Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools

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Note: Supplementary Data 1 is available on the Nature Methods website.

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Supplementary Figure 1. Ub-PSAQ standards. (**a**) Coomassie stained SDS-PAGE (4-20% gradient) gel showing Rsp5 autoubiquitination and purification. Purified recombinant GST-Rsp5 autoubiquitination reaction containing E1 enzyme, E2 enzyme and ^{15}N-Ub (see Methods), converts GST-Rsp5 to high molecular weight ^{15}N-Ub conjugates in the presence of ATP, as indicated. The conversion of full length GST-Rsp5 to higher molecular weight Ub conjugates was nearly quantitative after overnight room-temperature incubation. After auto-ubiquitination, GST-Rsp5 was purified by using Glutathione Sepharose affinity capture. Lanes labeled FT (flow through), Wash 1 and Wash 2 show unbound and non-specifically retained material, respectively. Specifically bound GST-Rsp5 conjugates were eluted with glutathione (lanes marked Elution 1 and Elution 2). Bound material was quantitatively eluted from beads. Glutathione elution 1 was used in all experiments as the polyubiquitinated protein standard. (**b**) Domain diagram of GST-Rsp5(196-809) with major sites of ubiquitin modification, as determined by LC-MS/MS analysis, shown. The 5 major sites include K27 and K113 in GST and K443 (K411 using Rsp5p numbering), K796 (K764), and K825 (K793). AQUA data in Supplementary Table 1 reveal that, on average, the standard has 1.3 polyubiquitin chains per molecule with an average of 12.5 Ub monomers per chain. (**c**) Mass spectra from extracted ion chromatograms showing ESTL ions derived from tryptic digests of pure ^{15}N Ub (left) and ^{13}C Ub (right) in the presence of the ESTL synthetic AQUA peptide. We estimate the extent of isotope incorportation as > 98% for both ^{15}N Ub and ^{13}C Ub. Consistent with the observed correct intact masses for fully labeled proteins (not shown), there was no detectable unlabeled ^{13}C Ub ESTL peptide and negligible (< 1%) unlabeled ^{15}N Ub ESTL peptide (black sphere, m/z = 356.6). As expected, ^{13}C labeled peptides (blue sphere, m/z = 372.3) are the mirror image of natural Ub, which is 99% ^{12}C and 1% ^{13}C. There is a discrete, ESTL ion at m/z = 366.26, which is the M-18 peak for the ^{13}C ESTL ion from the formation of pyro-Glu, which occurs to the same extent for the analytes and standards indicating that they form during the digestion-reaction or sample-work-up and have no effect on the accuracy of the results. (**d**) Mass spectra from extracted ion chromatograms showing sample and standard derived ESTL (left) ions and UbK63 (right) ions. Black circles signify unlabeled sample-derived Ub, open circles signify synthetic AQUA peptide standards with a single heavy isotope-labeled amino acid, red circles signify ^{15}N polyubiquitinated standard-derived Ub, blue circles signify ^{13}C free Ub standard-derived Ub, orange circles signify (^{15}N and ^{13}C) Ub-GFP standard-derived Ub.

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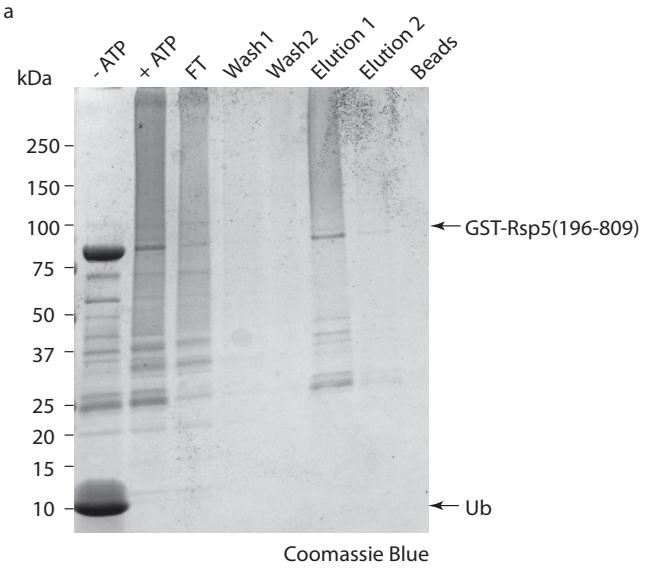

