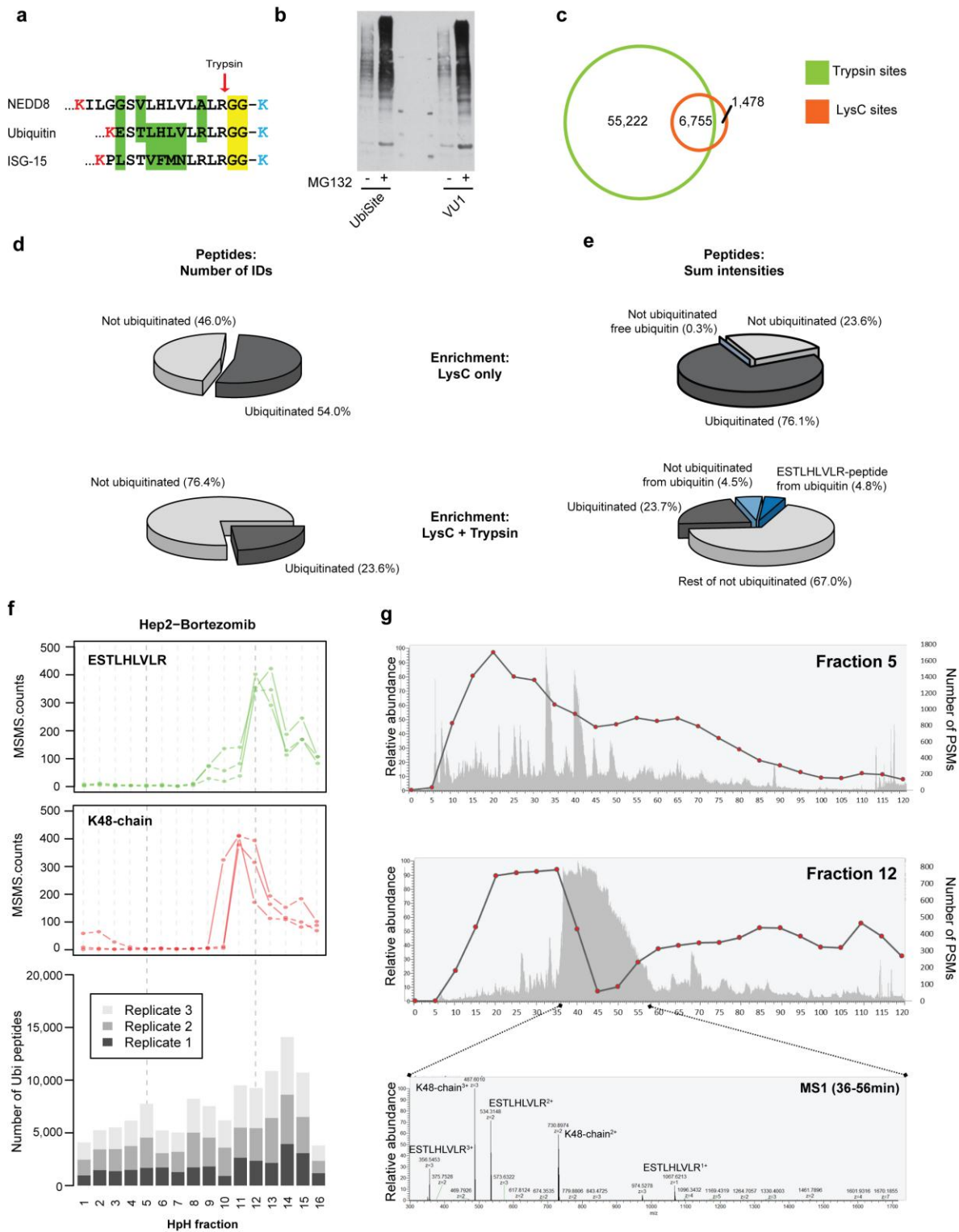


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UbiSite approach for comprehensive mapping of lysine and N-terminal ubiquitination sites

