

Life Sciences Reporting Summary

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For further information on the points included in this form, see [Reporting Life Sciences Research](#). For further information on Nature Research policies, including our [data availability policy](#), see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

► Experimental design

1. Sample size

Describe how sample size was determined.

A minimum of 3 independent biological replicates were performed for each experimental condition, which provides the sufficient grounds for the subsequent statistical significance calculations.

2. Data exclusions

Describe any data exclusions.

Mass spectrometric raw data were searched against a target/decoy (forward and reversed) version of the human Uniprot database at a false discovery rate (FDR) of 1% for peptide, protein and site identifications. All peptide matches to the reversed database were removed, as well as peptides with Andromeda scores of less than 25. All MS/MS spectra for potential protein N-terminal ubiquitinations were manually inspected and ambiguous identifications removed.

3. Replication

Describe whether the experimental findings were reliably reproduced.

All large-scale experiments were performed in biological triplicates as a minimum. Number of replicates for each experiment is clearly stated in the manuscript. 3. The validating experiments with the 10 N-terminally ubiquitinated candidate proteins were performed at least twice with similar results for each of the 10 proteins.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

There was no randomization for this in vitro study.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

There was no blinding for this in vitro study.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- ☐ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ A statement indicating how many times each experiment was replicated
- ☐ ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- ☐ ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- ☐ ☒ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- ☐ ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- ☐ ☒ Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

Spectronaut Pulsar v11.0.15038.15.23605 was used for the DIA analyses. For the rest of the analyses, MaxQuant (versions 1.5.3.17 & 1.5.5.31), DAVID (v 6.8), IceLogo (v 1.2), GProX (v 1.1.12), Cytoscape (v 3.4), R (versions 3.1.3 & 3.4.2) and Perseus (versions 1.5.5.3 & 1.5.5.6) software were used, as described in the Methods section of the manuscript.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

University of Southern Denmark has signed agreements with Millipore and LifeSensors for distribution of the UbiSite antibody.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

We generated a novel antibody, namely UbiSite (mouse monoclonal, isotype IgG1k, clone number 2G7B8), for enrichment of ubiquitinated peptides from complex biological samples.

Additional antibodies used in the study:

Anti-Ubiquitin mouse monoclonal antibody; Supplier: SANTA CRUZ

BIOTECHNOLOGY, INC; catalog number: sc-8017; clone name: P4D1; lot number: I2413; dilution: 1:1000; Validated: <https://datasheets.scbt.com/sc-8017.pdf>

Anti-Ubiquitin mouse monoclonal antibody; Supplier: LifeSensors; catalog number: VU101; clone name: VU-1; lot number: AB-40627.004; dilution: 1:1000; Validated: https://lifesensors.com/wp-content/uploads/2017/02/VU101_VU-1Antibody_datasheet_032113.pdf

Anti-HA rabbit polyclonal antibody; Supplier: Sigma-Aldrich; catalog number: H6908; clone name: polyclonal; lot number: 077M4854V; dilution: 1:1000; Validated: <https://www.sigmaaldrich.com/catalog/product/sigma/h6908?lang=en®ion=DK>

Anti-Flag mouse monoclonal antibody; Supplier: Sigma-Aldrich; catalog number: F1804; clone name: M2; lot number: SLBT7654; dilution: 1:1000; Validated: <https://www.sigmaaldrich.com/catalog/product/sigma/f1804?lang=en®ion=DK>

Amersham ECL Mouse IgG, HRP-linked whole Ab (from sheep); Supplier: GE Healthcare Life Sciences; catalog number: NA931V; clone name: NA; lot number: 9761190; dilution: 1:4000; <https://www.gelifesciences.com/en/dk/shop/protein-analysis/blotting-and-detection/blotting-standards-and-reagents/amersham-ecl-hrp-conjugated-antibodies-p-06260>

Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey); Supplier: GE Healthcare Life Sciences; catalog number: NA934V; clone name: NA; lot number: 9720457; dilution: 1:4000; <https://www.gelifesciences.com/en/dk/shop/protein-analysis/blotting-and-detection/blotting-standards-and-reagents/amersham-ecl-hrp-conjugated-antibodies-p-06260>

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Hep2 cell line was a kind gift from Dr. van Deurs (Copenhagen University). Jurkat and HEK293T cell lines was obtained from ATCC (the American Type Culture Collection).

b. Describe the method of cell line authentication used.

None of the cell lines have been authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

The three cell lines tested negative for mycoplasma contamination. Tests were performed in-house.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

Hep2 cells have been described in some instances to be contaminated with HeLa cells. However, our study did not aim at providing tissue/origin-specific research but rather to demonstrate the utility of the UbiSite antibody/approach for in-depth profiling and comparison of ubiquitinomes of any cells or tissues. Any cell lines could have been used for this purpose, irrespective of their origin or identities. We therefore chose two commonly used but distinct human cell lines: one adherent cell line, Hep2, and one suspension cell line, Jurkat.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used in this study.

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.