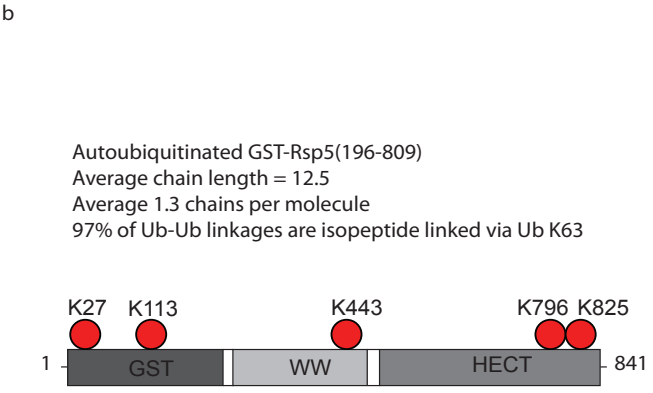

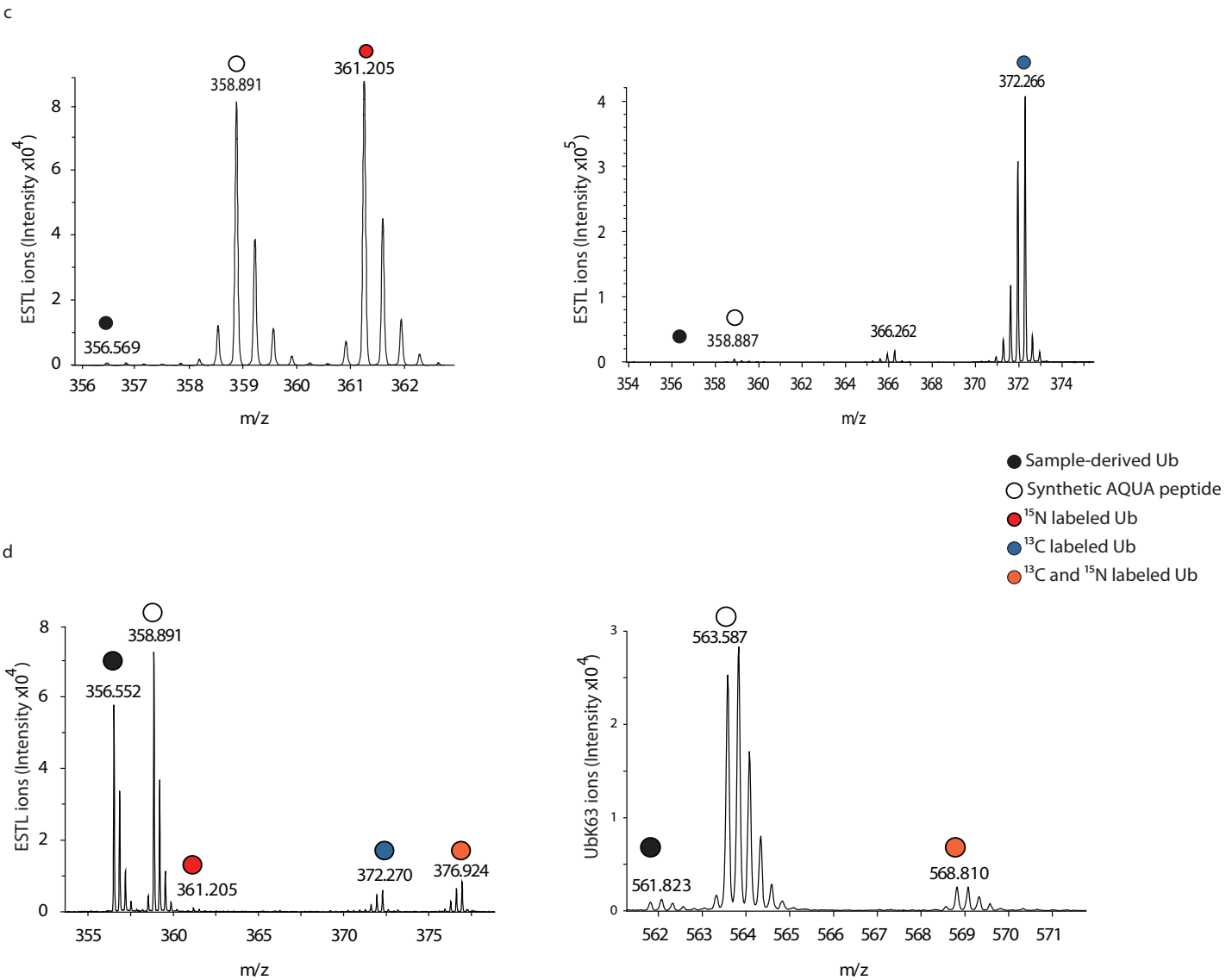

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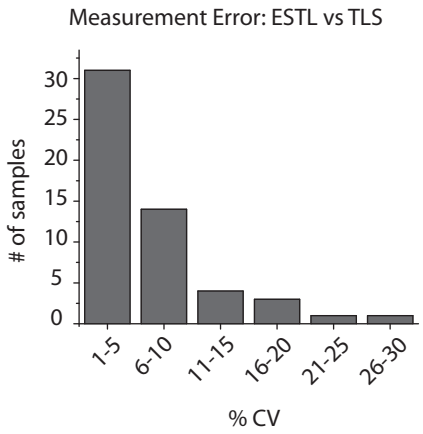
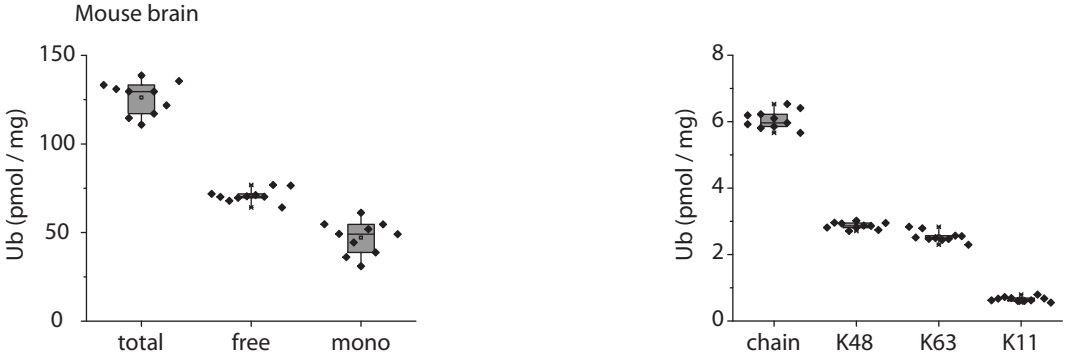
Supplementary Figure 2. AQUA measurement error. Ions derived from Ub tryptic peptides ESTLHLVLR and TLSDYNIQK independently measure the concentration of parent Ub molecules. We measure both in all studies and average the results. The measurement error between ESTL and TLS is plotted here for 54 HEK293 cell derived samples. The average fold difference between ESTL and TLS was 0.98 +/- 0.11 indicating no systematic error in these two measurements.



Supplementary Figure 3. Precise measurement of Ub pools in mouse brain. Ub-PSAQ measurement of Ub pools in mouse brain (n=10) shown as box plots with overlaid data. Averages describing the same data are presented in Table 1.



