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Interaction Between Two E3 Ligases, NEDD8ylated Cullin and HHARI

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By
Kheewoong Baek
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ABSTRACT

RBR (RING1-in between RING-RING2) is a special type of E3 ubiquitin ligase containing three zinc-binding RING (Really Interesting New Gene) domains, while adopting mechanisms of HECT (Homologous to E6-AP Carboxyl Terminus) for substrate ubiquitination. Most well known RBRs include Parkin and HOIP, which are associated with Parkinson's disease and innate immune deficiency. However, it is not well known how the RBR proteins gain activity, as they are known to be autoinhibited. Here I show that a specific F430A, E431A, E503A triple mutation of RBR protein HHARI (Human homologue of Ariadne) and its interaction with NEDD8ylated cullin RING ligase can both boost its activity and stabilize complex formation. Analytical size-exclusion chromatography, autoubiquitination, and electron microscopy reveal consistent behavior for this triple-mutant. Future structure-based studies will help elucidate the mechanism of the unsolved mystery of RBR activation and its interaction with NEDD8ylated cullin RING ligases.

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LIST OF ABBREVIATIONS

CRL   Cullin RING ligase

DTT   1,4-Dithiothreitol

HECT   Homologous to E6-AP Carboxyl Terminus

HHARI   Human homologue of Ariadne

NEDD8   Neural precursor cell expressed developmentally down-regulated 8

N8Cul1Rbx1   NEDD8ylated Cullin-1 Rbx1

N8Cul3Rbx1   NEDD8ylated Cullin-3 Rbx1

RBR   RING1- in between RING- RING2

Rbx   RING box protein

RING   Really Interesting New Gene

WT   Wild type

Ub   Ubiquitin

Ub-VME   Ubiquitin Vinyl Methyl Ester

CHAPTER 1.   INTRODUCTION

The Ubiquitin Pathway

Ubiquitination is a post-translational covalent modification of protein by a 76 amino acid ubiquitin (Ub) forming an isopeptide bond between the ubiquitin C-terminal glycine residue 76 and the substrate lysine. Ubiquitin can form monoubiquitin or polyubiquitin chains linked by the N-terminal methionine or seven lysine residues (K6, K11, K27, K29, K33, K48, K63) and its C-terminus GG is a required for its covalent modification to proteins (Hodgins et al., 1992; Komander, 2009). Different types of polyubiquitin chains allow participation of substrates in many important processes including proteosomal/lysosomal degradation, transcription, signal transduction, and protein trafficking (Komander and Rape, 2012).

In order to ubiquitinate target substrates, the pathway involves three sequential mechanisms (**Figure 1-1**). First, the C-terminus of ubiquitin must be activated by acyl-adenylation in the presence of ATP and Mg^{2+}. The catalytic cysteine of the E1 activating enzyme attacks the adenylated ubiquitin, producing a highly active E1~Ub thioester complex (Schulman and Harper, 2009). Then, the E2 ubiquitin conjugating enzyme accepts the ubiquitin from the E1 by transthioesterification. The ubiquitin-bound E2 interacts with the E3 ubiquitin ligase, which ultimately ubiquitinates target protein substrates (Streich and Lima, 2014). This step largely divides into two major categories of E3s. **First**, RING (Really New Interesting Gene) E3 ligases act as scaffolds for the E2~Ub and substrates, where the ubiquitin directly transfers to the lysine of substrate protein. Over 600 E3 ligases are known to be part of the RING family (Deshaies and Joazeiro, 2009). **Second**, HECT (Homologous to E6-AP Carboxyl Terminus) E3 ligases also act as scaffolds for the E2~Ub and substrates, but makes a thioester linked E3~Ub intermediate, where the Ub from the E2 transfers to the catalytic cysteine of the HECT E3, and then sequentially transfers the Ub to the substrate protein via its HECT domain (Huibregtse et al., 1995). There is a hybrid type of E3 ligases called RBRs (RING1-in between RING-RING2) where it contains RING domains but utilizes HECT mechanisms. Overall in the human genome, there are two E1s, tens of E2s, and hundreds of E3s that regulate ubiquitination of over thousands of substrates.

Cullin RING Ligases

Cullin RING ligases (CRL), a superfamily of RING E3s, are modular complexes composed of a catalytic RING subunit bound to cullin repeats. The RING domain interacts with two zinc ions by its cysteine and histidine residues to provide structural stability for protein-protein interactions by producing a globular platform (Borden et al., 1995). Each different cullins have variety of substrate receptors that allow participation of cullin RING ligases in majority of cellular processes (Deshaies and Joazeiro, 2009). Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5 along with their substrate receptors and RING box proteins (Rbx) yield around 500 different combinations of cullin RING ligases
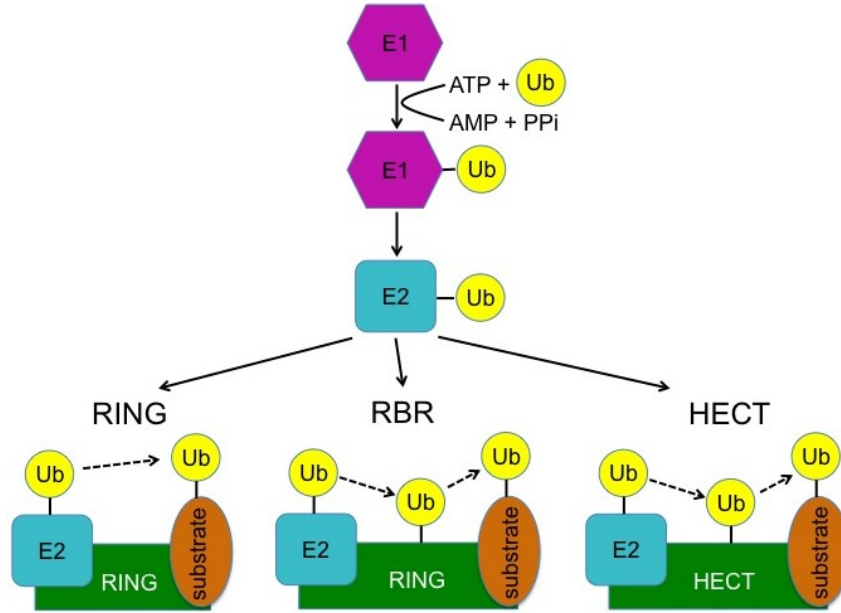
Figure 1-1.   The Ubiquitin Pathway.

