SI Text

Antibodies and peptides. Antigen affinity-purified sheep anti-SUMO-1 (1:1000) and anti-SUMO-2 (1:1000), antibodies were prepared in-house. Mouse anti-β-tubulin, mouse anti-Lamin A/C were obtained from Amersham Life Sciences and Sigma. The SIM peptide (sequence VDVIDLTIEEDE) (Yuan Chen, personal communication) was generated at >95% purity by HPLC (Enzo Life sciences).

Generation of polySUMO and polyubiquitin chains for matrix characterization. For the analysis shown in Supp. figure 1, stocks of unanchored polySUMO-2 chains linked by lysine 11, unanchored polySUMO-1(D15V) chains linked via lysine 16, polySUMO-2 chains linked via lysines 5 (or 7), 11 and 41 (conjugated to RanBP2(2633-2761)), and polyubiquitin chains linked via lysines 7, 11, 48 and 63 (conjugated to GST-Ubc5) were generated as described previously(3-6). The monomeric and dimeric SUMO-2 were separated from longer SUMO-2 polymers by Superdex 75 gel filtration (Fig. 1D).

Mass spectrometry. Protein bands were subjected to in-gel digestion with trypsin (Promega), essentially as described previously(7). Mass spectrometric analysis was performed by LC-MS/MS using a linear ion trap-orbitrap hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific) equipped with a nanoelectrospray ion source and coupled to a Proxeon nano-HPLC system (Proxeon Biosystems). Peptides were injected into a 75 mm reverse phase C_{18} column with a flow of 500 nl/min and eluted with a 120-min gradient from 73% solvent A (0.5% acetic acid in H_2O) to 85% solvent B (90% acetonitrile, 0.5% acetic acid in H_2O) and a flow of 200 nl/min (exact details of

gradient available upon request). Wideband activation was applied and the instrument was operated with the "lock mass" option to improve the mass accuracy of precursor ions and data were acquired in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Full scan spectra (m/z 300-1650) were acquired in the orbitrap with resolution R = 60,000 at m/z 400 (after accumulation to a target value of 1,000,000). The 5 most intense ions were fragmented by collision induced dissociation and recorded in the linear ion trap (target value of 5,000) based upon a low resolution (R = 15,000) preview of the survey scan.

Data analysis. The raw MS data files from the control condition and heat stressed conditions were processed with the quantitative MS processing software MaxQuant (version 1.0.13.13)(1, 2) and using the Mascot search engine (Matrix Science, version 2.2.2). Please see ref(1) for further details of the algorithms and principles applied in the MaxQuant package. Enzyme specificity was set to trypsin-P. Cysteine carbamidomethylation was selected as a fixed modification and methionine oxidation, protein N-acetylation and gly-gly adducts to lysine were searched as variable modifications. The data were searched against a target/decoy human IPI database (version 3.24)(8). Initial maximum allowed mass deviation was set to 7 parts per million (ppm) for peptide masses and 0.5 Da for MS/MS peaks. The minimum peptide length was set to 6 amino acids and a maximum of two missed cleavages. 1% false discovery rate (FDR) was required at both the protein and peptide level. In addition to the FDR threshold, proteins were considered identified, if they had at least one unique peptide.

Relative protein quantitation was based on extracted ion chromatograms (XICs) of contained peptide.

Raw MS files were processed using MaxQuant (1, 2), which not only identifies proteins but provides summed peptide intensities for each protein based on extracted ion chromatograms of peptides assigned to that protein. This can be used as an approximation of relative protein abundance when comparing the same protein in different samples. As such it is possible to determine which proteins were relatively enriched in the RNF4wt sample in comparison with the RNF4mut.

The SUMO-branched peptides were identified by modifying the MASCOT search database with the linear pseudo-branched peptides of the various SUMO-SUMO and SUMO-substrate branched peptides after tryptic digestion, which are then treated as individual proteins, applying a similar principle to a method already described(9).

Apparent molecular weight calculation and SUMOylation stoichiometry estimation. The apparent molecular weight (MW_{App}) of each identified protein was calculated according to the retention of that protein within the SDS polyacrylamide gel, using MaxQuant signal intensity of all peptides as a surrogate for protein abundance. Briefly, the relationship between retention factor (RF = distance of band from the well/distance of dye-front from the well) and protein MW_{App} was determined using direct analysis of the pre-stained molecular weight markers fractionated on the same gel as the experimental sample (Fig. 3A). The lanes containing the experimental samples were sliced into 9 sections, and the RF of each cut of the gel calculated, which provided upper and lower MW_{App} ranges for each section (as shown on Fig. 3A), and hence the expected average MW_{App} of a protein within that slice. For the data analysis of the raw MS files

