Supplemental Data:

Supplemental Figure S1: Schematic work flow of the algorithm used for data-independent acquisition (DIA).

Supplemental Figure S2: Relative proportion of fragment ions assigned to backbone sequence ions for different activation modes.

Supplemental Figure S3: Impact of the position of the modified lysine residue on the fragmentation of synthetic peptides with SUMO remnant chain.

Supplemental Figure S4: Evaluation of optimal ion storage conditions for data-independent acquisition (DIA).

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Supplemental Figure S6: MS/MS spectra of identified SUMO peptides using MS/MS edition tools (separate pdf file).

Supplemental Figure S7: Sequence motif surrounding modified Lys residues.

Supplemental Figure S8: Validation of SUMOylated Ubiquitin identification with synthetic peptide.

Supplemental Figure S9: Bioinformatics analyses for enriched GO terms representing SUMOylated proteins identified in this study.

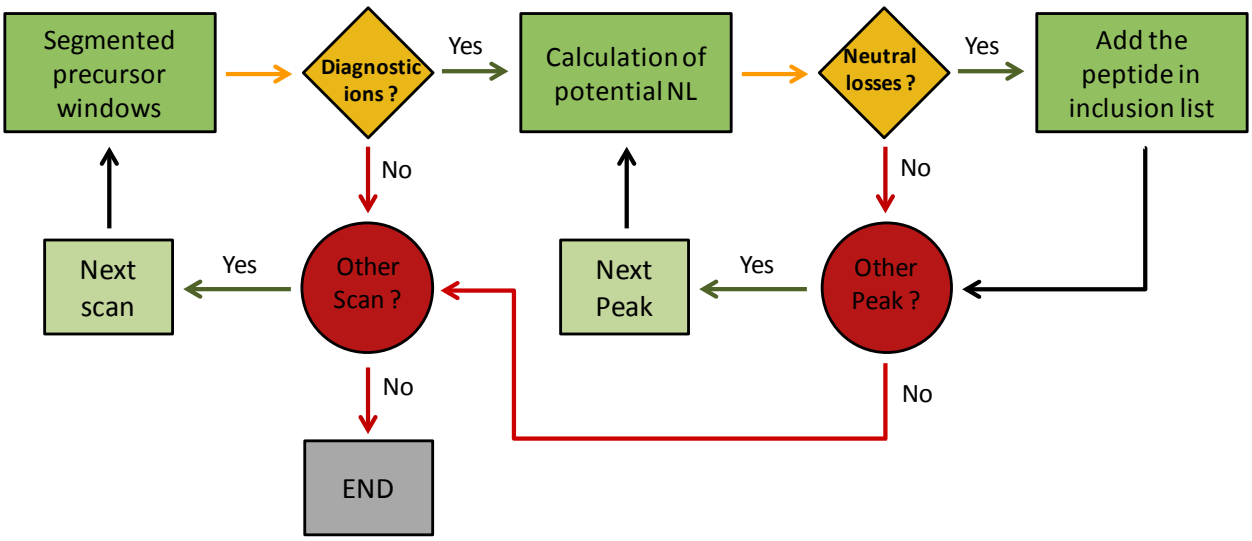
Supplemental Table S1: List of synthetic SUMO peptides identified using different activation modes (ETD, CID, HCD).

Supplemental Table S2: Characteristic fragment ions observed during HCD fragmentation and their respective distribution of intensities.

Supplemental Table S3: Distribution of scores for Mascot searches performed on HCD MS/MS spectra of synthetic peptides with and without removal of fragment ions associated with the SUMO remnant.

