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SUPPLEMENTAL INFORMATION FOR:

A Structurally Unique E2-Binding Domain Activates Ubiquitination by the ERAD E2, Ubc7p, Through Multiple Mechanisms

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SUPPLEMENTAL INVENTORY OF DATA

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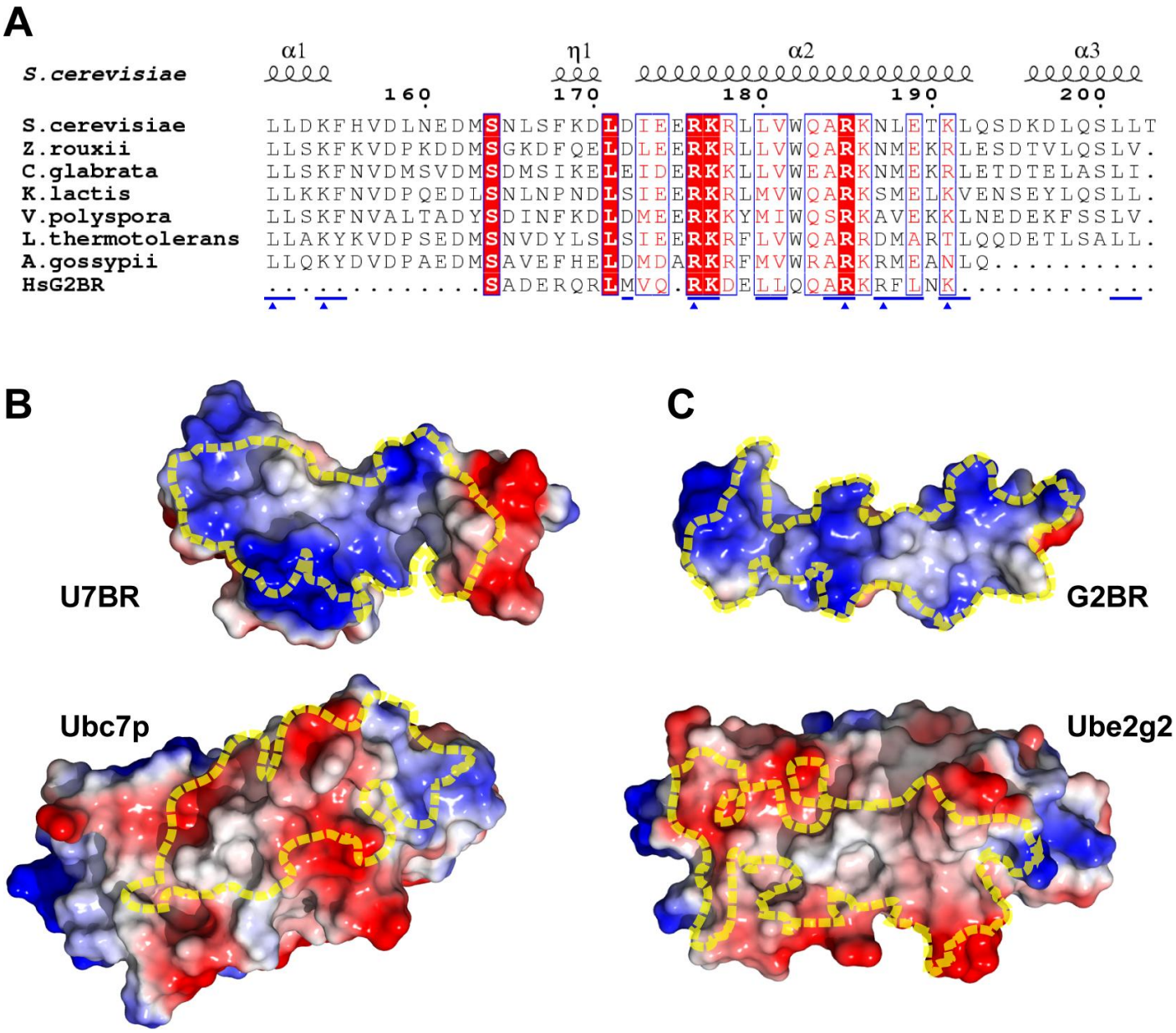
Supplemental Table 1. Yeast strains and yeast/bacterial expression plasmids used in this study