'Identify' was run in MaxQuant such that each gel slice was considered as a different experiment which provided a total signal intensity for each protein in each slice. The MW_{App} of a protein was then calculated using the average MW_{App} of each slice that the protein was detected in, weighted for the intensity of the peptides in those slices, as detailed in equation 1.

$$MW_{App(p)} = \sum_{i=1}^{9} MW_{App(i)} \left(\frac{I_i}{I_{Tot}} \right) \qquad (1)$$

Where $MW_{App(p)}$ is apparent molecular weight of protein p

i is slice number

$MW_{App(i)}$ is expected average MW_{App} of a protein within slice i

I_i is intensity of all peptides of protein p in slice i

I_{Tot} is the total intensity of all peptides of protein p in all slices

In this example nine slices were prepared

Note; due to gel-dependent differences in MW_{App}, the apparent MW of the protein markers shown (Biorad) was re-calibrated using markers whose MW_{App} was already defined in this system (New England Biolabs) (not shown).

The expected number of SUMO molecules attached to each protein (SUMOylation stoichiometry) can then be determined by the difference in molecular

weight (ΔMW) between the MW_{App} and the predicted molecular weight (MW_{Pred}) according to the protein amino-acid sequence (see equation 2).

$$\Delta MW = MW_{App} - MW_{Pred} \qquad (2)$$

As SUMO-2 has an MW_{App} of 15.8 kDa in the gel system used for this analysis (not shown), the following ΔMW boundaries were used to define SUMOylation stoichiometry: Unmodified; ΔMW <7.9 kDa, one SUMO; ΔMW = 7.9 to 23.7 kDa; two SUMOs; ΔMW = 23.7 to 39.5 kDa; three SUMOs; ΔMW = 39.5 to 53.3 kDa; four SUMOs; ΔMW 53.3 to 71.1 kDa, five or more SUMOs; ΔMW >71.1 kDa.

The RNF4 isolation efficiency of polySUMO2 K11 chains in was measured by spiking heavy SUMO2 K11 dimer into 4000 ug nuclear lysate or 0.8 ug RNF4 isolate. The samples were digested with Trypsin (Promega) in solution. Subsequently, they were measured with a Dionex Ultimate 3000 instrument HPLC and an LTQ-Orbitrap Velos mass analyzer (Thermo Fisher Scientific) and a gradient of 40 min. Quantitation of the incorporation was performed by Maxquant analysis. This method will be described in detail elsewhere (IM, RB, EGJ and RTH in preparation).

Supplementary Table

Target Protein	di-glycine modification site	Mascot Score
SUMO-2/-3	K32/33 and K34/35	49.96
Ubiquitin	K11	78.90
Ubiquitin	K48	64.22
Ubiquitin	K63	75.81

Supplementary Table 1. Summary of the Mass spectrometric analysis of di-glycine modification sites of the 339 putative polySUMO modified substrates and ubiquitin. Only SUMO2/3 and ubiquitin were found to be contain di-glycine linked lysines.

Supplementary file legends

Supplementary File 1. MaxQuant output file for analysis of the four RNF4 purifications as described in figure 3 and putative polySUMO2 substrates. Please refer to (Cox & Mann, 2008; Cox et al, 2009) for details of other column headers.

Supplementary File 2. Calculation of ΔMW and SUMO stoichiometry from MaxQuant analysis of the four RNF4 purifications as described in figure 3. Apparent MW, Delta MW and number of SUMOs were calculated and inserted as columns. Please refer to (Cox & Mann, 2008; Cox et al, 2009) for details of other column headers.

References

1. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26**,** 1367-1372.
2. Cox J*, et al*. (2009) A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat Protoc* 4**,** 698-705.