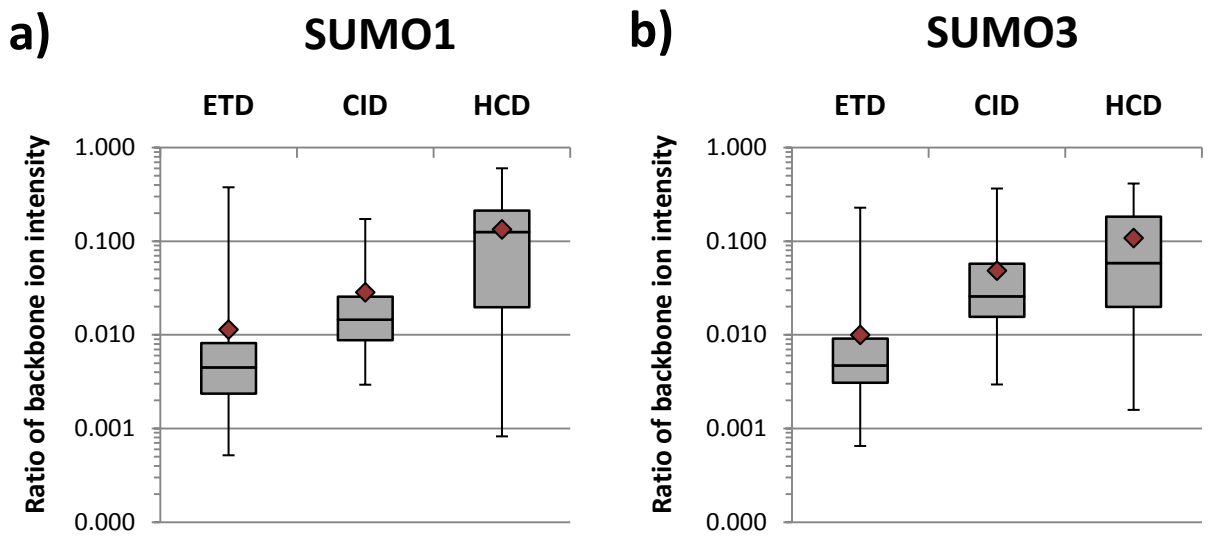
Supplemental Table S4: List of identified SUMO peptides from the LC-MS/MS analyses of tryptic digests from HEK293-SUMO3 cells.

Supplemental Table S5: Distribution of scores for Mascot searches performed on HCD MS/MS spectra of SUMO3 peptides from HEK293-SUMO3 cells with and without removal of fragment ions associated with the SUMO remnant.