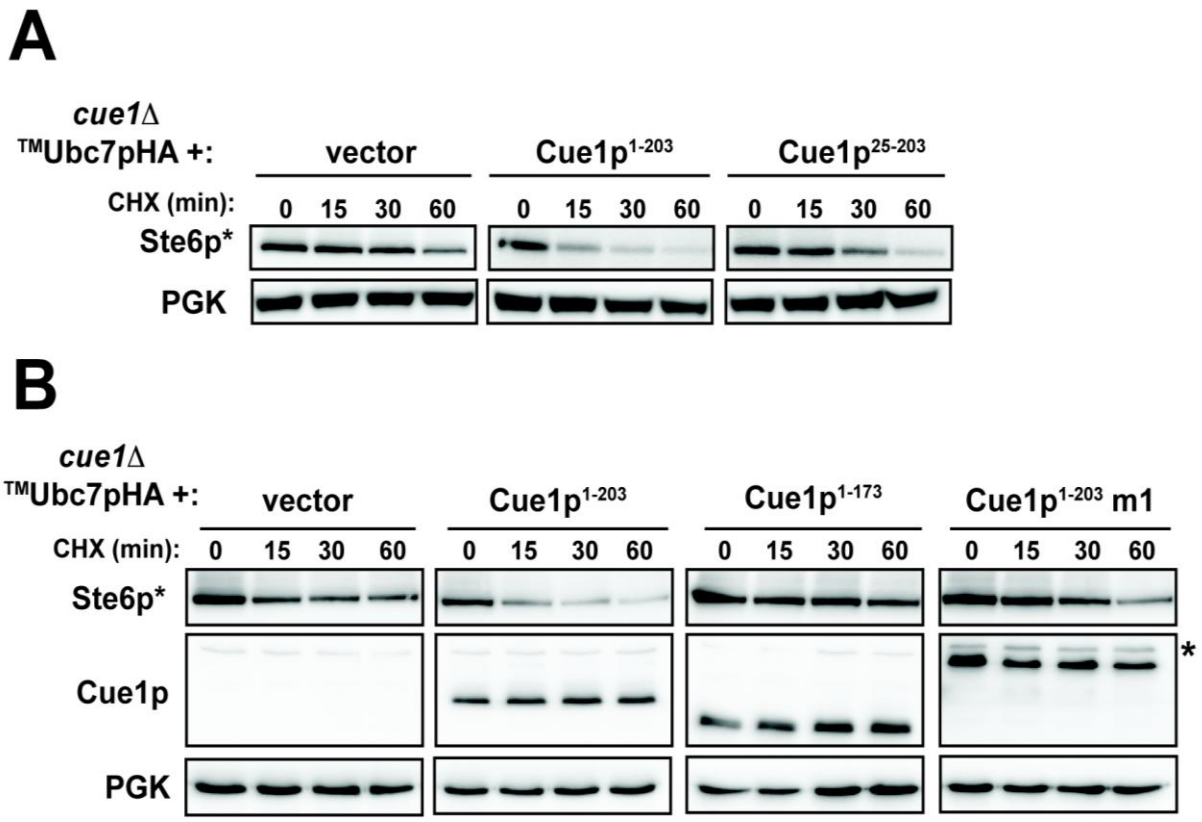
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SUPPLEMENTAL REFERENCES

