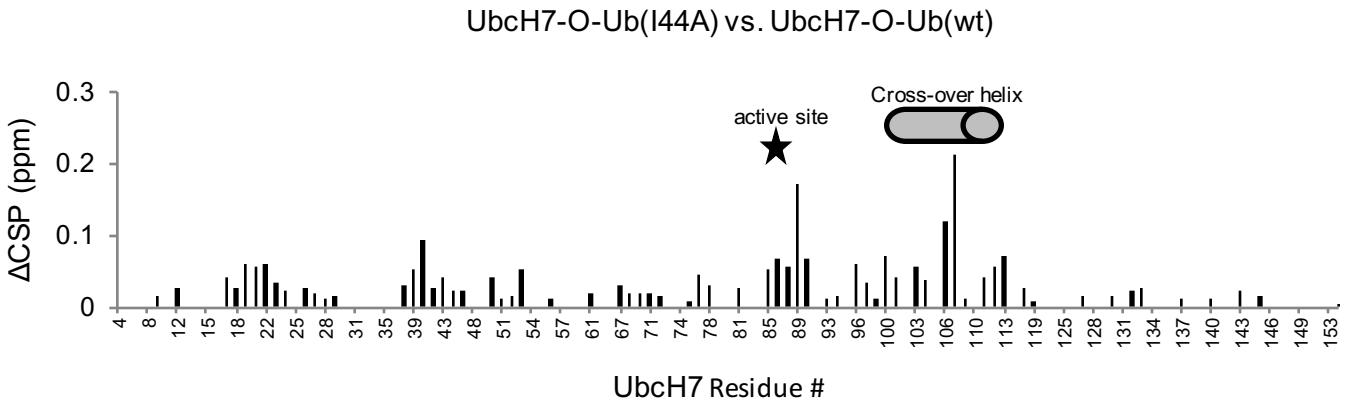
Table of Contents

Appendix Table S1

	Name used in manuscript	Residues	species	tags	Figure where construct was used
Canonical RINGs Constructs	BRCA1/BARD1	1-304/25-327	human	N-terminal Flag-tag on BRCA1	Fig 1A
	BRCA1/BARD1	1-100/26-140	human	none	Fig 3C
	E4BU	1142-1173	mouse	none	Appendix Fig S4
HHARI Constructs	$HHARI_{RBR}$	177-395	human	N-terminal GST-tag	Fig 1A, Fig 4B, Fig 5A&E, Fig 6C
	HHARI RING1	177-270	human	none	Fig 1B, Fig 3, Fig EV2A, Appendix Fig S3&5, Fig EV3
	HHARI RING2	325-396	human	none	Fig 6A&E
	HHARI RING2-ΔL	336-396	human	none	Fig 6A
Other RBR Constructs	$HOIP_{RBR-LDD}$	697-1072	human	none	Fig 5D, Fig EV4
	RNF144 RING1	2-108	human	none	Fig 3C
	$Parkin_{RBR}$	217-465	rat	N-terminal GST-tag	Fig 1A, Fig 4B, Fig 5A&C
	$Triad1_{\Delta ARI}$	1-348	human	N-terminal His-T7-tag	Fig 1A
Hybrid construct	UBR-hybrid	1142-1173 (E4BU)	mouse	N-terminal GST-tag	Fig 4
		271-396 (HHARI)	human		