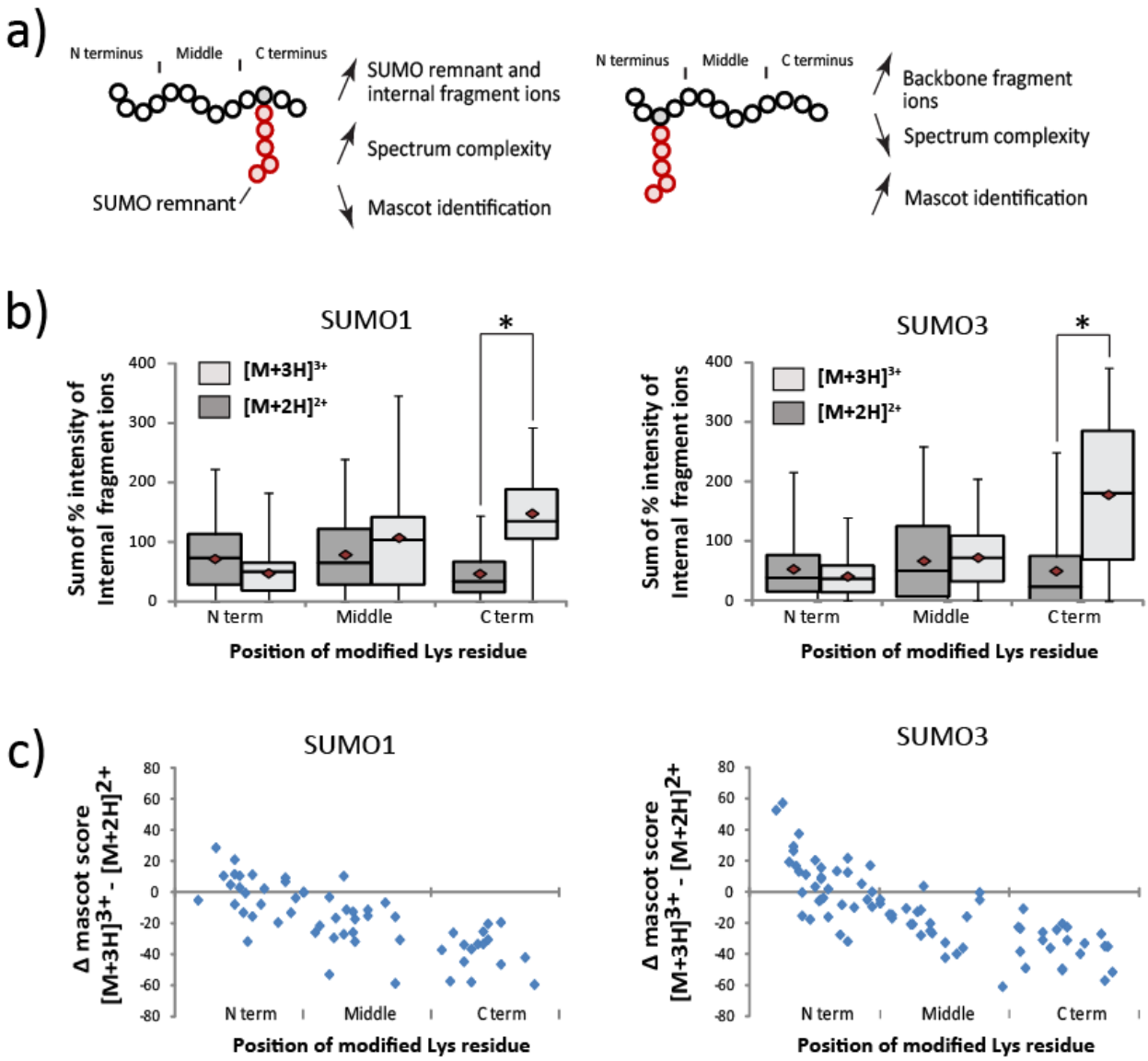


Diagnostic ions for SUMO3:	m/z 132.078 (c_1*), m/z 226.083 (b_2*-NH_3), m/z 243.109 (b_2*), m/z 344.157 (b_3*)
Neutral losses for SUMO3:	242.101 Da (NQ), 343.149 Da (NQT), 400.171 Da (NQTG), 457.192 Da (NQTGG)
Mass tolerance of ± 10 ppm is applied on fragment ions	

Supplemental Figure S1: Work flow for the algorithm used in data-independent acquisition (DIA). First, the algorithm searches for fragment ions characteristic to SUMO remnant chains (diagnostic ions) with intensities > 5000 counts obtained from each segmented precursor ion windows (mass tolerance ± 10 ppm). If at least two diagnostic ions are present in the scan, then the algorithm calculates potential neutral losses for all precursor ions observed in the survey scan. If two neutral losses are observed for the same precursor ion (mass tolerance ± 10 ppm), then the corresponding peptide mass is added to an inclusion list. The algorithm repeats this operation until all peaks and all scan are processed.

