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UbcH7 reactivity profile reveals Parkin and HHARI to be RING/HECT hybrids

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Abstract

Although the functional interaction between ubiquitin conjugating enzymes (E2s) and ubiquitin ligases (E3s) is essential in ubiquitin (Ub) signaling, the criteria that define an active E2–E3 pair are not well-established. The human E2 UbcH7 (Ube2L3) shows broad specificity for HECT-type E3s[1], but often fails to function with RING E3s *in vitro* despite forming specific complexes[2–4]. Structural comparisons of inactive UbcH7/RING complexes with active UbcH5/RING complexes reveal no defining differences[3,4], highlighting a gap in our understanding of Ub transfer. We show that, unlike many E2s that transfer Ub with RINGs, UbcH7 lacks intrinsic, E3-independent reactivity with lysine, explaining its preference for HECTs. Despite lacking lysine reactivity, UbcH7 exhibits activity with the RING-In Between-RING (RBR) family of E3s that includes Parkin and human homologue of ariadne (HHARI)[5,6]. Found in all eukaryotes[7], RBRs regulate processes such as translation[8] and immune signaling[9]. RBRs contain a canonical C3HC4-type RING, followed by two conserved Cys/His-rich Zn^{2+}-binding domains, In-Between-RING (IBR) and RING2 domains, which together define this E3 family[7]. Here we show that RBRs function like RING/HECT hybrids: they bind E2s via a RING domain, but transfer Ub through an obligate thioester-linked Ub (denoted '~Ub'), requiring a conserved cysteine residue in RING2. Our results define the functional cadre of E3s for UbcH7, an E2 involved in cell proliferation[10] and immune function[11], and suggest a novel mechanism for an entire class of E3s.

RING and U-box E3s facilitate Ub transfer directly from an activated E2~Ub to a lysine on a target protein. Therefore, E2s that function with RINGs must be catalytically competent to form an isopeptide bond between Ub and lysine. Previous characterization of E2 activity demonstrates that some E2s can transfer Ub to free lysine independent of an E3[12], providing a framework to examine E2 function. We compared the intrinsic, E3-independent reactivity of UbcH7~Ub and UbcH5c~Ub with free amino acids that represent Ub acceptors: lysine,

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Author Contributions:

D.M.W. performed all experiments. P.S.B. and A.L. contributed to the experimental design for Fig. 2 and Supplementary Fig. 5. D.M.W. and R.E.K. designed the overall study and wrote the manuscript with P.S.B.

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serine, threonine[13], cysteine[14], or arginine as a control (Fig. 1a). UbcH5c~Ub reacts completely with either cysteine or lysine, but not other amino acids, indicating the side chain functional group is the relevant nucleophile. Strikingly, UbcH7 reacts only with cysteine. Reaction time courses for UbcH7~Ub and UbcH5c~Ub with free lysine show that UbcH5c~Ub is nearly depleted after 15 minutes, while after 60 minutes UbcH7~Ub shows no detectable reaction (Fig.1b). UbcH7's lack of reactivity is lysine-specific and cannot be attributed to UbcH7~Ub being intrinsically more stable, as both E2s react equally rapidly with cysteine (Supplementary Fig 2a). Ube2k and Ubc13, E2s known to function with RINGs[15,16], both react with cysteine and lysine (Supplementary Fig. 2b), indicating that lysine reactivity is a general feature of RING-active E2s. The reactivity properties of UbcH7 are conserved, as the *C. elegans* orthologue Ubc18[17] also lacks lysine reactivity (Supplementary Fig. 3).

To determine which residues in E2s are important for lysine reactivity, the active site sequences of lysine-reactive E2s were aligned with that of UbcH7 (Fig 2a). Two residues in UbcH7 are distinctly different: D87 and D117 (in UbcH5c numbering) are proline and histidine, respectively, in UbcH7. To establish whether these residues contribute to lysine reactivity, each was mutated in UbcH5c and lysine reactivity was measured (Fig. 2b). The effect of substitution at position 87 ranges from no effect for the isosteric mutation D87N to complete loss of lysine reactivity for the charge-swapped D87K mutation. UbcH5c-D87E and -D87P have intermediate reactivities. Consistent with D87 playing a general role in lysine reactivity, mutation of the analogous residue in Ube2k (D94E) results in decreased lysine reactivity and impaired formation of free poly-Ub chains (Supplementary Fig. 4a,b). Substitution of D117 in UbcH5c with a histidine as found in UbcH7 greatly decreases lysine reactivity (Fig. 2b). A structurally analogous residue in the SUMO E2, (Ubc9-D127), has been shown to lower the pKa of a lysine approaching the active site[18]. The invariant active site asparagine residue (N77 in UbcH5c) is recognized for its role in isopeptide catalysis[19], and its mutation to serine abolishes UbcH5c~Ub lysine reactivity in our assay. Identification of several residues that affect an E2's intrinsic lysine reactivity suggests that the determinants are likely multi-factorial. Accordingly, we failed to convert UbcH7 into a lysine-reactive E2 by mutation (Supplementary Table).