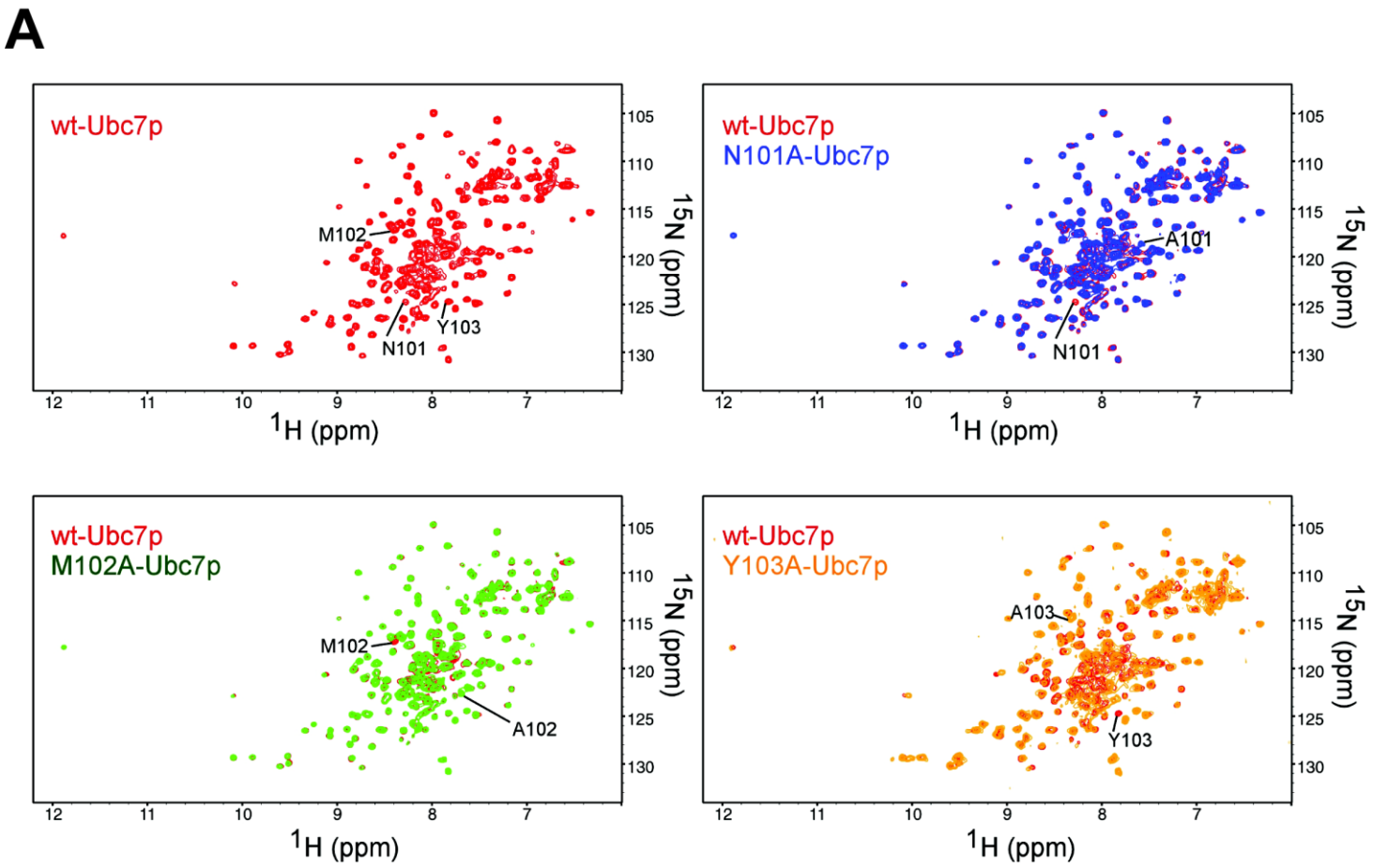
Metzger *et al.* Supplemental Figure 1

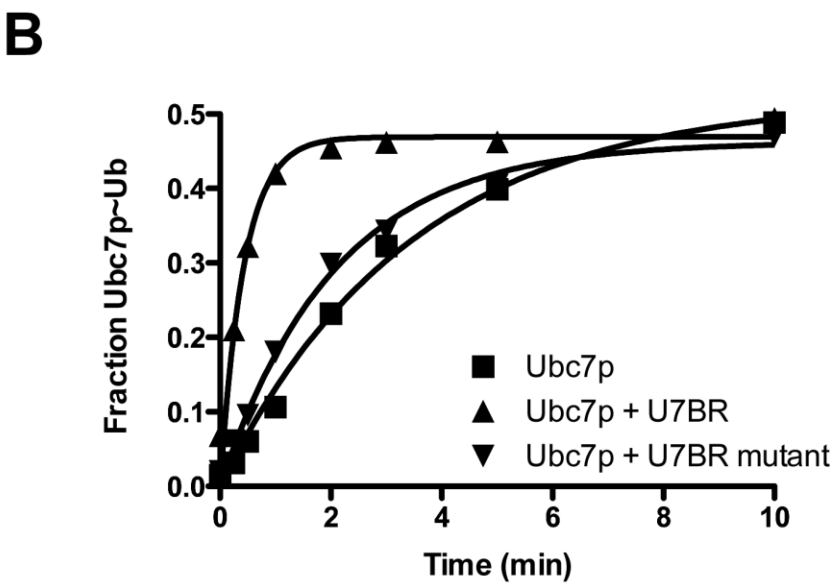


Metzger *et al.* Supplemental Figure 2

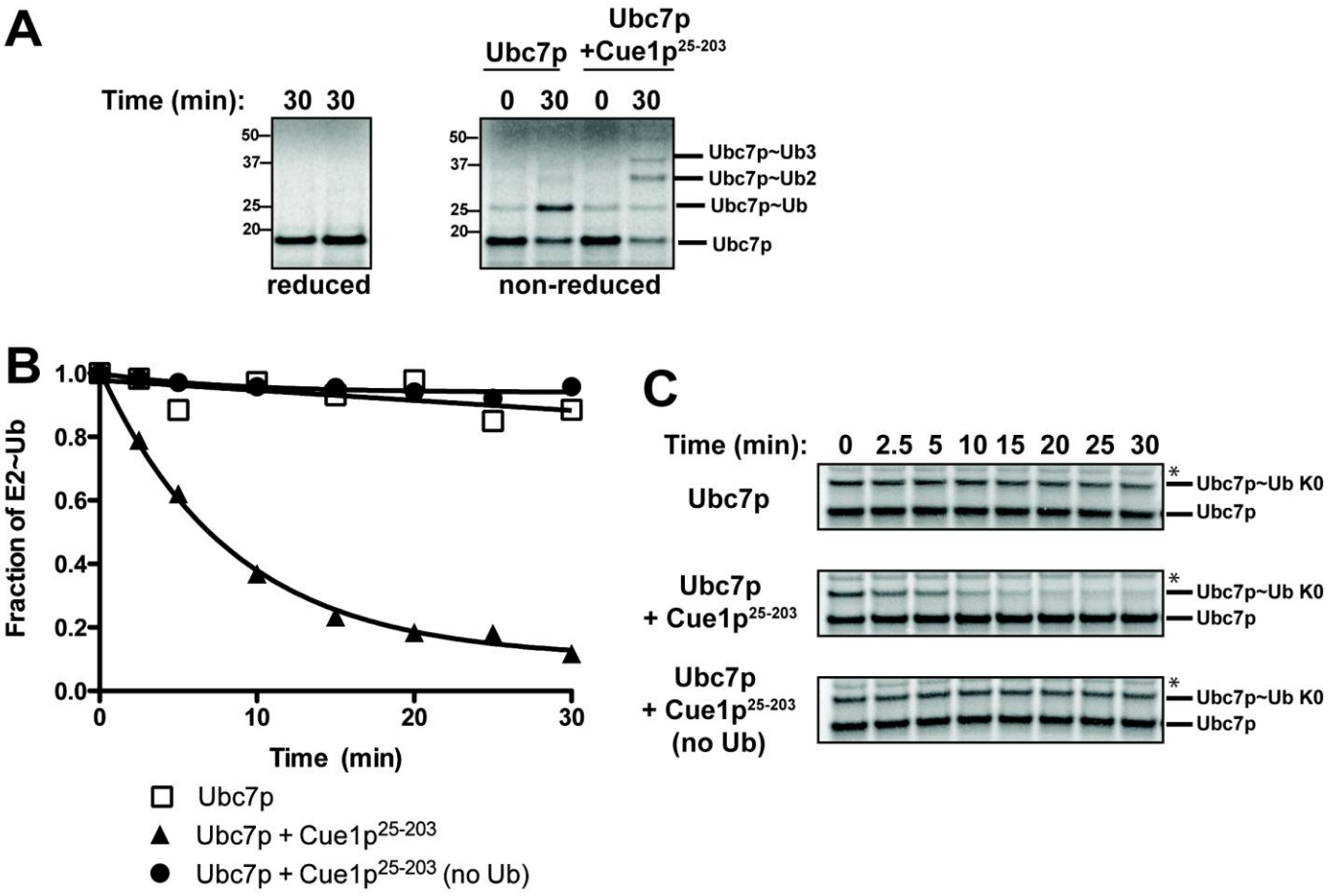


Metzger *et al.* Supplemental Figure 3

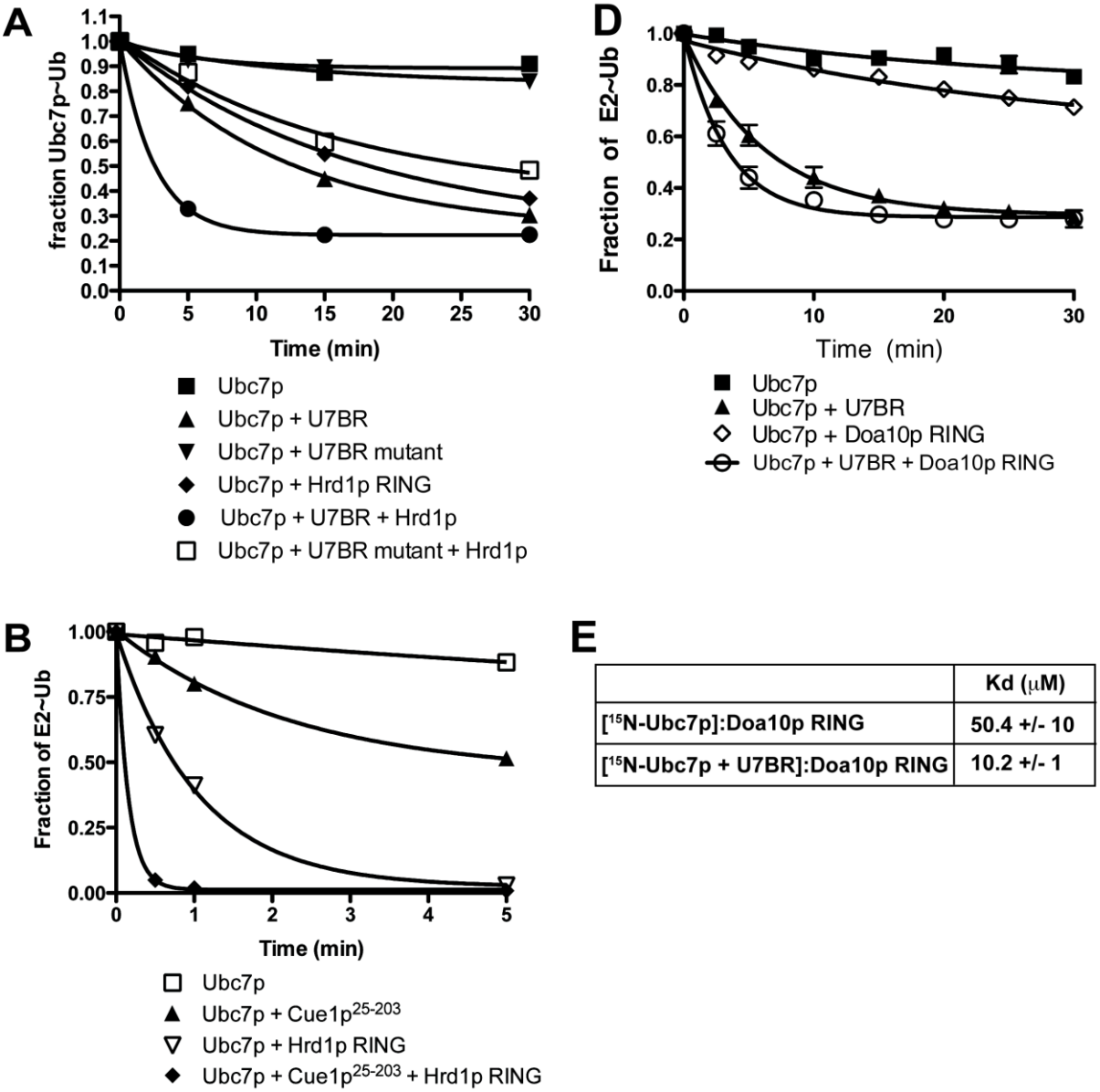




Metzger *et al.* Supplemental Figure 4



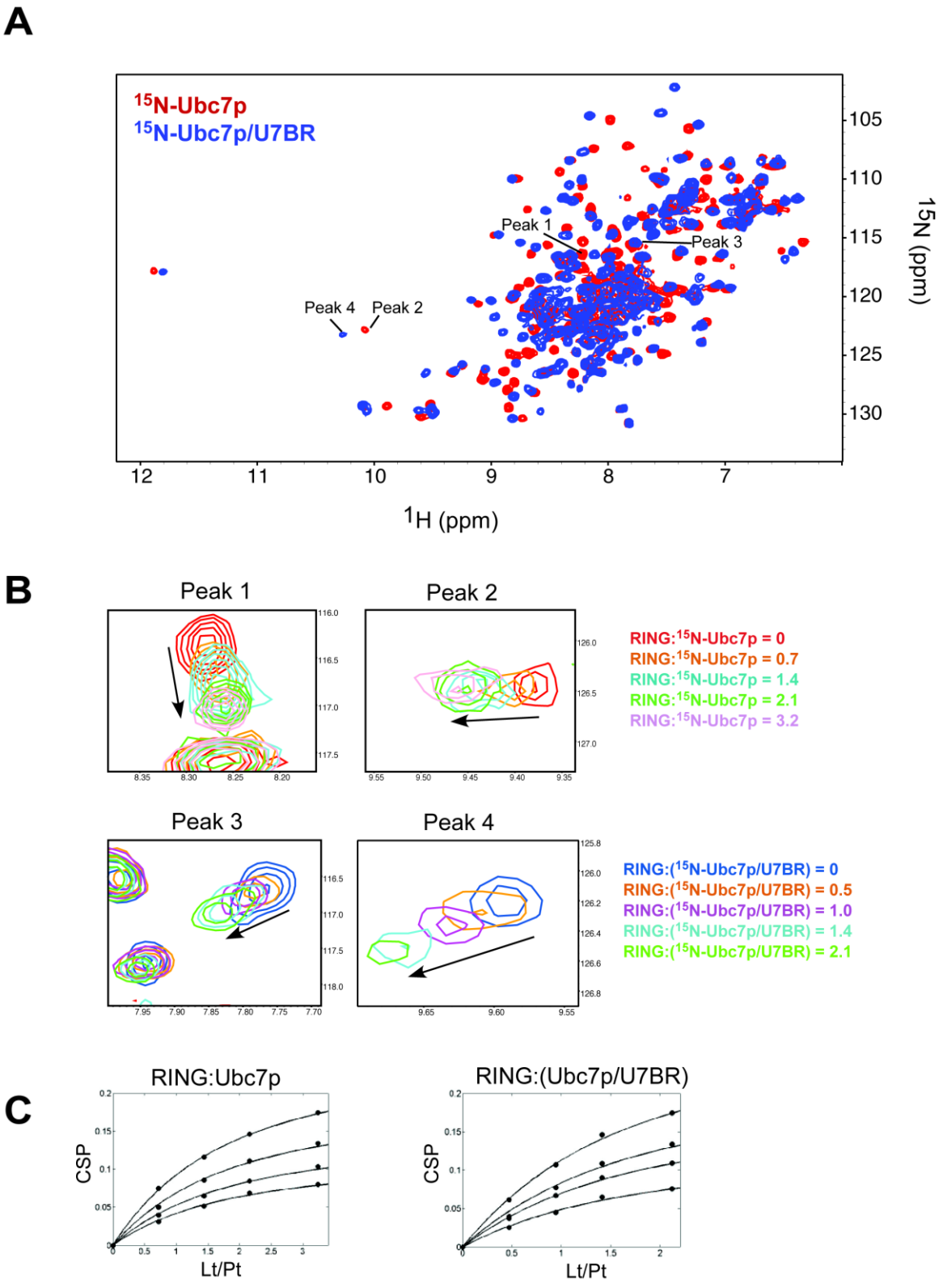
Metzger *et al.* Supplemental Figure 5



E

	Kd (μM)
[^{15}N-Ubc7p]:Doa10p RING	50.4 +/- 10
[^{15}N-Ubc7p + U7BR]:Doa10p RING	10.2 +/- 1

Metzger *et al.* Supplemental Figure 6



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1, related to Figure 1. A comparative analysis of the U7BR (A) Structure-based sequence alignment of U7BR homologues and the G2BR of mammalian gp78. Background in red indicates sequence identity; single-letter code in red indicates sequence similarity. The blue lines under the sequences indicate residues that are involved in Ubc7p-U7BR interactions and the blue triangles indicate residues that form hydrogen bonds or salt bridges with Ubc7p. (B, C) Surface electrostatic potential (blue for positive, red for negative) at the Ubc7p-U7BR (B) and Ube2g2-G2BR (C) interfaces. The contacting area on the surface (buried surface) of each molecule is outlined (dashed yellow lines). The buried surface is generated using program VASCo (Steinkellner et al., 2009) and visualized with Pymol (DeLano Scientific LLC).

Supplemental Figure 2, related to Figure 3. Degradation of the Doa10p ERAD substrate, Ste6p*, is inhibited by deletion of the Cue1p transmembrane domain or deletion or mutation of the U7BR (A) The turnover of Ste6p* was assessed by cycloheximide (CHX) chase at the indicated times points using a *cue1Δ* strain containing a chromosomal copy of ^{TM}Ubc7pHA transformed with a low copy plasmid encoding Ste6p*HA and a low copy plasmid encoding either Cue1p$^{1-203}$ or Cue1p$^{25-203}$, or vector (pRS315) as a control. Protein stability was analyzed by SDS-PAGE and immunoblotting by anti-HA antibodies (Ste6p*). PGK is used as a loading control. (B) As in (A) except with the indicated Cue1p-encoding plasmids. Immunoblotting was also done with polyclonal Cue1p antiserum to

assess the stability of the mutant proteins. The asterisk indicates a cross-reactive band seen with this antiserum.

Supplemental Figure 3, related to Figure 4. NMR assignment of residues for assessing β4α2 loop dynamics and stimulation of Ubc7p~Ub thioester formation by U7BR (A) Peak assignments of residues in the β4α2 loop were obtained by the analysis of individual point mutations of residues in this loop. The ^{15}N-edited HSQC spectrum of wild-type Ubc7p (red) was compared with that of Ubc7p-N101A (blue), Ubc7p-M102A (green), and Ubc7p-Y103A (orange). In each of the mutant spectra, the peak with a drastic shift is assigned as the mutated residue; the rest of the spectrum overlaps closely with that of the wild-type protein. (B) A binding-defective U7BR mutant does not stimulate Ubc7p ubiquitin thioester formation. A representative quantification of the rate of ubiquitin loading of *in vitro* transcribed and translated ^{35}S-labeled Ubc7p. ^{35}S-Ubc7p was incubated at 12°C with ubiquitination mix containing K0 ubiquitin and human E1 alone, or with cleaved Cue1p$^{151-203}$ wild-type or a form mutated in multiple contact residues throughout the U7BR (I173A R176A K177A A184G R185A L192A L201A L202A) for the indicated times. Data are graphed as the fraction of E2~Ub remaining at each time point.