Schematic of the ubiquitin pathway. Catalytic cysteine of E1 attacks adenylated ubiquitin, producing a highly reactive complex of E1~Ub. By transthioesterification, ubiquitin transfers from E1 to E2, and further onto substrate via 3 types of E3 ubiquitin ligases: RING, HECT, and RBR.

(Zimmerman et al., 2010). Among the CRL family includes the well-known Skp1-Cul1-F-box (SCF) ubiquitin ligase where the N-terminus of Cul1 binds Skp1, and the C-terminus binds Rbx1 for E2 interaction. The F-box protein directly binds to Skp1 by the F-box domain for substrate specificity (Cardozo and Pagano, 2004). The F-box proteins also contain protein-protein interaction motifs such as leucine-rich repeats (LRR) or WD repeats (Schulman et al., 2000). By different types of F-box proteins, the cullin RING ligases undergo different conformations. For instance, Fbw7 can dimerize Cul1Rbx1 complex by its D-domain, which is highly conserved in F-box proteins with WD40 repeat motifs (Tang et al., 2007). Cul3Rbx1 can also dimerize via MATH-BTB proteins (Zhuang et al., 2009).

NEDD8ylation

Many ubiquitin like proteins (Ubl) exist to regulate protein functions. NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8), a Ubl, is essential for the activity of cullin RING ligases. Human NEDD8 shares 60% sequence identity with ubiquitin and has a NEDD8ylation cascade. By its Nedd8 E1 APPBP1-Uba3, Nedd8 E2 Ubc12, and Nedd8 E3 Rbx1/2, Nedd8 is ultimately attached onto its substrate cullin (Huang et al., 2009). With its E3 RING box protein, NEDD8 conjugates onto the cullin scaffold lysine that activates the cullin RING ligase (Lydeard et al., 2013). Cullin NEDD8ylation enhances E2 recruitment by its selectivity towards ubiquitin charged E2s and improves substrate ubiquitination (Saha and Deshaies, 2008). NEDD8 ligation also causes a dramatic conformational change of the Cullin Rbx complex so that the E2~Ub positions proximally to the substrate lysine (Duda et al., 2008; Scott et al., 2014). DCN1 also enhances cullin NEDD8ylation as an auxiliary E3 by binding its PONY (potentiating NEDD8) domain with Cul1 and Ubc12. NEDD8ylated cullins are deNEDD8ylated by the multiprotein complex COP9 signalosome (CSN) (Enchev et al., 2012; Lyapina et al., 2001). Recent structural studies were able to observe the mechanism of CSN and its cullin RING ligase regulation (Cavadini et al., 2016; Mosadeghi et al., 2016). DeNEDD8ylation by CSN blocks substrate access and allows substrate receptor exchange by CAND1. CAND1 can only bind to the unNEDD8ylated form of cullins and this substrate receptor exchange allows cullin RING ligases to be involved in numerous substrate ubiquitinations (Pierce et al., 2013; Schmidt et al., 2009).

RBR E3 Ligases

There is a special family of E3 ligases, called RBRs (RING1-in between RING-RING2). RBRs are reported to be RING/HECT hybrid E3s. Unlike RING E3s, RBRs produce an E3~Ub intermediate, where the ubiquitin makes a thioester link on the catalytic cysteine of RING2, just as would occur on a HECT E3 (Wenzel et al., 2011). RBR E3s contain 3 RING domains each coordinating two zinc ions: RING1, in-between-RING, and RING2 (Hristova et al., 2009). However, the name today is controversial as the RING2 does not have the canonical RING E3 structure with only a single cysteine. So far, only 13 human RBRs and 2 yeast RBRs have been identified (Eisenhaber et al.,

2007). Most studied RBRs include the famous Parkinson's disease related Parkin, LUBAC (linear ubiquitin chain assembly complex), HOIP, HOIL-1L, Cul9, HHARI, and Triad1 (Spratt et al., 2014). Studies show that RBRs adopt an autoinhibitory conformation, where a specific domain masks the catalytic cysteine of RING2: Ubl domain for Parkin and Ariadne domain for HHARI (Chaugule et al., 2011; Duda et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). RBRs are also known to interact with kinases. PINK1(PTEN induced putative kinase 1) is reported to phosphorylate ubiquitin, sequentially activating parkin (Koyano et al., 2014).

