Expanded View Figures

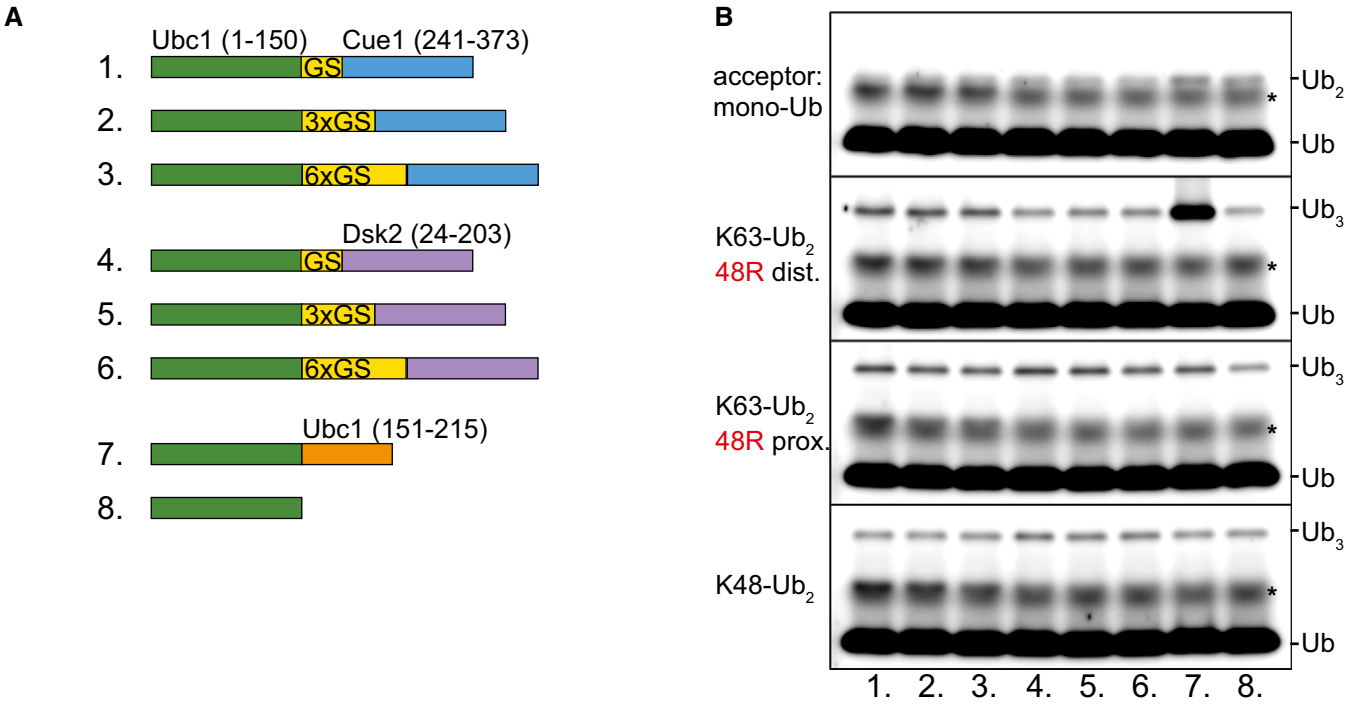


Figure EV1. Chimeric Ubc1 fusion proteins illustrate the specialized properties of the Ubc1 UBA domain.

A Fusion proteins with the UBC domain of Ubc1 (aa1-150), a linker region with one to six GlySer repeats, and either the CUE domain of Cue1 (aa24-203) or the UBA domain of Dsk2 (aa241-374) were created.

B Single turnover ubiquitination reactions in the presence of different acceptor ubiquitin molecules were performed with the fusion proteins described in (A) (enumerated 1–8). Final reaction products were analyzed by SDS–PAGE and fluorescence scan. Asterisk indicates free dye contaminant from fluorescently labeled ubiquitin.