To assess whether an E2's intrinsic reactivity is predictive of its functional E3 interaction, UbcH5c mutants were assayed with RING and HECT-type E3s. Of the D87 mutants, only UbcH5c-D87N (which has wild-type lysine reactivity) is able to function with the RING E3 BRCA1/BARD1 in an auto-ubiquitination assay; the other substitutions are inactive (Fig. 2c). The observed loss of activity results from E2 catalytic defects, as NMR binding experiments confirm UbcH5c-D87 mutants bind BRCA1 comparable to UbcH5c-WT (Supplementary Fig. 5). UbcH5c-D117H, which has impaired lysine reactivity, retains some ability to transfer Ub to BRCA1 (Fig. 2c). Given its position at the active site, D117 may provide substrate-specific lysine reactivity as a gating residue. As expected, UbcH5c-N77S is inactive with BRCA1. Previous studies show that mutation of E2 residue N77 abolishes Ub-transfer with RING-type ligases Mdm2[19], Rma1[20], CNOT4 and APC/C[21] but retains activity with HECT-type ligases E6AP, KIAA10[19], and NEDD4L[22]. This suggests that the catalytic requirements for trans-thiolation differ from those for isopeptide bond synthesis.

Accordingly, the ability to form E3~Ub thioesters with the HECT E3 E6AP is unaffected in all UbcH5c mutants (Supplementary Fig. 6).Taken together, our results indicate that E2 lysine reactivity is a prerequisite for transfer with RING E3s. Furthermore, lysine-unreactive E2s such as UbcH5c-N77S, can be diagnostic for differentiating between RING- vs. HECT-type Ub transfer mechanisms.

Although many E2s possess an intrinsic reactivity for lysine, most do not transfer Ub to protein substrates independent of an E3. To explore the contribution of E3s to E2 reactivity, we examined the intrinsic reactivity of E2~Ub in the presence of RING or HECT E3s. Interaction with BRCA1/BARD1 (RING) or E6AP (HECT) does not change the intrinsic reactivity of UbcH5c and UbcH7 with free amino acids (Supplementary Fig. 7a, b). However, BRCA1/BARD1 enhances UbcH5c~Ub lysine reactivity compared to a reaction containing the E2-binding mutant BRCA1-I26A[3,15] (Fig. 2d). Intriguingly, the lysine reactivity of UbcH5c-D87E, which retains both its ability to bind BRCA1/BARD1 and some intrinsic lysine reactivity, is not enhanced by BRCA1 (Fig. 2d). These results suggest that RING-binding to a lysine-reactive E2~Ub results in a thioester with enhanced reactivity to lysine and residues such as D87 in UbcH5c couple E3-binding and E2 activation. Notably, E6AP does not enhance UbcH5c~Ub lysine reactivity (Supplementary Fig. 8b), highlighting a mechanistic difference between RINGs and HECTs. In matched experiments with UbcH7, neither BRCA1/BARD1 nor E6AP enhance reactivity toward free cysteine (Supplementary Fig 8a, c.)

Besides HECT-type ligases, UbcH7 is reported to function with members of the RING E3 family known as RBRs that includes Parkin and HHARI[5,6]. This activity runs contrary to our conclusion that UbcH7 lacks lysine reactivity and is consequently restricted to Ub transfer involving trans-thiolation chemistry. Therefore, we examined the Ub ligase mechanism for HHARI and Parkin. The minimal ligase (RBR) domains of $HHARI_{R1\text{-}IBR\text{-}R2}$ and $Parkin_{R1\text{-}IBR\text{-}R2}$ (Supplementary Fig. 9 for schematic of constructs) show comparable auto-ubiquitination activity with either UbcH7 or UbcH5c (Fig. 3a,b). In contrast to our results with BRCA1/BARD1, UbcH5c-N77S exhibits Ub transfer with $HHARI_{R1\text{-}IBR\text{-}R2}$ and $Parkin_{R1\text{-}IBR\text{-}R2}$ (Fig. 3a, b). As UbcH5c-N77S activity is restricted to HECT-type ligases, its activity with Parkin and HHARI suggests that these ligases do not function via a typical RING-type mechanism.

A hallmark of HECT-type Ub transfer is the formation of an obligate E3~Ub thioester intermediate. Reactions designed to trap a HHARI~Ub conjugate were conducted on ice and quenched with SDS-loading buffer with or without reducing agent (βME) to distinguish reducible thioester-linked Ub from non-reducible isopeptide-linked Ub. A βME-sensitive band corresponding to the molecular weight of HHARI~Ub was detected at 10, 20, and 30 seconds post addition of pre-charged UbcH7~Ub (Fig. 3c). We next sought to determine the position of the HHARI active site cysteine. The N-terminal canonical RING1 of HHARI has been shown to be the principal E2-binding region[23], although RING2 is also required for ligase activity[5]. Cysteine 357 in RING2 is highly conserved across RBR ligases (Fig. 4a). C357 is not a Zn^{2+}-liganding residue, and mutation of C357 does not destabilize the RING2 structure (Supplementary Fig. 10 and Ref. 5), but does abolish the ability of HHARI to transfer Ub (Fig. 4b and Ref. 5). This suggests C357 may have a catalytic function.