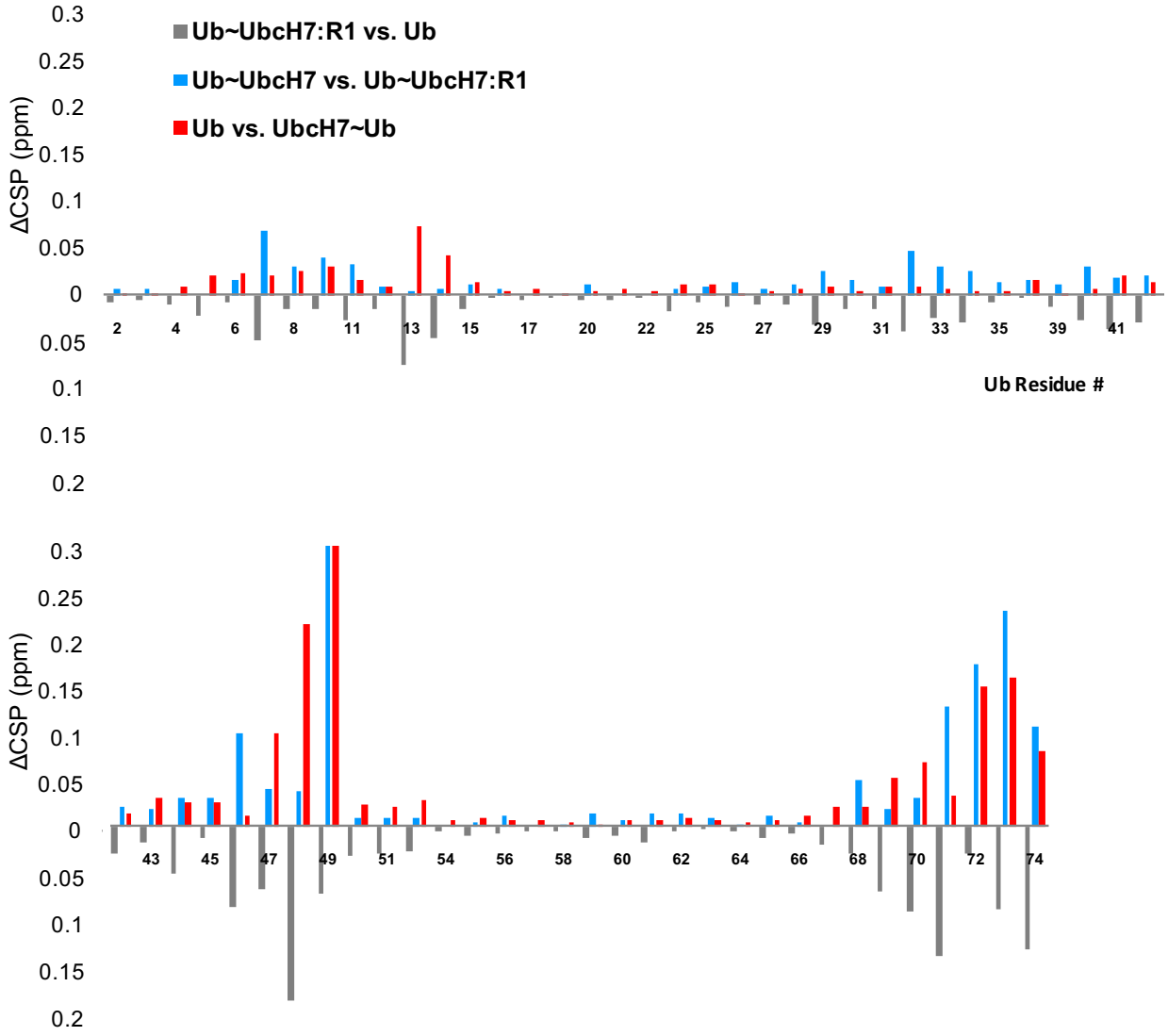
Appendix Table S1. Constructs of all RBR E3 ligases used in this study.