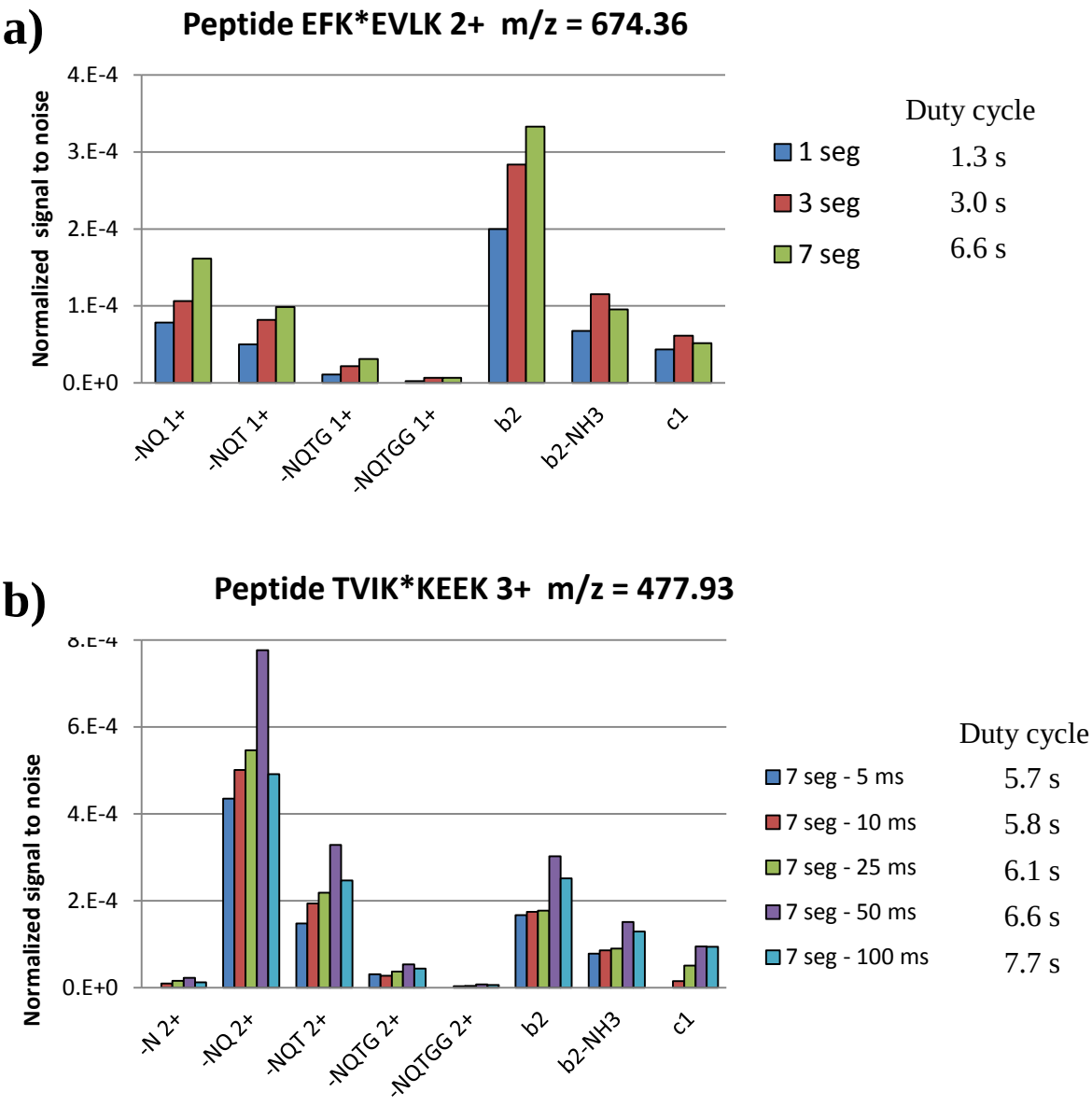


Supplemental Figure S2: Relative proportion of fragment ions assigned to backbone sequence ions for different activation modes. a) Intensity ratio of backbone sequence ions to all ions for SUMO1 (a) and SUMO3 (b). Intensity ratios correspond to the sum of backbone sequence ions (b- and y-type fragment ions) to the sum of all fragment ions. This ratio was obtained from the open source program Morpheus (J Proteome Res. 2013 Mar 1;12(3):1377-86) in which the proportion of backbone fragments (b- and y-type or c and z-type) to total spectrum abundance is provided as part of the score. The ratio of backbone fragment ions for MS/MS spectra shown in Figures 5c and 5e are typical of SUMO3 synthetic peptides and represent 20 and 8%, respectively. Each box plots identifies minimum value, lower quartile, median, upper quartile and maximum value. The diamond represents the mean value. Intensity ratios were determined for each synthetic SUMO remnant peptide. The distribution observed indicates that a larger proportion of fragment ions match backbone sequence ions when HCD is used compared to either CID or ETD. The larger proportion of assigned fragment ions using HCD is consistent with the increased Mascot scores observed for this activation mode.