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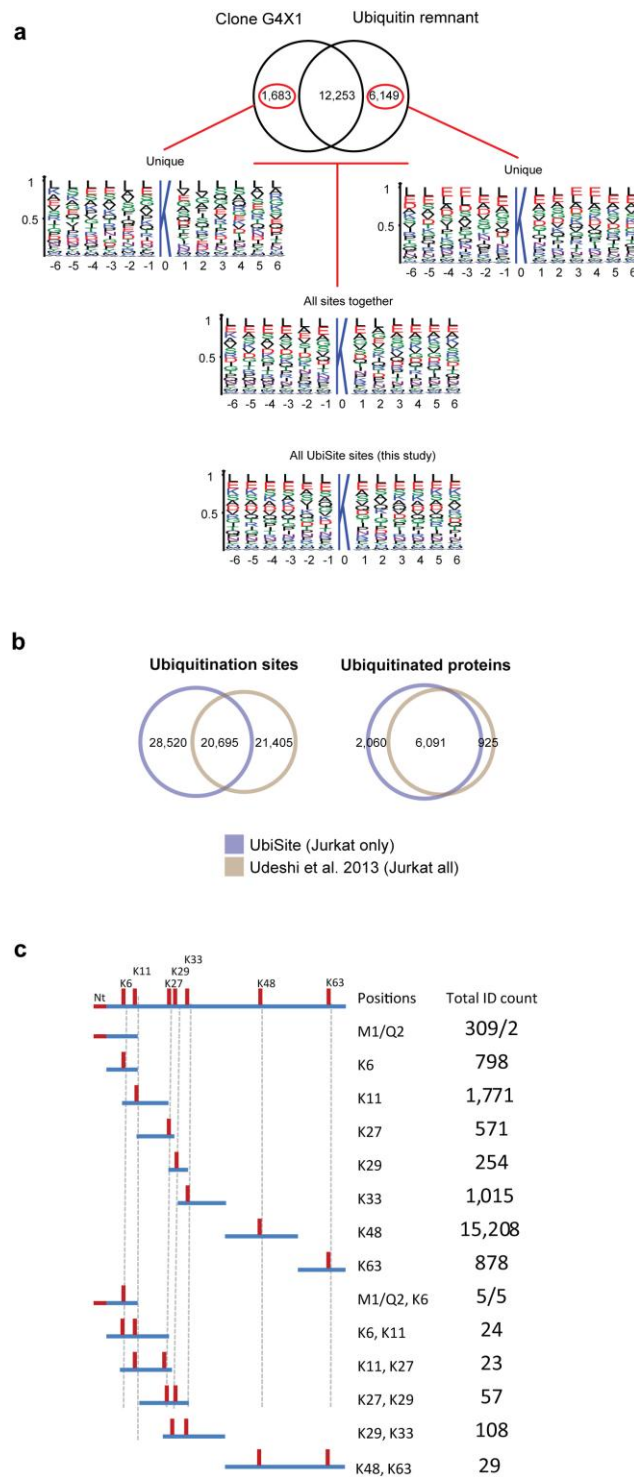


Supplementary Figure 1

Enrichment of ubiquitinated peptides by UbiSite.

a, Sequence alignment of remnants after LysC digestion of NEDD8, ubiquitin and ISG-15. **b**, Comparison of UbiSite and VU1 antibodies in recognizing ubiquitinated species in Hep2 cells treated with vehicle (DMSO) or proteasomal inhibitor (5 μ M, 8 h) by western blotting. Shown is a representative of three independent experiments. **c**, Overlap between unique ubiquitination sites in

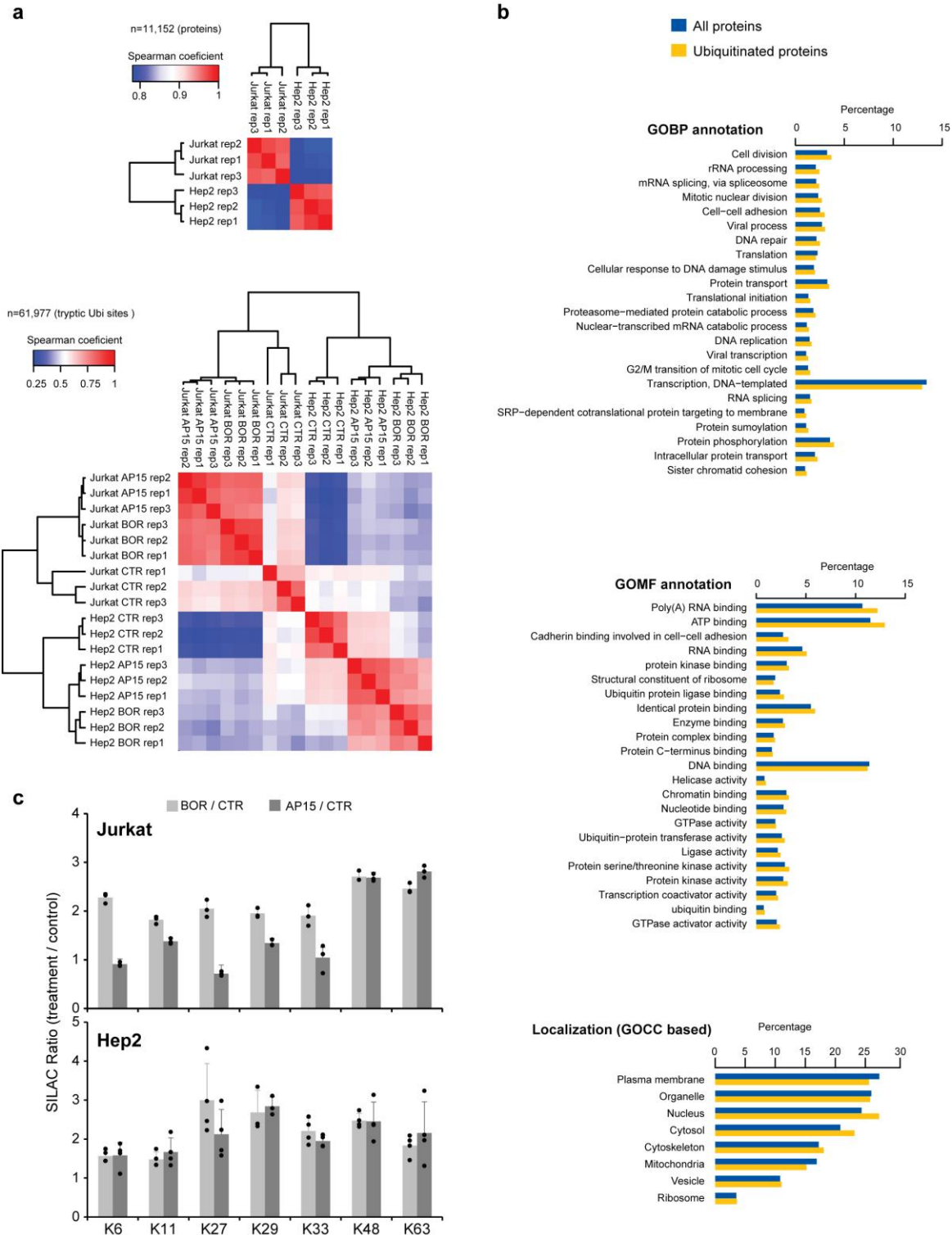
samples digested with LysC only or trypsin plus LysC. **d**, Proportions of identified ubiquitinated and non-ubiquitinated peptides in samples treated with LysC only or LysC followed by trypsin. **e**, Proportions of the summed peptide intensities in samples treated with LysC only or LysC followed by trypsin. **f**, Elution profiles of ESTLHLVLR and K48 ubiquitin chain peptides over all offline high-pH (HpH) fractions summarized for the three replicates of Hep2 cells treated with bortezomib. The bottom panel shows the number of identified ubiquitinated peptides within each fraction and replicate for the same cells and treatment. **g**, Drop in the number of peptide identifications during elution of the highly abundant ESTLHLVLR and K48 ubiquitin chain peptides. Top panels show the total ion chromatograms for HpH fractions 5 and 12, as indicated. Red dotted lines provide the number of peptide-to-spectrum matches (PSMs) throughout the LC gradient, binned in 5-min windows. The bottom panel shows the total MS1 spectrum for fraction 12 during the 20-min elution of the two abundant peptides.