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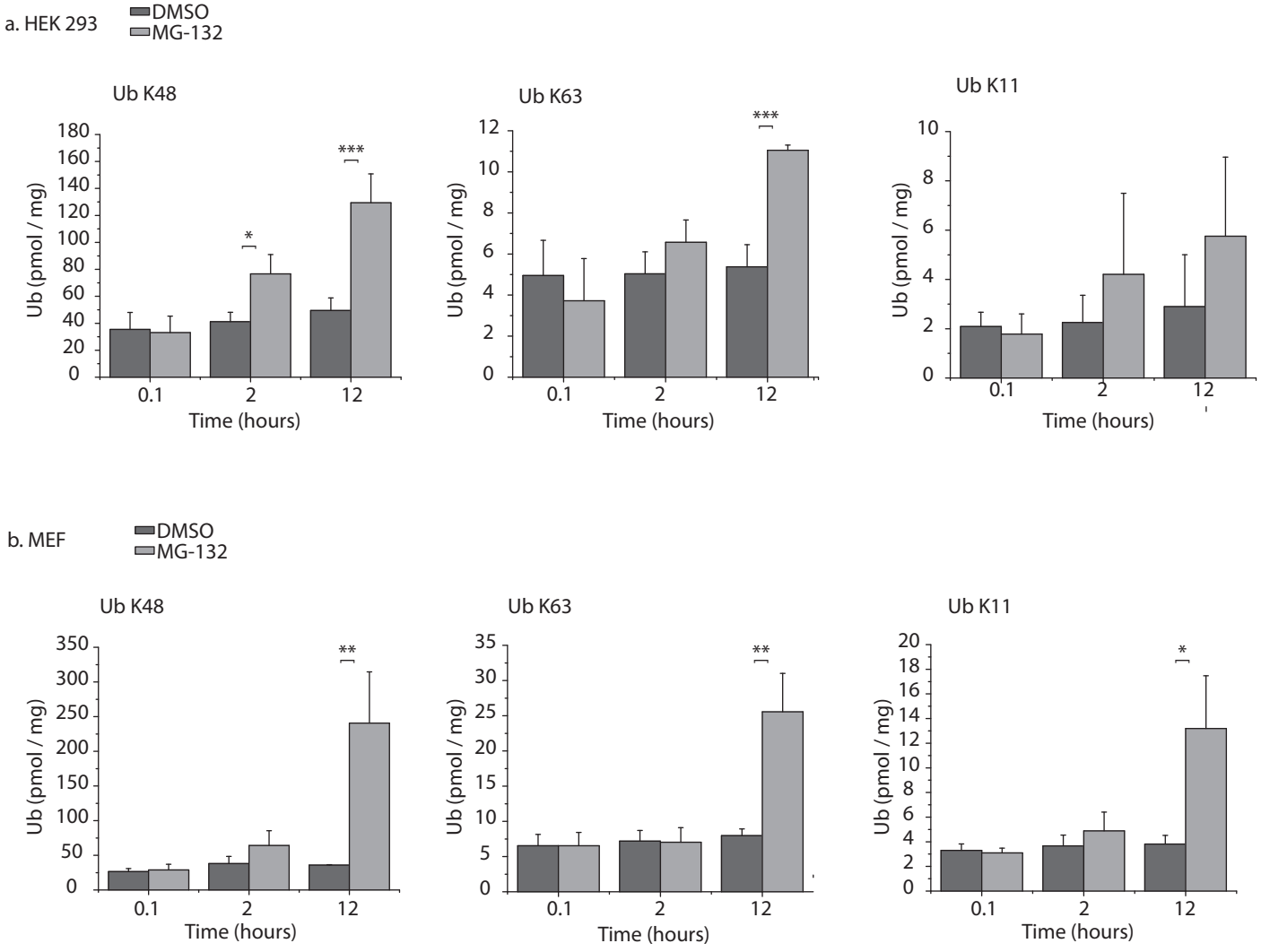
Supplementary Figure 4. Effects of acute proteasome inhibition on Ub chains in HEK293 and MEF cell lines. (**a** and **b**) In both cell lines, all detected Ub chains increase with MG-132 treatment. Error bars represent means ± s.d., n=3.