3. Tatham MH, *et al*. (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* 276**,** 35368-35374.

4. Tatham MH, *et al*. (2008) RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat Cell Biol* 10**,** 538-546.

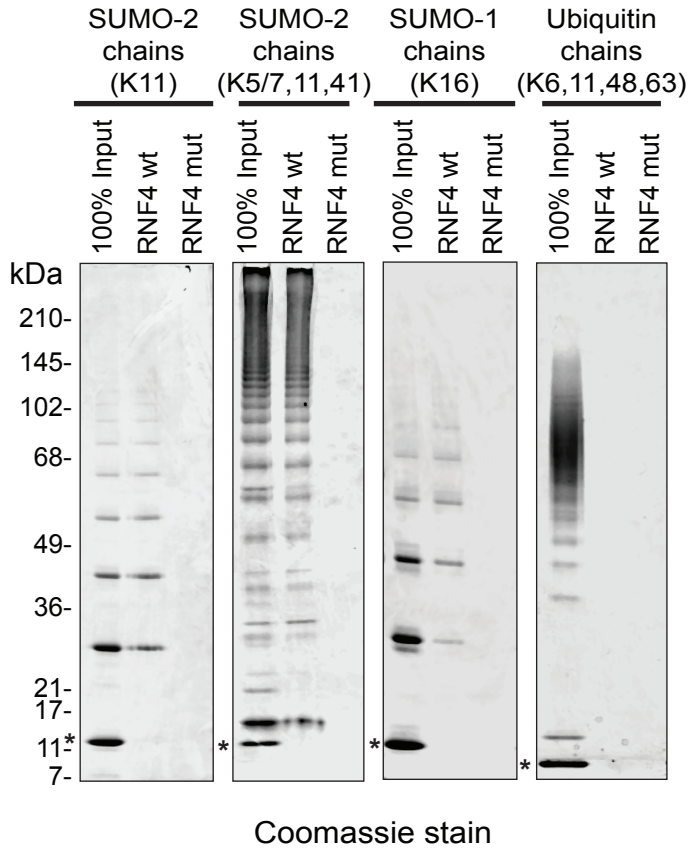
5. Cooper HJ, *et al*. (2005) Fourier transform ion cyclotron resonance mass spectrometry for the analysis of small ubiquitin-like modifier (SUMO) modification: identification of lysines in RanBP2 and SUMO targeted for modification during the E3 autoSUMOylation reaction. *Anal Chem* 77**,** 6310-6319.

6. Cooper HJ, *et al*. (2004) Identification of sites of ubiquitination in proteins: A Fourier transform ion cyclotron resonance mass spectrometry approach. *Anal. Chem*. In press.

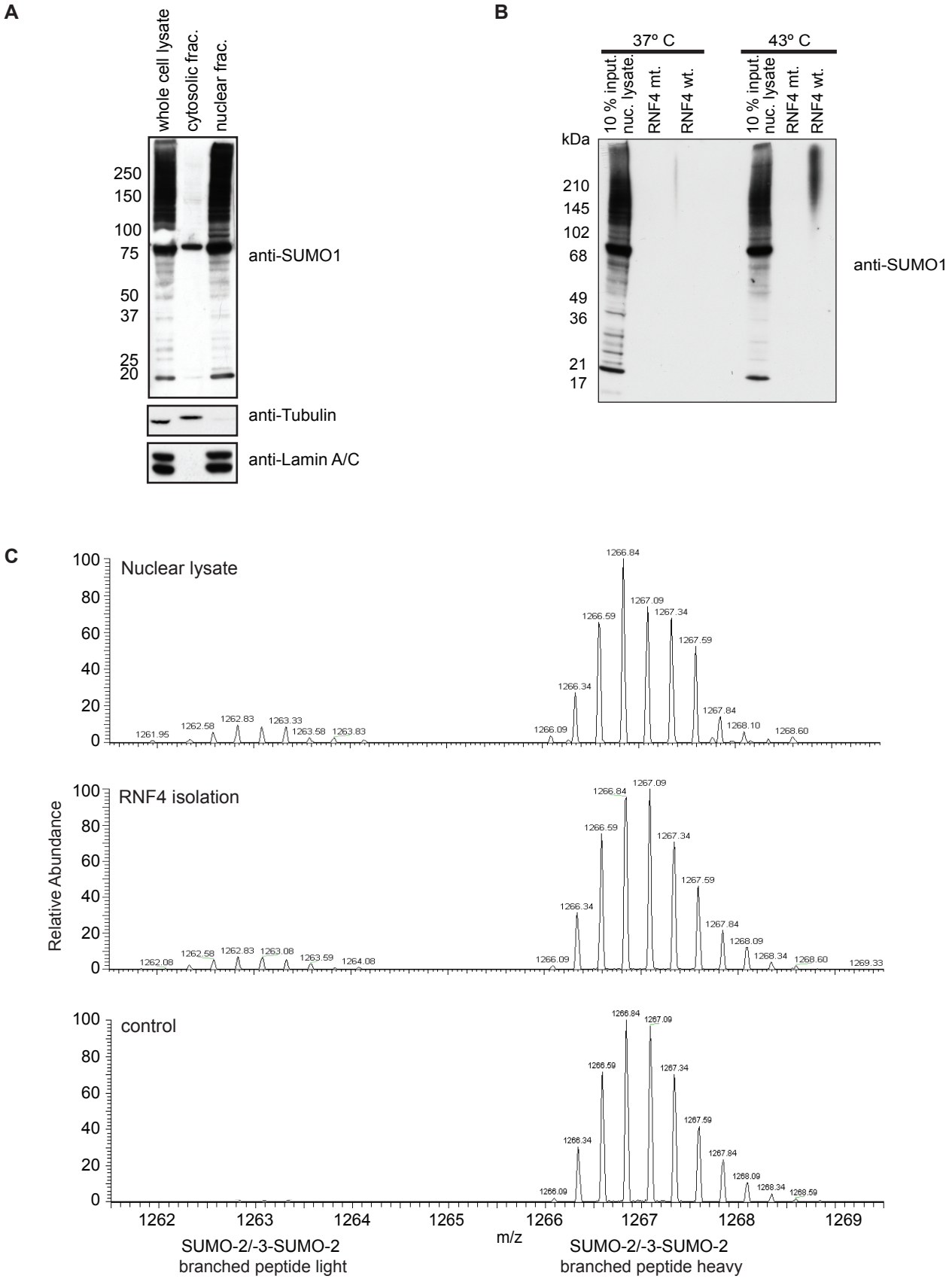
7. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1**,** 2856-2860.