Supplementary Figure 2

Ubiquitination sequence motifs and coverage of polyubiquitin chains.

a, Sequence motif analysis of ubiquitination sites from two different clones of the diGly-specific antibody (*Mol. Cell. Proteomics* **11**, 1578–1585, 2012) and UbiSite (lower panel). **b**, Overlap of diGly-lysine-modified sites and proteins from Jurkat cells identified in Udeshi et al. (*Mol. Cell. Proteomics* **12**, 825–831, 2013) with the ubiquitination sites and proteins identified in this study from the Jurkat cell line alone by the UbiSite approach. **c**, Ubiquitination sites found in ubiquitin and the number of times each peptide was identified with an Andromeda score higher than 25.

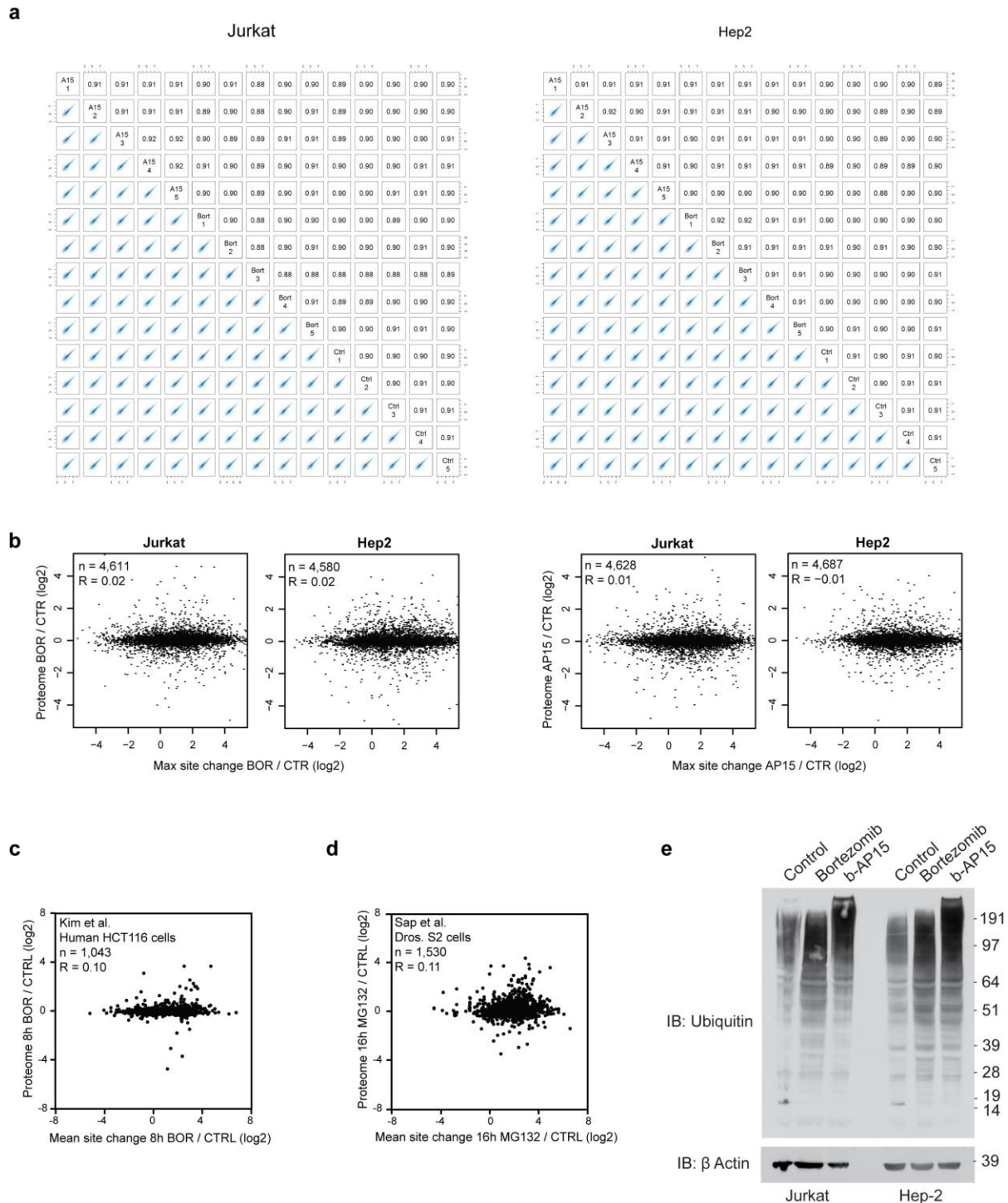


Supplementary Figure 3

Ubiquitination is distributed over the entire proteome.

