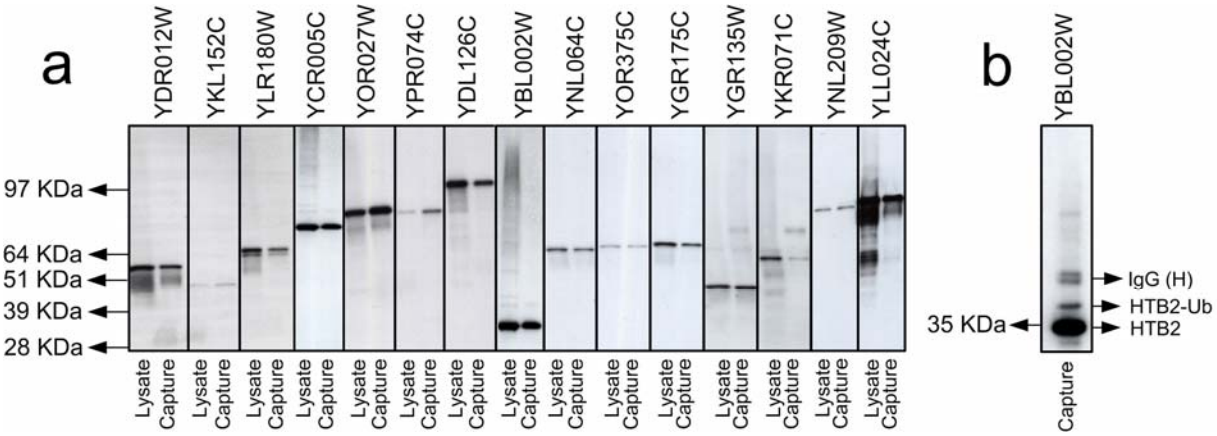
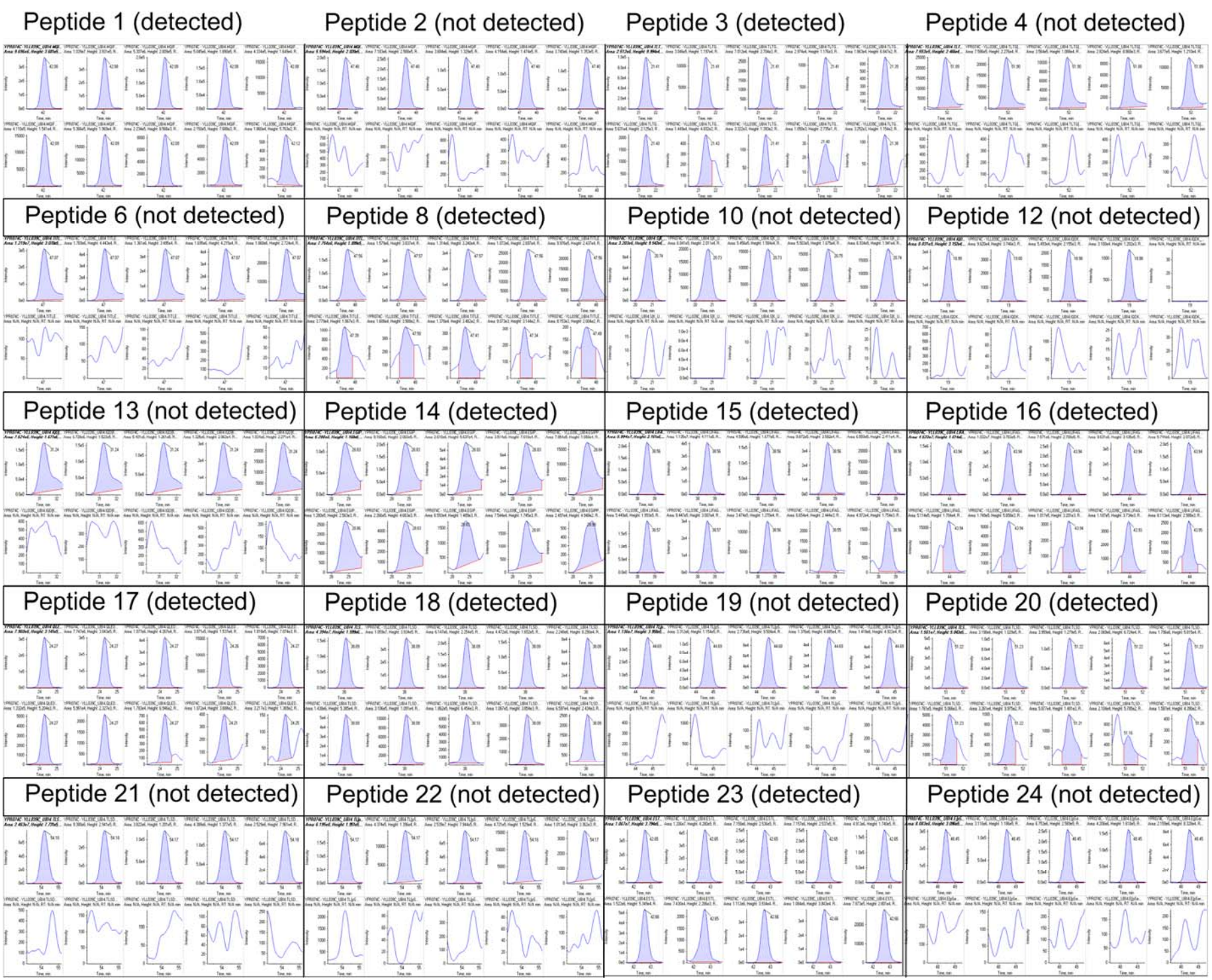
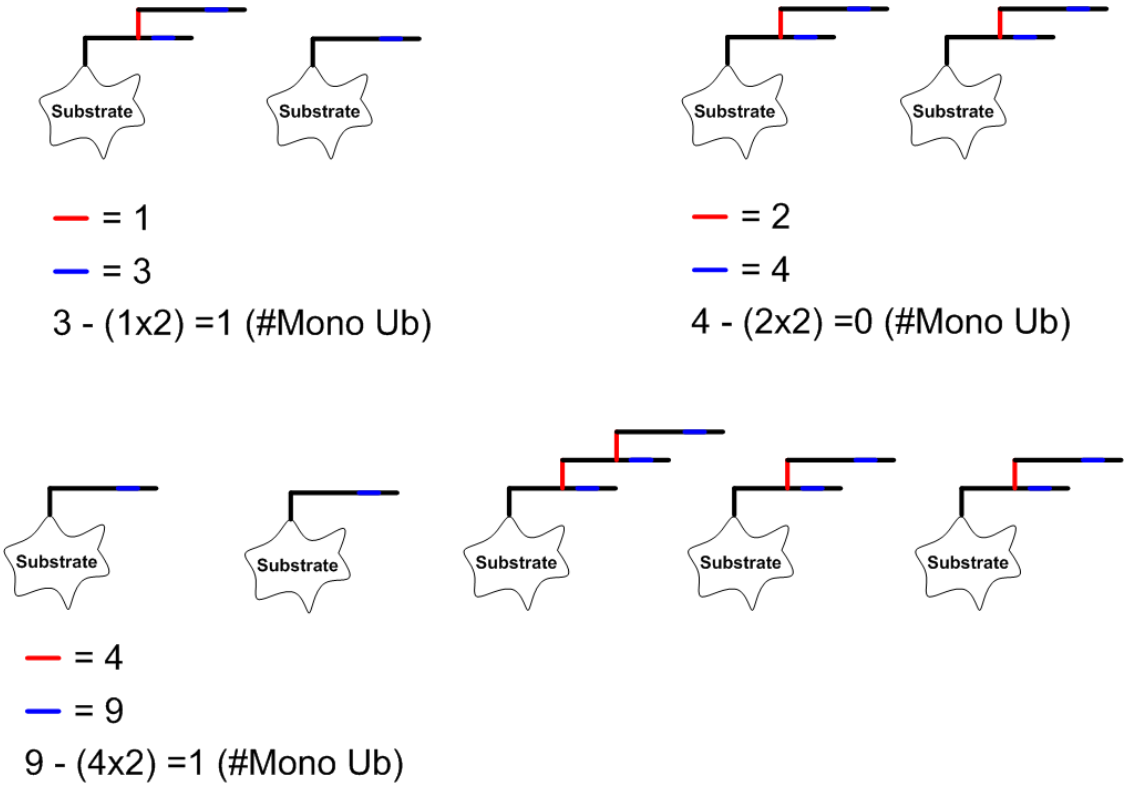