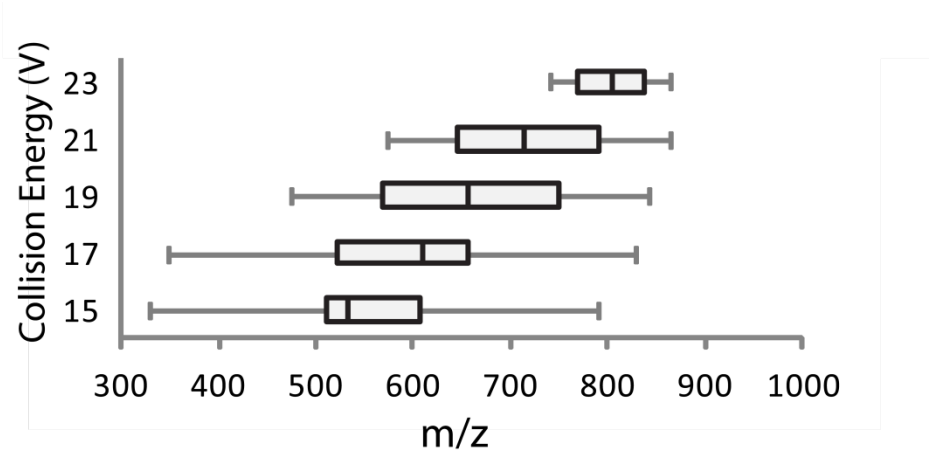
Supplemental Figure S3: Impact of the position of the modified lysine residue on the fragmentation of synthetic peptides with SUMO remnant chain. a) Tryptic peptides bearing a SUMO remnant chain on a C-term lysine residue increase the occurrence of internal fragment ions and render the interpretation of MS/MS more difficult. In contrast, tryptic peptides with SUMO remnant chain located on a N-term lysine residue produce a larger number of y-type fragment ions thereby facilitating the identification of backbone sequence with fewer internal fragment ions. b) Distribution of the cumulative intensity of internal fragment ions for SUMO1 and SUMO3 remnant chains observed for both doubly- and triply-protonated precursor ions. An increase in internal fragment ions is observed for $[M+3H]^{3+}$ precursor ions when the modified lysine residues are closer to the C terminus. No specific trend in the distribution of internal fragment ions with respect to the position of the modified lysine residue was observed for $[M+2H]^{2+}$ precursor ions (*p-value of 5.49E-6 for SUMO1 and 2.79E-5 for SUMO3). c) Variation of Mascot score for MS/MS spectra of triply- and doubly-protonated precursor ions as a function of the position of the modified lysine residue.

$$\text{Normalized signal to noise} = \frac{\text{Fragment signal}}{\text{Noise in MS/MS scan}} \text{x} \frac{1}{\text{Precursor signal}}$$