Human Homolog of Ariadne (HHARI)

Human homolog of Ariadne, HHARI, is an E3 RBR ligase consisting of an N-terminal acidic/glycine rich domain, UBA domain, RING1, IBR, RING2, and an Ariadne domain (**Figure 1-2**). In recent years, Duda et al were able to solve the crystal structure of HHARI, which provides tremendous insight into the mechanism of RBRs. The structure explains how HHARI is autoinhibited, as 30% of the RING2 surface including the catalytic cysteine is masked by the Ariadne domain (**Figure 1-3**). In order to study the activation of HHARI, Duda et al performed various combinations of mutational analyses, and discovered a set of mutations that significantly activates HHARI. Triple mutation of F430A, E431A, and E503A significantly activated HHARI by a autoubiquitination assay, which is known to be a good measurement of E3 ligase activity (Duda et al., 2013). Seen in the structure (PDB ID: 4KBL), all three residues closely interact with the catalytic cysteine residue 357, indicating that mutating these residues might facilitate opening of the RING2 for ubiquitin access.

Interaction Between HHARI and Cullin RING Ligase

Kelsall et al screened combinations of two E3 ligases, one RBR and one RING, for activity. With several combinations, they found that NEDD8ylated cullin RING ligases and RBRs have interaction, specifically the two members of the Ariadne subfamily HHARI and TRIAD. Autoubiquitination assays show that adding NEDD8ylated Cul1Rbx1 activated wild type HHARI significantly, which is normally autoinhibited (Kelsall et al., 2013). NEDD8ylation of cullin RING ligase is essential for the activity of CRLs, and Kelsall et al were able to discover that only the NEDD8ylated cullin RING ligases activate HHARI, and the unmodified form of CRLs have no effect. In fact, modification of CRL by the ubiquitin like protein NEDD8 causes dramatic reorientation of the structure allowing conformations capable of substrate polyubiquitination (Duda et al., 2008). The interaction between these two types E3 ligases opens up possibilities of a whole new mechanism of the function of E3s. In fact, another type of RBR Parc is known to be a fused form of Cul7 and an RBR Ariadne gene, which indicates that interaction of cullins and HHARI might have specific functions (Skaar et al., 2007).
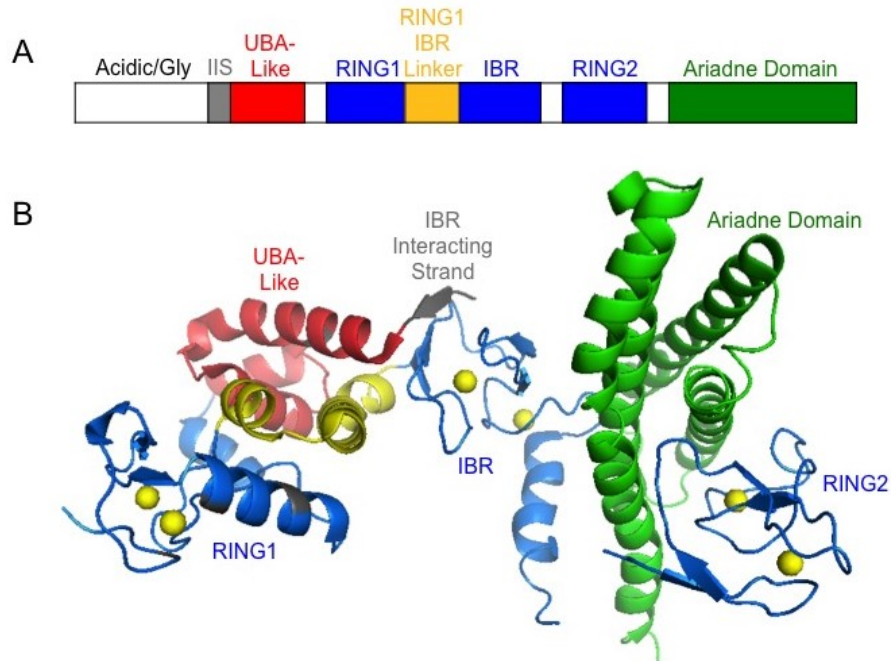
Figure 1-2. Human Homologue of Ariadne.

(A) Domains of HHARI, human homolog of Ariadne. HHARI has an Acidic/Gly rich domain, UBA domain, RING1, IBR, RING2, and an inhibitory Ariadne domain. (B) Structure of HHARI. The Ariadne domain covers about 30% of RING2, containing the catalytic site. Circles in yellow indicate zinc ions.

Data source: DOI: 10.2210/pdb4kbl/pdb
Duda, D.M., Olszewski, J.L., Schuermann, J.P., Kurinov, I., Miller, D.J., Nourse, A., Alpi, A.F., and Schulman, B.A. (2013). Structure of HHARI, a RING-IBR-RING Ubiquitin Ligase: Autoinhibition of an Ariadne-Family E3 and Insights into Ligation Mechanism. Structure 21, 1030-1041.

Figure 1-3.   FEE Mutation of HHARI.

Sites of FEE mutation of HHARI. F430A, E431A, and E503A are sites closely related to the catalytic cysteine C357. Circles in yellow indicate zinc ions.

