete workflow and specify the imputation function ('fun'), control sample name ('control') and type of contrasts to test ('type') by running the following command:

```
> results <- LFQ(proteins = df, expdesign = exp_design,
fun = "MinProb", control = "ctrl", type = "control")
```
? TROUBLESHOOTING

(v) Generate a report and results table by running the following command:
```
> report(results)
```

(vi) A new folder called 'Report' is generated in your current directory. This folder contains the results table ('results.txt') and an HTML and PDF report containing a summary of the analysis and different plots.

(C) Analysis of the data using the R command line and step-by-step functions

(i) Set your working directory to the folder containing your data and load the MaxQuant output table 'proteingroups.txt' and experimental design as in Step 46B(i–iii).
**? TROUBLESHOOTING**

(ii) Create unique names for all proteins after filtering your data for reverse hits and potential contaminants by running the following commands:
```
> filtered <- df[df$Reverse != "+" & df$Potential.contaminant != "+",]
> df_unique <- make_unique(filtered, "Gene.names", "Protein.IDs")
```

(iii) Create a SummarizedExperiment object based on the data and experimental design by running the following commands:
```
> columns <- grep("LFQ", colnames(df_unique))
> se <- make_se(df_unique, columns, exp_design)
```
? TROUBLESHOOTING
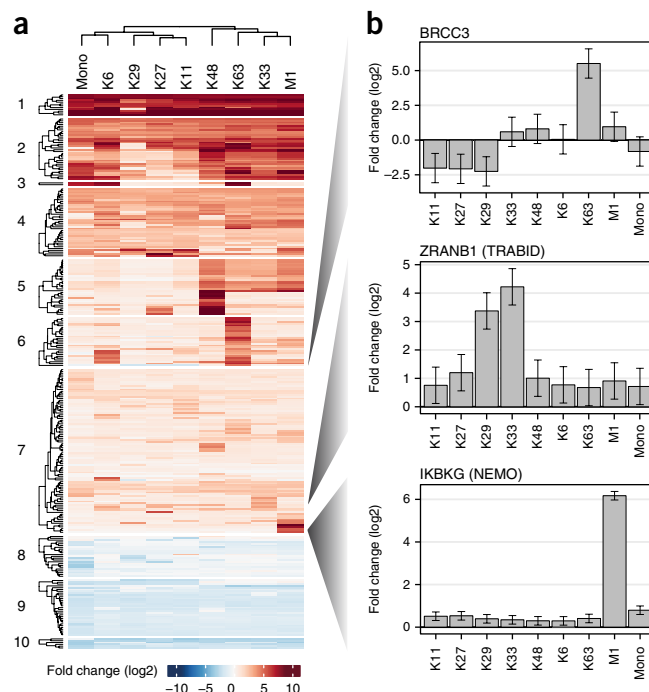
Figure 3 | Interactors of different diubiquitin linkages in HeLa cells. (**a**) Heatmap of ubiquitin interactors of different diubiquitin linkages. The rows represent different proteins and are clustered in ten groups by *k*-means clustering (numbers on the left). The columns depict the different diubiquitin linkages as compared with control. Rows and columns are hierarchically clustered on Euclidean distance. Colors represent enrichment in the diubiquitin linkage versus control (red: enriched; blue: depleted). (**b**) Bar plots depicting the enrichment of three known linkage-selective ubiquitin interactors by different diubiquitin linkages. Error bars represent the 95% confidence intervals.

(iv) Filter on missing values to retain proteins that were quantified in all replicates of at least one condition by running the following command:
```
> se_filt <- filter_missval(se,
thr = 0)
```
(v) The number of proteins left after these filtering steps can be visualized using the following functions (**Supplementary Fig. 1**):
```
> plot_numbers(se_filt)
> plot_frequency(se_filt)
> plot_coverage(se_filt)
```
(vi) Normalize the data using vsn[22] by running the following command:
```
> se_norm <- normalize_vsn(se_filt)
```
(vii) The effect of vsn normalization can be visualized using the following function (**Supplementary Fig. 1**):
```
> plot_normalization(se_filt,
       se_norm)
```
(viii) Before imputation of the remaining missing values, visualize the pattern of missing values by running the following commands (**Supplementary Fig. 1**):
```
> plot_missval(se_norm)
> plot_detect(se_norm)
```

Figure 4 | K48 diubiquitin interactors. (**a**) Volcano plot of the K48 diubiquitin pull-down as compared with the control. P values ($-\log_{10}$) are plotted against the fold changes ($\log_2$). Black points represent the differentially enriched proteins. (**b**) Bar plots of RAD23A visualizing its enrichment in different diubiquitin pull-downs. The fold changes for all baits are depicted as centered log2 intensities (top panel) or as log2 ratios of direct comparisons to the control (bottom panel). Error bars represent the 95% confidence intervals.