Supplementary Figure 1. a) This figure shows the western blot data for all 15 poly-ubiquitinated proteins that were enriched using the TAP tagged strains. Enriched proteins were probed using rabbit polyclonal antibody against ubiquitin protein conjugates. The protein A tag cross-reacts with anti Ub-antibody which explains the strong band at the MW corresponding to the tap-tagged proteins. The ubiquitinated forms of these proteins could not be detected by western blot. b) The western blot for the HTB2 detects the ubiquitinated forms of HTB2 in addition to the unmodified form.


Peptide 1 (detected)
Peptide 2 (not detected)
Peptide 3 (detected)
Peptide 4 (not detected)
Peptide 6 (not detected)
Peptide 8 (detected)
Peptide 10 (not detected)
Peptide 12 (not detected)
Peptide 13 (not detected)
Peptide 14 (detected)
Peptide 15 (detected)
Peptide 16 (detected)
Peptide 17 (detected)
Peptide 18 (detected)
Peptide 19 (not detected)
Peptide 20 (detected)
Peptide 21 (not detected)
Peptide 22 (not detected)
Peptide 23 (detected)
Peptide 24 (not detected)

Supplementary Figure 2. The top row of each panel shows the 5 SRM transitions (Q1/Q3) for Aqua peptides that were synthesized to assay the ubiquitin driven peptides. The bottom row of each panel shows the 5 SRM transitions for light peptides driven from tryptic digestion of ubiquitin in mono- and poly-chain forms (for the protein YPR074C in this case). If the light peptides were detected corresponding ion curves are integrated. If no integration is shown it means that peptide was not detected. Quantifications were based on the ratios between the area under the curve for heavy and corresponding light transitions.