a, Hierarchical clustering of pairwise Spearman's correlation coefficients between each replica and experiment. The results are plotted as heat maps for the total proteomes (top) and for all ubiquitination sites from the LysC followed by trypsin dataset (bottom). **b**, GO term biological processes (GOBP), molecular functions (GOMF) and cellular component (GOCC) enrichment of the total proteome and the UbiSite-enriched ubiquitinome. **c**, SILAC-based quantification of ubiquitin chain peptides in response to bortezomib (BOR) and b-AP15

(AP15) relative to control (CTR) for Jurkat and Hep2 cells based on three and four biologically independent experiments, respectively. Bars represent the corresponding SILAC ratios (mean + s.d.).

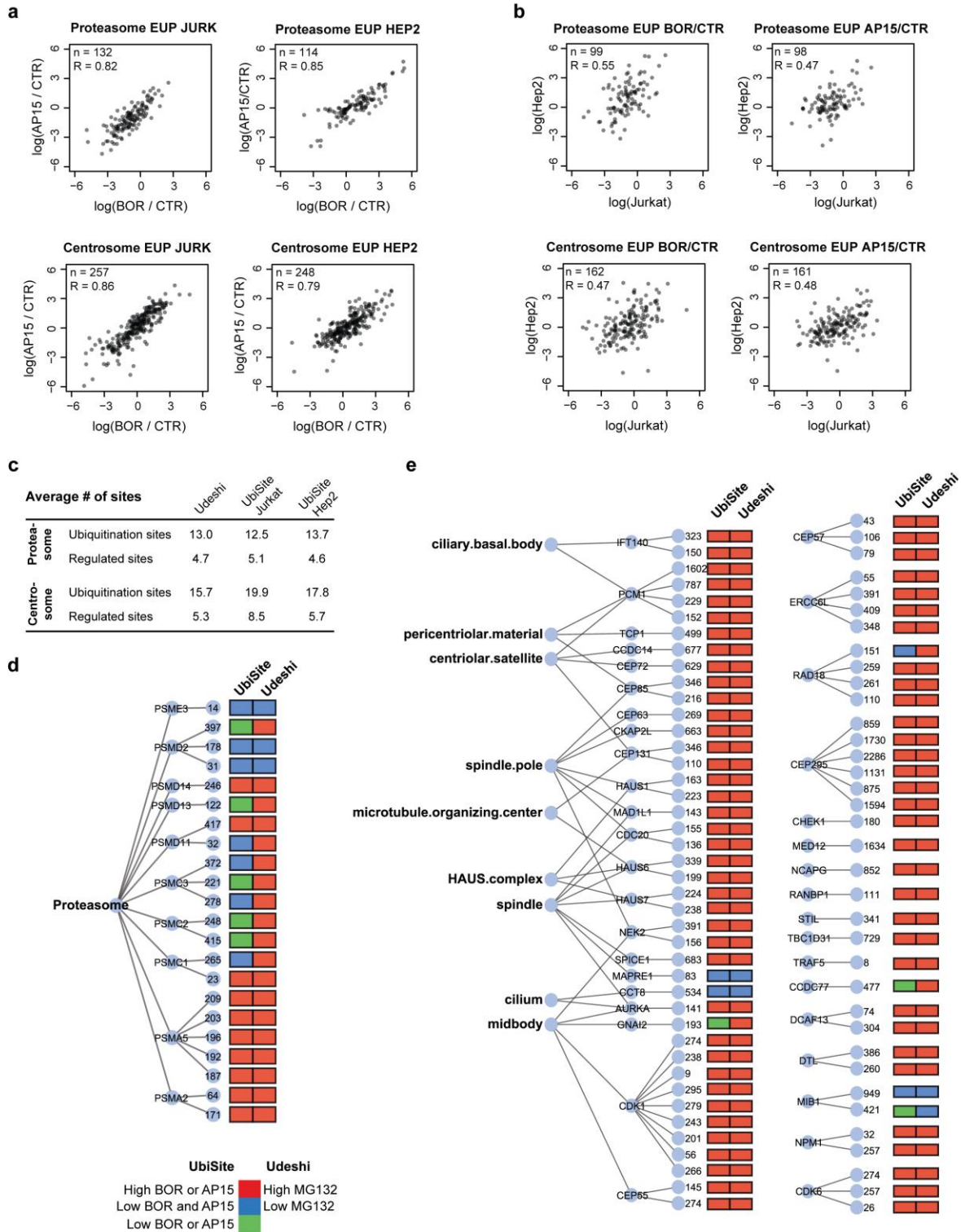


Supplementary Figure 4

Correlation analyses between protein abundance changes and the deviation in their ubiquitination sites in response to proteasomal inhibition.