(ix) In proteomics data, the missing values are likely to be missing because they were below or around the detection limit (as depicted in the last plot). Therefore, values are imputed that are close to the detection limit in the current experiment, i.e., left-censored imputation. To do this, run the following commands:
```
> se_imp <- impute(se_norm, fun = "MinProb", q = 0.01)
> plot_imputation(se_norm, se_imp)
```
(x) Use protein-wise linear models and empirical Bayes statistics to test for differentially enriched proteins by running the following command:
```
> se_diff <- test_diff(se_imp, control = "ctrl", type = "control")
```
? TROUBLESHOOTING
(xi) Classify proteins as differentially enriched proteins by user-defined cutoffs (alpha and lfc are the adjusted P value and the $\log_2$ fold change cutoffs, respectively) by running the following command:
```
> dep <- add_rejections(se_diff, alpha = 0.05, lfc = log2(1.5))
```
(xii) Generate a results table that is easy to read and export by running the following command:
```
> results_table <- get_results(dep)
```
(xiii) Visualize the differentially enriched proteins by running one of the following functions.
A heatmap depicting all differentially enriched proteins (**Fig. 3a**):
```
> plot_heatmap(dep, type = "contrast", kmeans = TRUE,
k = 10, col_limit = 8, row_font_size = 3)
```
A volcano plot depicting the enrichment and P value for proteins in a single contrast (**Fig. 4a**):
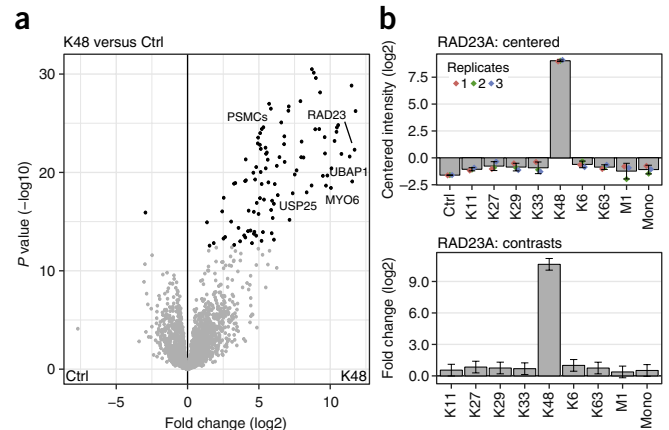```
> plot_volcano(dep, contrast = "K48_vs_ctrl")
```
A bar plot depicting the enrichment values of a single protein (**Figs. 3b** and **4b**):
```
> plot_single(dep, protein = "ZRANB1")
```
**? TROUBLESHOOTING**
(xiv) Export the results table to a tab-delimited text file by running the following command:
```
> write.table(results_table, "Results.txt", sep = "\t", row.names = FALSE)
```

**47|** The results table, which is obtained in the last substep of Step 46, contains all proteins that were reproducibly quantified. Inspect the quantified proteins together with their adjusted $P$ values and ratio/enrichment values. The columns and content present in the results table are described in the table below:

| Column | Description |
|---|---|
| name | Unique names for every protein |
| ID | The Uniprot identifier |
| [contrast]_p.adj | The adjusted $P$ values for this particular contrast |
| [contrast]_significant | Indicates whether the protein is differentially enriched in this particular contrast, as defined by the chosen cutoffs |
| significant | Indicates whether the protein is differentially enriched in any of the contrasts, as defined by the chosen cutoffs |
| [contrast]_ratio | The average log2 fold change in this particular contrast |
| [bait]_centered | The average log2 enrichment values per bait, scaled by protein-wise centering |

## ? TROUBLESHOOTING

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1 |** Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| **Box 1**, step 2 | The biotin-coupling mixture does not dissolve | Reagents may have been added in the wrong order | First dissolve biotin in NMP and add this solution to the HBTU/HOBT solution. If there is still some pre-cipitation, briefly sonicate the reaction mixture |
| **Box 1**, step 13 | Little or no precipitate is seen | The diethyl ether/pentane mixture is not cold enough | Put the diethyl ether/pentane mixture into a −20 °C freezer for 15 min before use |
| **Box 1**, step 18 | Ubiquitin does not dissolve in the amount of DMSO used | Ubiquitin solution is too concen-trated | Heat the DMSO in a water bath or by briefly using a heat gun. If this does not work, add more DMSO and repeat the heating steps. Alternatively, try placing it in an ultrasonic bath for ~5 min |
| **Box 1**, step 19 | The ubiquitin precipitates | Ubiquitin was added too quickly to the solution | Add Ub-DMSO solution to Milli-Q water and not vice versa. Add buffer slowly |