8. Kersey PJ, *et al*. (2004) The International Protein Index: an integrated database for proteomics experiments. *Proteomics* 4**,** 1985-1988.

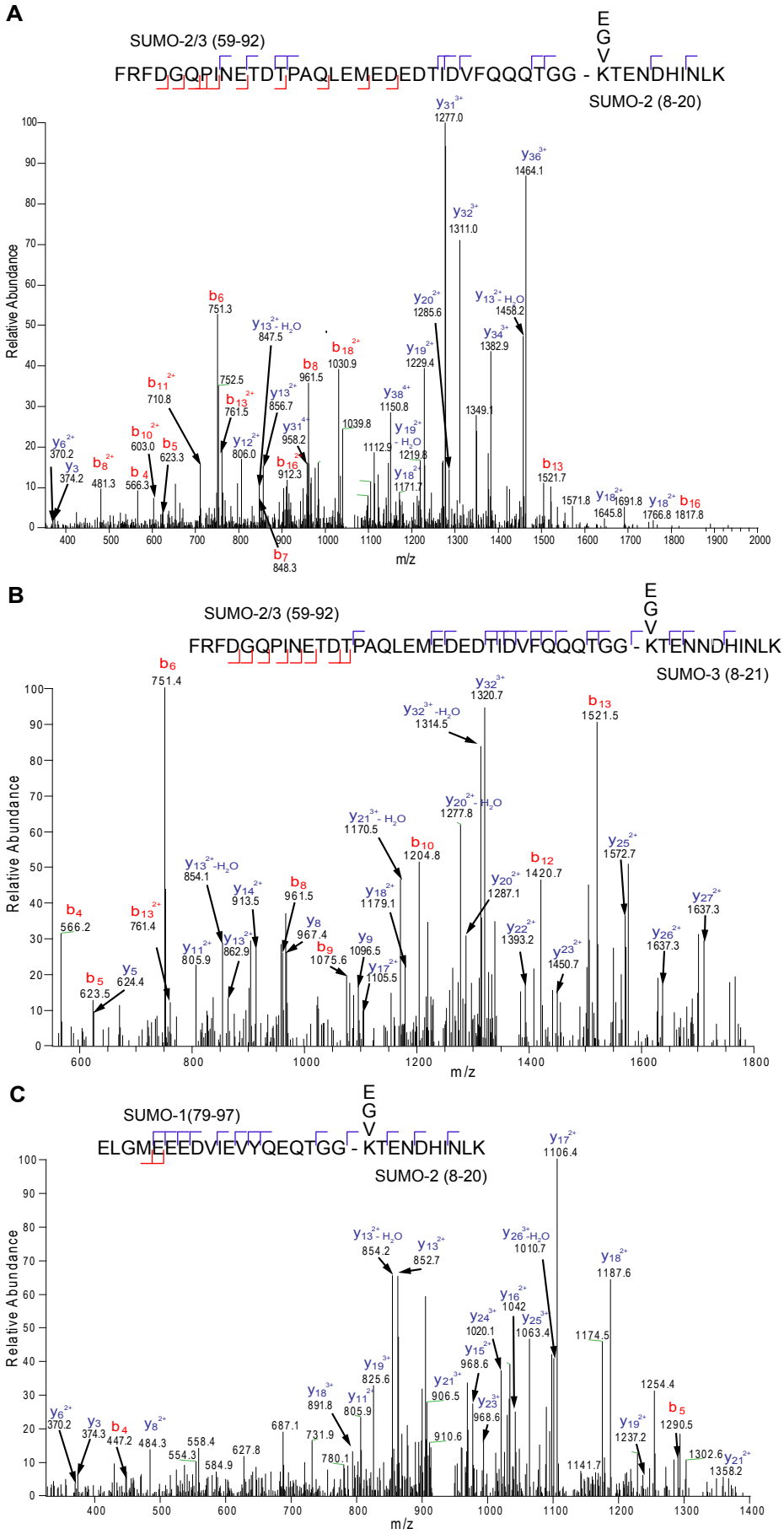
9. Maiolica A, *et al*. (2007) Structural analysis of multiprotein complexes by cross-linking, mass spectrometry, and database searching. *Mol Cell Proteomics* 6**,** 2200-2211.



