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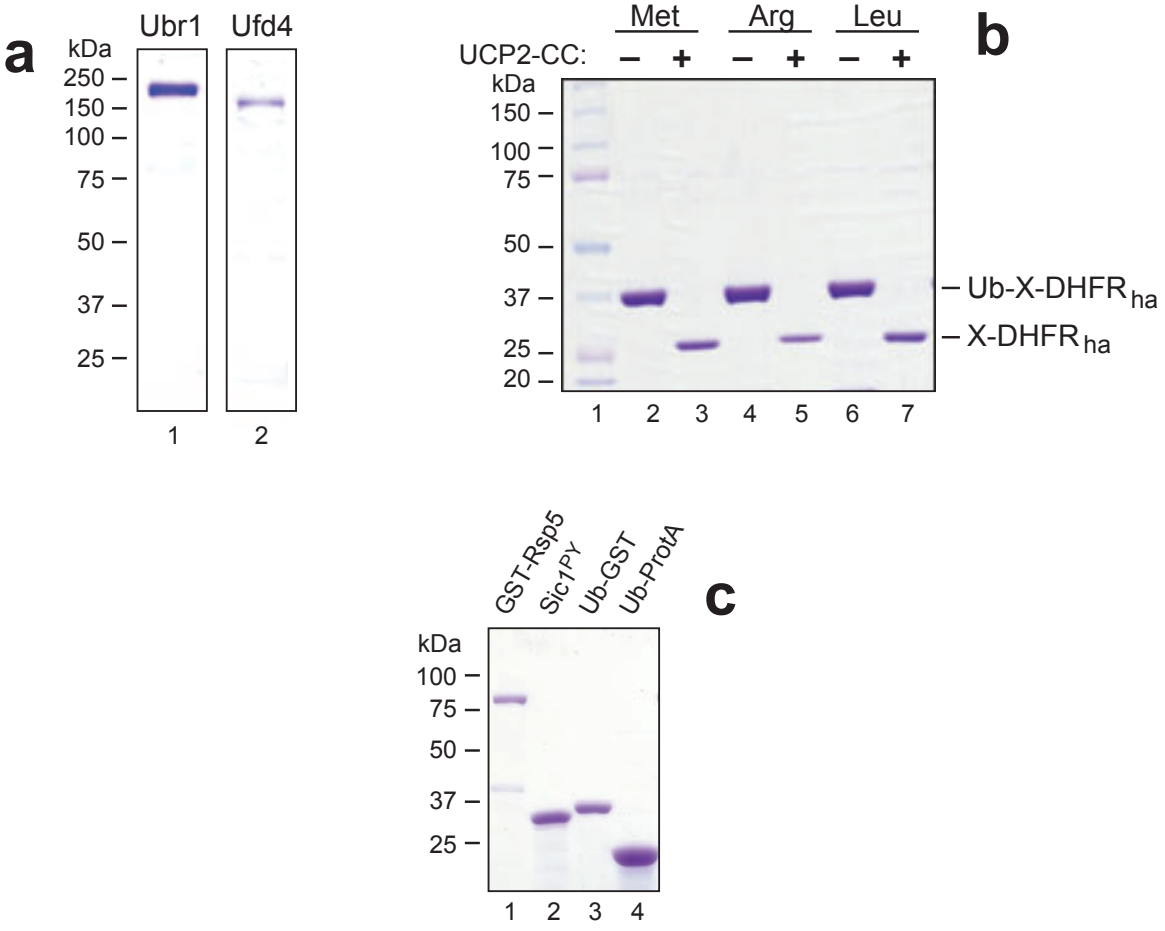

Figure S1 Purification of test proteins. (**a**) Purified *S. cerevisiae* ^{f}Ubr1 and ^{f}Ufd4 (see ref. 12). (**a**) Purification of reporter N-end rule substrates. His_{10}-tagged Ub-X-$DHFR_{ha}$ fusions (X=Met, Arg, Leu) [54,55] , denoted below (and in the main text) without e^{K}, were purified from extracts of KSP22 (*aatΔ*) *E. coli*, using the Usp2-cc DUB enzyme[55] (see Methods). Lane 1, molecular mass markers (staining with Coomassie). Lanes 2, 4 and 6, purified Ub-Met-$DHFR_{ha}$, Ub-Arg-$DHFR_{ha}$ and Ub-Leu-$DHFR_{ha}$, respectively. Lanes 3, 5 and 7, deubiquitylated Met-$DHFR_{ha}$, Arg-$DHFR_{ha}$ and Leu-$DHFR_{ha}$, respectively. (**b**) Coomassie-stained test proteins, purified as described in Methods and fractionated by SDS-PAGE. Lanes 1-4, GST-Rsp5, $Sic1^{PY}$, Ub-GST, and Ub-ProtA, respectively.