Although $HHARI_{R1\text{-}IBR\text{-}R2}$-C357A showed no ligase activity in an auto-ubiquitination assay, $HHARI_{R1\text{-}IBR\text{-}R2}$-C357S generated a single monoubiquitinated species. An analogous oxyester-linked Ub product can be generated for E2s whose active site cysteines have been mutated to serines[20, 22, 24]. Consistent with its identity as an oxyester (vs. isopeptide) bond, the Ub-adduct on $HHARI_{R1\text{-}IBR\text{-}R2}$-C357S is labile to alkaline treatment (Supplementary Fig. 11). Formation of an oxyester is unique to C357 as serine substitution of other conserved cysteines (both Zn^{+2}- and non Zn^{+2}-liganding) in RING2 does not stall the E3 at a single-Ub adduct but rather impairs (C375S) or abolishes (C367S, C372S) ligase activity (Supplementary Fig. 12). Similarly, the Parkin mutant C431S (analogous to HHARI C357) eliminates E3 ligase activity (Fig. 4c). We were unable to trap a $Parkin_{R1\text{-}IBR\text{-}R2}$-C431S Ub-adduct under our reaction conditions. However, we note that the Parkin mutation C431F has been consistently shown to abolish Parkin's ubiquitination of substrates[25–27] and genetically predisposes for Parkinson's disease[28]. The results presented above are not affected by the presence of a GST-domain, as several results were reproduced with non-GST versions of HHARI (Supplementary Fig. 13).

$HHARI_{R1\text{-}IBR\text{-}R2}$-C357A effectively binds E2 as GST-pulldowns with purified UbcH7 and constructs of GST-HHARI demonstrate that $HHARI_{R1\text{-}IBR\text{-}R2}$-C357A interacts with UbcH7 as efficiently as $HHARI_{R1\text{-}IBR\text{-}R2}$-WT (Fig. 4d). In contrast, a HHARI-RING1 mutant (I188A[23], analogous to BRCA1-I26A) does not interact detectably with UbcH7 in this assay. Furthermore, C357 is surface accessible and reactive, as $HHARI_{R2}$-WT but not $HHARI_{R2}$-C357A is readily derivitized by cysteine-modifying reagents (Supplementary Fig. 14).

In the absence of a bona fide substrate, anf *in vitro* product of HHARI-catalyzed Ub transfer is the non-reducible ubiquitination of UbcH7 (Fig. 4b). Ubiquitination of UbcH7 is E3-dependent as mutation of $HHARI_{R1\text{-}IBR\text{-}R2}$-C357 to serine or alanine abolishes the formation of this product (Fig. 4b). Our finding that $HHARI_{R1\text{-}IBR\text{-}R2}$-C357S forms an oxyester-linked Ub without subsequent transfer of Ub to UbcH7 suggests an ordered mechanism that involves formation of an E3~Ub prior to modification of UbcH7 (or substrates). Thus, HHARI, unlike other RING E3s, does not facilitate direct transfer from an E2~Ub to a target. We note that the HHARI oxyester-linked Ub conjugate accumulates in low yield compared to the available number of active sites. This is consistent with our failure to observe intrinsic serine reactivity for UbcH7, even at serine concentrations as high as 0.5 M (Fig. 1a and data not shown) and suggests that the unique chemical environment surrounding a target residue (in this case an enzyme active site) contributes to catalysis—a contribution that is absent in the nucleophile assay. Our combined results are consistent with HHARI and Parkin functioning via a HECT-like mechanism whereby RING1 harbors the E2-binding site and RING2 harbors the active-site cysteine.

Our characterization of the reactivity of UbcH7 resulted in two unexpected discoveries. First, while E2s known to work with RING-type E3s have E3-independent reactivity toward lysine, UbcH7's intrinsic reactivity is restricted to cysteine and consequently, its Ub transfer activity is restricted to HECT-type ligases. Second, while confirming reports that UbcH7 is active with E3s in the RBR family, we discovered that these RING-containing E3s function like HECTs in that they require an obligate trans-thiolation step during Ub transfer. Both

findings have important implications for guiding our understanding of ubiquitination pathways. RING1 of HHARI does not harbor catalytic activity, and neither $HHARI_{R1\text{-}IBR}$, nor $HHARI_{R2}$ enhance lysine reactivity of UbcH5c (Supplementary Fig. 15). Our results underscore the diversity of structures that facilitate thiol-based Ub transfer, enzymes that include bacterial HECT-like E3s that bear no homology to eukaryotic HECT counterparts[29]. Knockdown and over-expression studies indicate that UbcH7 regulates S-phase progression into G2, but neither the E3(s) nor targets responsible have been identified[10]. Our results suggest that the relevant E3(s) will be found among HECT or RBRs. Although it is possible that UbcH7 cooperates with RINGs such as BRCA1 to modify substrate cysteines, we have not observed such species.

Among human E2s, only five residue types are found at the position analogous to UbcH5c-D87: aspartate, serine, asparagine, glutamate, and proline. The tolerance for asparagine and serine at position 87 suggests that the negative charge of D87 may not be critical for its role in catalysis, but instead a hydrogen-bonding function seems likely, possibly interacting with the conjugated Ub. Of human E2s, only UbcH7 and UbcH8 have a proline at position 87. Although we did not test UbcH8 for lysine reactivity, it functions primarily with the HECT-type ligase HERC5 to transfer the Ub-like protein ISG15 to substrates[30]. Like UbcH7, we anticipate UbcH8 activity will be limited to HECT or RBR E3s. In conclusion, our effort to understand the mechanism of UbcH7-mediated Ub transfer highlights the predictive power of elucidating E2 mechanisms to understand the E3s with which they function.