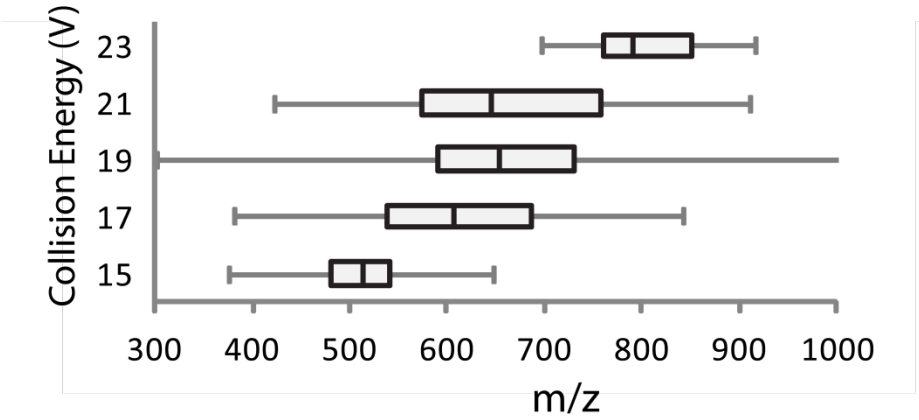


Supplemental Figure S4: Evaluation of optimal ion storage conditions for data independent acquisition (DIA). Normalized intensities of SUMO-specific fragment ions for different precursor windows (a) and accumulation times (b) for typical SUMO remnant peptides. Normalized intensity values were determined from the ratio of fragment ions to noise intensity divided by the intensity of the precursor ion abundance. Optimized conditions were obtained for an accumulation time of 50 ms with 7 segments of 100 m/z units spanning from m/z 300-1000.