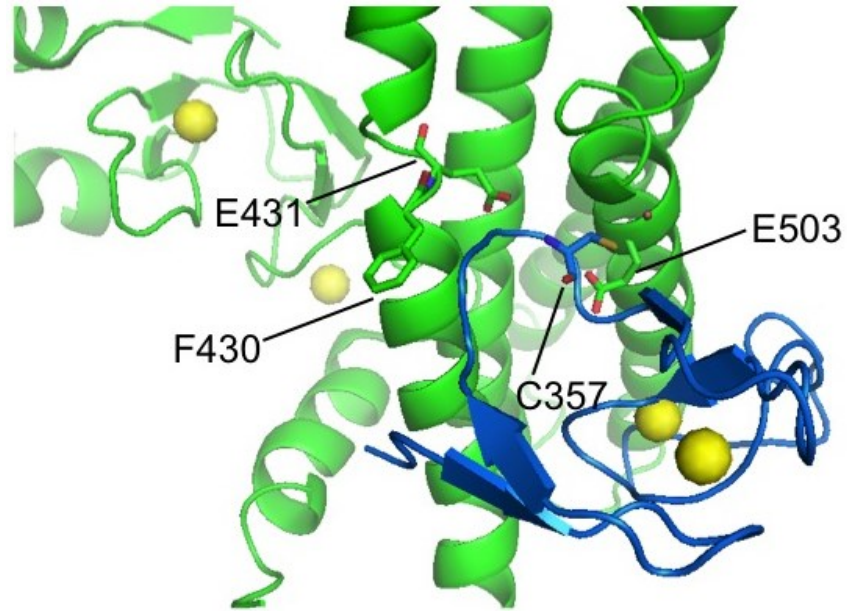Based on these evidences, my primary goal is to study this complex of two E3 ligases structurally to understand its mechanism. As technology for electron microscopy is on its prime time of development, I can take advantage of this to study the structural interaction between the two proteins. Now, electron microscopy can produce 3D images of macromolecules with resolutions close to that of X-ray crystallography or NMR spectroscopy. Another groundbreaking technology of EM microscopy called GraFix, which artificially fixes a complex during ultracentrifugation will facilitate greatly in trying to stabilize the complex during electron microscopy. Capturing the NEDD8ylated cullin and HHARI in action, along with the E2 and a potential substrate reveal the molecular basis for a fundamentally novel mechanism of E3 ligases.

CHAPTER 2.   METHODS

Protein Expression and Purification

All proteins used correspond to human sequences. Full length HHARI and F430A, E431A, E503A triple mutant were both cloned into pGEX4T1 (GE) modified with a TEV proteolytic cleavage site following GST. HHARI wt and FEE clones were transformed to BL21 (DE3) Gold competent cells for expression in *E.coli*. Cultures were grown in LB media in the presence of antibiotics at 37°C shaking at 200 rpm. At O.D. of 0.8, cells were induced with 0.6mM IPTG (isopropyl beta-D-thiogalactopyranoside) and grown overnight at 16°C. Proteins were purified by glutathione affinity chromatography, followed by overnight TEV proteolysis at 4°C to separate HHARI and GST. HHARI was purified away from GST by anion exchange with a 5ml High trap Q column (GE), and further purified by size exclusion chromatography in buffer conditions of 25mM HEPES pH 7.5, 150mM NaCl, 1mM DTT. After size-exclusion chromatography, proteins were pooled, concentrated, flash-frozen, and stored at −80°C. UbcH7, Uba1, and ubiquitin used in the autoubiquitination assays along with APPBP1-Uba3, Ubc12, and Nedd8 used in NEDD8ylation reactions were prepared in a similar manner.

Constructs of full-length Cul1 and Rbx1 were cloned into pFastBac vectors, with untagged Cul1 and TEV cleavable GST-TEV-Rbx1. Baculoviruses were made with bacmid DNA transformed into DH10alpha, and were amplified in Sf9 insect cells. Cul1 and Rbx1 was coexpressed in High Five insect cells, using viruses of Cul1 and GST-Rbx1. Cul1Rbx1 was purified by glutathione affinity chromatography, followed by overnight TEV proteolysis at 4°C. Further purification was done by cation exchange with 5ml High trap S column, and size-exclusion chromatography in buffer conditions of 25mM HEPES pH 7.5, 150mM NaCl, 1mM DTT. After size exclusion-chromatography, proteins were pooled, concentrated, and flash-frozen, and stored at −80°C.

Biochemical Reactions and Assays

NEDD8ylation reactions of Cul1Rbx1 and Cul3Rbx1 were done by incubating 100nM APPBP1-Uba3, 1μM Ubc12, 12μM Cul1Rbx1, 25μM Nedd8, in 25mM HEPES pH 7.8, 100mM NaCl, 2.5mM $MgCl_2$, 1mM ATP, for 9 min at room temperature. The reaction was done in 2ml volume, quenched with 10mM DTT. Sample was spun down 13,000rpm for 10 min and further purified by size-exclusion chromatography in 25mM HEPES pH 7.5, 150mM NaCl, 1mM DTT.

Autoubiquitination reactions were performed by incubating 200nM Uba1, 1μM UbcH7, 20μM Ubiquitin, 400nM HHARI, ±400nM N8Cul1Rbx1/N8Cul3Rbx1 in 50mM Tris-HCl pH 7.6, 50mM NaCl, 10mM $MgCl_2$, 5mM ATP at room temperature, and was quenched with 2X SDS-PAGE sample buffer containing 20mM DTT each time point. For reactions containing N8Cul1Rbx1/N8Cul3Rbx1, cullins were preincubated with HHARI for 20 min on ice. Reactions were initiated by adding Uba1 and were run on 4%-

12% gradient gels (Invitrogen), stained overnight by SYPRO-Ruby stain, and visualized by a Typhoon gel-imager.