METHODS SUMMARY

Plasmids, protein expression/purification, E3 auto-ubiquitination assays, and NMR experiments were performed as described previously[3, 15]. Modifications and details are described in Methods. For intrinsic reactivity assays, E2s were charged with Ub for 20–30 minutes at 37°C before addition of cysteine, arginine, lysine, serine, threonine or buffer (final concentration 50 mM, pH 7.0). After 15–20 minutes, reactions were quenched in non-reducing loading-buffer and visualized by Coomassie-stained SDS-PAGE. Reactivity time courses with lysine and cysteine were performed similarly with samples quenched at the indicated times.

Full Methods are available in the online version of the paper at www.nature.com/nature.

Methods

Multiple sequence alignments

Multiple sequence alignments were performed using Clustal W[31] with manual sequence adjustments based on E2 structures.

Plasmids, Protein Expression and Purification

Plasmid constructs, protein expression, and purification of wheat E1, Ub, Ubc13, UbcH5c, Ube2k, UbcH7, Flag-BRCA1(residues 1–304)/BARD1(residues 26–327), Flag-BRCA1(residues 1–112) /BARD1 (residues 26–140) were described previously[3, 15]. Point mutations were introduced using site-directed mutagenesis (Stratagene) and confirmed by

DNA sequencing. **PFastBac**-His-Human E1 was expressed in Hi5 cells and purified by Ni^{2+}-affinity chromatography, according to the manufacturer's instructions (Sigma), followed by gel-filtration using Superdex 200 resin (GE Health care Life Sciences). E6AP (residues 495–852) was expressed and purified as described previously[32]. Ubc18 was subcloned into **pHis** vector[33] in-frame with the N-terminal His-tag, and UbcH7 was subcloned into **pet24a** vector in-frame with a His-T7 N-terminal tag. His-T7-UbcH7, His-Ubc18, His-UbcH5c-N77S, HA-and T7-tagged Ub were purified by Ni^{2+} affinity chromatography followed by gel-filtration using Superdex 75 resin (GE Healthcare Life Sciences). Constructs of **pGEX-4T** Parkin and HHARI were expressed in BL21 *E. coli* (Invitrogen) in LB media supplemented with 2 mM ZnCl2 and purified using GSTrap FF columns (GE Healthcare and Life Sciences) eluted with 10 mM reduced glutathione. Glutathione was removed by dialysis against 50 mM Tris, 200 mM NaCl, 1 mM DTT, pH 7.6 (Parkin) and pH 8.0 (HHARI). $HHARI_{RING2}$ was subcloned into **pGEX-4T** in-frame with the Nterminal GST-tag. The GST-tag on $HHARI_{R1\text{-}IBR\text{-}R2}$ and $HHARI_{RING2}$ was removed by thrombin cleavage for NMR and cysteine modification experiments as well as to repeat activity assays shown in Supplementary Fig. 14.

GST pulldown assays

100μl binding reactions contained GST-HHARI (5 μM) with T7-UbcH7 (5 μM) and 50 μl of Glutathione Sepharose B resin (GE Healthcare) in the binding buffer: 50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 0.5 mM DTT pH 7.5. Binding reactions were incubated for 3 hours at 4°C, and resin was washed 5 times with 1.5 mLs of binding buffer before proteins were eluted with 80μl of reduced SDS-PAGE loading buffer. Reaction products were resolved on a 15% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (Bio-Rad). The membranes were probed simultaneously with rabbit antibody to GST (Affinity BioReagents) and mouse antibody to T7 (Novagen) followed by goat anti-mouse and goat anti-rabbit secondary antibody conjugated to Alexa Fluor 680 (Molecular probes) and IRdye 800 (Rockland Immunochemicals) respectively. Blotted proteins were detected using an Odyssey infrared imaging system (Licor).

E3 autoubiquitination activity assays

One hundred microliter reaction mixtures for BRCA1 autoubiquitination contained 2μM His-Flag BRCA1 (residues 1–304)/BARD1(residues 26–327), 2 μM UbcH5c, 20 μM T7-Ub, 0.5 μM wheat E1, and 10 mM $MgCl_2$. Reactions were initiated at 37°C by adding 10mM ATP and samples were quenched at the indicated time points by boiling in SDS sample buffer that contained βME. Ubiquitination products were visualized by Western blot, probing for T7(Ub) and Flag (BRCA1) epitopes simultaneously. Parkin and HHARI ubiquitination assays were performed similarly except products were visualized by probing for the GST tag on Parkin or HHARI or the HA-epitope on HA-Ub (mouse primary from Covance). HHARI assays with UbcH7, UbcH5c, and UbcH5c-N77S/D87K mutations were performed at higher concentrations (15 μM E2/E3, 50 μM Ub) and visualized by Coomassie staining on a 15% SDS-PAGE gel. One hundred microliter reaction mixtures for E6AP thioester formation assays included 15 μM E2, 15 μM E6AP, 30 μM Ub, and 10 mM $MgCl_2$. Reactions were initiated at 37°C by the addition of 10 mM ATP and gel samples were taken

in parallel at the indicated time points in loading buffer that lacked or contained the reducing agent βME.