Supplemental Figure 4, related to Figure 5. Cue1p$^{25-203}$ retains the RING-independent stimulatory effects of the U7BR (A) ^{35}S-Ubc7p was incubated at 30°C with ubiquitination mix containing wild-type ubiquitin, human E1, and with or

without cleaved Cue1p$^{25-203}$ for 0 and 30 min. Samples were reduced as indicated by the addition of 10% β-mercaptoethanol. (B) Quantification of a representative experiment analyzing the rate of discharge of the ^{35}S-Ubc7p thioester. Ubc7p was first loaded with K0 ubiquitin, followed by apyrase treatment to deplete ATP and inactivate E1 prior to the addition of wild-type ubiquitin (80 μM) and Cue1p$^{25-203}$ (4 μM), or both. (C) SDS-PAGE showing the representative discharge experiment quantified in (B). The asterisk indicates a Promega *E. coli* T7 S30 Extract System lot-dependent non-specific band.

Supplemental Figure 5, related to Figure 6. RING-dependent stimulatory effects of the U7BR and Cue1p$^{25-203}$ (A) A representative quantification of the discharge rate of the ^{35}S-Ubc7p thioester at the indicated time points in the presence of wild-type ubiquitin (80 μM) and combinations of 4μM cleaved wild-type U7BR (Cue1p$^{151-203}$) or the U7BR mutant described in Supplemental Figure 3B and the Hrd1p RING (5μM; amino acids 321-551), as indicated. Data are graphed as the fraction of E2~Ub remaining at each time point. (B) Quantification of a representative discharge experiment at the indicated time points performed as described in (A), except in the presence of wild-type ubiquitin (80 μM) alone, or plus Cue1p$^{25-203}$ (4 μM), plus the Hrd1p RING (5 μM), or plus both Cue1p$^{25-203}$ and RING. (C) SDS-PAGE showing a representative discharge experiment described in (B). The asterisk indicates a Promega *E. coli* T7 S30 Extract System lot-dependent non-specific band. (D) Quantification of discharge experiments at the indicated time points performed as described in (A), except in the presence of

wild-type ubiquitin (80 μM) alone, or plus the U7BR (4 μM), plus the Doa10p RING (5 μM; amino acids 1-114), or plus both U7BR and RING. Data are graphed as the fraction of E2~Ub remaining at each time point from the average of three independent discharge experiments. Error bars represent the standard errors. (E) K_d values for ^{15}N-Ubc7p alone or plus the U7BR for the Doa10p RING were calculated by NMR. Errors represent the standard deviation from mean; values were calculated over all peaks that shifted upon binding to the Doa10p RING.

Supplemental Figure 6, related to Figure 6. NMR assessment of altered affinity of Ubc7p: Hrd1p RING with U7BR (A) ^{15}N-edited HSQC spectrum of U7BR bound ^{15}N-labeled Ubc7p (blue) is superimposed with that of free ^{15}N-labeled Ubc7p (red). Shifts are observed in several peaks when the U7BR binds, indicating that although the overall UBC fold is maintained, minor environmental changes get transmitted throughout Ubc7p upon U7BR binding. (B) Trajectories of two Ubc7p (peaks 1 and 2) or U7BR-bound Ubc7p (peaks 3 and 4) peaks that shift upon titration with the Hrd1p-RING domain. (C) NMR titration data for four peaks in Ubc7p and Ubc7p:U7BR spectra, which has been fit to a 1:1 model to obtain the dissociation constants given in Figure 6.

Supplemental Table 1. Yeast strains and yeast/bacterial expression plasmids used in this study