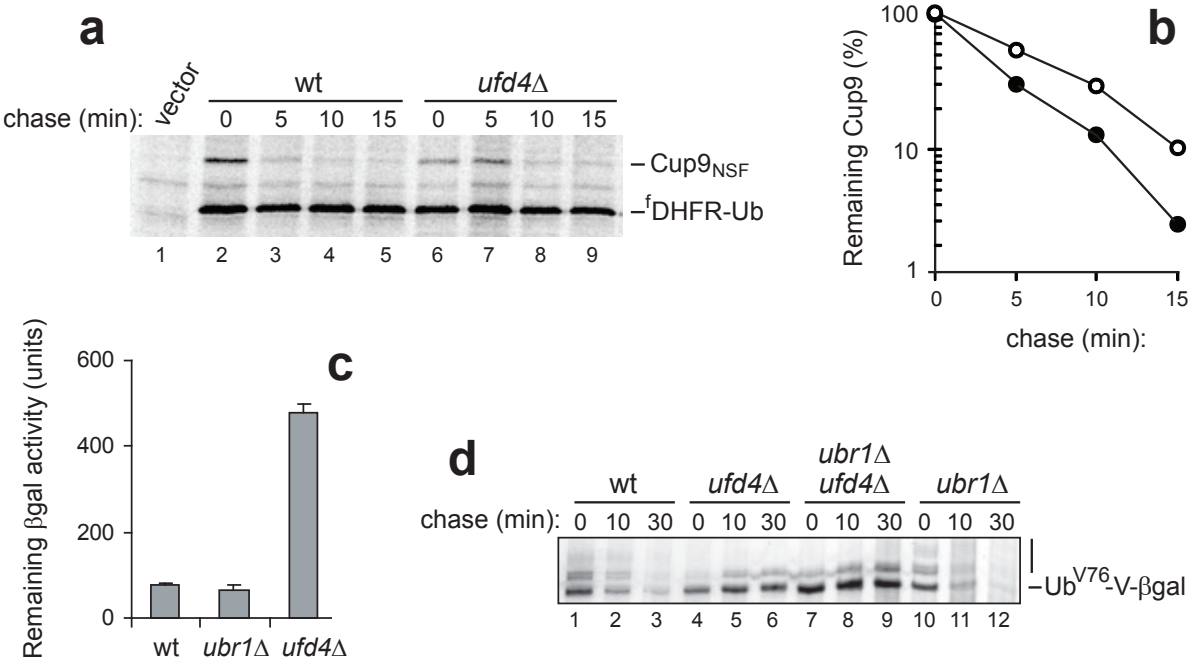

Figure S2 Pulse-chase assays with Cup9 and a UFD substrate. (**a**) Because overexpression of wild-type Cup9 is toxic in *S. cerevisiae*, these experiments utilized $CUP9_{NSF}$, a previously characterized, nontoxic, single-residue mutant with a reduced affinity to its cognate DNA sites but no significant changes in the rate of Cup9 degradation *in vivo*[5]. $Cup9_{NSF}$ was expressed as ^{f}DHFR-Ub^{K48R}-$Cup9_{NSF}$, in which ^{f}DHFR was the N-terminally flag-tagged mouse DHFR moiety. DUBs cotranslationally cleave this fusion at the Ub^{K48R}-$Cup9_{NSF}$ junction[5,27], yielding the long-lived reference protein ^{f}DHFR-Ub^{K48R} and the test protein $Cup9_{NSF}$. *S. cerevisiae* JD52 (wild-type) (lanes 2-5), and CHY194 (*ufd4Δ*) (lanes 6-9) expressing ^{f}DHFR-Ub^{K48R}-$^{f}Cup9_{NSF}$ were grown at 30°C to A_{600} of ~0.8, followed by labeling for 5 min with ^{35}S-methionine/cysteine and a chase (in the presence of cycloheximide) for 0, 5, 10 and 15 min at 30°C. Cell extracts were immunoprecipitated with anti-flag, followed by SDS-PAGE and autoradiography. The bands of $Cup9_{NSF}$ and ^{f}DHFR-Ub^{K48R} are indicated on the right. Lane 1, vector alone (control). (**b**) Quantitation of ^{35}S-patterns in **a**. Open and closed circles, relative levels of pulse-labeled $Cup9_{NSF}$ in wild-type and *ufd4Δ* cells, respectively. Note a partial stabilization of $Cup9_{NSF}$ in *ufd4Δ* cells. (**c**) Relative levels (enzymatic activity) of Ub^{V76}-V-βgal in extracts of *S. cerevisiae* RJD347 (wild-type), AVY26 (*ubr1Δ*) and CHY251 (*ufd4Δ*). Cells were grown to A_{600} of ~0.8 in a SRGal medium at 30°C, followed by measurements of βgal activity in cell extracts. (**d**) No significant contribution by the Ubr1 E3 N-recognin to the rate of *in vivo* degradation of Ub^{V76}-Val-βgal, a substrate of the UFD pathway. *S. cerevisiae* JD52 (wild-type) (lanes 1-3), CHY194 (*ufd4Δ*) (lanes 4-5), CHY195 (*ubr1Δ ufd4Δ*) (lanes 7-9), and JD55 (*ubr1Δ*) (lanes 10-12) that expressed Ub^{V76}-Val-βgal were grown in SRGal medium to A_{600} of ~0.8 at 30°C, and were labeled with ^{35}S-methionine/cysteine for 5 min, followed by a chase for 0, 10 and 30 min (in the presence of cycloheximide), immunoprecipitation with anti-βgal, SDS-6% PAGE, and autoradiography. Bands of polyubiquitylated Ub^{V76}-Val-βgal are indicated on the right. Although Ub^{V76}-Val-βgal is virtually completely stabilized in both *ufd4Δ* and *ubr1Δ ufd4Δ* cells (lanes 4-6, 7-9)[38], it is still partially monoubiquitylated in these cells, by an unknown E3.