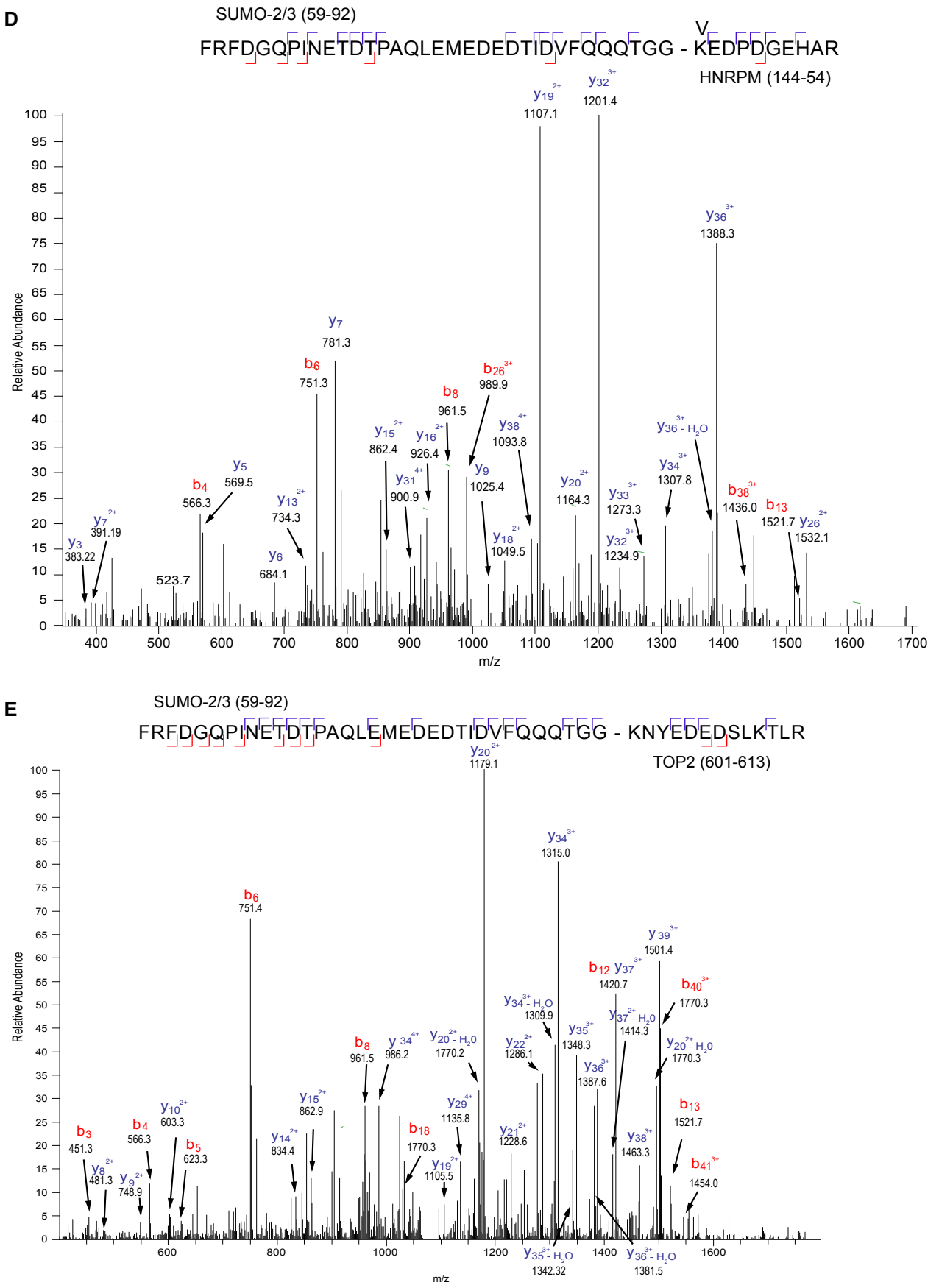
Bruderer et al Supplementary Figure 1. *RNF4 selectivity* RNF4-dependent purification of recombinant SUMO-2 polymers conjugated via internal lysine 11, SUMO-2 polymers conjugated via internal lysines 11, 41 and 5 or 7 on RanBP2(2633-2761), SUMO-1(D15V) polymers via internal lysine 16, and ubiquitin polymers via internal lysines 6, 11, 48 and 63 on GST-Ubc5. Bound proteins eluted by boiling in Laemmli's sample buffer. Asterisks indicate position of unmodified SUMO or ubiquitin.