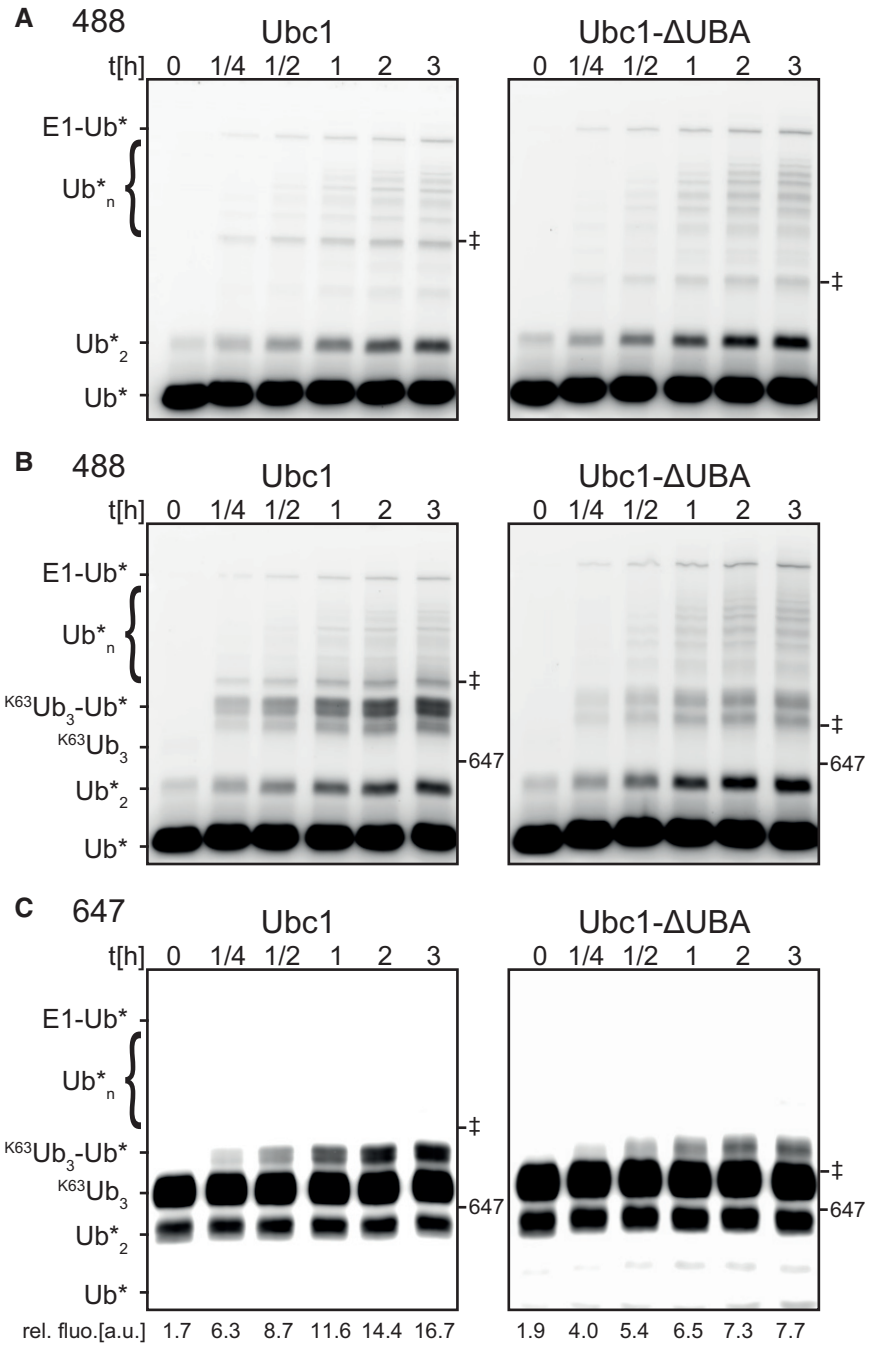


Figure EV2. Individual fluorescence scans of the in vitro ubiquitination assays comparing *de novo* chain synthesis and branching by Ubc1 in Fig 6.

A–C Images show scans from (A) Fig 6B, (B) Fig 6C (Alexa 488), and (C) Fig 6C (Alexa 647). The relative fluorescence (rel fluo.) of Ub_4 was quantified as product over total fluorescence per lane in the Alexa 647 channel. A double cross (‡) indicates the apparent molecular weight of ubiquitinated E2 enzyme. For the overlay in Fig 6C, the image from the 647 channel was cropped below the point indicated by "647".