| **Box 2**, step 7 | A gel forms and/or the solu-tion does not become clear | The ubiquitin is too concentrated and does not dissolve | Add more DMSO and heat the Eppendorf tube contain-ing the sample in a heating block at 95 °C or briefly warm using a heat gun |
| **Box 2**, step 8 | A precipitate is seen | The ubiquitin is added too quickly to the 8 M urea solution | Add a few microliters of ubiquitin–DMSO solution at a time and vortex the solution regularly between addi-tions |
| **Box 2**, step 10 | The click reaction does not work | Cu$^+$ is oxidized | Make sure that all solutions are freshly prepared and are degassed. Make sure that the starting material is of high purity |
| | | The concentration of reactants is not high enough, or too little of one of the reactants was added | Add more Ub$_{76}$-N$_3$: 0.5–1 equiv., depending on how far the reaction has proceeded |
| **Box 2**, step 15 | The ubiquitin precipitates | Ubiquitin was added too quickly to the solution | Add Ub-DMSO solution to gel filtration buffer and not vice versa. Vortex regularly to ensure proper mixing |
| 7 | Protein yield is lower than expected | Inefficient cell detachment | Check by microscopy to determine if the cells are detached from the dishes after trypsinization |

(continued)

**TABLE 1 |** Troubleshooting table (continued).

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 7 | | Inefficient cell lysis | In Step 6, vortex longer (up to 1 min) to completely resuspend the cell pellet |
| 12 | No depletion of ubiquitin chains | No biotin-tag at the N terminus of the distal ubiquitin | Prepare the ubiquitin chains again |
| | | Old stock of streptavidin beads | Buy new streptavidin beads |
| 30–34 | Liquid does not pass, or passes very slowly through the StageTip | Peptide samples contain streptavidin beads | Make sure to avoid pipetting any streptavidin beads in Step 33 |
| | | C18 plugs were inserted using too much force | Make new StageTips but use less force |
| 42 | No or weak ion signals | Wrong buffer A or buffer B | Make new buffer A and buffer B |
| | Too much polymer signal | Use of autoclaved tips and bottles | Use Rainin tips for each step, and make new buffers in Duran bottles. Do not autoclave buffers |
| | | IGEPAL CA-630 is not washed out well | Make sure to wash the beads twice with PBS at Step 18, and remove the remainder completely at Step 19 |
| | Too many large peptides, trypsinization is not efficient | Not enough or old trypsin | Make sure to add the recommended amount of trypsin. Obtain new trypsin |
| | | pH of elution buffer is not suitable for trypsin | Make sure that the pH value of the 2 M urea elution buffer is between 8 and 8.5 |
| | | DTT is old | Use fresh DTT that has not undergone freeze–thaw cycles |
| | | IAA (iodoacetamide) or CAA (2-choroacetamide) is old | Make new IAA and CAA |
| | | Alkylation is not performed in the dark | Cover the thermoshaker with aluminum paper after adding IAA or CAA |
| | | Digestion temperature is not correct | Make sure that the whole digestion procedure is performed at 25 °C |
| 44 | Errors while loading different Bioconductor packages | Incorrect R or package versions were used | Download and install the most recent version of R and/or reinstall Bioconductor packages |
| 46A(i, vi), 46B(i, iv), 46C(i, iii) | Error in the data-analysis workflow | Incorrect R or package versions were used | Download and install the most recent version of R and/or reinstall Bioconductor packages |
| | | The data and/or experimental design files do not contain the required columns | Check whether the MaxQuant output table contains 'LFQ intensity' columns and whether the right gene names, protein IDs and columns to filter on were annotated. Check whether the column names of the experimental design match the required names as depicted in Step 45 |
| 46C(x) | Error in the linear model analysis | Incorrect control name | Check for the exact control condition in the experimental design |