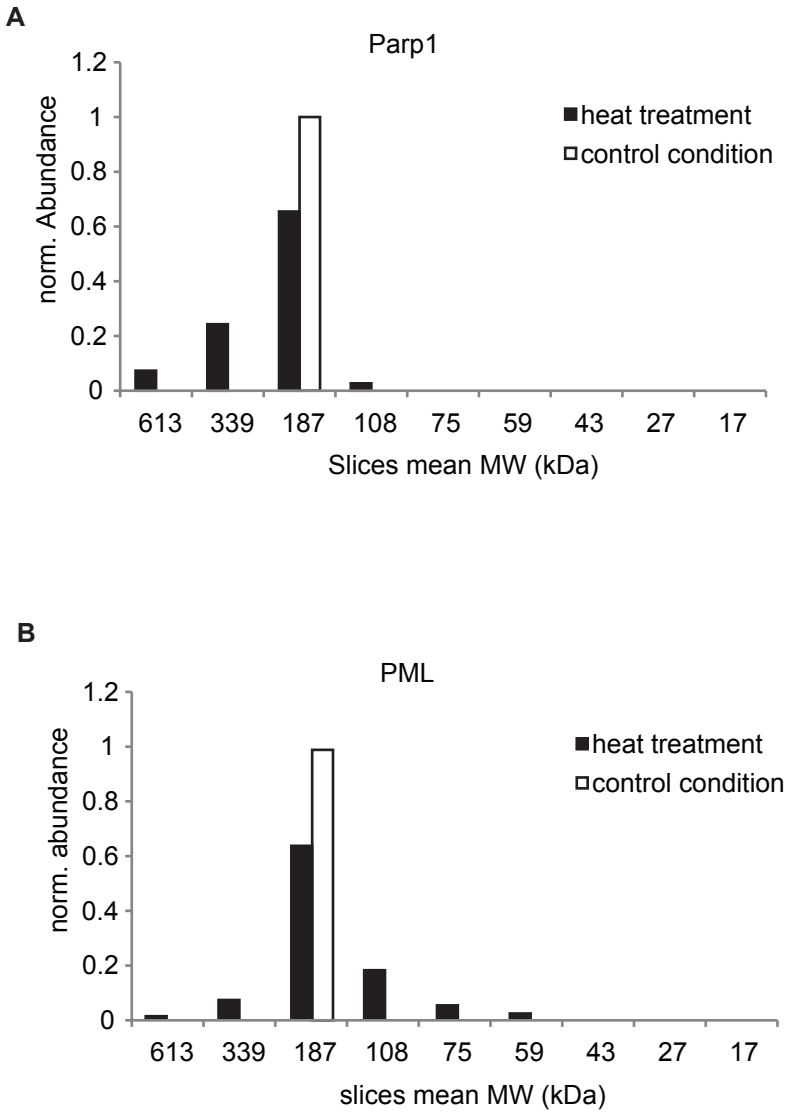
Bruderer et al Supplementary Figure 2. SUMO-1 conjugates are purified by RNF4-pulldowns. (**A**) Native Fractionation of HeLa cells into cytoplasmic and nucleoplasmic lysate. Fractionation efficiency is monitored by anti-Tubulin and anti-Lamin A/C antibodies. Preservation of SUMO-1 protein modification is monitored by anti-SUMO-1 antibodies. (**B**) Isolation of SUMO modified proteins from HeLa nuclear lysate with RNF4 beads was peformed. Samples were analysed by western blotting with anti-SUMO-1 antibodies. Note the predominant band in the nuclear lysates at about 75kDa is RanGAP1 modified with SUMO-1. (**C**) Heavy labelled SUMO2 K11 branched peptide spiked into 4000 ug input HS nuclear lysate (top), 0.8 ug RNF4 purification (middle) and buffer (bottom) followed by quantitative analysis by mass spectrometry. The SILAC pairs of the quadrupol charged precoursor ions are shown.