Analytical Size-Exclusion Chromatography

Analytical size-exclusion chromatography was used to examine association between HHARI and NEDD8ylated cullin RING ligases. Proteins were mixed at equimolar concentrations of 10µM in 200µl volumes on ice for 30 min. Each mix was spun down at 13000 rpm for 10 minutes and loaded onto a SD200 10/300 column (GE), in buffer conditions of 25mM HEPES pH 7.5, 100mM NaCl, 1mM DTT. 500µl fractions were collected and run on 10% SDS gels, and visualized by Coomassie staining.

GraFix and Negative Staining

Protein samples were mixed at equimolar concentrations of 10µM in 100µl. N8Cul1Rbx1, Skp1Fbw7, HHARI FEE mutant were mixed on ice for 30 min. The sample was spun down 35,000rpm for 15 hr in a 5ml 10%-30% (w/v) sucrose gradient made by 25mM HEPES pH 7.5, 200mM NaCl, 1mM DTT. The sucrose gradient contained 0.05% gluteraldehyde for fixation. 205µl fractions were taken from the top and mixed with 5µl of 0.5M Sodium Aspartate pH 8.0 for each fraction. Fractions were measured with 1x Biorad protein assay on a 96well plate, and desired fractions were collected and flash frozen before use. 30µl of each fraction were used for making negative stain grids and fixed with 2% Uranyl acetate on carbon film.

CHAPTER 3.   RESULTS

NEDD8ylated Cullin RING Ligases Activate HHARI

I sought to understand the mechanisms of how E3 ligases gain activity, specifically RBR type E3 ligases. Studies by Duda et al gave us insight into how the F430A, E431A, E503A triple mutation helps activate HHARI. Further studies from Kelsall et al showed that adding NEDD8ylated cullin RING ligase results in HHARI activity. It might be possible that the interaction between the two E3 ligases might produce an entire new form of E3 that has its own certain mechanism for substrate ubiquitination. Therefore, I first tested whether the FEE mutation enhances interaction with the NEDD8ylated cullins by looking at the autoubiquitination activity of HHARI. HHARI FEE clearly showed activity compared to wild type HHARI by formation of ubiquitin chains on HHARI (**Figure 3-1A**). Adding either NEDD8ylated Cul1Rbx1 or Cul3Rbx1 both slightly activated HHARI WT. As HHARI FEE activates so quickly, there is not a noticeable difference in autoubiquitination when NEDD8ylated cullins are added (**Figure 3-1B, C**). However, it was clear from the wild type activation that the NEDD8ylated cullins influence HHARI activation.

NEDD8ylated Cullin RING Ligases Bind with HHARI

In order to understand how the NEDD8ylated cullin RING ligases interact with HHARI, I performed analytical size exclusion chromatography to assess complex formation. If the interaction enhances HHARI activity, it is possible that the two proteins have structural interaction. First, the complex formed with wild type HHARI and N8Cul1Rbx1 produced a higher molecular weight complex, but also produced a big shoulder peak that seemed to have a partial product. However, HHARI FEE and N8Cul1Rbx1 made a larger complex, with a smaller shoulder peak (**Figure 3-2A**). Seen by coomassie staining of gels run on same fractions, both WT or FEE HHARI make a complex with N8Cul1Rbx1, but the FEE complex makes a larger form of almost two fractions (**Figure 3-2B**). This suggests that it is possible the HHARI FEE makes a tighter complex with N8Cul1Rbx1 compared to HHARI WT.

An analogous experiment showed that N8Cul3Rbx1 also helps activate HHARI WT on its autoubiquitination. However, surprisingly there were no differences between the sizes of the complexes made between N8Cul3Rbx1 and HHARI WT or HHARI FEE (**Figure 3-3A, B**). Therefore, the N8Cul3Rbx1 did not differentiate whether HHARI has the FEE mutation or not. Overlap of all peaks showed that the largest complex made was between N8Cul1Rbx1 and HHARI FEE (**Figure 3-3C**).
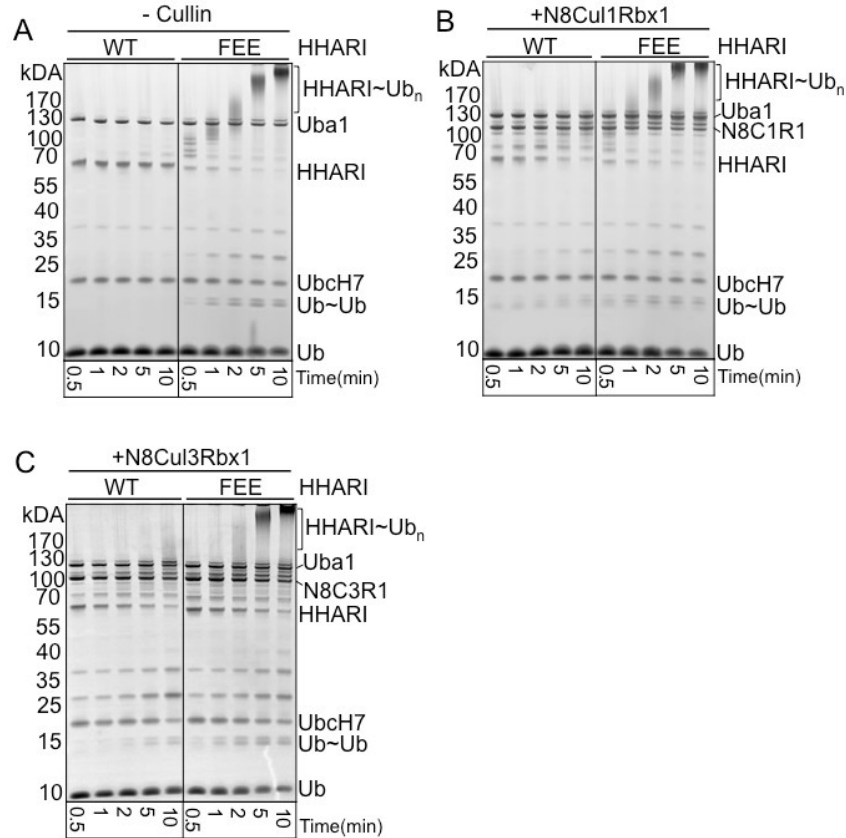
Figure 3-1.   FEE Mutation of HHARI and the Addition of N8Cul1Rbx1 Both Activate HHARI.