HHARI thioester detection

Reactions containing 20μM UbcH7, 20μM HA-Ub, 0.5μM E1, 10 mM $MgCl_2$/ATP were incubated at 37°C for 30 minutes to form UbcH7~HA-Ub and chilled on ice. Reactions with HHARI were initiated by diluting 2 μl of charged E2 with 18 μl of 2 μM $HHARI_{R1\text{-}IBR\text{-}R2}$ on ice and incubating for the indicated times. Reactions were quenched by the addition of SDS-loading buffer that either contained or lacked the reducing agent βME. HHARI thioesters were visualized by Western blot probing for the HA-epitope on Ub.

Nucleophile reactivity assays

Reaction mixtures for amino acid reactivity assays contained 20 μM E2, 20 μM Ub, 0.5 μM E1 and 10 mM $MgCl_2$/ATP in 25 mM NaPi, 150 mM NaCl, pH 7.0 buffer. Amino acids were purchased from Sigma except cysteine (Nutritional Biochemicals Corporation). 500 mM stock solutions of L-lysine monohydrochloride, L-Arginine monohydrochloride, L-Cysteine, L-Serine, and L-Threonine were prepared in reaction buffer and pH was checked by pH paper to be ~7. E2s were charged for 20–30 minutes at 37°C before being mixed with 50 mM of cysteine, arginine, lysine, serine, threonine or buffer and incubated for 15-20 minutes at 37°C. Samples were quenched in non-reduced loading-buffer and visualized by Coomassie stained SDS-PAGE. Reactivity timecourses with lysine and cysteine were performed similarly except that samples were taken at several time points during the reaction.

In reactions containing E3, 20 μM E3 (His-Flag-BRCA1 residues 1–112 [WT or I26A] / BARD1 residues 26–140 or E6AP C820A residues 495–852 or GST-$HHARI_{R1\text{-}IBR\text{-}R2}$, or GST-$HHARI_{R2}$) was added and mixed to the precharged E2 just prior to incubation with amino acids. UbcH5c was precharged with Ub where all lysines were mutated to arginines (K0) in reactions with BRCA1 to prevent transfer of Ub. Reactivity reactions visualized by Western were performed similarly except concentrations were 10 μM E2 or E3, and 5 μM HA-Ub.

HHARI oxyester detection

Ubiquitination reactions contained 15 μM $HHARI_{R1\text{-}IBR\text{-}R2}$-C357S, 150 μM HA-Ub, 1.5 μM E1, 10 mM MgCl2 and 15 μM UbcH7. Reactions were initiated by the addition of 10 mM ATP, and incubated at 37°C for 30 minutes before being quenched in reduced SDS-loading buffer. Reactions were then incubated for 20 minutes at 37°C with 0.14 N NaOH before being boiled and loaded on a 15% SDS-PAGE gel. Reaction products were visualized by Western, simultaneously blotting for the HA (Ub) and GST (HHARI) epitopes. For controls, parallel ubiquitination reactions with Ubc13 and Ubc13 C86S were performed. Ubc13 readily auto-ubiquitinates itself (via an isopeptide) and the Ubc13 mutant C86S forms an oxyester-linked Ub conjugate[24].

NMR

For the production of ^{15}N-labelled proteins, bacteria were grown in minimal MOPS medium supplemented with [^{15}N] ammonium chloride (Cambridge Isotope Labs). NMR data was collected on a Bruker DMX 500 MHz spectrometer. Samples of ^{15}N-His-Flag BRCA1(residues 1–112)/BARD1(residues 26–140) and UbcH5c mutants were prepared as described previously[15]. Samples of $HHARI_{R2}$ were prepared as reported previously[5]. Spectra were processed using NMRPipe[34]/NMRDraw[35].