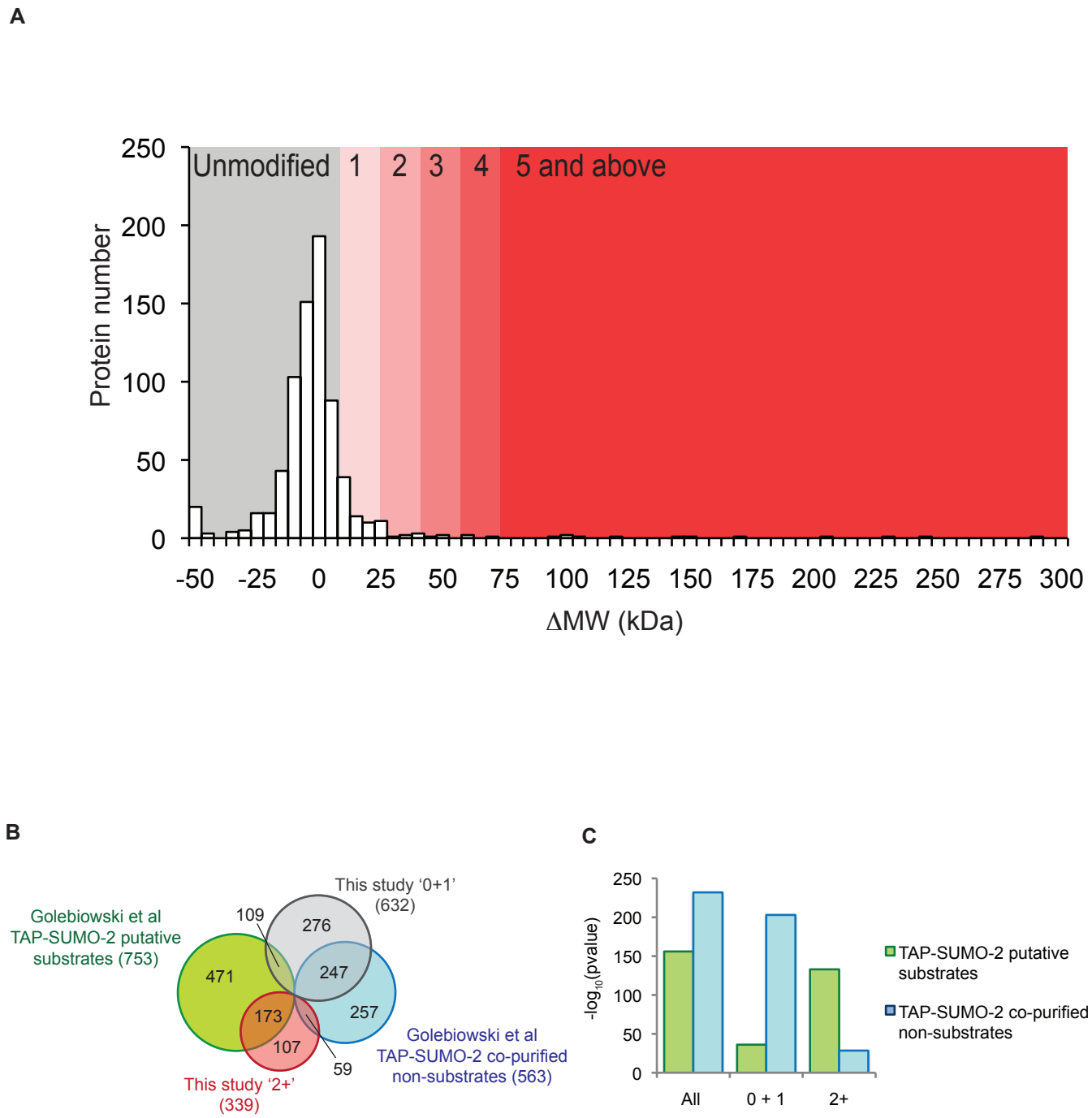
Bruderer et al. Supplementary Figure 3. Endogenous SUMO chain branched peptides identified in RNF4 isolated fractions. Proteins were separated by SDS-PAGE and digested in gel. The precursor ions were analyzed in the orbitrap mass spectrometer ((**A**) SUMO-2/-3-SUMO3 m/z 1370.634 (4+); mass deviation, -0.73 ppm, (**B**) SUMO-2/-3-SUMO-2 m/z 1070.701 (5+); mass deviation, -0.08 ppm and (**C**) SUMO-1-SUMO-2 m/z 941.376 (4+); mass deviation, -0.25 ppm) and subsequently fragmented by collision induced dissociation. The resulting fragmentation spectra were acquired in the LTQ analyzer.