a, DIA analyses of the total proteomes of bortezomib- (Bort), b-AP15- and mock-treated (Ctrl) Jurkat and Hep2 cells. Scatterplots showing correlations among the five independent replicates for each condition and cell line. Minimum and maximum sample sizes for Jurkat cells were 6,021 and 6,433 proteins, respectively. Minimum and maximum sample sizes for Hep2 cells were 6,120 and 6,527 proteins, respectively. Numbers in each box show the corresponding Pearson correlation coefficients. **b**, Scatterplots displaying the change in protein abundance versus protein's ubiquitination site with the biggest change in response to bortezomib (BOR) and b-AP15

(AP15) as compared to mock-treated cells (CTR). *R* refers to Pearson correlation. **c**, Scatterplot showing the change in protein abundance versus protein's mean ubiquitination site in response to bortezomib (BOR) (data extracted from *Mol. Cell* **44**, 325–340, 2011). *R* refers to Pearson correlation coefficient. **d**, Scatterplot displaying the change in protein abundance versus protein's mean ubiquitination site in response to MG132 (data extracted from *J. Proteome Res.* **16**, 2848–2862, 2017). *R* refers to Pearson correlation coefficient. **e**, Accumulation of high-molecular-weight protein–ubiquitin conjugates upon treatment of cells with proteasomal inhibitors detected by immunoblotting on the same cellular lysates that were used for the DIA measurements. Shown is a representative of three independent experiments.

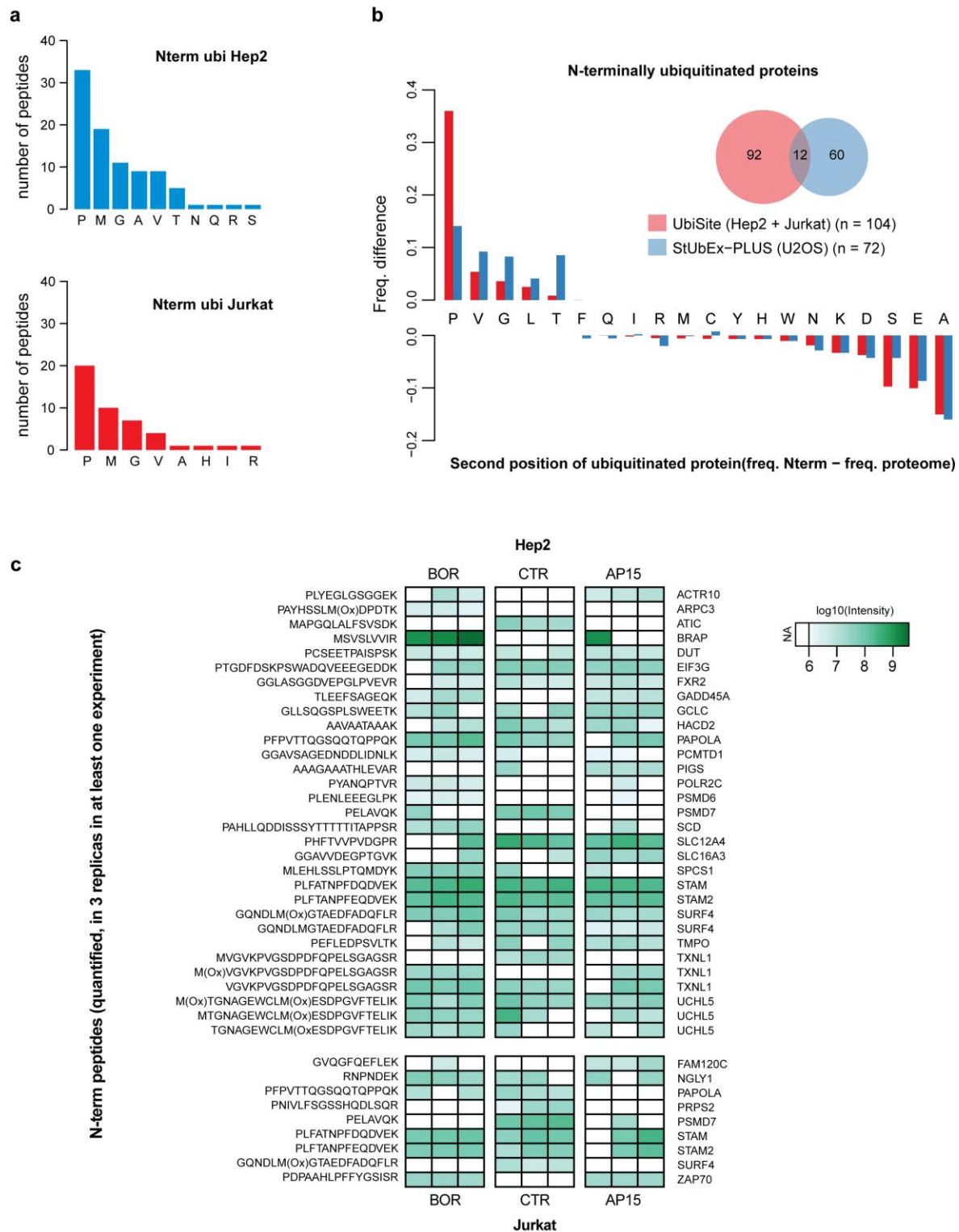


Supplementary Figure 5

Extensive ubiquitination of proteins from the non-membrane-bound organelle proteasome and centrosome.