Cysteine Modification of $HHARI_{R2}$

100 μl cysteine modification reactions contained 100 μM $HHARI_{R2}$-WT and $HHARI_{R2}$-C357A and 500 μM 4-(2-Iodoacetamido)-TEMPO (Sigma). Stock solutions of 4-(2-Iodoacetamido)-TEMPO were prepared at 60 mM in DMSO. Cysteine modification reactions were incubated overnight at 4°C. Samples for MALDI-TOF were diluted 1:10 in MALDI matrix (saturated sinapinic acid (Sigma) in 40% acetonitrile, 0.1% TFA) and masses were quantitated by MALDITOF spectrometry on a Bruker AutoFlex II spectrometer, using insulin and apomyoglobin as standards.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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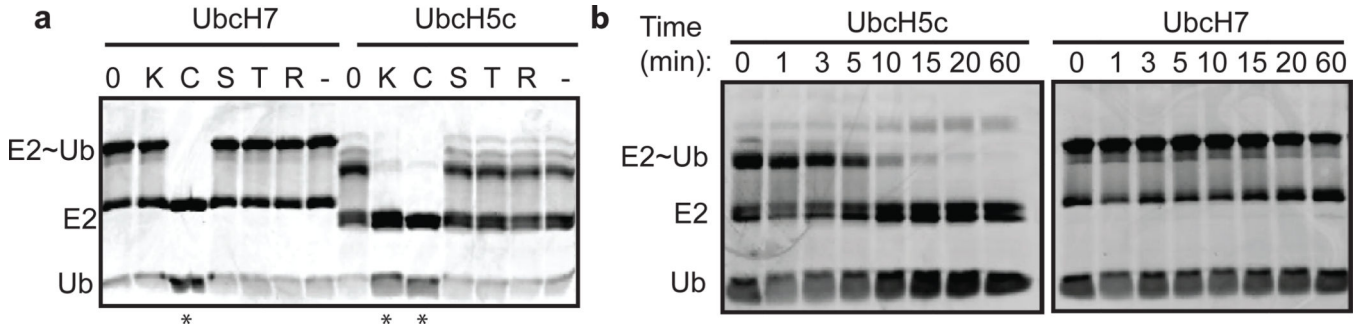

Figure 1. UbcH7 does not react with free lysine

A) Coomassie-stained SDS-PAGE of UbcH7~Ub (left) and UbcH5c~Ub (right) incubated with amino acids lysine, serine, threonine, arginine, or buffer (-). Reactions were quenched in non-reducing loading buffer. '0' indicates starting amounts of E2~Ub prior to amino acid addition. Reactivity with amino acids is indicated by loss of E2~Ub and concurrent increase in free Ub (denoted by asterisks) and free E2. B) Time course assays of UbcH5c~Ub and UbcH7~Ub incubated with lysine. Reactions were quenched in non-reducing loading buffer at the indicated times.