Appendix Figure S1



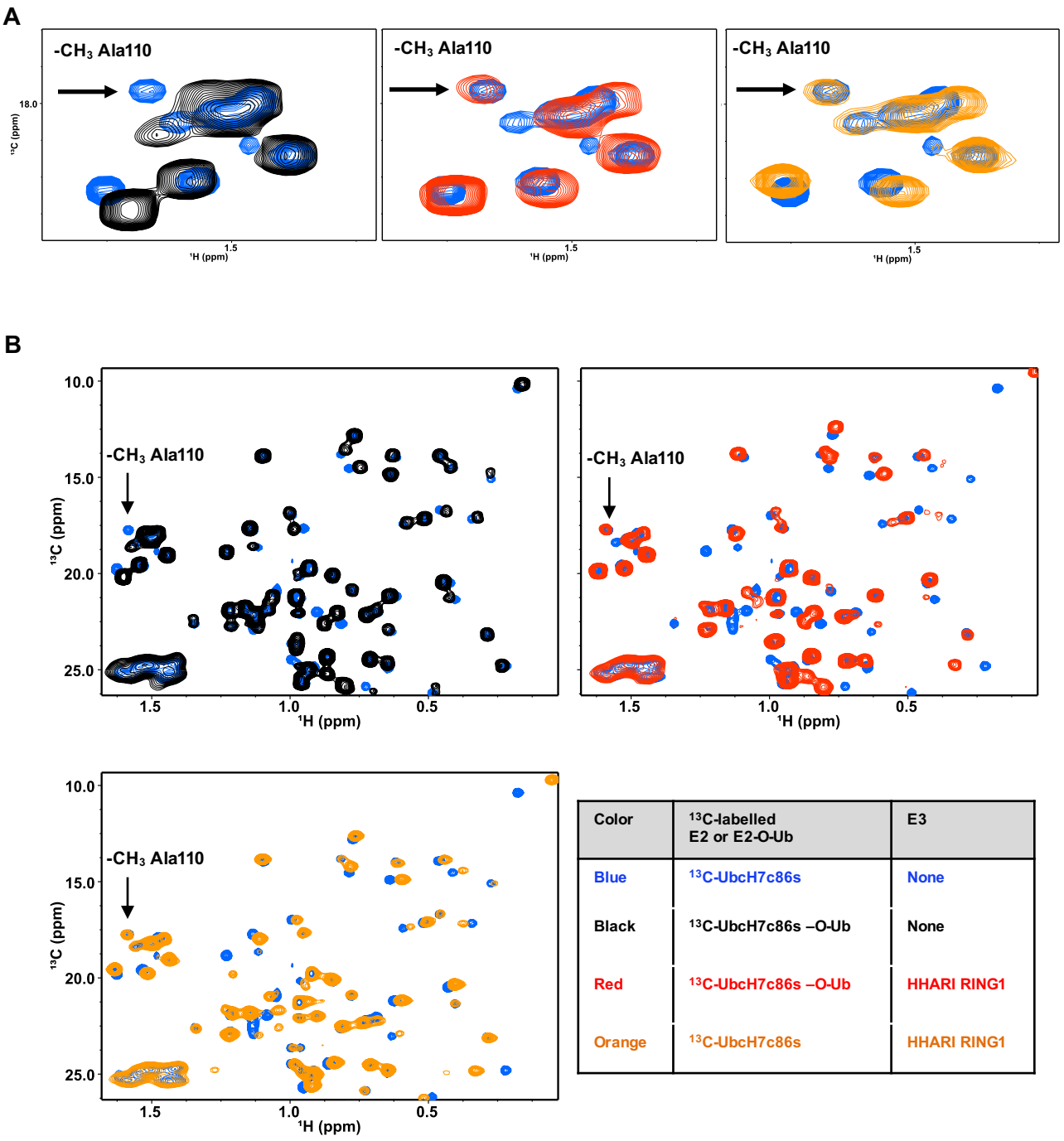
Appendix Figure S1. I44A-Ub disrupts UbcH7~Ub closed conformations. Histogram of CSPs between ^{15}N-UbcH7-O-Ub-I44A and ^{15}N-UbcH7-O-Ub-WT. Residues that are initially perturbed upon Ub conjugation to UbcH7 (Fig 2A) are *less* perturbed by conjugation of I44A-Ub to UbcH7 (Fig 2B). As a consequence, the histogram (above) displaying CSPs for ^{5}N-UbcH7-O-Ub-I44A vs. ^{15}N-UbcH7-O-Ub-WT highlights UbcH7 residues that are involved in the formation of closed conformations. The active site serine residue is indicated with a star and cross-over helix residues (101-113) are marked with a gray cylinder.

Appendix Figure S2



Appendix Figure S2. HHARI RING1 disrupts closed UbcH7~Ub conformations. The histogram shown summarizes comparisons of the three NMR spectra shown in Fig3: free ^{15}N-Ub, UbcH7-O-^{15}N-Ub, and HHARI RING1-bound UbcH7-O-^{15}N-Ub. CSPs that Ub residues undergo upon conjugation to UbcH7 are shown as red bars. These CSPs are characteristic of formation of closed UbcH7-O-Ub formations. CSPs that Ub residues undergo when HHARI RING1 binds to UbcH7-O-Ub are shown as blue bars. Most, though not all, Ub residues that shift upon conjugation (red bars) also shift upon RING1 binding (blue bars), consistent with disruption of closed UbcH7-O-Ub by RING1 binding. The grey bars (inverted for readability) compare resonance positions in free Ub to Ub in the context of HHARI RING1-bound UbcH7-O-Ub.