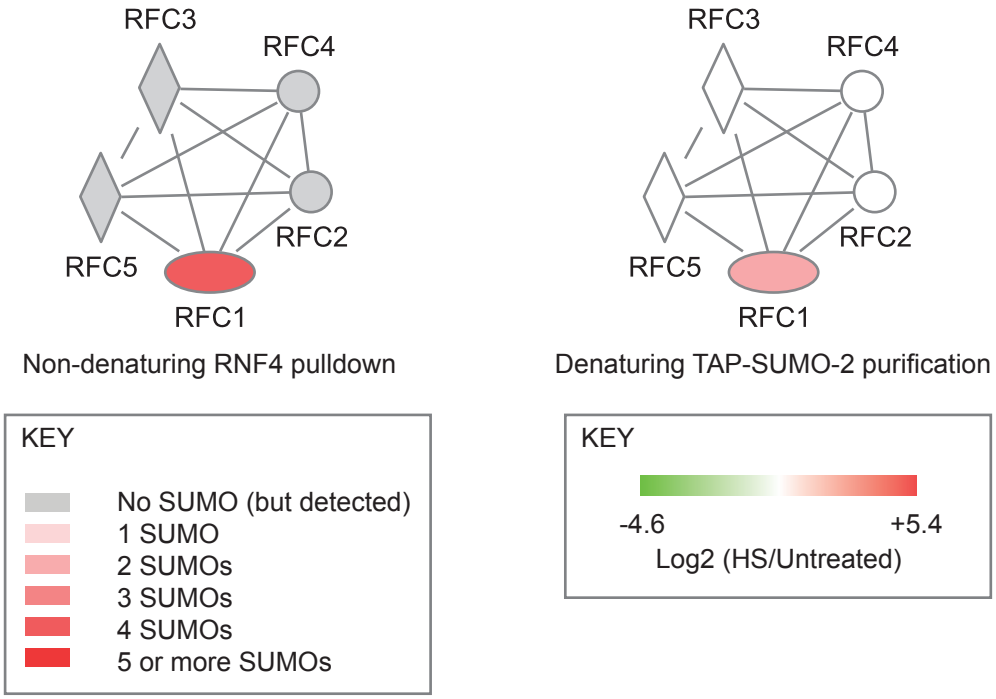
Bruderer et al. Supplementary Figure 3**D**+**E**. Endogenous SUMOylation of HNRNPM at Lys[145] and Top2b at Lys[601] identified in RNF4 isolated fractions. Proteins were separated by SDS-PAGE and digested in gel. The precursor ions were analyzed in the orbitrap mass spectrometer and subsequently fragmented by collision induced dissociation. The resulting fragmentation spectra were acquired in the LTQ analyzer. a MSMS spectrum (of 790 to 436 kDa slice of HS RNF4wt pulldown) of SUMO2/3-HNRPM with m/z 1025.0644 (5+); mass deviation, 0.74 ppm. The HNRPM (51 kDa) protein has 50.7% sequence coverage and was detected in the four top slices of the HS RNF4wt pulldown b MSMS spectrum (of 436-241 kDa slice of HS RNF4wt pull-down) of SUMO2/3-TOP2 m/z 1093.5074 (5+); mass deviation, 2.6 ppm). The TOP2 (183 kDa) protein has 21% sequence coverage and was detected in the top three slices of the HS RNF4wt pulldown.



Bruderer et al. Supplementary Figure 4. Changes in the SUMOylation status of Parp1 and PML after heat stress. (**A**) peptides of Parp1 were detected in the control and HS condition. Its molecular weight from sequence is 113.08 kDa, but was mostly detected in the HS experiment in the slices ranging from from the top down to 133 kDa. In the non-HS control Parp1 was detected in the range 230 to 133 kDa (mean 187 kDa), showing Parp1 undergoes increased polySUMOylation upon HS. **(B)** PML was detected in the control and HS conditions peaking in the slice ranging from 240 to 133 kDa in both cases. By amino-acid sequence the redicted MW for PML is 98 kDa indicating that polySUMOylated PML is detected at 37 ºC and 43 ºC, but polymer length does not significantly change.



Bruderer et al Supplementary Figure 5. (**A**) Less than 5% of proteins thought not to be SUMO conjugates have MW_{App} consistent with polySUMO conjugation. Data taken from proteins rejected from a TAP-SUMO-2 purification as 'non-substrate contaminants'. Note compare the largley Gaussian distribution around an average of 0 kDa in comparison with the RNF4wt purification data (Fig 3*B*). (**B**) Statistical comparison of the "0 +1" and "2+" subsets, with protein datasets of putative SUMO substrates or non-substrate co-purified proteins as defined in a previous independent study (Golebiowski et al, 2009). (**C**) Significance of overlap of the proteins identified in this study, with proteins identified in reference (Golebiowski et al, 2009). p-values are calculated using the Fishers exact test through 'Ingenuity pathways analysis' (www.ingenuity.com). *Entire datasets were not included in analysis due to redundancy filtering and numbers of protein IDs recognised by the software.



Bruderer et al Supplementary Figure 6.
(**A**) Panther biological process gene ontology (GO) analysis of the entire dataset and the "2+" set, and comparison with the TAP-SUMO-2 substrates (substrates) and co-purified non-substrates from reference (Golebiowski et al, 2009). (**B**) RNF4-mediated SUMO-2 pulldowns facilitate complex purification. Comparison between non-denaturing RNF4 pulldown results from this study (left), with those of denaturing TAP-SUMO-2 purification (Golebiowski et al 2009) for the identification of components of the replication factor C pentameric complex. The non-denaturing RNF4 affinity