Supplementary Figure 3. This figure shows how the minimum ubiquitin concentration is calculated. In a mixture of mono- and poly-Ub chains, the true concentration of mono-Ub cannot be calculated because the most distal Ub in a poly-Ub chain produces the same peptides as a mono-Ub chain, but a minimum concentration for mono-Ub can be calculated. Minimum mono-Ub concentration is defined as a lowest concentration of mono-Ub detectable by SRM. Minimum mono-Ub concentration is always equal or larger than the true mono-Ub concentration.

Description	Copy/cell	MQIFVK (1)	TLTGK (3)	(GG) (4)	TITLEVESSDTIDNVK (6)	(GG) (8)	EGIPPDQQR (14)	LIFAGK (15)	(GG) (16)	QLEDGR (17)	TLSDYNIQK (18)	(GG) (20)	ESTLHLVLR (23)	Mono-Ub Min Conc.	Total Ub. Conc.
YDR012W	151454	47.6±0.3	N/D	N/D	N/D	N/D	55±6	33±2	3.2±0.1	34*±6	30.7±0.3	9.5±0.5	31.2±0.2	19.6±8.6	45±8
YKL152C	171982	58.3±0.1	184*±46	N/D	N/D	N/D	75±1	50±2	6.0±0.1	52*±11	59.0±0.6	16.0±0.6	55±2	22±11.7	66±11
YLR180W	103237	91±2	170*±61	N/D	N/D	N/D	106±2	57.6±0.4	4.7±0.1	66*±8	63.6±0.3	10±1	64.9±0.5	53.6±17.1	83±16
YCR005C	2313	85±1	126*±77	8±2	N/D	N/D	139±8	67.0±0.6	8.4±0.5	56*±14	76±1	23±2	76.8±0.1	21.2±22.5	100±18
YOR027W	67559	7.5±0.1	N/D	N/D	1.3*±0.4	N/D	2.1±0.1	3.7±0.1	0.5±0	N/D	1.6±0.4	2.1±0.1	2.5±0.1	Can't confirm	4±1
YPR074C	40272	19.5±0.5	136*±2	20.0±0.6	N/D	4.9*±0.2	41±3	36.7±0	6.0±0.6	57*±13	43.9±0.3	19±0.6	39.0±0.6	Can't confirm	41±8
YDL126C	78351	78±1	395*±162	8±2	N/D	N/D	96±2	48±2	7.5±0.1	85*±7	60±2	10.6±0.2	61±1	22.8±16.3	75±14
YBL002W	443201	22.1±0.2	146±69	N/D	1.6*±0.1	N/D	N/D	14.4±0.3	0.7±0.1	7.0*±0.6	6.6±0.5	4.0±0.6	7.9±0	6.6±4.7	16±4
YNL064C	118525	123±2	296*±13	N/D	N/D	N/D	134±14	87±1	6.9±0.3	84*±0.3	85±1	16±1	84.4±0.5	67.2±22.3	113±21
YOR375C	77488	33.9±0.5	84*±20	N/D	N/D	N/D	45.0±0.6	22.2±0.1	3.1±0.2	36*±3	29.2±0.4	4.7±0.5	25.5±0.2	18.4±7.7	34±7
YGR175C	65384	70±1	155*±5	8±2	N/D	N/D	87±7	43.0±0.6	4.8±0.2	53*±6	46±1	13.2±0.5	41.4±0.3	13±14.7	65±12
YGR135W	17078	122±1	685*±180	N/D	N/D	N/D	191±13	89.7±0.3	19.8±0.2	117*±3	117±1	25.3±0.5	119±1	50.8±26.7	141±26
YKR071C	1047	34.0±0.6	97*±13	N/D	N/D	N/D	46±3	24.5±0.2	2.4±0.1	25*±0.1	30±0.6	7.0±0.6	28.2±0.2	17.2±7.7	36±7
YNL209W	103902	53±1	80*±14	N/D	N/D	N/D	76.0±0.6	41±1	N/D	59*±10	46.3±0.5	16.0±0.6	45.5±0.3	32±9.6	64±9
YLL024C	364128	11.6±0.1	42*±17	N/D	N/D	N/D	18±2	9.5±0.5	1.8±0	7.0*±0.5	12.3±0.1	3.5±0.3	11.5±0	3.4±3.3	14±3

Supplementary Table 1. Proteins used for poly-Ub characterization by SRM are listed. These proteins vary significantly in concentration in terms of copy/cell. Peptides detected by SRM in at least one protein are listed. Total Ub concentration was calculated by averaging the summation of the concentration of linkage bearing peptides and their preceding and following linear peptides. Values superscripted with a star (*) were detected outside the established linear dynamic range and were not used for total Ub concentration. All concentrations are reported in fmol/μg of digest.