a, Correlation of ubiquitination site changes on proteasomal and centrosomal proteins between bortezomib (BOR) and b-AP15 (AP15) treatment within the same cell line. R refers to Spearman correlation. **b**, Correlation of bortezomib- or b-AP15-induced changes on the proteasomal and centrosomal ubiquitination sites between the two cell lines. R refers to Spearman correlation. **c**, An average number of ubiquitination sites per protein on the EUPs from the proteasome and centrosome in the current UbiSite dataset and in the study by

Udeshi et al. (*Mol. Cell. Proteomics* **12**, 825–831, 2013). The average numbers of significantly regulated sites per protein in response to proteasomal inhibition are indicated as well. **d,e**, Comparison of the significantly regulated ubiquitination sites on proteasomal (**d**) and centrosomal (**e**) EUPs from the two studies.



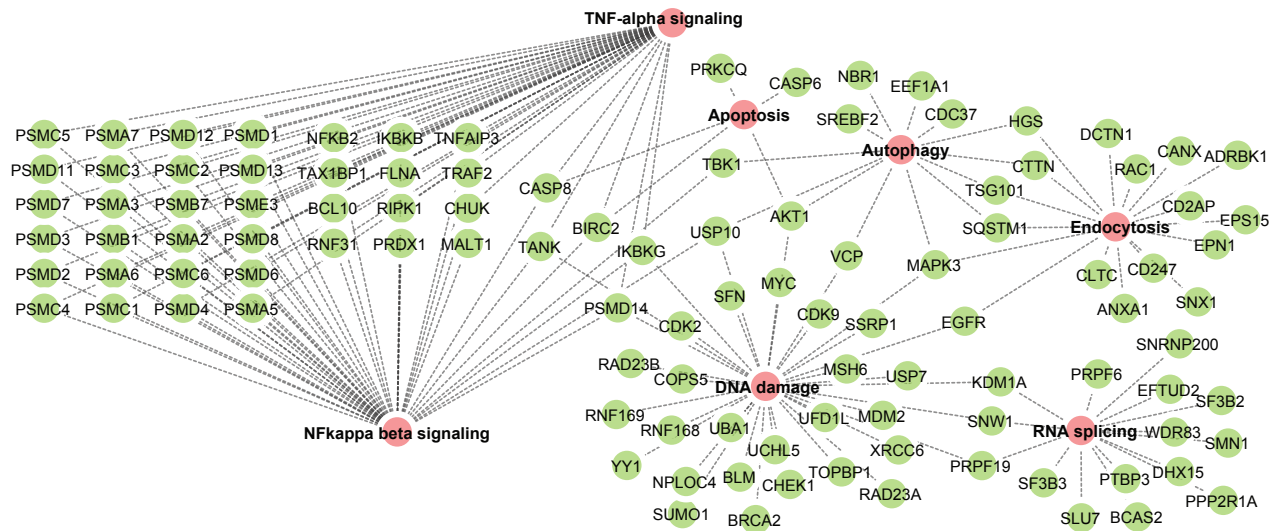
Supplementary Figure 6

N-terminal protein ubiquitination is not affected by proteasomal inhibition.