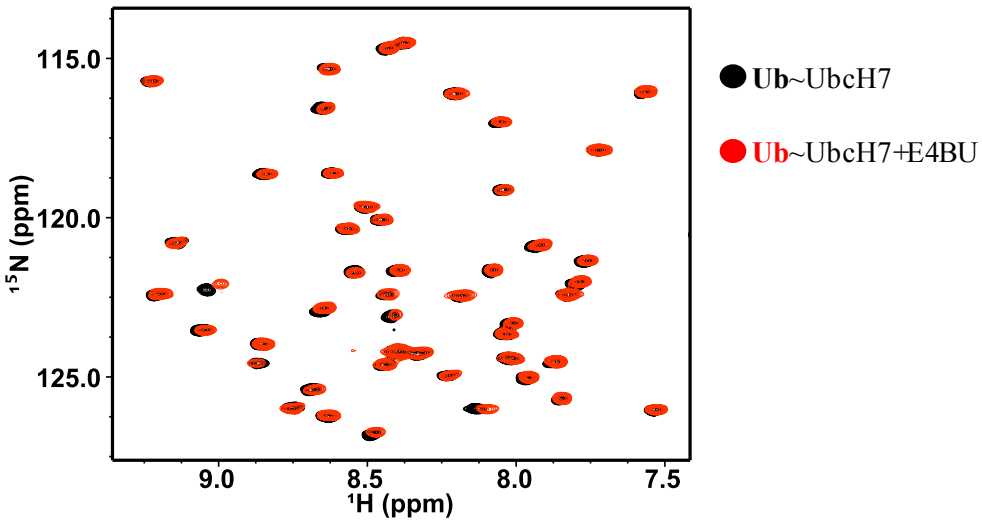
Appendix Figure S3



Color	^{13}C-labelled E2 or E2-O-Ub	E3
Blue	^{13}C-UbcH7c86s	None
Black	^{13}C-UbcH7c86s –O-Ub	None
Red	^{13}C-UbcH7c86s –O-Ub	HHARI RING1
Orange	^{13}C-UbcH7c86s	HHARI RING1

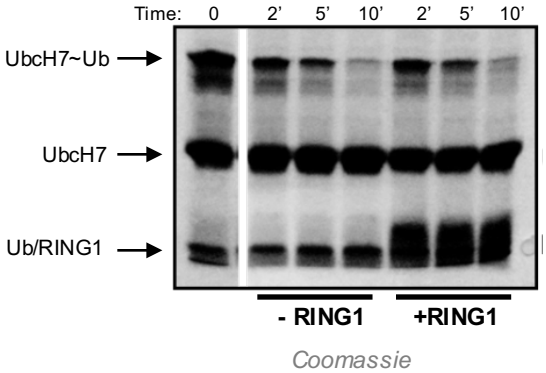
Appendix Figure S3. HHARI RING1 disrupts closed UbcH7~Ub conformations. a) Regions of ^{1}H-^{13}C-HSQC spectral overlays that contain sidechain methyl resonance of Ala110 are shown: free ^{13}C-UbcH7 (black), ^{13}C-UbcH7-O-Ub (red), HHARI RING1-bound ^{13}C-UbcH7-O-Ub (orange), and HHARI RING1-bound ^{13}C-UbcH7 (blue) are shown. The methyl resonance of cross-over residue Ala 110 (red arrow) shifts to an unknown position when Ub is conjugated to UbcH7 (red spectrum), indicating Ub interacts with the sidechain of Ala110. The peak reappears in its original position when HHARI RING1 binds to UbcH7-O-Ub (orange spectrum), consistent with disruption of closed UbcH7~Ub conformations in the complex. The Ala110 resonance is not perturbed when unconjugated UbcH7 binds to HHARI RING1 (blue spectrum), showing that that effect observed is due specifically to the presence of conjugated Ub and not to RING1 directly. b) Full methyl resonance-containing region of spectra shown in a) are provided.

Appendix Figure S4



Appendix Figure S4. E4BU Ubox binding does not disrupt closed UbcH7~Ub conformations. ^{1}H-^{15}N-HSQC-TROSY spectra of UbcH7-O-^{15}N-Ub in the absence (black) and presence (red) of 3 mol equiv. Ubox domain of E4BU. Similar to BRCA1/BARD1 binding, E4BU has little effect on the Ub spectrum while HHARI RING1 and RNF144 RING1 significantly perturb Ub resonances upon binding to UbcH7-O-^{15}N-Ub (Fig 3c). Together these data demonstrate that RBR RING1s, but not canonical RING-type E3s disrupt closed UbcH7~Ub conformations.

Appendix Figure S5



Appendix Figure S5. HHARI RING1 does not increase Cys reactivity of UbcH7~Ub. Cys reactivity assays of UbcH7~Ub were performed in the absence (left) or presence of excess HHARI RING1 (right). Reactions were quenched upon the addition of non-reducing SDS-page load dye. The zero min time point was taken immediately before addition of free Cys. The reaction timecourse shows the disappearance of the conjugate following addition of Cys, monitored by Coomassie-stained SDS-PAGE.