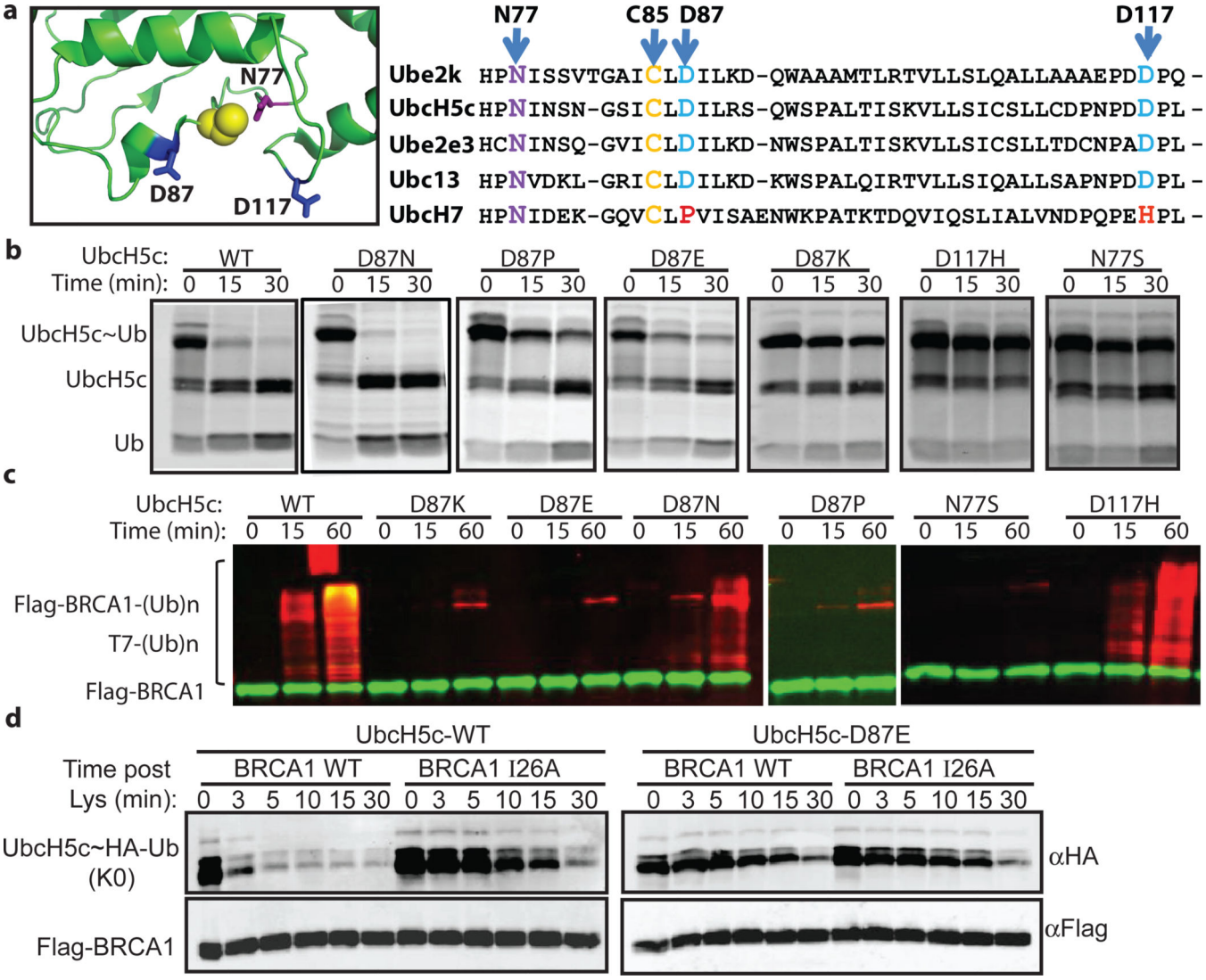

Figure 2. Lysine reactivity is multifactorial

A) Alignment of E2 active site residues (right). Structure of UbcH5c (green; PDB ID 2FUH) with active site residues represented as sticks and active site cysteine as spheres (left). B) Wildtype (WT) or mutant UbcH5c~Ub incubated with lysine as in Fig. 1B. C) Western blot of Flag BRCA1(1–304)/BARD1(26–327) (green) auto-ubiquitination with indicated E2 and T7-Ub (red). Time is measured as minutes post ATP addition. D) Same as in Fig 1B, except a 1:1 (E3:E2) equivalent of Flag-BRCA1(1–112)/BARD(26–140) was added to either UbcH5c-WT (left) or D87E(right) charged with HA-Ub-K0. Reactions were visualized by Western for HA (K0-Ub) and Flag (BRCA1) epitopes.