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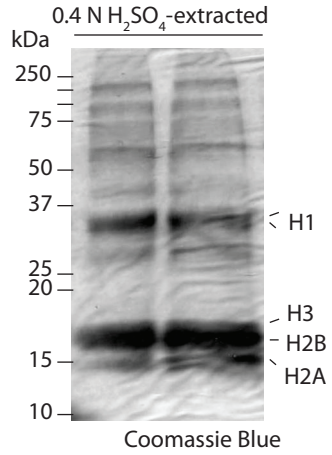
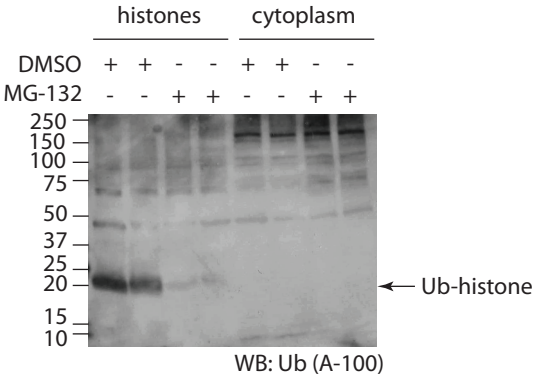
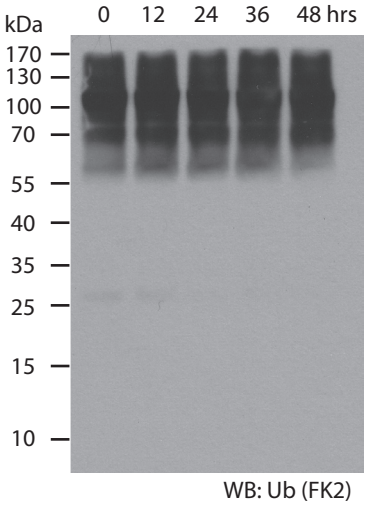
Supplementary Figure 5. Gel analysis of histone-enriched fraction. After hypotonic lysis, nuclei were pelleted, extracted with 0.4 N H_2SO_4 and dialyzed as outlined in Methods.



Supplementary Figure 6. Deubiquitination of histones by acute proteasome inhibition. Western blot of histone and cytosolic fractions showing depletion of ubiquitinated histone by treatment with 10 μM MG-132 for 6 hrs.



Supplementary Figure 7. Analysis of Ub conjugate stability in lysate. Western blot of time course after lysis of mouse brain showing stability of Ub conjugates over the timeframe of pulldowns.



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Supplementary Table 1. AQUA analysis of polyubiquitin chain standard. Polyubiquitinated GST-Rsp5 protein standard was analyzed by synthetic peptide AQUA as previously described (10) to quantify the molar concentrations of GST and Ub species in the standard. 91% of the Ub on the GST-Rsp5 standard is in the form of Ub chains and ~9% is present either as monoUb or at the ends of Ub chains (which cannot be distinguished by this method). Of Ub chains, 97% are linked via Lys63-linked isopeptide bonds, 2% via K11 and 1% via K48. Utilizing equations thoroughly presented in reference (10) these data enable the estimation of the number of ubiquitination sites per molecule and of average chain length. Although there is a wide distribution of chain lengths (see Supplementary Figure 1a), on average the standard has 1.3 chains / molecule with an average chain length of 12.5 Ub monomers per chain.

	pmol Ub / μl	% linked
ESTL	2.1	
Ub K48	0.17	1%
Ub K63	16	97%
Ub K11	0.35	2%
GST	1.2	

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