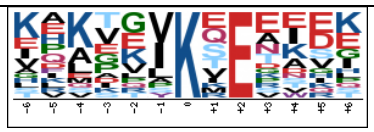
a) **Neutral loss: NQ**

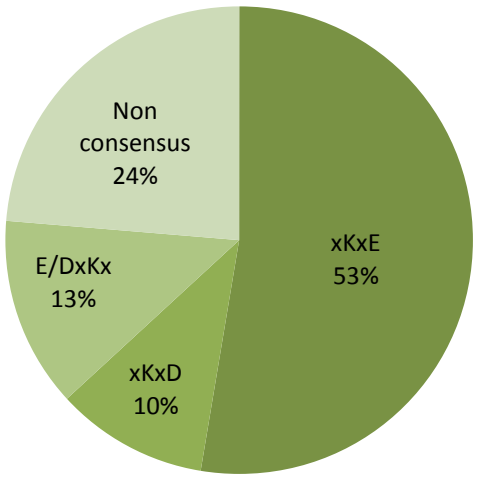


b) **Neutral loss: NQT**

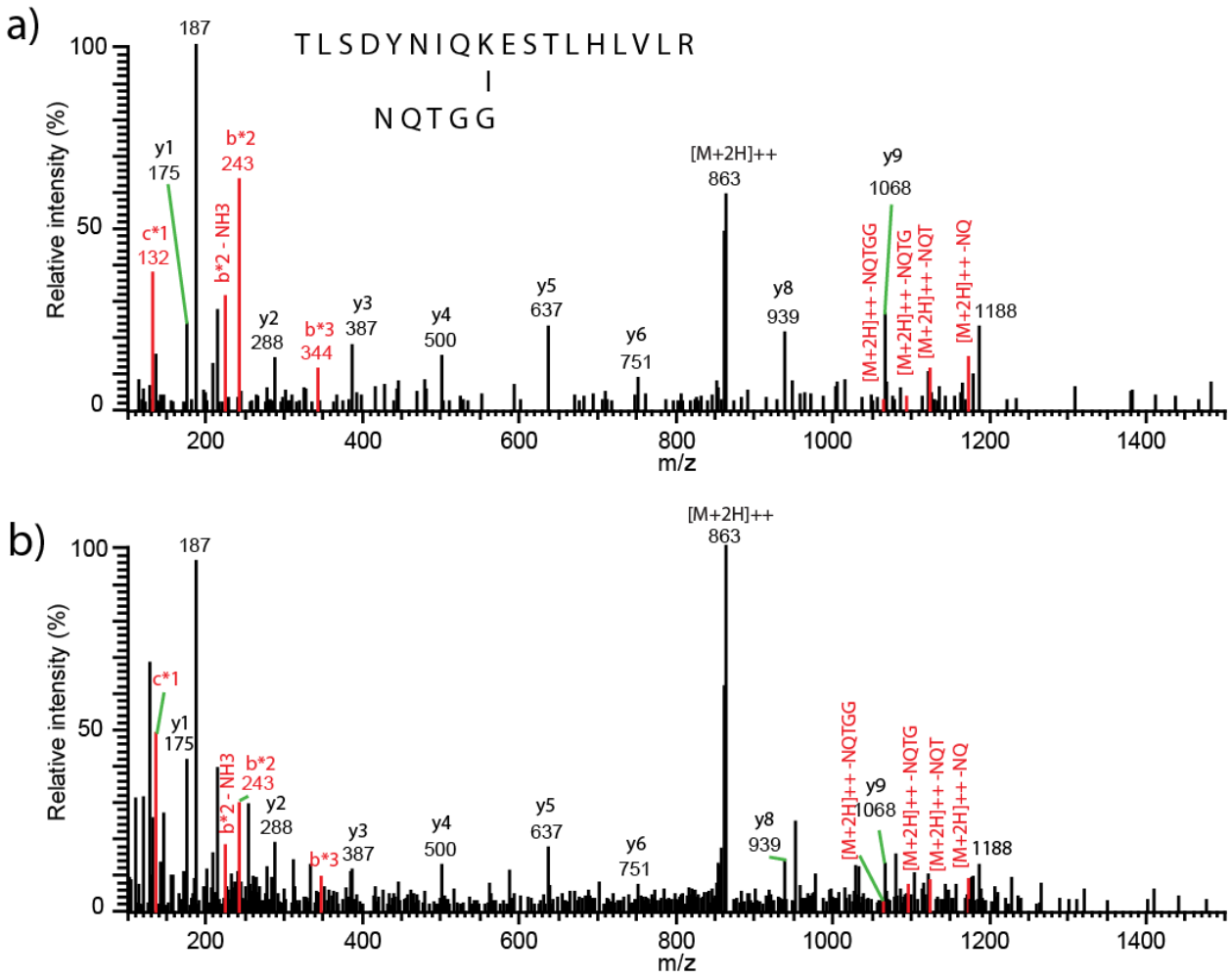


Supplemental Figure S5: Optimization of collision energy for the formation of neutral loss fragment ions from SUMO remnant peptides. Box-plot distribution of collision energies for precursor ions giving rise to maximum intensity of NQ (a) and NQT (b) neutral losses. A library of 96 synthetic SUMO3 peptides was used for LC-MS/MS experiments performed at collision energy ranging from 15-30 V. Relevant portion of the distribution is shown for convenience. These experiments enabled the identification of optimal collision energies for different precursor ion m/z values. LC-MS/MS experiments performed with DIA used a collision energy ramped according to precursor ion m/z.