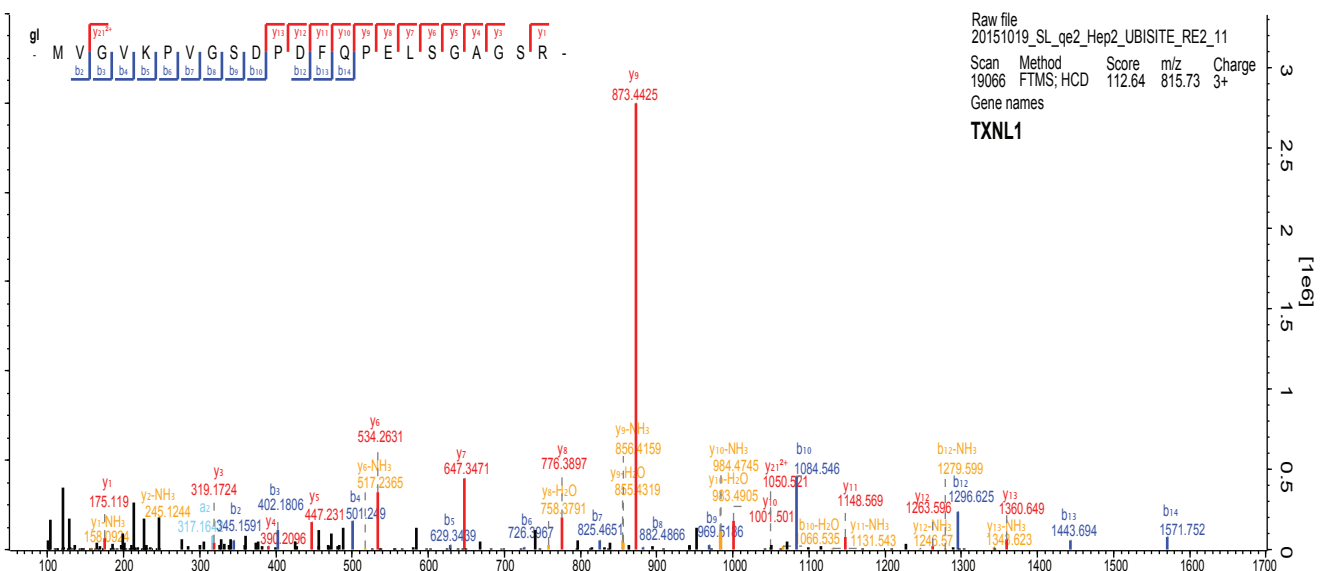
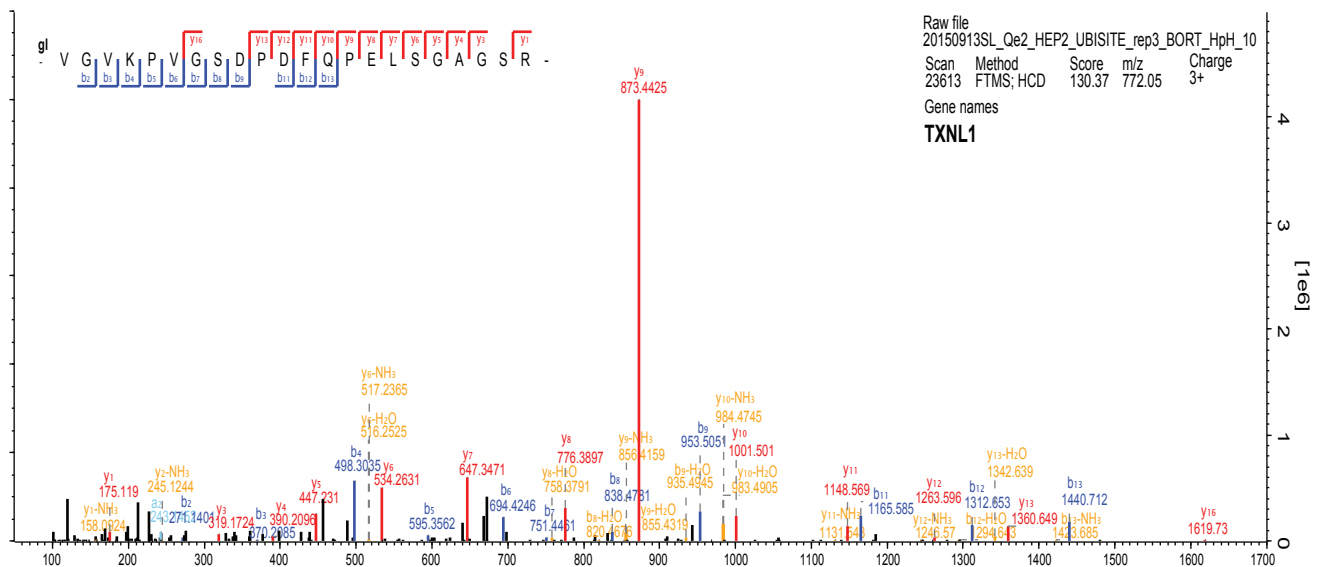
a. Prevalence of amino acids at the second position of N-terminally ubiquitinated proteins in Hep2 (top) and Jurkat (bottom) cells. **b.** Comparison of the N-terminally ubiquitinated proteins identified by the StUbEx PLUS approach in U2OS cells (blue) (data extracted from *J. Proteome Res.* **17**, 296, 2018) with those identified in the current UbiSite study (red). **c.** log₁₀ intensities of N-terminally

ubiquitinated peptides in untreated cells or upon proteasomal inhibition with bortezomib (BOR) or b-AP15 (AP15). Peptides quantified in all three replicates of at least one treatment are shown.