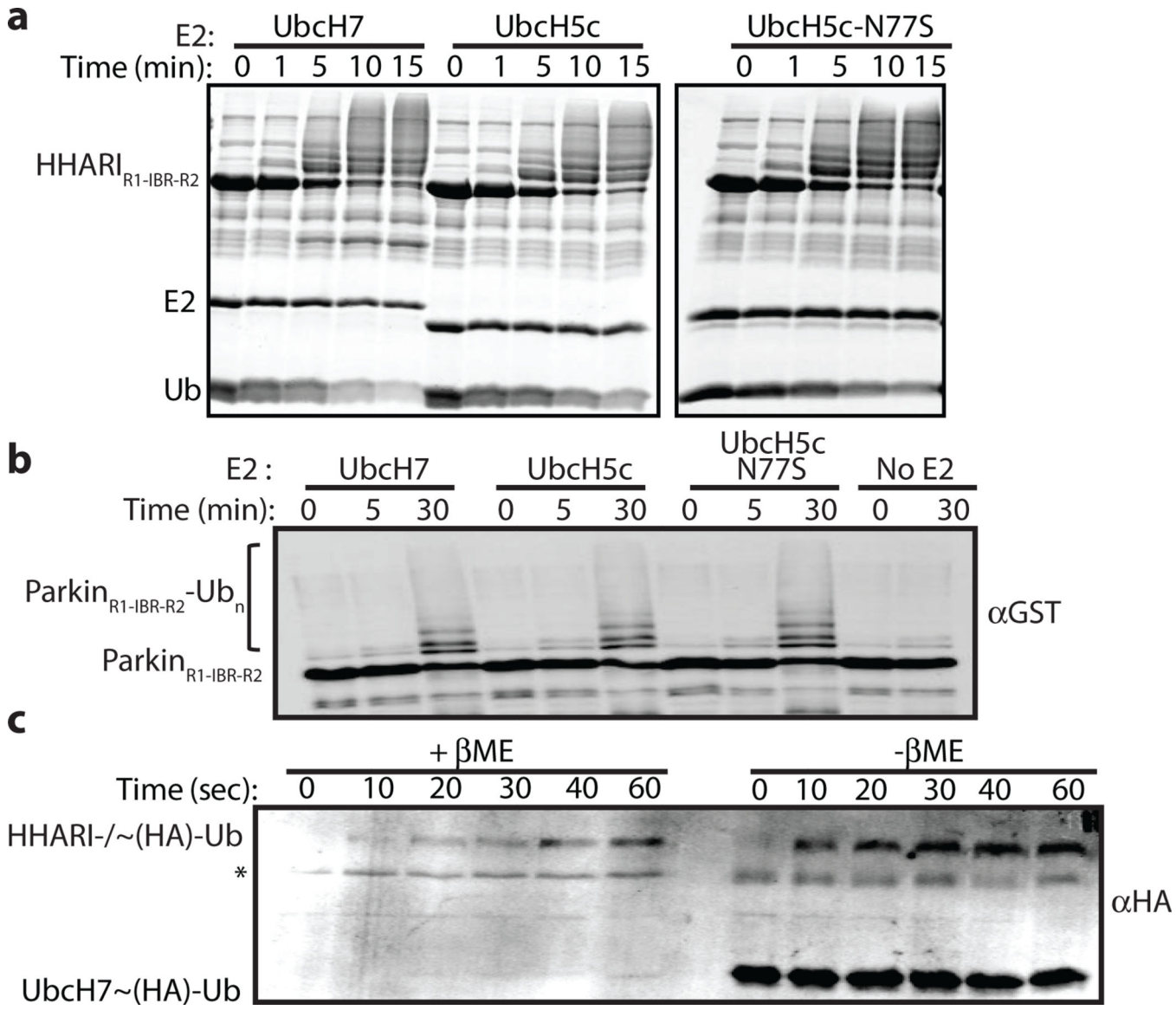

Figure 3. RBR E3s function via a HECT-like mechanism

A) $HHARI_{R1\text{-}IBR\text{-}R2}$ auto-ubiquitination assays with the indicated E2 were visualized on a Coomassie-stained, reducing, SDS-gel. Time is measured as minutes post ATP addition. B) Auto-ubiquitination assay of $Parkin_{R1\text{-}IBR\text{-}R2}$ with the E2s indicated. Products were visualized by Western blotting for the GST-tag on Parkin. C) UbcH7 was pre-charged with HA-Ub and mixed with $HHARI_{R1\text{-}IBR\text{-}R2}$. Reactions were quenched in SDS-buffer under reducing (+βME) and non-reducing conditions (−βME) and visualized by Western blotting for the HA-epitope on Ub. A βME-sensitive HA-Ub band corresponding to the molecular weight of $HHARI_{R1\text{-}IBR\text{-}R2}$-Ub appears at 10, 20, and 30 seconds post addition of UbcH7~Ub. Asterisk denotes a cross-reactive band.

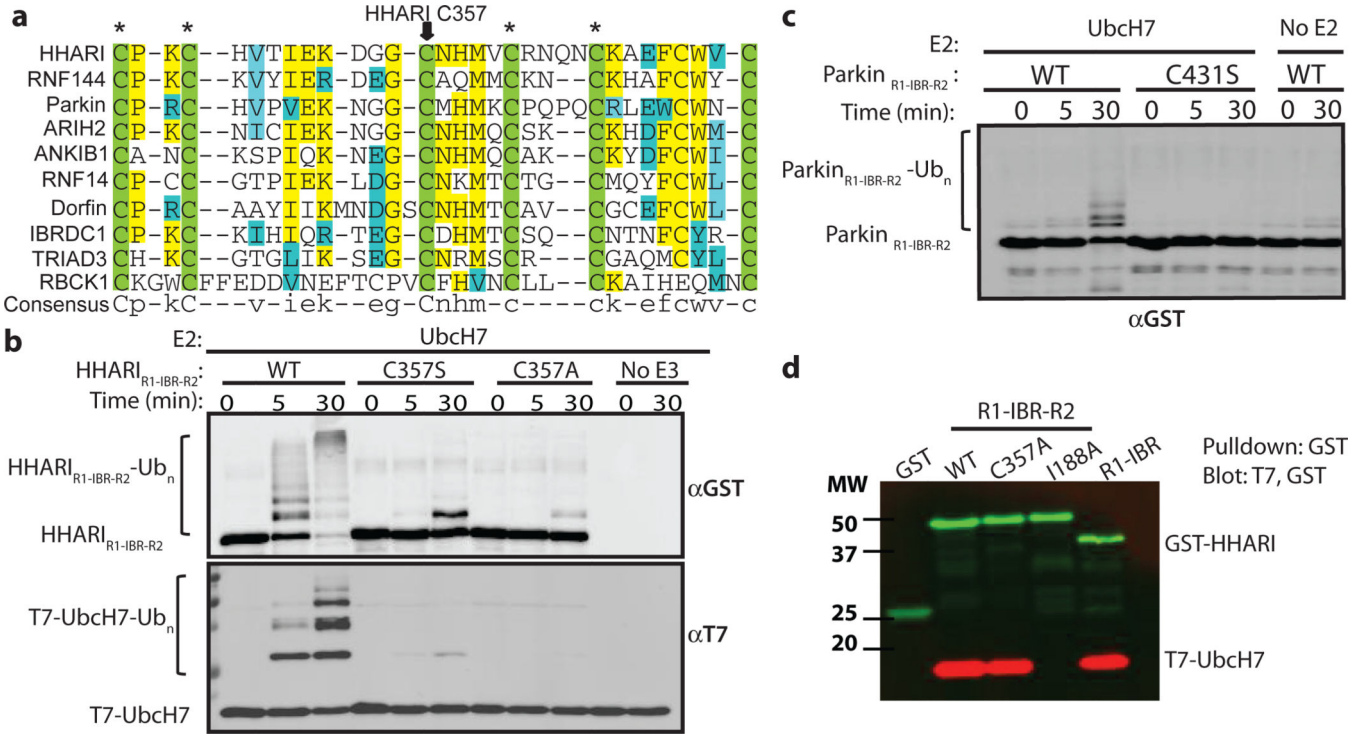

Figure 4. Cysteine C357 is the active site of HHARI

A) Sequence alignment of RING2 domains from human RBR ligases. Conserved cysteine residues are colored green. Asterisks indicate Zn^{2+}-liganding cysteines in the $HHARI_{R2}$ structure[5]. B) Auto-ubiquitination assays of T7-tagged UbcH7 and the indicated construct of HHARI. Ubiquitination is measured as time post ATP-addition. Reaction products were visualized by Western blotting simultaneously for the GST-tag on HHARI (top panel) and the T7-tag on UbcH7 (bottom panel). C) E3 auto-ubiquitination assay with UbcH7 and the indicated construct of Parkin. D) GST-pulldowns of purified constructs of GST-tagged HHARI and T7-tagged UbcH7. Bound protein was eluted and visualized by Western blotting for T7 and GST epitopes simultaneously.