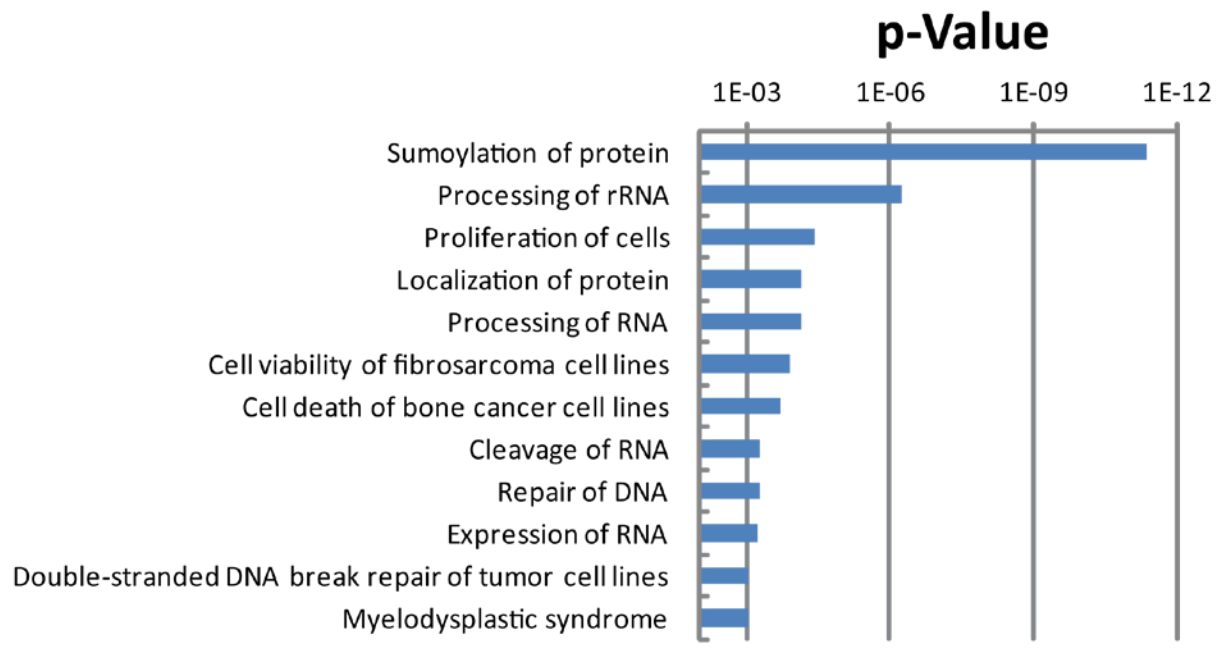
		Non consensus
.....K.E....	**.....K.D....**	**.....E/D.K.....**
AQKVQIKQETIES KPKEGVKTENNDH KPKEGVKTENDHI KAKIKVKVEEEEE IEKTVIKKEEKIE GKKKHIKEEPLSE EKKPKIKEEAVKE KEKGKYKEETIEK VAATEIKMEEESG HHSVEIKIEKTVI HIINPIKAEDVGY KQLPGVKSEGKRK EVAVPVKQEAEGL PIPRAVKPEPTNS IIVDELKQEVIST VHMGLLKSEDKVK GDETGAKVERADG TFQKRPKEEEWDP LRASTSKSESSQK SLLAVVKREPAEQ	MKPRKIKEDDAPR GRPLKVKEDPDGE LRGEKRKRDAEDD NKLTEDKADVQSI	**Non consensus** RKQLATKAARKSA CAIPGKKGTPWEG SDYNIQKESTLHL MADEKPKEGVKTE GETGKEKLPRYYK RHTPLSKLMKAYC GGKAPRKQLATKA RARLQEKLSPPYS PFDFTWKMLKDKF



Supplemental Figure S7: Sequence motif surrounding modified Lys residues. Distribution of amino acids flanking the modified Lys residue with a consensus motif for ψKxE/D (top) and relative proportion of sites with consensus and non-consensus SUMO motifs (bottom). SUMOylation motif obtained with Motif-X (http://motif-x.med.harvard.edu/motif-x.html)



Supplemental Figure S8: Validation of identified SUMOylated Ubiquitin with a synthetic peptide. a) Synthetic peptide corresponding to SUMOylated Ubiquitin on K63 (TLSDYNIQKESTLHLVLR; SUMO3-K9; Deaminated-Q8). b) Peptide identified in the cell extract as TLSDYNIQKESTLHLVLR SUMOylated on K9 and deaminated on Q8.



Supplemental Figure S9: Bioinformatics analyses for enriched GO terms representing SUMOylated proteins identified in this study. SUMOylated proteins were enriched in GO terms associated with RNA processing, p-value: 5.50E-07 (NOP58, NPM1, RPL26, RPS6) or double-strand repair of DNA, p-value: 4.01E-04 (NPM1, PARP1, SUMO1, TRIM28). Ingenuity Pathway Analysis was used defined enriched GO terms.