| 46C(xiii) | Error during data visualization | Incorrect contrast (for volcano plot) or protein (for single-protein plot) names | Check the exact contrasts and protein names in the error statement or in the results table |

(continued)

**TABLE 1 |** Troubleshooting table (continued).

| Step | Problem | Possible reason | Solution |
|------|---------|-----------------|----------|
| 47 | Too much keratin contamination | Keratin comes from the experimenter | Wear gloves throughout the protocol |
| | Too many interactors for empty streptavidin beads | Beads are not washed well in Step 17 | Make sure that the wash buffer contains 1% (vol/vol) IGEPAL CA-630, and the beads are resuspended well in each wash step |
| | Wrong interactions between ubiquitin chains and UBPs | Samples are swapped | Make sure to clearly label the tubes at each step |
| | Fewer linkage-selective interactors than expected | Too much IGEPAL CA-630 in lysis or wash buffer | Make sure that the lysis and wash buffers contain the recommended concentration of IGEPAL CA-630 |
| | | Cell lysis is not complete | Make sure that the cell lysis is efficient by checking the concentration of cell lysates. It should be ~10 mg/ml |

● **TIMING**
Steps 1–7, preparation of whole-cell extracts: ~4 h
Steps 8–13, coupling of ubiquitin chains to streptavidin beads: ~5 h
Steps 14–18, capture of ubiquitin interactors: ~20 h
Steps 19–27, on-bead digestion: ~1 d
Steps 28–35, stage-tipping: ~1 h
Steps 36–39, peptide elution: ~1 h
Steps 40 and 41, LC–MS/MS measurement: ~6.5 d
Steps 42 and 43, protein identification and quantification using MaxQuant: ~8 h
Steps 44–47, differential enrichment analysis using R: ~0.5 h
**Box 1**, production of alkyne- (distal) and azide (proximal)-functionalized ubiquitin: ~16.5 d
**Box 2**, nonhydrolyzable diubiquitin synthesis: ~3.5 d

**ANTICIPATED RESULTS**
UbIA-MS typically results in the identification of dozens to hundreds of ubiquitin interactors. Here, we exemplify the protocol by identifying interactors for diubiquitin linkages in HeLa whole-cell lysates. In this experiment, ~240 proteins were enriched in ubiquitin pull-downs over controls (**Fig. 3a**). These include many known linkage-selective UBPs. For example, BRCA1-A complex member BRCC3 binds selectively to K63 linkages, TRABID binds to K29 and K33 linkages, and NEMO selectively interacts with methionine 1 (M1) linkages (**Fig. 3b**). The interactors of the well-studied K48 linkage are also recapitulated in our screen (**Fig. 4a**). These interactors include components of the proteasome (PSMCs), the K48- and K63-selective deubiquitinase USP25 and UV excision repair protein RAD23 (**Fig. 4b**).

>50 ubiquitin interactors bind to each bait in the HeLa data set (**Fig. 5**). The monoubiquitin bait enables identification of general UBPs, and the exclusion of monoubiquitin interactions results in the identification
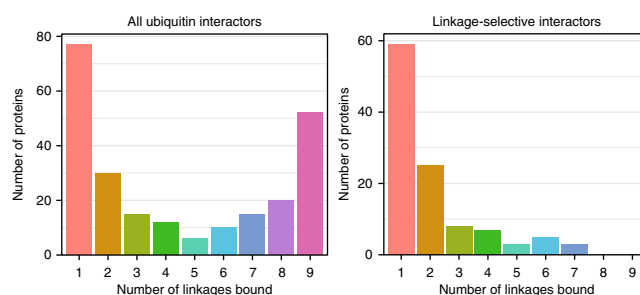


**Figure 5 |** Distribution of the number of diubiquitin linkages bound by each interactor. Histograms of the number of interactors that bind to a certain number of linkages for all ubiquitin interactors (left) and all linkage-selective interactors (right).

of ~110 linkage-selective interactors (**Supplementary Fig. 2**). Of these 110 interactors, >80 proteins recognize only one or two ubiquitin-linkages (**Fig. 5**).

We have previously shown that cell-type-selective ubiquitin interactors can be identified using UbIA-MS in mouse embryonic stem cells and neuronal precursor cells[5]. In addition, our previous work revealed DNA damage–induced ubiquitin interactions. These examples indicate that the ubiquitin interactome is dynamic, and that context-specific ubiquitin interactions are to be anticipated in future experiments.

**AUTHOR CONTRIBUTIONS** X.Z. and M.V. designed the method with input from A.H.S., G.B.A.v.T., W.H. and H.O. A.H.S. prepared figures, and developed and maintained the R package. X.Z., A.H.S., G.B.A.v.T. and M.V. wrote the manuscript with input from H.O. and W.H.

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