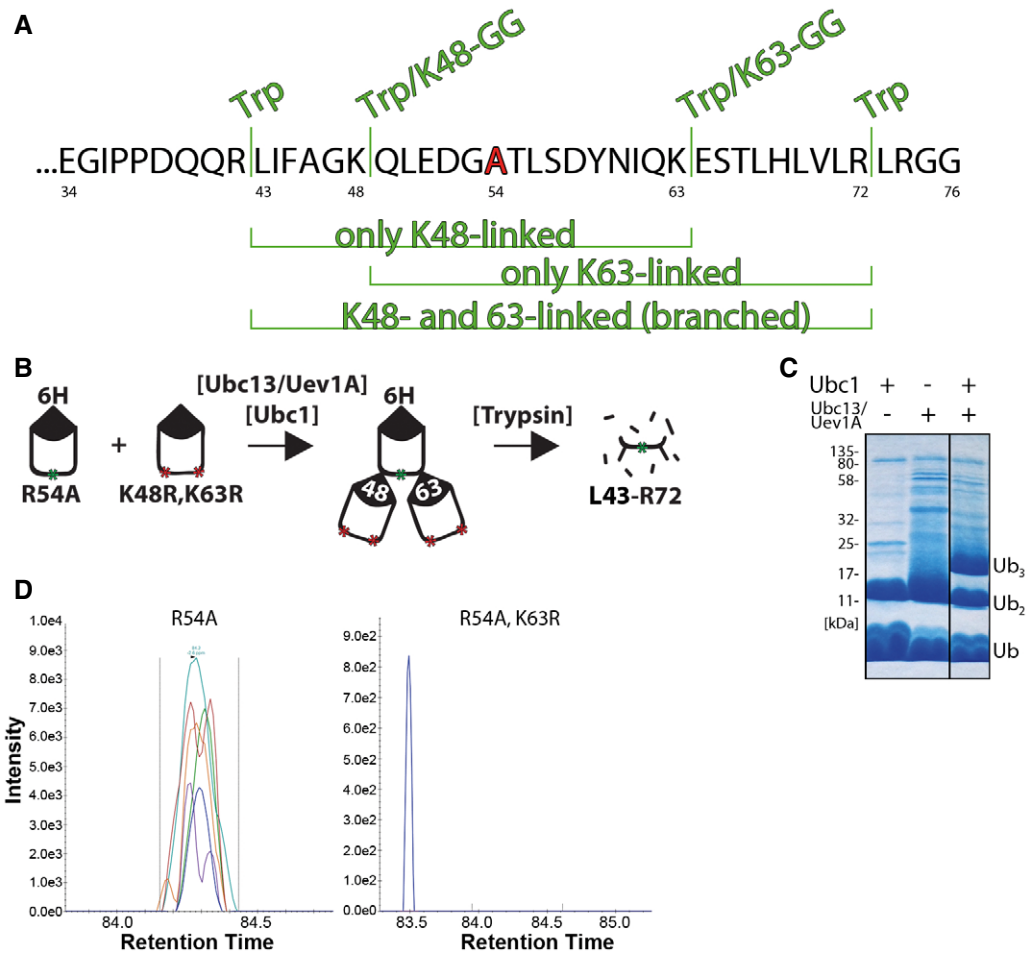


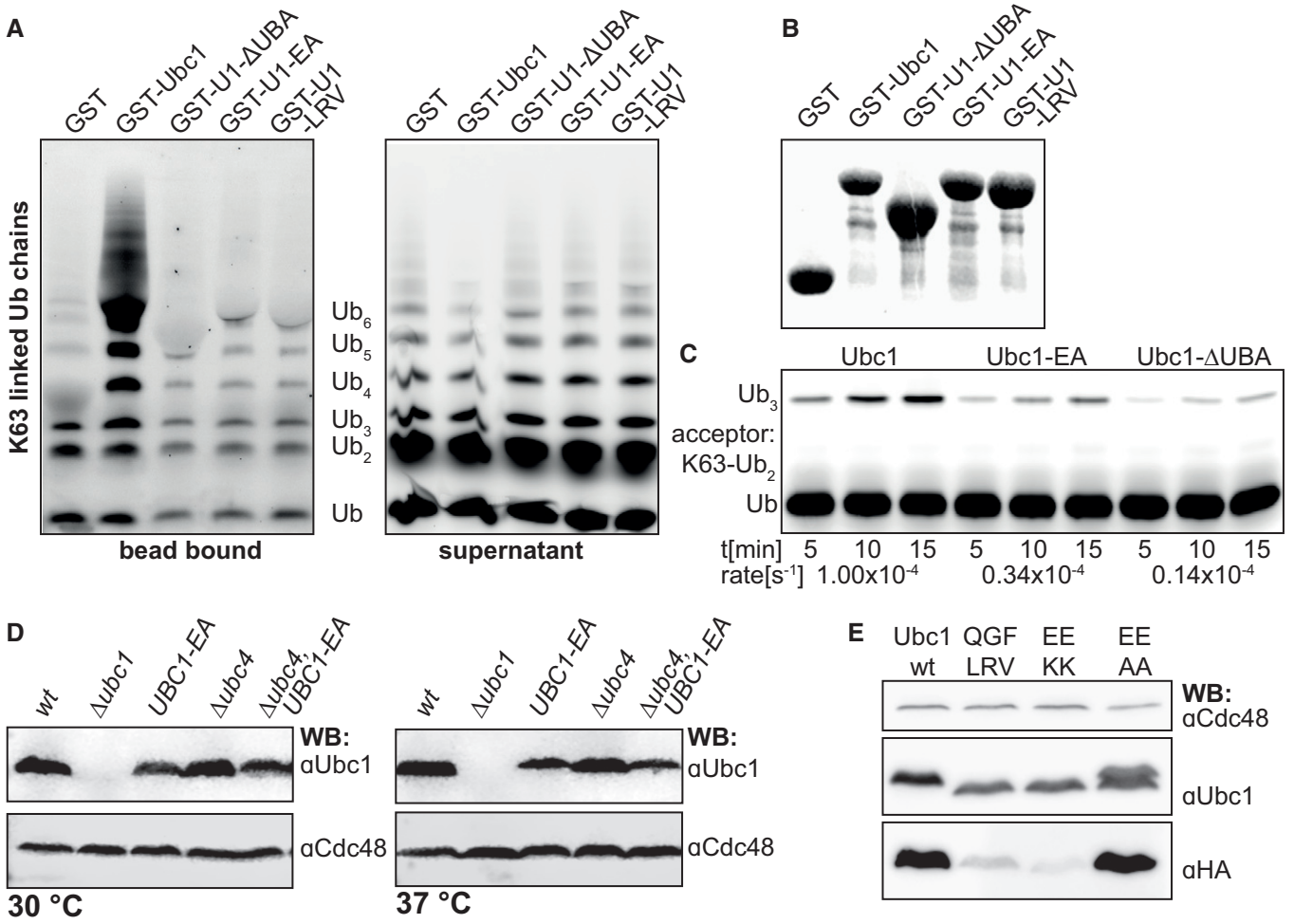
Figure EV3. Mass spectrometric detection of K48/K63-branched chains from yeast lysate by parallel reaction monitoring (PRM).