(A) WT HHARI stays inactivate as no autoubiquitin chains are built by time. However, FEE mutation of HHARI induces autoubiquitination activity starting from 0.5 min building autoubiquitin chains, and almost fully saturated by 5min. **(B)** N8Cul1Rbx1 promotes wild type HHARI activation, while enhancing the activity of HHARI FEE mutant. **(C)** N8Cul3Rbx1 also promotes wild type HHARI activation, while enhancing the activity of HHARI FEE mutant. All assays are observed by SYPRO-Ruby staining.
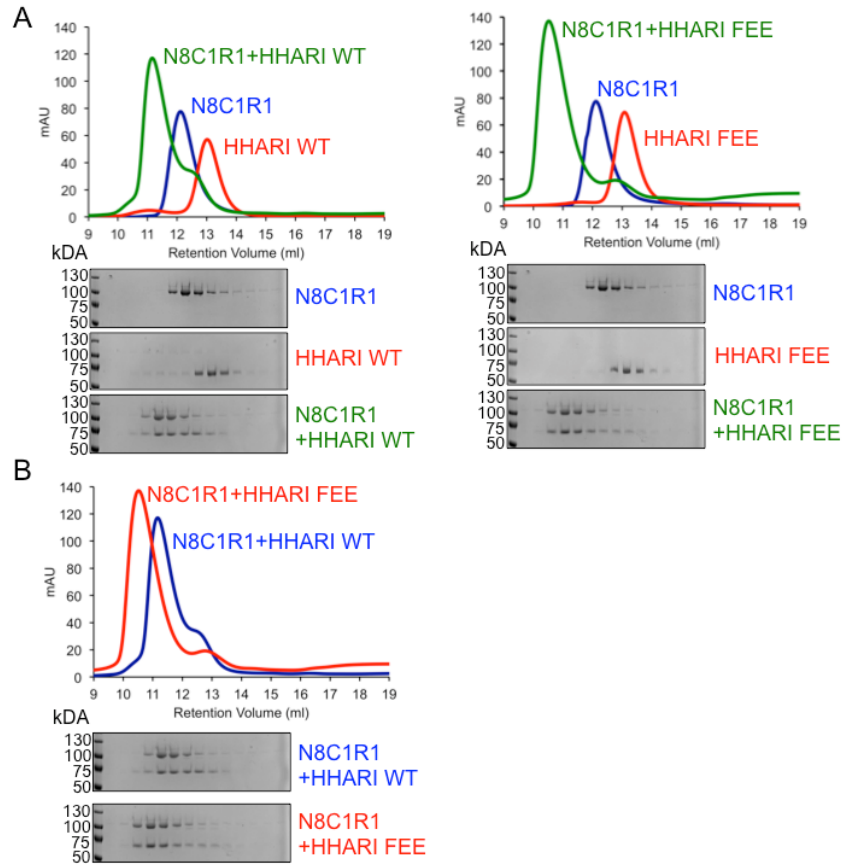
Figure 3-2. **Analytical Size-Exclusion Chromatography Shows Interaction Between N8Cul1Rbx1 and HHARI.**

(A) Wild type HHARI forms a complex with N8Cul1Rbx1, but makes a big shoulder peak of an intermediate complex. HHARI FEE mutant forms a larger complex with almost very little intermediate product, indicating equilibrium shifting more towards complex formation. **(B)** Overlay of two complexes made either by wild type or FEE mutant HHARI. FEE mutant clearly makes a better complex with N8Cul1Rbx1.

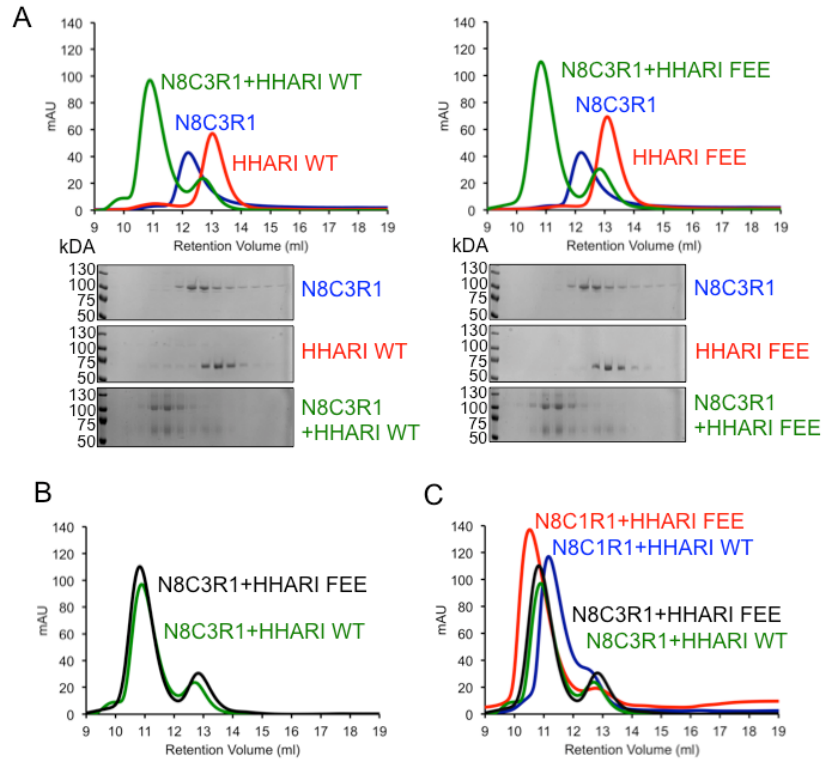**Figure 3-3. Interaction Between N8Cul3Rbx1 and HHARI.**