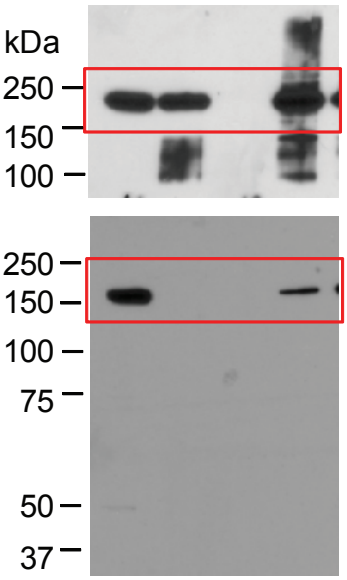

Fig. 3a

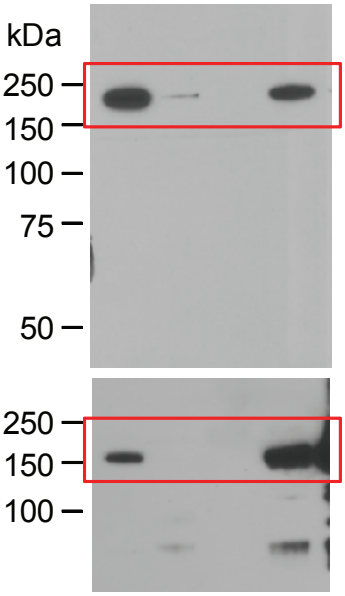

Fig. 3b

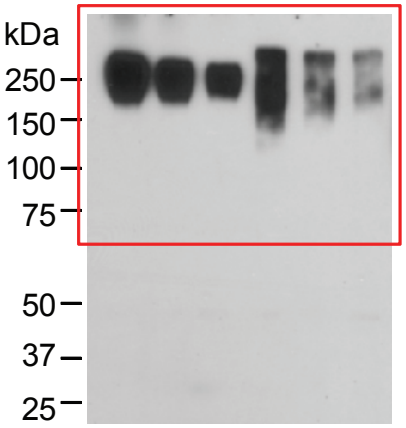

Fig. 4d

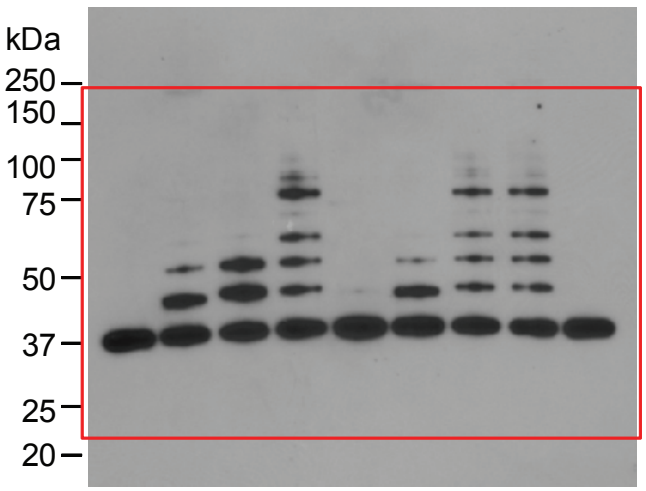

Fig. 6b

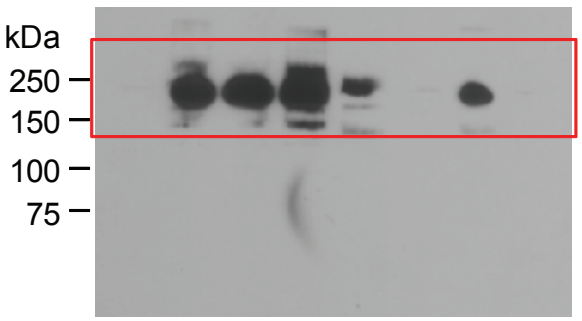

Fig. 6c

Figure S3 Uncropped images of key immunoblots shown in individual figures.