A Amino acid sequence of Ub(R54A). "Trp" indicates trypsin cleavage sites. Modification of lysine residues with ubiquitin protects these from tryptic digest (K48-GG/K63-GG). Marker peptides for homotypic chains and K48/K63-branched chains are indicated below the amino acid sequence.

B Reaction scheme for preparative assembly of K48/K63-branched triubiquitin.

C Reaction products of Ubc1 and/or Ubc13/Uev1a, respectively, were analyzed by SDS–PAGE and Coomassie staining. Branched triubiquitin was further purified and used to create a spectral library.

D Identification of K48/K63-branched ubiquitin chains by PRM from lysate of *S. Cerevisiae* expressing only either Ub(R54A) or Ub(R54A,K63R) using a modified strain of sub328, which harbors only a single copy of a ubiquitin-encoding gene (Spence *et al*, 1995). Graphs display excerpts of the chromatogram at peak retention time from the six most abundant fragment ions observed for Ub(R54A) and Ub(R54A,K63R), respectively. The dot product, as a measure to express spectra similarity, was at 0.79 for R54A when comparing the spectra from the *in situ*-generated reference and the yeast lysate. This indicates a correct identification of the peak in R54A and absence of the peak in R54A, K63R.



F

Peptide	ratio *wt*/Δ*ubc1*	ratio Δ*ubc1*/*wt*	ratio *wt*/*UBC1-EA*	ratio *UBC1-EA*/*wt*
K48/K63-branched	4.10	0.36	1.07	1.02
K48-linked(unbranched)	3.22	0.42	1.28	0.95
K63-linked(unbranched)	3.23	0.41	1.06	0.93
not-K11-linked	2.78	0.40	1.27	1.02
K11-linked	2.07	0.69	1.06	0.96

G

	UBC1-EA/*wt*			
	rep1	rep2	*37°C*	Δ*ubc4*
Ssa4	1.40	1.61	0.90	1.17
Hsp12	0.78	1.00	1.20	0.65

Figure EV4. Characterization of the ubiquitin-binding-deficient Ubc1 variant Ubc1-EA (E211A, E212A).

A A ubiquitin-binding experiment was performed as in Fig 1A/Appendix Fig S1 including GST-Ubc1-EA. Analysis by SDS–PAGE and fluorescence scan.

B Loading controls for the GST-fusion proteins used in (A) were analyzed by SDS–PAGE and Coomassie staining.

C A single turnover ubiquitination experiment as in Fig 2 with K63-Ub_2 as acceptor was performed including Ubc1-EA. Initial rates ("rate") were determined as described above.

D Expression levels of Ubc1 and Cdc48 as loading control were assessed from total cell lysate for various yeast strains at 30°C and at 37°C. Analysis by SDS–PAGE and Western blotting using the indicated primary antibodies.

E Ubc1 variants with QGF179-181 mutated to LRV or EE211-212 mutated to either KK or AA were cloned together with the endogenous promoter into the pRS416 plasmids containing a C-terminal HA-tag. Expression levels of Ubc1 variants in *S. cerevisiae* grown in minimal medium with selective amino acid mix were tested. Complete lysate was analyzed by SDS–PAGE and Western blotting using the indicated antibodies.

F Fold changes for the ubiquitin marker peptides quantified in the targeted proteomics experiments (Fig 7A–C).

G Fold changes for the proteins Ssa4 and Hsp12 after growth of yeast cells at 30°C (rep1, rep2), at 37°C, or at 30°C in a *Δubc4* deletion background as determined by shotgun proteomics experiments (Fig 7D and E).