(A) Both wild type and FEE mutant HHARI forms a complex with N8Cul3Rbx1 in a similar manner, making similar shoulder intermediates. **(B)** Overlay of two complexes made either by wild type or FEE mutant HHARI. They are identical. **(C)** Overall overlay all type of complexes. N8Cul1Rbx1 and HHARI FEE form the largest complex with the least intermediate.

GraFix and Negative Staining

We sought to study the newly formed complex by structural studies. However, due to the current limits of electron microscopy, a protein complex of less than 200 kDa would not produce an ideal resolution. I tried to enhance contrast by making the complex bigger by adding an adaptor protein of N8Cul1Rbx1. Skp1Fbw7 is a substrate adaptor protein of N8Cul1Rbx1 that dimerizes, suggesting the entire complex might be able to dimerize by Skp1Fbw7. However, adding Skp1Fbw7 to HHARI FEE and N8Cul1Rbx1 did not form a large complex when analyzed over size-exclusion chromatography (figure not shown). Therefore, another way to generate a robust complex was to artificially trap it. GraFix, a well known technique used to chemically lock a protein complex, was used for making samples for electron microscopy (Kastner et al., 2008). This allowed me to make a complex that contain all three proteins, HHARI FEE, N8Cul1Rbx1, and Skp1Fbw7, possibly in a dimer form. The percentage of gluteraldehyde added, speed and time of centrifugation were optimized, calculated by the theoretical molecular weight. The peaks of Biorad protein assay traveled to an optimal fraction after ultracentrifugation (**Figure 3-4A**).

Negative staining of samples of Fraction#18 produced semi-homogenous molecular images (**Figure 3-4B**). We can definitely see the multiple circular shapes with a hollow center, and cryo EM is currently in progress. If the results produce a high resolution 3D image, it will give tremendous information on structural interaction between NEDD8ylated cullins and RBRs.
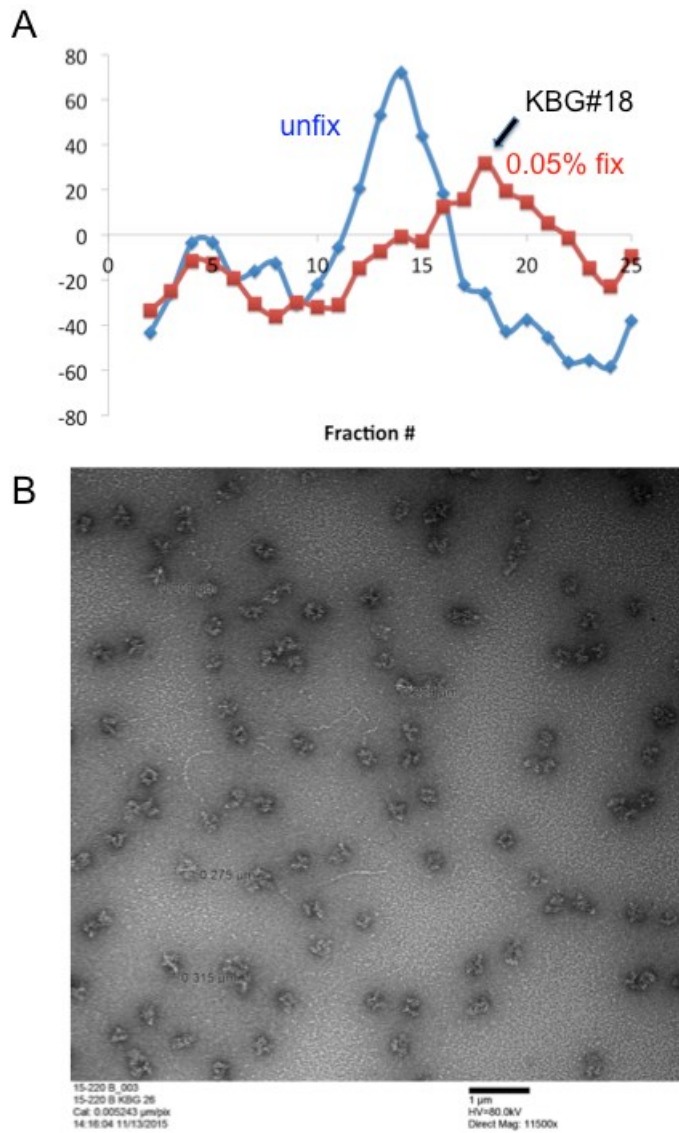
Figure 3-4. Electron Microscopy of HHARI FEE, N8Cul1Rbx1, and Skp1/Fbw7.