	Relevant genotype/protein product	Reference
Yeast strains		
W303-1C *ubc7Δcue1Δ*	*MAT**a** ura3-1 leu2-3,112 his3-11,15 ade2-1 trp1-1 can1-100 prc1-1*	(Kostova et al., 2009)
BY4741 *cue1Δ* ^{TM}Ubc7pHA	*MAT**a** his3Δ leu2Δ ura3Δ cue1::KanMX* ^{TM}*Ubc7pHA*	This study
Yeast plasmids		
pRS314 (CEN/*TRP1*)		(Sikorski and Hieter, 1989)
pRS314-GFP*CUE1*$^{110-203}$	pRS314-GFP-Cue1p$^{110-203}$	(Kostova et al., 2009)
pRS316 (CEN/*URA3*)		(Sikorski and Hieter, 1989)
pRS315 (CEN/*LEU2*)		(Sikorski and Hieter, 1989)
pRS315-*CUE1*$^{1-203}$	pRS315-Cue1p$^{1-203}$	This study
pRS315-*CUE1*$^{25-203}$	pRS315-Cue1p$^{25-203}$	This study
pRS315-*CUE1*$^{1-173}$	pRS315-Cue1p$^{1-171}$	This study
pRS316-^{TM}*UBC7*HA	pRS316-^{TM}Ubc7pHA	(Kostova et al., 2009)
pRS316-*UBC7*HA	pRS316-Ubc7pHA	(Kostova et al., 2009)
pMM21	pRS413-Ste6p*	This study
pMM50	pRS315-Cue1p$^{1-203-m1}$ (L151A L152A K154A F155A)	This study
pMM115	pRS314-GFP-Cue1p$^{110-203-m1}$ (L151A L152A K154A F155A)	This study
pMM116	pRS314-GFP-Cue1p$^{110-203-m2}$ (I173A R176A K177A)	This study
pMM117	pRS314-GFP-Cue1p$^{110-203-m3}$ (L180A V181A)	This study
pMM118	pRS314-GFP-Cue1p$^{110-203-m4}$ (A184G R185A)	This study
pMM119	pRS314-GFP-Cue1p$^{110-203-m5}$ (N187A L188A E189A)	This study
pMM120	pRS314-GFP-Cue1p$^{110-203-m6}$ (L192A L201A L202A)	This study
Bacterial plasmids		
pMM4	pGEX-6P-1-GST-Ubc7p	This study
pMM14	pETDuet-1-GST-Ubc7p + Cue1p$^{151-203}$	This study
pMM18	pGEX-6P-1-GST- Cue1p$^{25-203}$	This study
pMM20	pGEX-6P-1-GST-Cue1p$^{151-203}$	This study
pMM23	pGEX-4T-1-GST-Cue1p$^{151-203}$ L151A L152A F154A K155A (U7BR^{m1})	This study
pMM24	pGEX-4T-1-GST-Cue1p$^{151-203}$ L192A L201A L202A (U7BR^{m2})	This study
pMM28	pGEX-6P-1-GST-Cue1p$^{151-203}$ I173A R176A K177A (U7BR^{m3})	This study
pMM30	pGEX-6P-1-GST-Cue1p$^{151-203}$ L180A V181A (U7BR^{m4})	This study
pMM32	pGEX-6P-1-GST-Cue1p$^{151-203}$ A184G R185A (U7BR^{m5})	This study
pMM34	pGEX-6P-1-GST-Cue1p$^{151-203}$ N187A L188A E189A (U7BR^{m6})	This study
pMM43	pET3a-Ubc7p	This study
pMM44	pGEX-6P-1-Doa10p$^{1-114}$	This study
pMM65	pGEX-6P-1-GST-Cue1p$^{151-203}$ I173A R176A K177A A184G R185A L192A L201A L202A (U7BR mutant)	This study
pMM72	pET3a-Ubc7p^{C39A}	This study
pMM73	pET3a-Ubc7p^{C89A}	This study
pMM74	pET3a-Ubc7p^{C141A}	This study
pMM75	pET3a-Ubc7p$^{C39A\ C141A}$	This study
pMM96	pGEX-6P-1-GST-Hrd1p$^{321-551}$	This study
pMM154	pET3a-Ubc7p-N101A	This study
pMM155	pET3a-Ubc7-M102A	This study
pMM156	pET3a-Ubc7p-Y103A	This study
GB1-His6-Thr-Ubc7p	pET3a-GB1-His6-Thrombin-Ubc7p	This study

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast strains, media, and growth conditions

The *S. cerevisiae* strains used in this study are listed in Supplemental Table 1. Cells were cultured at 30°C in SD medium supplemented with the appropriate amino acids.

Plasmid Constructs

The plasmids used in this study are listed in Supplemental Table 1. The relevant regions of all constructs were confirmed by sequencing. Ubc7p was subcloned via the BamHI and XhoI sites from pGEX-4T-1*UBC7* (Kostova et al., 2009) into pGEX-6P-1 (GE Healthcare) to generate pMM4. pMM14 was generated by first PCR amplifying GST from pGEX-6P-1 with flanking NcoI and BamHI sites and then cloning into pETDuet-1 (Novagen) cut at the same sites, replacing the endogenous HIS tag with GST. Next, Ubc7p was PCR amplified from pGEX-4T-1*UBC7* with flanking BamHI and NotI sites and cloned into the same sites in the first multiple cloning site of pETDuet-1-GST. Finally, Cue1p$^{151\text{-}203}$ was PCR amplified from pGEX-4T-1*cue1*$^{151\text{-}203}$ with flanking NdeI and XhoI sites and added to the same sites in multiple cloning site two to generate pMM14. Cue1p$^{25\text{-}203}$ and Cue1p$^{151\text{-}203}$ were subcloned from pGEX-4T-1*cue1*$^{25\text{-}203}$ and pGEX-4T-1*cue1*$^{151\text{-}203}$ (Kostova *et al.* 2009), respectively, using the BamHI and XhoI sites into the same sites in pGEX-6P-1 to generate pMM18 and pMM20. pMM43 was created by PCR amplifying Ubc7p from pDEST47-*UBC7* (Kostova *et al.* 2009), adding flanking NdeI and BamHI sites, and subcloning it into pET3a using the

same sites. Hrd1p$^{321-551}$ was PCR amplified from yeast genomic DNA with flanking BamHI and XhoI sites and subcloned into the same sites in pGEX-6P-1 to generate pMM96. pMM23, pMM24, pMM28, pMM30, pMM32, pMM34, pMM65, pMM72, pMM73, pMM74, pMM75, pMM154, pMM155, and pMM156 were created using Stratagene's QuikChange® XL Site-Directed Mutagenesis Kit. pRS314-GFP*CUE1*$^{110-203}$ mutant plasmids (pMM115, pMM116, pMM117, pMM118, pMM119, and pMM120) were all generated by recombinational cloning in yeast. Each mutation-containing Cue1p$^{110-203}$ insert was PCR amplified from the respective pGEX-6P-1-Cue1p$^{110-203}$ mutant plasmid and recombined *in vivo* into pRS314-GFP*CUE1*$^{110-203}$ digested with ClaI.