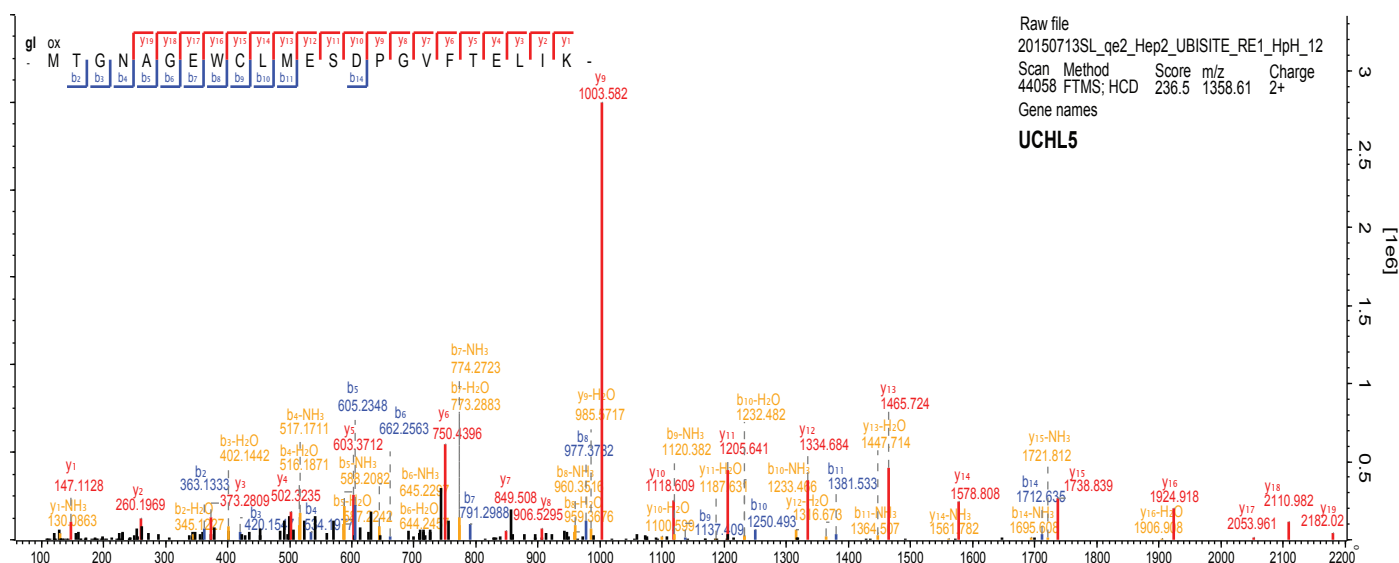
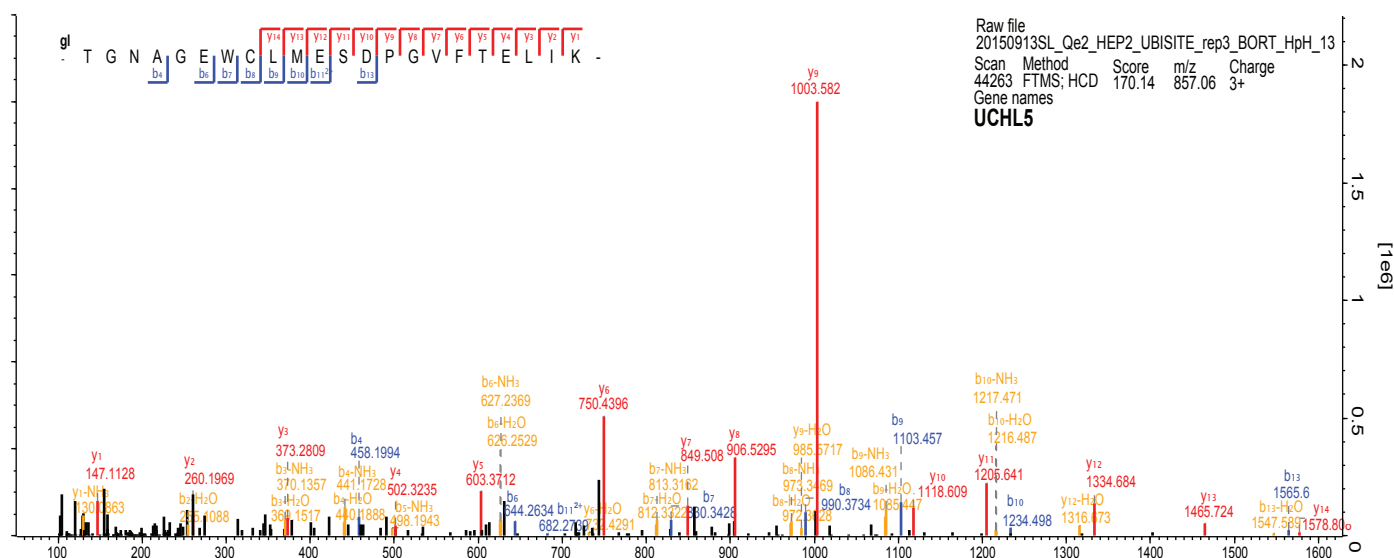
Supplementary Notes



Supplementary Note 1. Annotation of extensively ubiquitinated proteins (EUPs) interacting with Ubiquitin Binding Proteins. Biological processes (red dots) with highest prevalence of EUPs (green dots) known to interact with ubiquitin-binding proteins.



Supplementary Note 2a. Annotated fragment spectra of the peptides derived from the N-terminally ubiquitinated TXNL1. MS/MS spectra of the tryptic peptides derived from the N-terminally ubiquitinated TXNL1, without (top) or with (bottom) the initiating methionine being present; 'gl' indicates the di-Gly remnant of ubiquitin. Annotations of the spectra were done with MaxQuant Viewer (version 1.5.3.17).



Supplementary Note 2b. Annotated fragment spectra of the peptides derived from the N-terminally ubiquitinated UCHL5. MS/MS spectra of the tryptic peptides derived from the N-terminally ubiquitinated UCHL5, without (top) or with (bottom) the initiating methionine being present; 'gl' indicates the di-Gly remnant of ubiquitin. Annotations of the spectra were done with MaxQuant Viewer (version 1.5.3.17).