(A) 10-30% (w/v) sucrose gradient ultracentrifugation fraction profile with or without 0.05% Gluteraldehyde. Fraction#18 was used for negative staining. **(B)** Negative stain image of Fraction#18 of complex with HHARI FEE, N8Cul1Rbx1, and Skp1/Fbw7.

CHAPTER 4.   DISCUSSION

Here, I studied the interaction between two E3 ligases, HHARI and N8Cul1Rbx1. I showed that NEDD8ylated cullin RING ligases are able to make a complex with HHARI. A specific set of HHARI mutations, F430A, E431A, and E503A, was not only able to activate its autoubiquitination, but also produce a complex with N8Cul1Rbx1. Understanding the mechanism of the two E3 ligases' interaction may reveal a novel mechanism of the ubiquitin pathway and providing structural insights will be the key.

Microscopic Reversibility of Protein Complex

Microscopic reversibility states that there is always a reverse reaction by time, therefore a heterogenous substance can constantly exist in subgroups composed of intermediate states for every process (Lewis, 1925). So far, the biggest challenge in our efforts to make a stable complex with HHARI and N8Cul1Rbx1 was that whenever the complex was run on size exclusion chromatography, it would produce a 70% bound form, with a shoulder peak that would indicate an unbound form or an intermediate. This resulted in many attempted failures on trying to crystallize the complex, and the crystals obtained were only one part of the complex, either HHARI or NEDD8ylated Cul1Rbx1. Therefore the microscopic reversibility of the complex of N8Cul1Rbx1 and HHARI WT has been an issue as the constant association and dissociation produced an unstable complex suitable for crystal formation. If we are able to somehow shift the equilibrium to where it is more favorable in a bound form, that would enable us to study its structure either through crystallography or electron microscopy.

Stabilizing the Complex

From preliminary studies (not shown), we know that the N-terminal 90 residues of HHARI, which are highly acidic and glycine rich, are necessary for binding to N8Cul1Rbx1. I believe the acidic regions are capable of making interactions but the abundance of glycines makes the structure not stable enough for a single, specific interaction. Therefore, this might be the reasoning behind the dynamic equilibrium state, producing intermediate products. It was surprising to find out that introducing the F430A, E431A, E503A triple mutation of HHARI was able to make a larger complex from size exclusion chromatography, while having a very little shoulder peak. This indicated that the equilibrium greatly shifted to the complex formation side and producing less intermediate forms than previous experiments. It is possible that the triple mutation opens up a secondary docking site for the NEDD8ylated cullin to bind. With the equilibrium shift, I was able to gain confidence that HHARI FEE form a much more stable complex compared to HHARI WT.

Ub-VME as a Chemical Warhead

Chemically modified ubiquitins were initially introduced as probes for reactivity of deubiquitinating enzymes (DUB) (Borodovsky et al., 2002). In previous studies of Parkin, chemically modified ubiquitin-vinyl sulfone (Ub-VS) was implemented as a probe that covalently modifies the catalytic cysteine of Parkin without the help of E2 (Riley et al., 2013). Kelsall et al also implemented an electrophilic Ubiquitin-vinyl methyl ester (Ub-VME) to find that the Ub-VME binds to the catalytic cysteine of HHARI by either removing the Ariadne domain or the addition of NEDD8ylated cullins (Kelsall et al., 2013). In fact, there have been structural studies using the Ub-VME to study molecular contacts for ubiquitin recognition (Sheedlo et al., 2015). Implementing Ub-VME on structural studies of the complex with NEDD8ylated cullins and HHARI will have a modified ubiquitin bound to the active site cysteine, producing an active form of the complex. Further on, if this active site bound Ub-VME interacts with multiple sites on either HHARI or NEDD8ylated cullin, it can act as a chemical linker that locks the complex in action.

Targets for Electron Microscopy

Currently, size is a very important factor in achieving high-resolution 3D images for electron microscopy. Therefore, dimerizing the complex would significantly improve EM studies. F-box protein Fbw7 of Cul1Rbx1 would theoretically produce a dimer. Also, Cul3Rbx1 binds with a BTB protein that is known to interact with substrate adaptors. Most importantly they are known to dimerize (Furukawa et al., 2003; Zhuang et al., 2009). As Cul3Rbx1 also interacts with HHARI, dimerization of Cul3Rbx1 and its substrate adaptor will likely cause dimerization of the potentially HHARI bound complex as well, producing a sizable protein suitable for electron microscopy. However, according to the analytical size-exclusion chromatography experiments (**Figure 6B**), N8Cul3Rbx1 did not make a difference in the interaction between either HHARI WT or FEE mutant. Therefore, it is not clear whether N8Cul3Rbx1 would interact in a similar way with N8Cul1Rbx1, but it will definitely give insight towards how the cullin RING ligases interact with HHARI.

According to protein interaction studies, HHARI is seen to interact with Cul1, Cul2, Cul3, and Cul4A, which can also be tried for structural studies. Another Ariadne family protein TRIAD1 can also be used, as Kelsall et al used to study interactions with Cul5. One option to study a complex between RBR and cullins would be Parc (Cul9) as this is known as a cullin and Ariadne RBR fused protein. So far, there have been no structural studies on this protein, which can potentially provide great insight on the cullin RBR complex. From these studies I hope to learn a novel mechanism of two different E3 ligases working together. It is possible that the study might reveal a new category of E3 ligases, as there have been no studies on this interaction mechanism. Further on, as an E3 ligase, its substrate regulation will be most important in its participation in cellular processes. It will be interesting to study what this new complex regulates downstream,

whether it will affect substrates of NEDD8ylated cullins, HHARI, or affect entirely new substrates.

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