Expression and Protein Purification

For purification of Ubc7p or the Ubc7p:U7BR (Cue1p$^{151-203}$) complex, *E. coli* BL21 star cells (Invitrogen) carrying the appropriate plasmid were grown at 37°C in 2x YT media until OD_{600}=0.8-1.0. Protein expression was induced with 200 μM IPTG for 18 hours at 30°C prior to harvesting. Cells were lysed using an APV-2000 homogenizer (Aluminum Plant & Vessel Company Limited) and protein supernatant was collected by centrifugation. GST was cleaved from GST-fusion proteins by incubation with PreScission protease (GE Healthcare), after first binding to Glutathione Sepharose™4B (GE Healthcare). Cleaved protein was collected and purified on a HiPrep 16/60 Sephacryl S-300 HR sizing column (GE Healthcare).

Hrd1p$^{321-551}$ and Doa10p$^{1-112}$ were expressed and purified as above, except 50 μM ZnOAc was added prior to IPTG induction at 23°C. Cue1p$^{151-203}$ wild-type and mutants were also expressed and purified as above, except they were expressed in Rosetta 2(DE3)pLysS cells (Novagen) and induced with IPTG at 23°C. Ubc7p$^{C39A\ C141A}$ was expressed and purified as wild-type Ubc7p above, except IPTG induction was carried out at 23°C and the protein pellet was saved after centrifugation and refolded in 4 M urea before purification on the S-300 sizing column. Ubiquitin K0 expressed from pET3a in Rosetta 2(DE3)pLysS cells was purified as described (You *et al.* 1999).

Cycloheximide chase

Yeast cells were grown at 30°C in selective media to OD_{600}=0.8-1.0. Cycloheximide was added at a final concentration of 100 μg/mL and cells were removed at the indicated time points into an equal volume of 20 mM sodium azide. Cells were lysed in 1% β-mercaptoethanol/250 mM NaOH and proteins were precipitated in 5% trichloroacetic acid (TCA). Protein pellets were resuspended in TCA sample buffer (3.5% SDS, 0.5 M DTT, 80 mM Tris pH8.8, 8 mM EDTA, 15% glycerol, 0.1 mg/mL bromphenol blue) and analyzed by SDS-PAGE and immunoblotting with rat monoclonal anti-HA-Peroxidase (Roche), mouse monoclonal anti-GFP (Santa Cruz), rabbit anti-Cue1p (Kostova et al., 2009), and mouse monoclonal anti-phosphoglycerate kinase (PGK; Molecular Probes) antibodies and visualization with SuperSignal® West Pico Luminol Enhancer Solution (Thermo Scientific) and a G:box (Syngene).

***In vivo* co-immunoprecipitation**

For *in vivo* co-immunoprecipitations, 25 OD_{600} units of yeast cells grown at 30°C in selective media to OD_{600}=0.8-1.0 were resuspended in 300 μl of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 5% glycerol, 1x Complete Protease Inhibitor Cocktail (Roche), 1 mM PMSF). Cells were lysed by vortexing with acid-washed glass beads (Sigma) 10x for 30 seconds each, with incubation on ice in between. Unbroken cells were removed by centrifuging twice at 3,000 rpm for 5 min at 4°C. Lysate was incubated with 1% Triton X-100 for 30 min on ice and detergent-solubilized proteins were collected by centrifugation at 30,000 x *g* for 15 min at 4°C. Approximately 4 mg of total protein was bound to mouse monoclonal anti-HA affinity matrix (Sigma) in lysis buffer containing 1% Triton X-100 for 18 hours at 4°C. Beads were washed in lysis buffer containing 1% Triton X-100. Immunoprecipitated proteins were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE, along with 10% of each input lysate, and visualized by immunoblotting using rat monoclonal anti-HA-Peroxidase (Roche) and rabbit anti-Cue1p (Kostova *et al.* 2009) antibodies and visualization with SuperSIgnal® West Pico Luminol Enhancer Solution (Thermo Scientific) and a G:box (Syngene).

***In vivo* labeling and immunoprecipitation**

The labeling and immunoprecipitation of CPY* was performed as described previously (Kostova et al., 2009), except cells were labeled for 10 min with 300

μCi ^{35}S EasyTag™ EXPRESS^{35}S Protein Labeling Mix (Perkin Elmer). Radioactive signal was quantified using ImageQuant™ Software and half-lives were calculated from exponential curve fits using Excel (Microsoft).

In vitro binding assays

Ubc7p was *in vitro* transcribed and translated from pMM43 (pET3a-Ubc7p) in an *E. coli* T7 S30 Extract System (Promega) using [^{35}S]Methionine (GE Healthcare) and used in a binding assay as previously described (Kostova et al., 2009). Relative binding was quantified using ImageQuant™ Software and calculated by comparing the signal for each mutant to the wild-type signal (normalized to 100%).

In vitro ubiquitination assays

In vitro ubiquitination assays in the presence of E3 were carried out as described previously (Das et al., 2009; Kostova et al., 2009) only using ~20 pmol GST-Hrd1p$^{321-551}$ or Doa10p$^{1-112}$, ~30 pmol Ubc7p, and ~2 μM cleaved Cue1p$^{151-203}$ wild-type or mutant.

SUPPLEMENTAL REFERENCES

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Steinkellner, G., Rader, R., Thallinger, G.G., Kratky, C., and Gruber, K. (2009). VASCo: computation and visualization of annotated protein surface contacts. BMC Bioinformatics *10*, 32.